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Research article

CHEMICAL CONSTITUENTS, ANTIMICROBIAL AND ANTIBIOFILM ACTIVITIES OF ESSENTIAL OILS AND ETHANOLIC EXTRACTS OF *ORIGANUM FLORIBUNDUM* MUNBY, *EUCALYPTUS CITRIODORA* HOOK AND *CYMBOPOGON SCHOENANTHUS* (L.) SPRENG.

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ABSTRACT

This study explores the chemical composition, antimicrobial, and antibiofilm activities of essential oils (EOs) and ethanolic extracts (EEs) from *Origanum floribundum* (OF), *Eucalyptus citriodora* (EC), and *Cymbopogon schoenanthus* (CS). The EOs analyzed by GC-MS revealed the presence of p-cymene (38%), citronellal (40%), and piperitone (52.1%) as main components in OF, EC, and CS, respectively. The antimicrobial activity was tested against seven microorganisms, including two Gram-positive and three Gram-negative bacteria, one yeast and one fungus. The EOs demonstrated notable antimicrobial activity, particularly OF, which exhibited broad-spectrum antimicrobial effects against all tested germs. Compared to EOs, EEs showed lower antimicrobial efficiency. All EOs and EEs displayed antibiofilm activity against *Staphylococcus aureus*. Evaluating antimicrobial activity for EO combinations revealed synergistic effects of OF-EC and EC-CS against Gram-positive bacteria. In contrast, other combinations demonstrated additive activity or indifferent effect compared to the individual oils. Additionally, most EO combinations exhibited antagonistic interactions against fungal species. In conclusion, EOs of OF and EC could be potential natural preservatives in the food industry.

1. Introduction

The scientific and pharmaceutical communities are now interested in the potential antimicrobial qualities of plant-derived substances, an underutilized source of antimicrobial chemotypes used in traditional medicine across various nations, due to the increasing prevalence of drug-resistant pathogens (Mokhtar *et al*., 2023). Medicinal and aromatic plants have been used to treat or

prevent infectious diseases. Previous research has indicated their abundance in polyphenols, including tannins and flavonoids, which exhibit antibacterial properties (Ben Othman *et al*., 2017).

Essential oils are complex combinations of volatile secondary metabolites that are generated by plants. They exibit biological activities against fungi, bacteria, and algae (Kalemba & Kunicka, 2003). These natural compounds are well known for their strong biological activities, and they have been frequently used in the pharmaceutical, medicinal, cosmetic, and agri-food industries (Hazzit *et al*., 2015).

Secondary metabolites, particularly phenolic compounds, are significant among plant constituents because of to their reported medicinal activities, including antioxidant, antibacterial, anticancer, antidiabetic, and anti-inflammatory effects (Sohaib *et al*., 2022). Numerous components found in plant oils and extracts have potent antibacterial activity against biofilms and multidrug-resistant microorganisms (Burt, 2004; Ahire *et al.*, 2011). Microorganisms, including Gram-positive and Gram-negative bacteria and fungi, are important sources of infection in humans and spoiled food (Yildiz, 2016). The growing issue of antimicrobial resistance has rendered the efficacy of conventional antibiotics ineffective, prompting a global shift towards discovering new antimicrobial solutions (Brochot *et al*., 2017). Essential oils derived from aromatic and medicinal plants have emerged as promising candidates for antimicrobial applications owing to their diverse and potent bioactive properties. Numerous studies have established the antibacterial efficacy of these oils and their constituents, showing notable activity against a range of pathogens associated with both human and animal diseases (González-Lamothe *et al*., 2009; Murbach Teles Andrade *et al*., 2014).

These plant-derived essential oils contain numerous bioactive compounds, such as aldehydes and phenolic compounds, which confer robust antibacterial activity and enhance their potential for practical applications (Lai & Roy, 2004). The broad-spectrum antimicrobial capabilities of essential oils and their components have been well established (Burt, 2004). Notably, the multi-component nature of essential oils makes it more challenging for bacteria to develop resistance compared to conventional antibiotics, which are often composed of single chemical entities (Değirmenci *et al*., 2020).

The present study aimed to investigate the chemical composition, antimicrobial and synergistic interactions, and antibiofilm activities of essential oils (EOs) and ethanolic extracts (EEs) from *O. floribundum*, *E. citriodora*, and *C. schoenanthus* grown in Algeria. Investigations of the antimicrobial and antibiofilm characteristics of essential oils and extracts from these plants are scarce, especially for *E. citriodora* and *C. schoenanthus*. This study aimed to enhance the existing knowledge base and fill this gap. The antibiofilm action of essential oils and extracts from these Algerian plants has not been documented.

2. Materials and methods

2.1. Materials

2.1.1 Plant material

O. floribundum before the flowering stage and *E. citriodora* were collected in June 2022 from the region of Hamame Melouane (40 km south of Algiers) and the botanical garden of the National Higher School of Agronomy (ENSA), Algiers, respectively. *C. schoenanthus* was obtained from Tamanrasset (2000 km south of Algiers) in April 2022. Plants were identified by Pr Hasen Abdelkrim, Professor of Weed Science and Plant Ecology, Department of Botany at the National Higher School of Agronomy (ENSA).

2.2. Methods

2.2.1. Essential oils and ethanol extracts isolation

Leaves (100 g), dried in the shade, were separately subjected to essential oil extraction using a Clevenger apparatus for 2 h. The EOs obtained were stored in amber glass vials at a low temperature of 4°C. The EEs were prepared by solid-liquid extraction with 96%

ethanol in a Soxhlet apparatus for 6 h. The resulting extracts were lyophilized to obtain powders, which were weighed and stored at 4°C until further use.

2.2.2. Gas chromatography-mass spectrometry (GC-MS) analysis

The gas chromatography-electron ionisation mass spectrometry (GC-MS) analysis was conducted using a Hewlett-Packard computerised system that included an HP 5MS apolar column (30 m × 0.25 mm × 0.25 µm film thickness) and a 6890 gas chromatograph connected to a 5973A mass spectrometer. Helium served as the carrier gas in the GC-MS experiment, with a flow rate of 0.5 mL/min, split mode (1:25), 0.2 µL (1/10 in hexane v/v) of injected volume, and an injection temperature of 250°C. Electronic ionization at 70 eV was used to ionize the samples over a 30-550 atomic mass unit scan range by comparing the GC retention indices (RI) of the essential oil constituents, which were calculated for a homologous series of C8-C20 n-alkanes with those of available authentic standards and literature (Adams, 2007; Babushok *et al*., 2011; Benchaa *et al*., 2018; Hadjadj & Hazzit, 2020). The identification was confirmed by comparing their mass spectral fragmentation patterns with those in the MS database (NIST 2005 and Wiley 7N libraries) and mass spectra data from published sources (Adams, 2007). The relative concentrations of the components were directly determined from the GC peak regions using GC-FID.

2.2.3. Determination of total phenols and flavonoid contents

The total phenolic and flavonoid contents were quantified using the methodology outlined by Douar-Latreche *et al*. (2018). The total phenolic components were quantified using the Folin-Ciocalteu technique and reported as mg Gallic Acid Equivalents (GAE) per gram of extract.

The aluminium tri-chloride ($AlCl_3$) method was employed to determine the total flavonoid content. Values were quantified and expressed in milligrams of quercetin equivalents (QE) per gram of extract. All experiments were conducted in triplicate, and the average data were documented.

2.2.4. Antimicrobial activity

The disc diffusion method for antimicrobial screening and the direct contact technique in an agar medium to ascertain the minimum inhibitory concentration (MIC) were used to evaluate the sensitivity of bacterial strains to essential oils.

2.2.4.1. Bacterial strains

A variety of bacterial strains were used in this investigation, including Gram-positive bacteria, such as *Staphylococcus aureus* (ATCC 6538) and *Bacillus subtilis* (ATCC 6633), and Gram-negative bacteria, including *Salmonella enterica serovars' Typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 9027), and *Escherichia coli* (ATCC 8739). The investigation also included the fungal strains *Candida albicans* (ATCC 10231) and *Aspergillus brasiliensis* (ATCC 16404). ATCC strains were obtained from the CRD (Research and Development Centre, SAIDAL Algiers). The inoculum in all studies achieved a microbiological density of approximately 10^7_10^8 colony-forming units (CFU) /mL (Benahmed *et al*., 2019)

2.2.4.2. Disc diffusion method

Whatman discs (9 mm in diameter) were saturated with 10 µl of pure essential oil or a 100 mg/ml concentration of ethanolic extract diluted in dimethyl sulfoxide (DMSO) and positioned on the surfaces of the inoculated Mueller-Hinton agar (MHA) for bacteria and Sabouraud dextrose agar (SDA) for yeast, using a sterile cotton swab. After two hours at 4°C, the plates were incubated for 24 h at 37°C for bacterial cultures and for 48 h at 25°C for yeast cultures. The study examined triplicate sets, which included DMSO as the negative control and gentamicin (40 mg/mL) and fluconazole (150 mg/mL) as positive controls. A digital caliper was used to measure the inhibition zones surrounding the discs to evaluate the antibacterial activity, which was then expressed as the mean of the inhibition diameters obtained (in millimeters) (Hazzit *et al*., 2015; Benahmed *et al*., 2019).

2.2.4.3. Determination of the minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBC)

The agar dilution method was employed in this experiment, following the protocol approved by the NCCLS with slight modifications. Microbial suspensions with concentrations ranging from 10^7 to 10^8 CFU/mL were prepared from young bacteria and yeast cultures. EOs and EEs were serially diluted, ranging from 4% (v/v) to 0.015% (v/v) in MHA for bacteria and SDA for fungal strains, both supplemented with a 0.5% (v/v) concentration of Tween 80 after autoclaving to enhance oil solubility, 1–2 µl aliquots of each microorganism, containing approximately 10^4 CFU, were used to inoculate filter paper discs. The MIC was the lowest following incubation, preventing visible microbial growth. Discs lacking growth were aseptically transferred to the culture media for the minimum bactericidal concentration assessment. (Hammer *et al*., 1999). The MBC/MIC ratio was calculated by classifying substances with a ratio ≤ 4 as bactericidal and > 4 as bacteriostatic (Dzoyem *et al.,* 2018).

***2.2.4.4.* The synergistic effect between EOs**

To evaluate the synergistic effect between the EOs, combinations of OFEC (*O. floribundum* and *E. citriodora*), OFCS (*O. floribundum* and *C. schoenanthus*), and ECCS (*E. citriodora* and *C. schoenanthus*) were studied. For each combination, the EOs were mixed in equal parts, and the antibacterial effect was determined using the disk diffusion method to measure the diameters of the inhibition zones (IZ), as well as their MIC, and MBC. The combined impacts of EOs were quantified and represented as the fractional inhibitor concentration index (FICI) using the formula delineated by Nikkhah *et al.* (2017):

$$\sum \text{FICI} = \text{FIC}_{(A)} + \text{FIC}_{(B)} \quad (1)$$

$$FIC(A) = \frac{MIC(A) in\ combination}{MIC(A) alone}$$

$$FIC(B) = \frac{MIC(B) in\ combination}{MIC(B) alone} \quad (2)$$

Where:

The obtained results were interpreted as follows: synergistic effect (FICI ≤ 0.5) additive effect (0.5 < FICI ≤ 1) indifferent effect (1 < FICI ≤ 4) and antagonistic effect (FICI > 4).

2.2.5. Antibiofilm activity

2.2.5.1. Detection of biofilm formation

For this evaluation, the method described by Ramadan *et al.* (2017) was adapted with some modifications. For bacterial strains, fresh microbial cultures cultivated on Tryptic Soy Agar (TSA) were diluted to 10^6 CFU/mL in Brain Heart Infusion Broth (BHIB) supplemented with 2% glucose (w/v) and Tryptic Soy Broth (TSB) supplemented with 2% glucose, and for *Candida albicans*, in Sabouraud Dextrose Broth (SDB) and Potato Dextrose Broth (PDB). This procedure was specifically carried out to identify the optimal growth medium that enabled maximum biofilm formation for each tested strain. The strains selected for biofilm optimization were the same as those previously used to evaluate the antimicrobial activity in this study. A 96-well plate was filled with 150 µL of the microbial dilution in each well. A negative control was established for each culture medium, with six replicates for each condition. Following a 24 hours incubation at 37 °C without agitation, the supernatant was discarded and the wells were washed twice with Phosphate-Buffered Saline (PBS) to remove non-adherent cells. The plates were air-dried, and surface-adherent bacterial cells were stained with 200 µL of 0.1% crystal violet for 30 min. After removing the crystal violet, the plates were cleaned with distilled water, and 200 µL of 96% ethanol was used to fix the reaction mixture. A Bio-Tek ELx800 microplate reader was used to measure the coloured adherent bacteria's optical density (OD) of the colored adherent bacteria at 540 nm. The values acquired for the treatment wells (OD) and control wells (ODc) were used to assess biofilm formation according to the

classification established by Stepanovic *et al.* (2004) as follows: OD ≤ ODc no biofilm producer, ODc < OD ≤ 2 × ODc weak biofilm producer, 2 × ODc < OD ≤ 4 × ODc moderate biofilm producer, and 4 × ODc < OD strong biofilm producer.

2.2.5.2. Determination of antibiofilm activity

The bacterial strains S. aureus ATCC 6538 and P. aeruginosa ATCC 9027, both of which were previously confirmed as strong biofilm producers, were selected for this study. The antibiofilm activity of EOs and EEs was assessed using, for each strain, the culture medium previously determined to support optimal biofilm formation for each strain. To evaluate the antibiofilm activity, the cultures grown on TSA were diluted to a concentration of 10^6 CFU/mL in BHIB supplemented with 2% glucose (w/v) for *S. aureus* and in TSB enriched with 2% glucose for *P. aeruginosa*. These media were specifically chosen because of their ability to facilitate robust biofilm development in these bacterial strains. Subsequently, 75µL of this bacterial dilution was added to each well of a 96-well plate, followed by 75 µL of EOs and EEs previously dissolved in dimethyl sulfoxide (DMSO) and diluted in the appropriate medium over a concentration range from 4% to 0.008%. A growth control was prepared by seeding 150 µL of culture medium seeded with the appropriate microbial dilution, and a negative control was prepared with culture medium supplemented with DMSO. Following a 24 hours incubation at 37°C, the supernatant was removed, the wells were washed twice with PBS, and the bacterial cells adhering to the surface were stained with 200 µL of 0.1% crystal violet. The optical density of the stained cells was measured at 540 nm following the removal of crystal violet and the fixation of the biofilms using ethanol. As Boumghar *et al.* (2019) reported, the biofilm inhibition percentage inhibition of essential oils and ethanol extracts was obtained using the following formula:

$$BPI(\%) = \frac{OD\ control - OD\ sample}{OD\ control} X\ 100 \quad (3)$$

BPI: Biofilm Percentage Inhibition; OD: Optical Density

2.3. Data analysis

All experiments were conducted in triplicates (n = 3). Data are expressed as mean ± standard error (SE). One-way analysis of variance (ANOVA) was performed using SPSS software (version 22), followed by Tukey's post-hoc test for pairwise comparisons. Statistical significance was defined as $p < 0.05$. Principal Component Analysis (PCA), Agglomerative Hierarchical Clustering (AHC), and population projection analyses were conducted using Statistica software (version 12) to explore the patterns and relationships in the antimicrobial and antibiofilm activity data.

3. Results and discussions

3.1. Essential oils yield and chemical composition

The dried leaves of the studied plants yielded EOs at rates of 3.5 ± 0.65%, 2.73 ± 0.15%, and 2.13 ± 0.3% (v/w dry matter) for OF, EC, and CS, respectively. For OF, these results were in line with previously documented levels for the species (2.8-4.5%) as reported by Kerbouche *et al*. (2015), Mir *et al.* (2022), and Hadjadj and Hazzit (2020). The yield for EC falls within the range reported by Tolba *et al*. (2015) and Benchaa *et al*. (2018), who found 2.6% and 3.4% yields, respectively, for EC grown in Algeria. However, Manika *et al*. (2012) found a 1.0%-2.1% range for EC grown under subtropical conditions in northern India. The 2.13% yield of CS was within the range of those reported for Algerian samples (0.5-3.7%) (Malti *et al.,* 2020; Aous *et al*., 2019), but it was superior to the values reported by Sawadogo *et al*. (2022) from Nigeria (0.95%), and Mohamed Abdoul-Latif *et al.* (2022) from Burkina Faso (1.16%). These variations can be attributed to the climatic and edaphic conditions (Mehalaine & Chenchouni, 2021).Chromatographic analysis of the studied plants (Table 1) led to the identification of a total of 33 components in the OFEO; p-cymene (38%), carvacrol (16.5%), thymol (15.6%) and γ-terpinene (4.8%) were found to be the major compounds, primarily belonging to the classes

of monoterpene hydrocarbons (51%) and oxygen-containing monoterpenes (45.9%). The predominance of p-cymene in this Origanum species has been previously reported by previous studies (Daoudi-Merbah et al., 2016; Boulaghmen et al., 2019; Hadjadj & Hazzit, 2020). However, thymol was reported by Kerbouche et al. (2015) as the predominant component, whereas other researchers found carvacrol to be the most prominent component (Baser et al., 2000; Fertout-Mouri et al., 2023). Certain substances in a plant are influenced by genetic traits, age, geographical location, environmental conditions, weather, climate, plant variety, ecotype, and harvest stage. Extraction procedures may also substantially affect the quantities of essential oil components and the growth conditions (Kerbouche *et al*., 2015; Benchaa *et al*., 2018). Different authors have reported that the main components found in this study for OF to have some biological activities. Thus, p-cymene has various pharmacological properties, including antimicrobial, anti-inflammatory, antiparasitic, antiviral, antioxidant, antidiabetic, and antitumor activities (Bonjardim *et al*., 2012; Marchese *et al*., 2017; Balahbib *et al*., 2021; Shakeel & Tabassum, 2022; Baginska *et al*., 2023). In addition, the monoterpenes thymol and carvacrol have received significant attention in recent years. These compounds are found in many plants, including oregano, thyme, sweet basil, black cumin, and savory, and plants rich in these compounds have been used in traditional medicine across various cultures (Rathod *et al*., 2021). Carvacrol, a key component of EOs, has gained increasing attention because of its extensive biological properties. It is known for its broad-spectrum antimicrobial activity and antiprotozoal (Can Baser *et al*., 2008), antinociceptive (Guimarães *et al*., 2010), antibacterial (Nostro *&* Papalia, 2012), antifungal (Lima *et al*., 2013), cardioprotective (Chen *et al*., 2017), antioxidative (Sharifi-Rad *et al.*, 2018), anti-inflammatory (de Carvalho *et al.*, 2020), anticarcinogenic (Sampaio *et al*., 2021), neuroprotective effects (Azizi *et al*., 2022) and antidiabetic (Aljelehawy *et al*., 2023), attributed to its unique structural characteristics that combine hydrophobic and hydrophilic properties (Memar *et al.*, 2017). Moreover, sesquiterpene hydrocarbons and oxygenated sesquiterpenes are rare in this EO, and are represented only by trans-β-caryophyllene (0.1%) and caryophyllene oxide (0.4%). In contrast, 36 constituents were identified in the EO of *E. citriodora* (Table 1). Oxygenated monoterpenes displayed the highest contribution (82.3%), among which citronellal was the most abundant compound (40%), followed by isopulegol (24.4%), and citronellol (10.5%). According to Manika *et al.* (2012), the most common EO component in samples taken from EC leaves is citronellal, which is present at significant levels (69.7%-87.4%). Tolba *et al*. (2015) identified 22 chemicals in these samples, with citronellal (69.77%), citronellol (10.63%), and isopulegol (4.66%) being the predominant components. Insuan and Chahomchuen (2020) also reported that the primary components were citronellal (60.55 ± 0.07%), followed by Dl-isopulegol (10.57 ± 0.02%) and citronellol (9.04 ± 0.03%).

For *C. schoenanthus* EO, the major volatile component was piperitone, comprising 52.1% of the EO; this is followed by elemol at 8.9% and δ-2-carene at 7.5%, with a total of 96.1% identified constituents. Oxygen-containing monoterpenes accounted for 55.3% of the total composition, whereas oxygen-containing sesquiterpenes accounted for 23.8%. Our results align with recent findings reported by Bayala *et al*. (2023) in Burkina Faso and El-Bassossy *et al*. (2023) in Egypt. Aous *et al*. (2019) observed that the EOs of *C. schoenanthus* from the Algerian Sahara exhibited heterogeneity among samples collected from various areas. The investigation categorized into three groups based on their chemical profiles. One group was distinguished by a high piperitone content (55.1%–63.2%), demonstrated the strongest insecticidal action against *Callosobruchus maculatus* and showed noteworthy antibacterial efficacy, particularly against *Candida albicans*.

Table 1. Chemical composition (%) of essential oils of *Origanum floribundum*, *Eucalyptus citriodora*, and *Cymbopogon schoenanthus.*

N°	Compounds[a]	ERI[b]	LRI[c]	%			Identification[d]
				OF	EC	CS	
1	2-Heptanone	891	892	T	-	-	RI-MS
2	Tricyclene	922	923	**T**	**-**	-	RI-MS-St
3	α-Thujene	928	928	1.1	0.1	-	RI-MS-St
4	α-Pinene	937	936	1.0	0.3	T	RI-MS-St
5	α-Fenchene	948	949	0.1	-	-	RI-MS
6	Camphene	950	950	0.2	-	-	RI-MS-St
7	Verbenene	964	963	T	-	-	RI-MS
8	Sabinene	973	973		T	-	RI-MS-St
9	β-Pinene	978	977	0.2	0.8	-	RI-MS-St
10	1-Octen-3-ol	981	980	0.8	-	-	RI-MS
11	3-Octanone	985	985	0.5	-	-	RI-MS
12	β-Myrcene	989	989	1.0	0.1	-	RI-MS-St
13	δ-2-Carene	1003	1003	-	0.7	**7.5**	RI-MS
14	α-Phellandrene	1005	1004	0.1	-	0.2	RI-MS-St
15	δ-3-Carene	1012	1011	0.1	-	-	RI-MS-St
16	α-Terpinene	1016	1017	2.0	-	0.1	RI-MS-St
17	p-Cymene	1024	1024	**38.0**	0.4	0.5	RI-MS-St
18	Limonene	1029	1030	2.1	0.5	2.2	RI-MS-St
19	1,8-Cineole	1032	1031	-	1.1	-	RI-MS-St
20	cis-β-Ocimene	1036	1037	-	0.1	0.1	RI-MS
21	Melonal	1039	1039	-	0.6	-	RI-MS
22	γ-Terpinene	1058	1060	**4.8**	0.3	T	RI-MS-St
23	cis-Sabinene hydrate	1066	1067	3.0	-	-	RI-MS
24	p-Mentha-3,8-diene	1068	1068[a]	-	0.2	-	RI-MS
25	L-Fenchone	1084	1086	-	-	0.1	RI-MS
26	α-Terpinolene	1088	1087	0.3	0.1	0.1	RI-MS-St
27	trans-Sabinene hydrate	1096	1098	0.2	-	-	RI-MS
28	Linalool	1099	1099	1.0	0.2	-	RI-MS-St
29	cis-Rose oxide	1111	1112	-	0.3	-	RI-MS
30	cis-p-Menth-2-en-1-ol	1125	1123	-	-	1.0	RI-MS
31	trans-Rose oxide	1128	1128	-	0.1	-	RI-MS
32	Terpinene-1ol	1137	1136	-	-	0.6	RI-MS
33	Isopulegol	1143	1145	-	**24.4**	-	RI-MS
34	Borneol	1166	1166	0.6	-	-	RI-MS-St
35	Citronellal	1156	1154	-	**40**	-	RI-MS-St
36	(iso)-Isopulegol	1155	1155	-	1.5	-	RI-MS
37	Terpinen-4-ol	1176	1177	1.1	-	-	RI-MS-St
38	p-Cymen-8-ol	1185	1184	0.6	-	-	
39	α-Terpineol	1192	1190	0.4	T	2.1	RI-MS-St
40	trans-Dihydrocarvone	1201	1201	0.1	-	-	RI-MS
41	trans-Piperitol	1205	1205	-	-	0.4	RI-MS
42	Thymol methyl ether	1233	1234	0.3	-	-	RI-MS-St
43	Citronellol	1228	1228	-	**10.5**	-	RI-MS-St
44	Cumin aldehyde	1236	1238	0.1	-	-	RI-MS-St
45	Carvone	1243	1242	-	0.5	-	RI-MS-St

46	Nerol	1228	1229	-	-	0.1	RI-MS
47	Carvacrol methyl ether	1244	1243	**6.4**	-	-	RI-MS-St
48	Piperitone	1254	1254	-	0.2	**52.1**	RI-MS
49	Isopulegyl acetate	1273	1274	-	0.1	-	RI-MS
50	Citronellyl formate	1277	1276	-	0.1	-	RI-MS
51	Thymol	1291	1290	**15.6**	-	-	RI-MS-St
52	Carvacrol	1300	1300	**16.5**	-	-	RI-MS-St
53	Citronellyl acetate	1352	1352	-	3.3	-	RI-MS-St
54	β-Bourbonene	1387	1384	-	-	T	
55	β-Elemene	1391	1390	-	0.1	0.9	RI-MS
56	cis-Jasmone	1394	1394	-	0.2	-	RI-MS
57	trans-β-Caryophyllene	1420	1420	0.1	**3.2**	0.4	RI-MS-St
58	Allo-aromadendrene	1461	1460	-	0.2	-	RI-MS
59	β-Gurjunene	1432	1431	-	-	0.2	RI-MS
60	α-Humulene	1453	1453	-	0.2	-	RI-MS
61	γ-Gurjunene	1471	1472	-	-	0.2	RI-MS
62	Germacrene D	1480	1480	-	0.1	0.3	RI-MS
63	β-Selinene	1485	1486	-	-	0.1	RI-MS
64	cis-β-Guaiene	1489	1489	-	-	0.3	RI-MS
65	α-Selinene	1493	1493	-	-	0.5	RI-MS
66	Bicyclogermacrene	1494	1494	-	0.1	-	RI-MS
67	α-Muurolene	1497	1498	-	-	0.3	RI-MS
68	Germacrene A	1501	1502	-	-	0.1	RI-MS
69	γ-Cadinene	1513	1513	-	-	0.5	RI-MS
70	δ-Cadinene	1522	1523	-	-	1.1	RI-MS
71	cis-Cadina-1,4-diene	1530	1531	-	-	0.1	RI-MS
72	α-Cadinene	1533	1533	-	-	0.1	RI-MS
73	α-Calacorene	1540	1540	-	-	0.1	RI-MS
74	Elemol	1547	1548	-	-	**8.9**	RI-MS
75	Spathulenol	1576	1576	-	0.4	-	RI-MS
76	Caryophyllene oxide	1582	1581	0.4	0.9	0.4	RI-MS
77	γ-Eudesmol	1631	1631	-	-	2.2	RI-MS
78	t-Cadinol	1634	1635	-	-	1.5	RI-MS
79	β-Eudesmol	1650	1650	-	-	**4.0**	RI-MS
80	α-Eudesmol	1651	1652	-	-	**4.6**	RI-MS
81	Intermedeol	1666	1666	-	-	2.2	RI-MS
	Total identification (%)			98.7	91.9	96.1	
	Monoterpenes hydrocarbons			51	3.4	10.7	
	Oxygenated monoterpenes			45.9	82.3	55.3	
	Sesquiterpene hydrocarbons			0.1	3.9	5.2	
	Oxygenated sesquiterpenes			0.4	1.5	23.8	
	Others			1.3	0.8	1.1	
	Yield % (v/w)			3.5±0.65^{B}	2.73±0.15^{B,A}	2.13±0.3^{A}	

a Components quantified on the HP 5MS capillary column and listed in the order of elution from the same column
b ERI: Experimental retention indices relative to n-alkanes C8-C22 on non-polar column HP 5MS
c LRI: Literature retention index.

d Identification
OF*: Origanum floribundum*; EC: *Eucalyptus citriodora*; CS: *Cymbopogon schoenanthus*
St, comparison with authentic compounds (standards)
MS, and comparison of mass spectra with MS libraries (NIST 2005 and Wiley 7N)
RI, comparison of retention index with the literature (Babushok *et al*.,2011; Benchaa *et al*., 2018; Hadjadj & Hazzit, 2020)
t = trace (concentration < 0.1%); main components (>3.2%) are marked in bold
Data followed by different capital letters for yields indicate significant differences between the values of different groups by Tukey's multiple range test ($p< 0.05$)

3.2. Ethanolic extracts yields, total phenol and flavonoid contents

The yield, phenolic content, and flavonoid content of the ethanolic extracts from OF, EC, and CS (Table 2) showed notable variations in their chemical profiles. EC exhibited the highest extraction yield (39.46%) and phenolic content (217.35 mg GAE/g extract) among the extracts. In contrast, OF was distinguished by its high flavonoid content (39.80 mg QE/g extract). Conversely, CS had the lowest values across all evaluated parameters. Hadjadj and Hazzit (2020) reported a methanol extract yield of 18.3% for *O. floribundum*, along with a high flavonoid content (55.3 mg QE/g extract). However, their study noted a lower total phenolic content of 66.4 mg GAE/g extract. In comparison, Koudoro *et al*. (2014) document lower phenolic and flavonoid contents for *E. citriodora*, reporting 4.52 mg GAE/g extract and 78.76 mg QE/g extract, respectively.

In contrast, our results revealed that *C. schoenanthus* contains a total phenolic content of 28.21 ± 1.22 mg GAE/g extract and a flavonoid content of 8.53 ± 0.09 mg QE/g extract. Najjaa *et al*. (2020) report significantly higher phenolic content (63.86 ± 0.01 mg GAE/g DW) but a lower flavonoid concentration (7.09 ± 0.07 mg QE/g DW) in various Tunisian samples. Similarly, Ibrahim and El-Khateeb (2013) observe lower levels, with phenolic content at 13.25 mg GAE/g and flavonoid content at 3.5 mg QE/g in *C. schoenanthus* subsp. *proximus*. Falleh *et al*. (2008) suggested that such variations could be influenced by environmental conditions at the collection site and the growth stage of the plant, as the distribution of secondary metabolites changes throughout plant development. Furthermore, extreme weather conditions may also stimulate the synthesis of secondary metabolites, including phenolic compounds.

Table 2. Ethanolic extracts yields and total phenol and flavonoid contents of *O. floribundum, E. citriodora and C. schoenanthus* (mean ± SD)

Samples	Yields (%, g/g DW)	Total phenols (mg GAE/g Extract)	Flavonoids (mg QE/g Extract)
O. floribundum	13.9 ± 0.36^{b}	151.54 ± 7.79^{b}	39.80 ± 0.31^{c}
E. citriodora	39.46 ± 0.50^{c}	217.35 ± 2.97^{c}	24.05 ± 0.81^{b}
C. schoenanthus	7.93 ± 0.40^{a}	28.21 ± 1.22^{a}	8.53 ± 0.09^{a}

GAE: gallic acid equivalent; DW: dry weight; QE: quercetin equivalent.
***** Results are presented as the mean ± standard deviation from three distinct experiments. Values denoted by different letters within the same experiment and column are significantly different ($p<0.05$).

3.3. Antimicrobial Activity

3.3.1. Diameter of microbial inhibition zones of EOs and EEs

The results of the sensitivity of microbial strains to EOs and EEs, studied using the disk diffusion method (Table 3), showed a variability in the inhibition zones (IZ) according to the samples and bacterial strains. These differences can be related to the nature of the microbial strains and the different chemical compositions of the samples, especially their main components. Hence, *O. floribundum* EO exhibited the highest efficiency against all tested microorganisms. According to the Meena and Sethi (1994) scale, this EO had strong inhibition against Gram-

positive bacteria and fungal strains (IZ: 53.3–66.7 mm) and the Gram-negative bacteria *S. Typhimurium* (IZ: 35.5 mm), while showing moderate activity against the two other Gram-negative bacteria *E. coli* and *P. aeruginosa* (IZ: 26.93 and 16.07 mm, respectively). The potent activity of *O. floribundum* EO can be ascribed to its richness in p-cymene and the two oxygenated monoterpenes (thymol and carvacrol), which are reported to be mighty antimicrobial components alone (Braga, 2006; Sasso *et al*., 2006; Yang *et al*., 2014; Shu *et al*., 2016; Marchese *et al*., 2016 and Marchese *et al*., 2017) or in combination (Xu *et al*., 2008; Du *et al*., 2015 and Memar *et al*., 2017). Thymol and carvacrol demonstrated significant antibacterial activity by damaging bacterial cell membranes. Their hydrophobic characteristics enable their integration into the lipid bilayer, resulting in structural changes and enhanced permeability, which results in leakage of cellular contents and cell death. In addition, they can inhibit biofilm formation and reduce bacterial motility. Owing to these effects, thymol and carvacrol are considered potential natural alternatives for addressing bacterial infections, and may augment conventional antibiotic efficacy when combined. However, the efficiency of an oil results from all the components that act synergistically or additively to produce effects through many mechanisms at various cellular locations (Kachur & Suntres, 2020). By altering the phospholipid layers, fatty acids, and polysaccharides, EO can alter bacterial structures by disrupting cell walls and cytoplasmic membranes, which frequently increases their permeability. Breakdown of these structures is associated with ion leakage, ATP reservoir depletion, membrane potential dissipation, proton pump dysfunction, and macromolecular damage. EO can damage cellular lipids and proteins, leading to cytoplasmic coagulation (Bensid *et al*., 2022).

Table 3. Diameter of the microbial inhibition zone in mm (mean value ± SD; n=3) of EOs, EEs from *O. floribundum*, *E. citriodora*, and *C. schoenanthus*, along with positive controls (Gentamicin and Fluconazole)

Samples	Diameter in mm of the inhibition zone, including the diameter of the disc (9mm)						
	E. coli	***S. Typhimurium***	***P. aeruginosa***	***S. aureus***	***B. subtilis***	***C. albicans***	***A. brasiliensis***
OFEO	$26.93 \pm 0.78^{c,B}$	$35.50 \pm 1.10^{c,C}$	$16.07 \pm 0.61^{c,A}$	$53.30 \pm 0.30^{f,E}$	$50.17 \pm 1.35^{c,D}$	$66.70 \pm 1.15^{e,F}$	$64.30 \pm 0.17^{e,F}$
ECEO	$12.53 \pm 0.45^{a,A}$	$11.70 \pm 0.92^{a,A}$	$10.07 \pm 0.25^{a,A}$	$23.05 \pm 1.36^{c,d,C}$	$20 \pm 0.8^{b,B}$	$33.20 \pm 0.62^{b,E}$	$26.30 \pm 1.61^{b,D}$
CSEO	$13.70 \pm 1.6^{a,B}$	$12.32 \pm 0.82^{a,A,B}$	$10.75 \pm 0.67^{a,b,A}$	$23.84 \pm 0.87^{d,D}$	$19.70 \pm 0.26^{b,C}$	$35.02 \pm 0.11^{c,E}$	$44.53 \pm 0.15^{d,F}$
OFEE	$19.33 \pm 0.25^{b,D}$	$12.2 \pm 0.30^{a,B}$	$18.3 \pm 0.17^{d,C}$	$21.33 \pm 0.61^{c,E}$	$18.37 \pm 0.21^{b,C}$	$R^{a,A}$	$R^{a,A}$
ECEE	$13.53 \pm 0.35^{a,C}$	$12.67 \pm 0.45^{a,C}$	$11.57 \pm 0.55^{a,b,B}$	$11.57 \pm 0.42^{a,B}$	$11.03 \pm 0.38^{a,B}$	$R^{a,A}$	$R^{a,A}$
CSEE	$14.3 \pm 0.26^{a,C}$	$15.07 \pm 0.74^{b,C}$	$12.53 \pm 0.55^{b,B}$	$16.60 \pm 0.36^{b,D}$	$19.53 \pm 0.78^{b,E}$	$R^{a,A}$	$R^{a,A}$
Gentamicin*	$35.23 \pm 0.70^{d,A}$	$40.00 \pm 0.52^{c,B}$	$48.13 \pm 1.36^{e,C}$	$40.68 \pm 0.98^{e,B}$	$49.67 \pm 0.49^{c,C}$	-	-
Fluconazole*	-	-	-	-	-	45.39 ± 0.95^{d}	39.7 ± 1.69^{c}

***Gentamicin (40 mg/mL) was used for bacterial strains, whereas Fluconazole (150 mg/mL) served as a reference for fungal strains. **R**: resistant strain; **OFEO**: *Origanum floribundum* essential oil; **ECEO**: *Eucalyptus citriodora* essential oil of, **CSEO**: *Cymbopogon schoenanthus* essential oil; **OFEE**: *Origanum floribundum* ethanolic extract*;* **ECEE**: *Eucalyptus citriodora* ethanolic extract*;* **CSEE**: *Cymbopogon schoenanthus* ethanolic extract*;* Data accompanied by distinct lowercase letters for columns and varying uppercase letters for rows indicate significant differences among group values as determined by Tukey's multiple range test ($p < 0.05$).

E. citriodora EO showed weak inhibition against Gram-negative bacteria (IZ: 10.07-12.53 mm) and a more or less moderate activity against the rest of the microorganisms (IZ: 20-33.2 mm). According to several studies, the effectiveness of *E. citriodora EO* is mainly attributed to citronellal and citronellol (Low *et*

al., 1974; Mulyaningsih *et al*., 2011; Elaissi *et al*., 2011).

The inhibitory activity of the EO of *C. schoenanthus* was similar to that of *E. citriodora* except for that recorded against *A. brasiliensis* (IZ: 44.5 –26.3 mm respectively). Our results are in agreement with those reported by Aous *et al*. (2019) for samples with similar chemical composition from the same region.

Notably, the fungi were sensitive to the three studied oils. This is based on previous studies on these oils (Su *et al*., 2006; Ghaffar *et al*., 2015; Ksouri *et al*., 2017; Tolba *et al*., 2018; Sawadogo *et al*., 2022; Fertout-Mouri *et al*., 2023).

Gram-positive bacteria are generally more sensitive to EOs than Gram-negative bacteria, which is consistent with the findings of several previous studies (Cimanga *et al*., 2002; Aelenei *et al*., 2016; Semeniuc *et al*., 2017; Tkaczenko *et al*., 2023). This increased sensitivity can be attributed to the structural characteristics of the peptidoglycan layer, which facilitate the penetration of antimicrobial agents into the cell, resulting in protein denaturation and disruption of the cell membrane. In contrast, the lipopolysaccharide layer and periplasmic space in Gram-negative bacteria provide a protective barrier, conferring greater resistance to these organisms (Ewunkem *et al*., 2024).

The Gram-negative bacterium *P. aeruginosa* was the most resistant to the examined extracts, likely because of its extensive virulence factors and intrinsic resistance mechanisms against various antibacterial agents (Ribeiro *et al*., 2023). This resistance is attributable to its extremely restrictive outer membrane barrier, which makes it particularly resistant to synthetic medicines (Tolba *et al*., 2018).

The ethanolic extracts were not active against all fungi and were moderately weakly active toward the other bacteria. These results partially agreed with those reported by Kerbouche *et al*. (2015) for OF and Khalil *et al*. (2017) for CS. Other studies on other microorganisms have reported contradictory results, which could be due to the use of different extraction solvents and methods used (Fiori *et al*., 2000; Jabeen & Javaid, 2008; Javed *et al*., 2012).

A Principal Component Analysis (PCA) biplot (Figure 1._A) displays the relationships among the microbial strains and their responses to the tested EOs and EEs. The first two principal components accounted for 76.13% of the total variance and 20.37% of the variance owing to the variability in antimicrobial activity. Gram-positive bacteria and fungi clustered closely in the PCA space, indicating higher susceptibility to the tested EOs. In contrast, Gram-negative bacteria, such as *P. aeruginosa,* were positioned farther away, reflecting their higher resistance, consistent with previous findings. The projection of sample cases (Figure 1._B) indicated clear separation of EOs and EEs based on their inhibitory profiles. *O. floribundum* EO (OFEO) appeared to be, in comparison to the others, the most potent, well away from the origin of Factor 1, which was a clear indication of its superior antimicrobial efficacy. ECEO and CSEO, which are located close to each other on the factor plane, showed similar antimicrobial activity patterns against most microorganisms. Gram-negative bacteria exhibited limited inhibition zones in these two samples. In contrast, the EEs were close to the origin, suggesting low or no activity against most of the microbial strains tested. The agglomerative hierarchical clustering (AHC) dendrogram (Figure 1._C) grouped microbial strains based on the similarity in their sensitivity profiles into two major clusters: the first cluster was formed by fungal strains and Gram-positive bacteria, exhibiting high susceptibility to many EOs, while the second cluseter included Gram-negative bacteria, which were more resistant. Notably, *P. aeruginosa* showed the highest resistance and hierarchical clustering independently within this group, emphasizing the robust defence mechanisms described above.

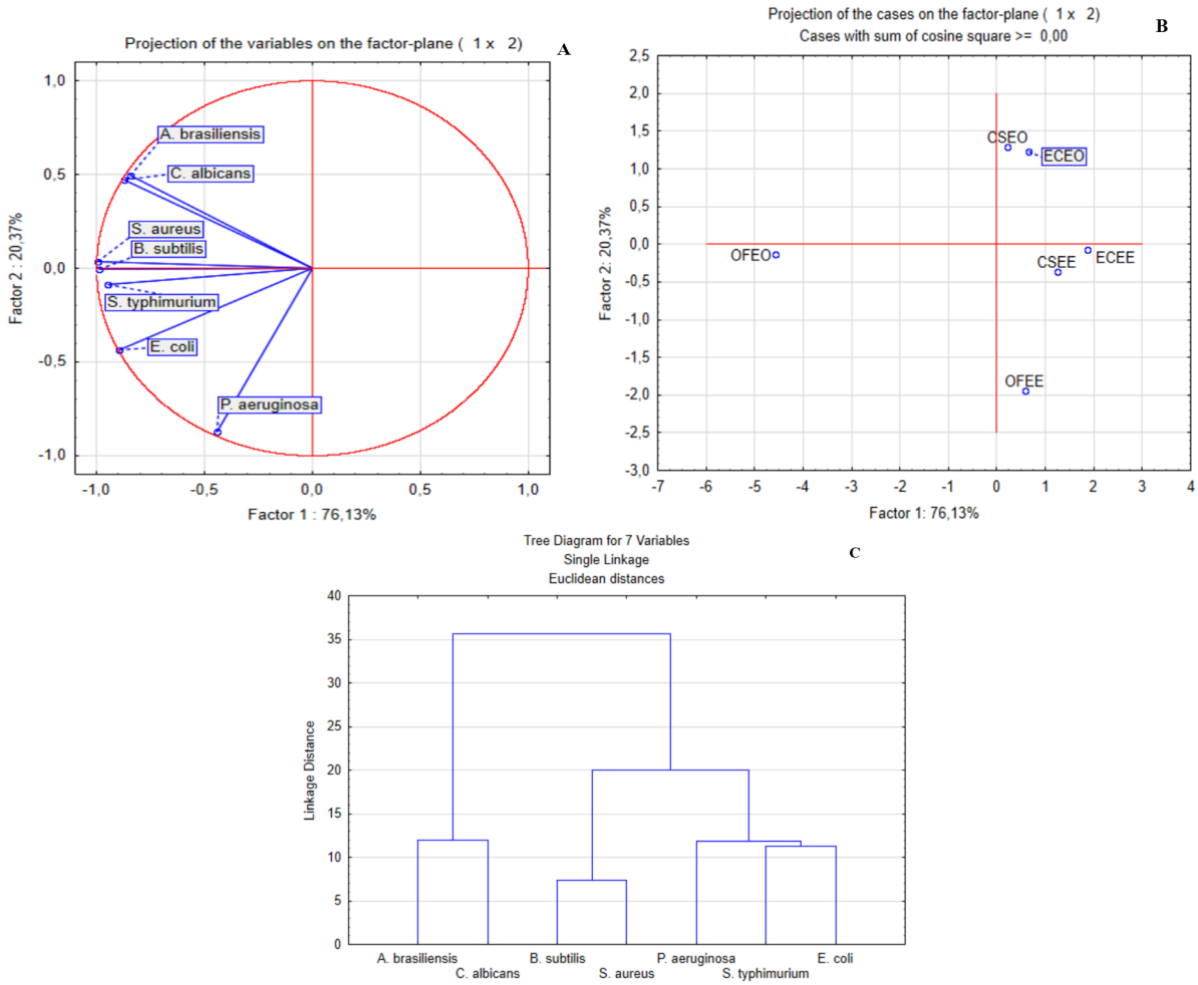


Figure 1. (A) Principal component analysis (PCA), **(B)** Projection of Samples in the PCA Space and **(C)** agglomerative hierarchical clustering (AHC) of diameter of IZ of EOs and EEs

3.3.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) Values of EOs and EEs

To further confirm antimicrobial activity, the MIC and MBC values were measured and are presented in Table 4. These values were consisent with the findings of the inhibition zone study. The MIC results revealed that the lowest concentrations of the tested EOs capable of inhibiting microbial growth were observed with fungal strains (MIC = 0.03–0.25%), with *O. floribundum* EO showing excellent inhibitory activity against *C. albicans* (MIC = 0.03%). This EO consistently exhibited superior activity against bacterial and fungal strains compared to the other EOs and EEs examined. In contrast, the MBC of OFEO for the yeast *A. brasileinsis* was significantly higher than its MIC (MBC=1%; MIC= 0.125 %), resulting in an MBC/MIC ratio > 4, which indicates a bacteriostatic effect of *O. floribundum* EO. Gram-negative bacteria exhibited the highest MICs and MBCs, confirming previous observations. Conversely, EEs showed limited antibacterial activity, with MIC values ≥ 4% against all tested microbial strains, indicating inferior efficacy compared to EOs. These findings underscore the enhanced bactericidal activity of EOs and highlight their antimicrobial potential for more effective medicinal and industrial applications (Chouhan *et al.*, 2017; Aziz *et al.*, 2018; Hou *et al.*, 2022).

Table 4. MIC and MBC (% v/v) values of essential oils and ethanol extracts from *O. floribundum*, *E. citriodora*, and *C. schoenanthus*

Strains	***E. coli***		***S. Typhimurium***		***P. aeruginosa***		***S. aureus***		***B. subtilis***		***C. albicans***		***A. brasiliensis***	
Samples	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
OFEO	0.5	0.5[a]	1	1[a]	1	1[a]	0.25	0.5[a]	0.25	0.25[a]	0.03	0.03[a]	0.125	1[b]
ECEO	2	2[a]	2	4[a]	2	2[a]	1	2[a]	1	>2 [ND]	0.25	0.25[a]	0.25	0.25[a]
CSEO	1	1[a]	1	1[a]	2	>2 [ND]	0.5	1[a]	0.5	0.5 [a]	0.06	0.125[a]	0.125	0.125[a]
OFEE	4	4[a]	8	8[a]	4	8[a]	4	4[a]	4	4[a]	R	R	R	R
ECEE	4	8[a]	8	8[a]	4	8[a]	4	4[a]	4	4[a]	R	R	R	R
CSEE	4	4[a]	>8	>8[ND]	8	>8[ND]	4	8[a]	4	4[a]	R	R	R	R

a : Bactericidal effect b : Bactériostatic effect ND : Not identified R: Resistant

3.3.3. Study of synergistic antimicrobial activity of EOs

The combination of EO chemicals can yield four potential effects: indifferent, additive, antagonistic, or synergistic (Bassolé *et al*., 2012). An additive impact occurs when the total effect equals the sum of the separate effects. Antagonism occurs when the combined effect of one or both substances is diminished compared with solo applications. Synergism occurs when the combined effect of substances exceeds the sum of their separate effects, and a lack of interaction is termed indifference (Burt, 2004).

The Fractional Inhibitor Concentration Index (FICI) values for the dual combinations of essential oils examined in this study are presented in Table 5. The most effective combinations, including *O. floribundum* / *E. citriodora* (OFEC), exhibited a synergistic effect against *B. subtilis* (FICI = 0.15) and *S. aureus* (FICI = 0.30), corresponding with their large inhibition zones (IZ = 49.05 mm and 39.23 mm, respectively). A synergistic effect was also observed for the combination of *E. citriodora* / *C. schoenanthus* (ECCS) against *B. subtilis* (FICI = 0.38) with an IZ of 53.2 mm. However, an additive effect was recorded for ECCS against *S. aureus* (FICI = 0.75), with an IZ of 24.17 mm. These results correlated with the low minimum inhibitory concentrations (MIC) of 0.25% and 0.06% observed for the *E. citriodora* / *C. schoenanthus* (ECCS) and *O. floribundum* / *E. citriodora* (OFEC) combinations, respectively. Gram-negative microbial strains showed indifferent interactions for all tested combinations, as indicated by the FICI values. Additionally, the *O. floribundum* / *C. schoenanthus* combination (OFCS) exhibited indifferent effects against both Gram-positive and Gram-negative bacterial strains. For the fungal species, most of the essential oil combinations tested yielded FICI values greater than four, suggesting antagonistic interactions. However, antagonistic effects of certain combinations underscore the importance of selecting appropriate essential oil pairs to avoid compromising their efficacy.

Gutierrez *et al*. (2009) and Bassolé *et al*. (2011) investigated combinations of *Origanum vulgare* essential oil with various other EOs and found that most combinations exhibited additive effects. Numerous studies have linked phenolic and alcoholic substances to both their additive and synergistic effects. Typically, drugs with analogous structures exibit additive effects rather than synergistic effects (Bassolé *et al*., 2012). The interaction between non-oxygenated and oxygenated monoterpene hydrocarbons has been associated with antagonistic effects (Hammer *et al*., 1999; Goñi *et al*., 2009). These findings highlight the complexity of EO interactions and emphasize the need for careful evaluation of EO combinations to optimize antimicrobial effectiveness. Combining antimicrobial agents aims to reduce drug resistance and microbial toxicity while achieving synergistic antibacterial effects (Azeredo & Soares, 2013;

Liu *et al*., 2019; Álvarez-Martínez *et al*., 2021). Given the difficulty in finding new antibiotics, the synergistic effect of several antimicrobial combinations is a possible strategy to address microbial drug resistance. One mechanism underlying these synergistic interactions is the membrane permeabilization induced by EOs, which disrupts the bacterial outer membrane and enhances the penetration of other antimicrobial agents (Rai *et al*., 2017).

Table 5. Diameter of IZ (mm), MIC, MBC (% v/v), and FICI classification of the synergistic combinations of EOs tested

	Strains	*E. coli*	*S. Typhimurium*	*P. aeruginosa*	*S. aureus*	*B. subtilis*	*C. albicans*	*A. brasiliensis*
OFEC	**IZ**	24.00 ± 0.70^{c,C}	17.6 ±0.40^{b,B}	9.60 ±0.61^{a,A}	39.23 ±0.90^{b,D}	49.05 ± 4.05^{a,E}	28.20 ±1.57^{b,C}	36.36 ±0.17^{a,D}
	MIC	1.00	2.00	1.00	0.06	0.03	0.25	0.50
	MBC	1.00	2.00	2.00	0.25	0.03	0.50	0.50
	FICI	2.50^{iii}	3.00^{iii}	1.50^{iii}	0.30^{i}	0.15^{i}	9.33^{iv}	6.00^{iv}
OFCS	**ZI**	19.15 ±0.05^{b,C}	15.90 ± 0.66^{a,B}	9.73 ± 0.68^{a,A}	46.07 ±1.99^{c,F}	50.43 ±0.42^{a,G}	22.27 ±0.35^{a,D}	43.23 ±0.60^{b,E}
	MIC	1.00	1.00	2.00	0.25	0.25	0.13	0.50
	MBC	1.00	1.00	>2	0.25	0.25	0.13	0.50
	FICI	3.00^{iii}	2.00^{iii}	3.00^{iii}	1.50^{iii}	1.50^{iii}	6.25^{iv}	8.00^{iv}
ECCS	**ZI**	12.2 ±0.00^{a,A}	18.87 ±0.21^{c,B}	11.30 ±1.50^{a,A}	24.17 ±0.45^{a,C}	53.2 ±1.60^{a,F}	35.45 ±1.77^{c,D}	47.57 ±0.90^{c,E}
	MIC	2.00	2.00	2.00	0.25	0.13	0.13	0.50
	MBC	2.00	2.00	2.00	0.25	0.13	0.25	0.50
	FICI	3.00^{iii}	3.00^{iii}	2.00^{iii}	0.75^{ii}	0.38^{i}	2.48^{iii}	6.00^{iv}

OFEC: the combination between essential oils of *Origanum floribundum* and *Eucalyptus citriodora;* **OFCS**: combination between essential oils of *Origanum floribundum* and *Cymbopogon schoenanthus;* **ECCS***:* combination between essential oils of *Eucalyptus citriodora* and *Cymbopogon schoenanthus;* **IZ:** inhibition zone**; MIC:** minimum inhibitory concentration; **MBC:** minimum bactericidal concentration; **FICI**: fractional inhibitor concentration index; **i**: Synergistic effect (FICI ≤ 0.5); **ii**: Additive effect (0.5 < FICI ≤ 1); **iii**: Indifferent effect (1 < FICI ≤ 4); **iv**: Antagonistic effect (FICI > 4); Data marked with specific lowercase letters for columns and different uppercase letters for rows signify substantial variations across group values as established by Tukey's multiple range test ($p < 0.05$).

The PCA biplot (Figure 2._A) demonstrated the correlations between microbial strains and their susceptibility to EO combinations as determined by FICI values. The initial two main factors (Factor 1: 77.13% and Factor 2: 22.87%) contained the entirety of the total variance, highlighting the variability in the combination effects. The initial principal component (Factor 1) mainly captured the collective response of bacterial strains, particularly *S. aureus, E. coli, P. aeruginosa, B. subtilis,* and *A. brasiliensis*. These microorganisms were positively associated and showed similar responses to the studied EO combinations. *C. albicans* was differentiated from this group and was closely associated with the second principal component (factor 2), indicating its unique response profile. *S. Typhimurium* demonstrated a negative relationship with the bacterial group along Factor 1, signifying an inverse trend in susceptibility, and it showed no significant correlation with *C. albicans,* as evidenced by its center position on the plot. The projection of the EO combinations (Figure 2._B) differentiated them based on their inhibitory abilities. OFEC was the most efficient combination for bacterial strains, situated far along the positive axis of Factor 1. The AHC dendrogram (Figure 2._C) validates the PCA findings by classifying the bacteria based on their susceptibility profiles. Two significant clusters were identified: *C. albicans* and *A. brasiliensis* formed a discrete cluster,

signifying their consistently high FICI values and unique reaction patterns to the EO combinations. The second cluster contained both Gram-positive and Gram-negative microorganisms. In this cluster, *S. Typhimurium* and *E. coli* were strongly linked, indicating analogous susceptibility patterns. Conversely, *B. subtilis* and *S. aureus* demonstrated reduced FICI values, signifying more synergistic interactions with the evaluated essential oil combinations.

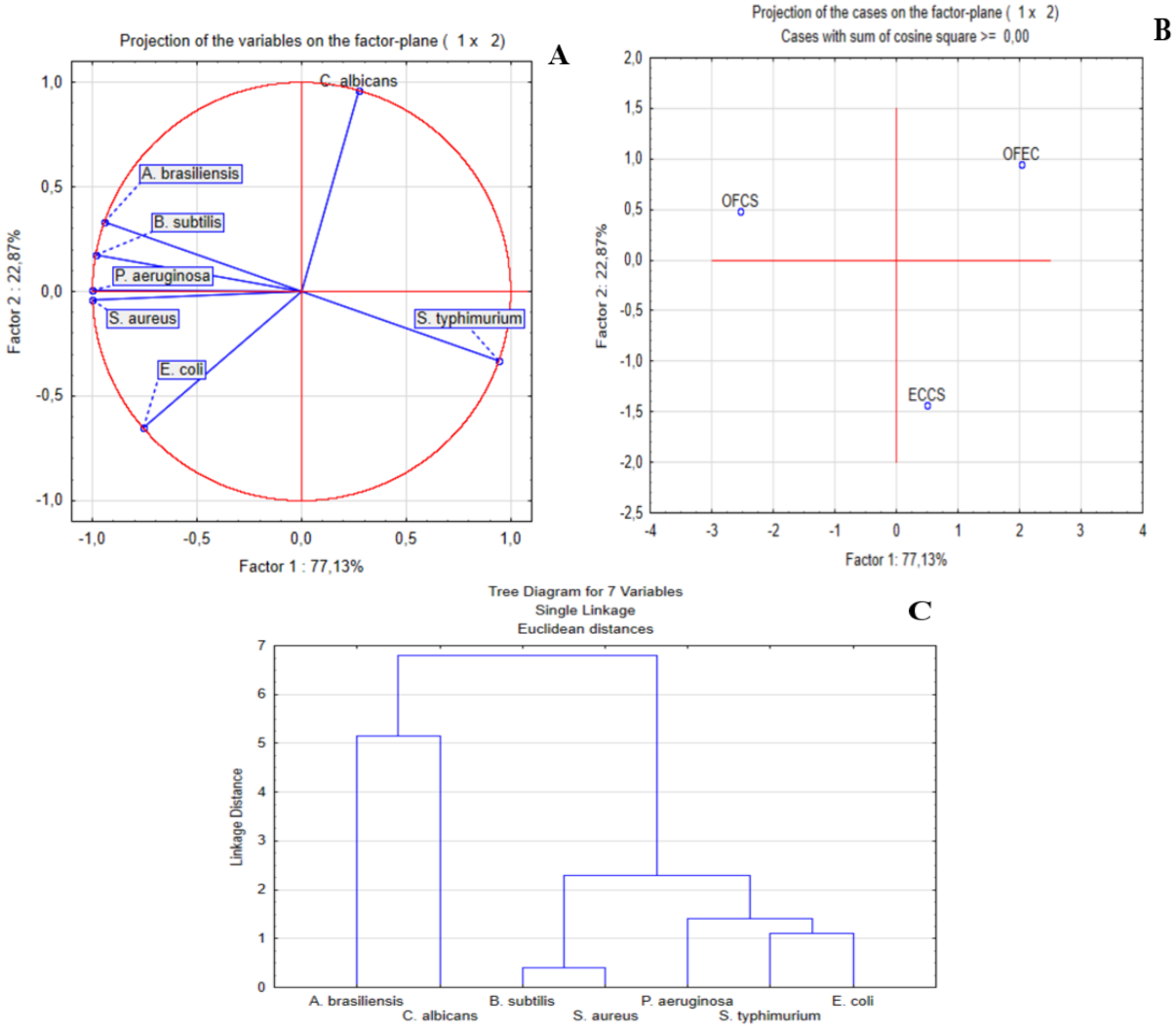


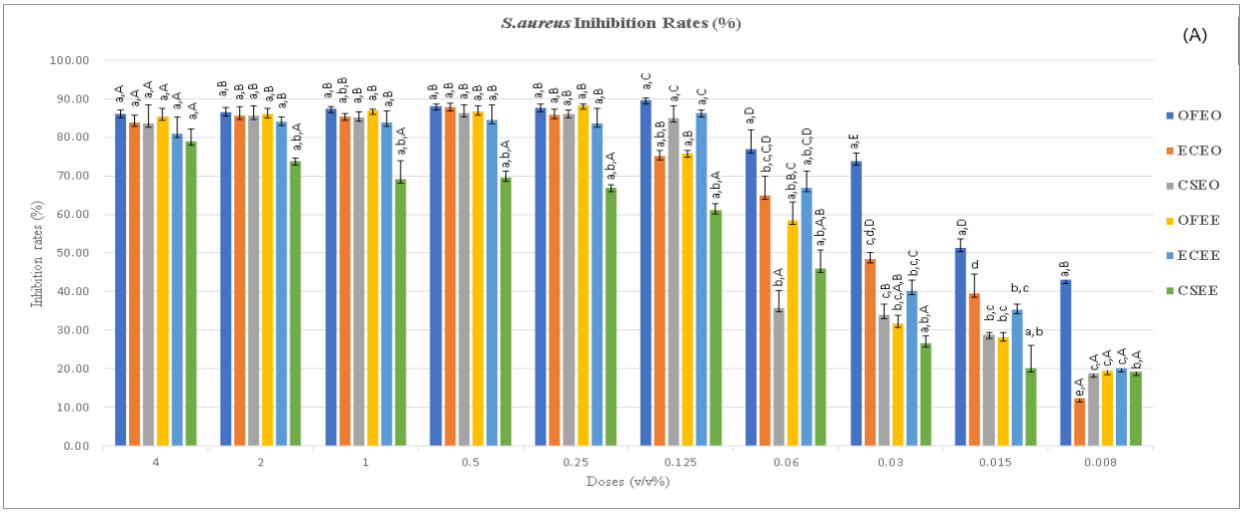
Figure 2 (A) Principal Component Analysis (PCA) biplot of microbial sensitivity based on FICI values, (B) Projection of EO combinations in PCA space, and (C) Agglomerative Hierarchical Clustering (AHC) dendrogram of microbial sensitivity to EO combinations.

3.4. Antibiofilm activity

Biofilms constitute the predominant microbial lifestyle and are present in various habitats, paricularly in healthcare, industrial settings, food processing, and drinking water distribution systems. The microbial composition of biofilms is essential for their pathogenicity, as this structure offers increased resistance to antimicrobial agents, resulting in significant challenges (Boumghar *et al*., 2019). The antibiofilm activities of EOs and EEs were tested against *S. aureus* and *P. aeruginosa*, both known for their strong biofilm-forming abilities, at different concentrations (4-0.008%). The data revealed variable inhibition rates depending on the concentration, extract

type, and the bacterial strain. For *S. aureus* (Figure 3._ A), EOs and EEs demonstrated significant inhibition rates at low concentrations exceeding 85% at a concentration of 0.25% for most of the studied samples. More notably, for EOOF, the inhibition rate surpassed 50% at 0.015% and reached 89.53 ± 0.76% at 0.125% concentration. These effects are attributed to phenols, which are well-known antibacterial compounds (Burt, 2004). Studies conducted by Nostro *et al*. (2007) and Oral *et al*. (2010) confirmed the effectiveness of oregano essential oil and reported that its major components, thymol and carvacrol, were responsible for inhibiting *S. aureus* biofilm formation. Boumghar *et al*. (2019) asserted that inhibiting the adhesion of planktonic microbial cells to a substrate is easier than removing an established biofilm. This is ascribed to various variables, including restricted medication penetration, inactivation of antimicrobial agents, existence of persistent strain subpopulations, and fluctuating physiological condition of microbes within the biofilm (Ceri *et al*., 1999; Donlan, 2000; Cerca *et al*., 2005).

For *P. aeruginosa* (Figure 3._ B), these essential oils applied at a 2% concentration demonstrated significant efficacy, with inhibition rates of 80.11 ± 1.95%, 82.70 ± 5.41%, and 92.07 ± 2.42% for *O. floribundum*, *E. citriodora*, and *C. schoenanthus* EOs, respectively. However, the efficacy decreased at lower concentrations, which is consistent with the findings of Lambert *et al*. (2001) and Sánchez *et al*. (2016), who highlighted the necessity of adequate concentrations for optimal biofilm inhibition. The EEs demonstrated significant inhibition only at the highest concentration studied (4%), with inhibition rates ranging from 49.80 ± 2.24% to 94.65 ± 0.90% for ECEE and OFEE. Lower concentrations generally resulted in reduced inhibition rates. Ayaz *et al*. (2021) reported that a methanolic extract of *Origanum haussknechtii* demonstrated significant antibiofilm activity. It inhibited biofilm formation *P. aeruginosa* at 80.53%, which was notably higher than the inhibition of other tested microorganisms such as *S. aureus* (77.33%) and *E. coli* (41.33%).



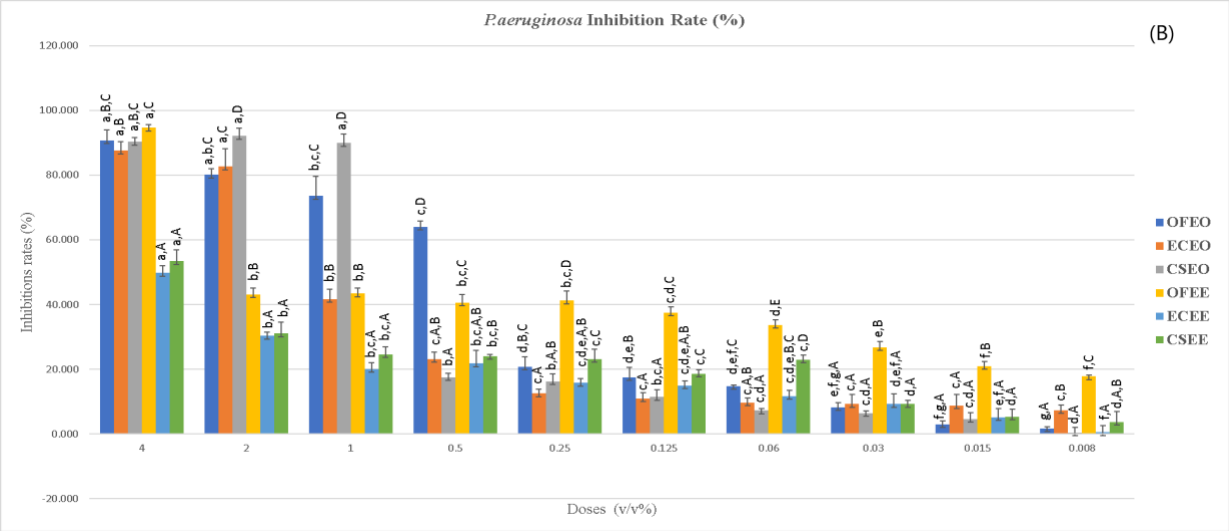


Figure 3. Antibiofilm activity of essential oils and ethanolic extracts against *Staphylococcus aureus* **(A)** and *Pseudomonas aeruginosa* **(B)** (mean inhibition rate ± SD %; n=3).
Different lowercase letters for the same samples and different capital letters for the same doses indicate significant variations between results, according to Tukey's multiple range test ($p < 0.05$).

Additionally, Marinkovic *et al.* (2021) demonstrated that the essential oils of *E. citriodora* markedly inhibited biofilm formation. Specifically, *S. salivarius* ATCC 9222 strains showed more pronounced biofilm inhibition, reaching 84_89% at an MIC/2 concentration. Compared to other essential oils, *E. citriodora* was the most effective antibiofilm agent among the tested oils. *C. schoenanthus* EO and its major compounds have also been shown to prevent biofilm formation, demonstrating antimicrobial activity and potential as antibiofilm agents (Piasecki *et al.*, 2021). The antibiofilm mechanisms of essential oils vary and are not fully understood (Rossi *et al.*, 2022). Recently, Vidaković Knežević *et al.* (2024), report that the principal constituents of essential oils, including cinnamaldehyde, eugenol, carvacrol, p-cymene, and thymol, are mainly responsible for their antibiofilm properties. However, the minor constituents of EOs exert a considerable synergistic role. Compared with cells shielded by biofilms, planktonic cells are generally more vulnerable to EOs. These findings validate the prospective use of these extracts in biofilm prevention and management, corroborating the assertions of Bassolé and Juliani (2012) regarding the efficacy of essential oils and plant extracts as natural antibacterial agents.

The PCA biplot results (Figure 4.A), paired with hierarchical clustering, demonstrated notable disparities in the susceptibility profiles of *S. aureus* and *P. aeruginosa* to EOs and EEs, respectively. The first principal component (Factor 1), accounting for 79.16% of the variation, represents the significant inhibitory effects of EOs and EEs on *S. aureus*. The clustering of combinations such as OFEO/SA, ECEO/SA, and CSEO/SA on the positive side of Factor 1 signified comparable and effective inhibitory patterns. Conversely, Factor 2 (13.70% of the variation) clarified the variability in the reaction of *P. aeruginosa*, as demonstrated by the scattered placements of combinations such as OFEO/PA, CSEO/PA, and ECEO/PA. The population projection in the PCA space (Figure 4._B) highlighted the concentration-dependent characteristics of antibiofilm activity. The positioning of data points on Factor 1 indicated the strength of the inhibitory effects, with points closer to the positive axis of Factor 1 signifying greater inhibition rates, especially for *S. aureus*. Conversely, points distributed along Factor 2 indicated variability in the

performance of specific EOs and EEs at diminished concentrations, which is frequently linked to reduced inhibition rates.

The dendrogram (Figure 4.C) illustrated the hierarchical clustering of 12 variable combinations based on their inhibitory effects against *S. aureus* and *P. aeruginosa*, using Euclidean distances and single linkage as the clustering method. Two distinct clusters were evident, reflecting differences in antibacterial activity between the tested EOs and EEs. The first cluster group combinations with strong and consistent activity, primarily targeted *S. aureus*, indicating a uniform antibacterial profile. In contrast, the second cluster included combinations with more variability and generally lower activity, predominantly associated with *P. aeruginosa*. This clustering highlighted the differential susceptibility of the bacterial strains to the tested extracts and confirmed the distinct patterns of inhibitory performance.

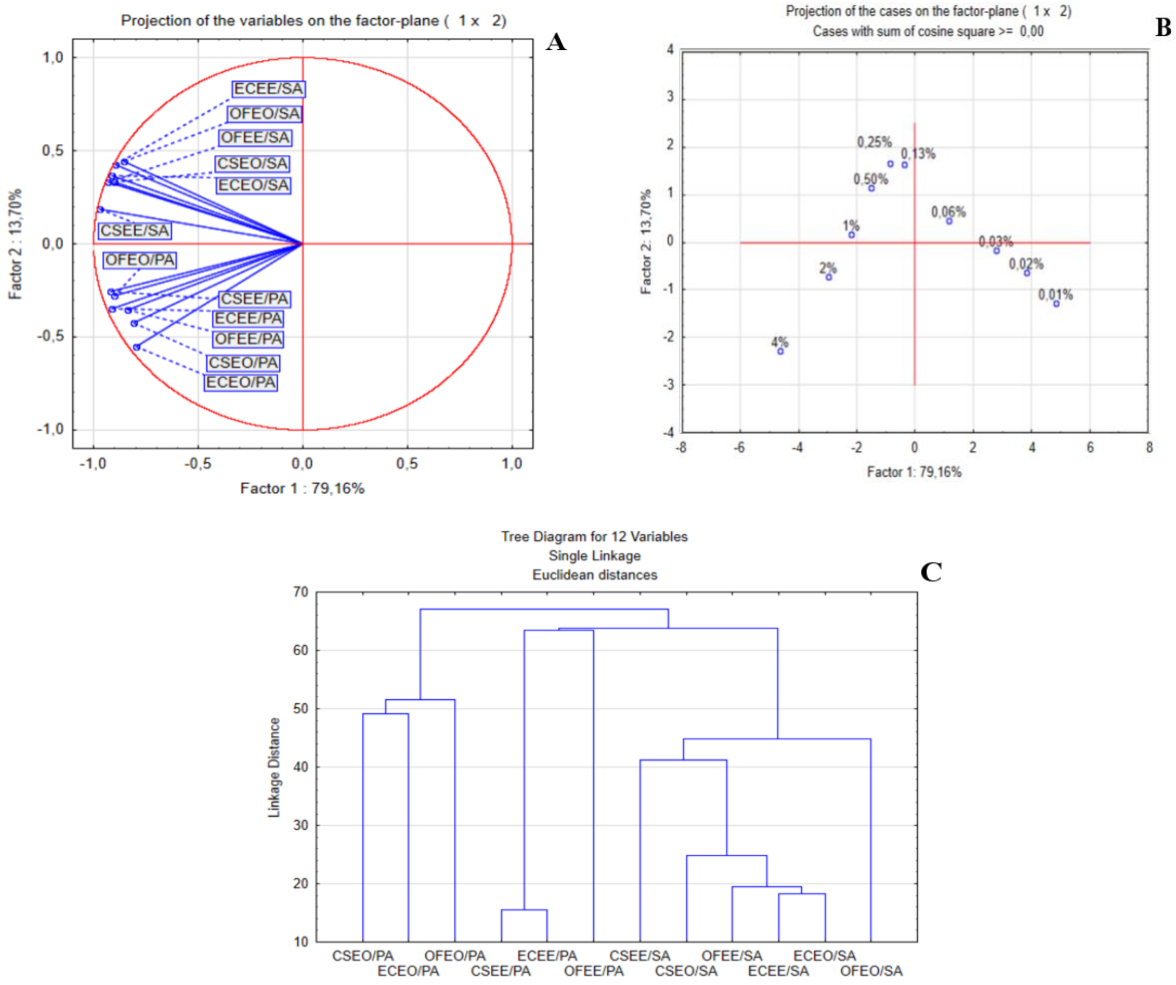


Figure 4 (A) Principal Component Analysis (PCA) biplot of antibiofilm activity of EOs and EEs against *S. aureus* (SA) and *P. aeruginosa* (PA), (B) projection of different doses in the PCA space, and (C) Agglomerative Hierarchical Clustering (AHC) dendrogram of microbial sensitivity to EOs and EEs.

4.Conclusions

This study provides a comprehensive evaluation of the chemical composition and antimicrobial, and antibiofilm activities of essential oils (EOs) and ethanolic extracts (EEs) from *O. floribundum*, *E. citriodora*, and *C. schoenanthus*, along with the synergistic effects of EO combinations on antimicrobial activity. Among the EOs, *O. floribundum* was distinguished by its potent antimicrobial

activity against all the tested microbial strains. In contrast, the other two oils displayed weaker activity against Gram-negative bacteria but demonstrated moderate to strong effects against other microorganisms. Ethanolic extracts of *O. floribundum* showed moderate antimicrobial activity against different bacterial strains, whereas *E. citriodora* and *C. schoenanthus* extracts were less effective. The combination of essential oils demonstrated distinct antimicrobial effects: the OFEC and ECCS combinations exhibited synergistic effects against Gram-positive bacteria, with an additive effect observed for ECCS against *S. aureus*, while indifferent effects were noted for Gram-negative strains. The OFCS combination showed indifferent effects across all bacterial strains tested. For fungal strains, most of the EO combinations displayed antagonistic effects. Regarding antibiofilm activity, the studied EOs and EEs demonstrated effective inhibition at low concentrations for *S. aureus*, with significant activity observed only at higher concentrations against *P. aeruginosa*. Further research is needed to isolate and identify the specific bioactive compounds responsible for these antibiofilm effects. Overall, *O. floribundum* essential oil demonstrated the strongest antimicrobial and antibiofilm efficacy among the tested extracts, particularly against foodborne pathogens and biofilm-forming strains. These findings support the potential of *O. floribundum* as a natural antimicrobial preservative, making it a promising candidate for application in food and pharmaceutical industries.

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