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Antifungal and antibacterial activities of Lippia callicarpifolia Kunth essential oils and influence of phenological stage on chemical composition

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Abstract

Background: *Lippia callicarpifolia* Kunth (Verbenaceae) is an aromatic perennial shrub popularly known as “flor de aire”, “mostranzo”, and “rosa de castilla” in the community of Tonatico, State of Mexico, Mexico. This study examined the influence of phenological stage on the chemical composition of essential oils (EOs) obtained from aerial parts collected at three different developmental periods and the impact on their antimicrobial activity against bacteria and yeasts of medical importance, including microorganisms isolated from clinical cases.

Methods: GC–MS, retention indices, and standard compounds were employed to identify 38 constituents. The antimicrobial activity of the EOs was evaluated following the Clinical and Laboratory Standard Institute M100 guidelines (2018).

Results: During the pre-flowering stage (September), the essential oil of fresh leaves/stems (LSEP) was composed mainly of β-caryophyllene. In contrast, β-phellandrene, β-caryophyllene, and α-pinene were the major compounds in the EOs of leaves/stems and flowers collected during full bloom in November (LNOV and FNOV, respectively) and at the end the flowering stage in February (LFEB and FFEB, respectively). All EOs studied exhibited antibacterial and anticandidal activities. Determination of minimal inhibitory concentrations (MICs) and bactericidal or fungicidal concentrations (MBCs or MFCs) revealed that the most significant antimicrobial activity was observed for the essential oils LNOV and FNOV. LNOV showed the highest level of antifungal activity, significantly inhibiting the growth of *Candida glabrata* (with an MIC value of 0.5 mg/mL). In addition, the essential oil of flowers FNOV exhibited a broader antibacterial spectrum, effectively inhibiting the development of all gram-negative and gram-positive bacteria studied, including multidrug-resistant strains isolated from clinical cases. The LSEP, LNOV, and FNOV EOs inhibited *C. glabrata* biofilm formation.

Conclusions: The EOs of *L. callicarpifolia* Kunth may be a viable alternative source of naturally occurring antimicrobial agents for effectively combating infectious diseases that offer an affordable approach to treatment. This study examines for the first time the chemical composition of *L. callicarpifolia* EOs during different growth stages, including vegetative and flowering stages, along with their antimicrobial activity.

Introduction

The antimicrobial resistance (AMR) phenomenon continues to evolve and disseminate beyond all limitations. Consequently, infectious diseases have become increasingly complex or even unmanageable, resulting in an upsurge in morbidity and mortality [1]. This is exemplified by the varying rates of resistance to ciprofloxacin, a frequently prescribed antibiotic for urinary tract infections, which range from 8.4–92.9% for *Escherichia coli* and from 4.1–79.4% for *Klebsiella pneumoniae* [2].

Fungal infections also significantly contribute to the overall burden of infectious disease-related fatalities worldwide. *Candida* species represent the prevalent aetiology of invasive mycotic diseases among patients with severe immunodeficiency [3–5]. Among the members of the *Candida* genus, *Candida albicans* has been identified as the predominant pathogenic microorganism in humans. It is closely followed by *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and the recent addition of *C. auris* [6–7]. The persistent escalation in the emergence of fungal pathogens resistant to antibiotics has reduced the effectiveness of many currently available antifungals [7].

Medicinal plants are extensively used for the treatment of various infectious diseases and are renowned for providing therapeutic agents with diverse chemical structures and biological efficacy [8]. Extensive research has been conducted on medicinal plant oils, and these studies have demonstrated that oils possess excellent antimicrobial properties [9–10]. The presence of various compounds in these oils makes them suitable for use as antimicrobial agents with a low risk of inducing resistance development [11].

The Verbenaceae family in Mexico comprises 26 genera and 286 species, and some species are used in traditional medicine as herbal medicinal resources [12]. The genus *Lippia* is a collection of plants distributed in tropical America, mainly Central and South America, with some species found in African territories [13–14], and Brazil as the epicentre of the diversity of the genus with 98 species [15]. These plants are renowned for their elevated levels of essential oils, which possess significant pharmacological potential [16]. Some species of *Lippia* are used in Mexican traditional medicine [14], and many of these remedies have been traditionally utilized for gastrointestinal and respiratory health purposes [12].

Ethnobotanical investigations have revealed that members of this genus possess medicinal properties and are traditionally employed as treatments for maladies affecting the gastrointestinal and respiratory systems [17]. In Central America, *L. graveolens*, also known as “mountain oregano” and “marjoram” is utilized for the treatment of gastrointestinal, respiratory, and digestive diseases and as an antiseptic and anti-inflammatory [18]. Moreover, *L. alba* is used as a sedative in folk medicine in Northeast Brazil [19–21]. In Colombia, traditional medicine relies on *L. origanoides* to cure respiratory and gastrointestinal disorders [22]. Furthermore, some species exhibit

antimalarial, antihypertensive, and antiviral activities [23–24]. In most instances, the parts of the plant employed are the aerial parts including the leaves and flowers. The most common preparation method is an infusion or decoction that is administered orally [25–26].

Lippia callicarpifolia Kunth (Verbenaceae) is a medicinal plant popularly known as “flor de aire”, mostranzo, and “rosa de castilla” in the town of Tonatico, State of Mexico, Mexico [12]. This plant is native to Mexico and is mainly distributed in the central states of the country. In locations within the State of Mexico, the leaves, stems, and flowers are infused to alleviate stomach aches and for bathing by women after labour. The Digital Library of Traditional Mexican Medicine also notes the use of this medicinal species to treat diarrhoea in the state of Morelos [27]. Reports from the town of Tonatico suggest that *L. callicarpifolia* is used as a medicinal plant, with the flowers infused for the treatment of stomach infection, stomach gas, and postpartum baths, according to López-Villafranco et al. [12]

L. callicarpifolia is a species of local medicinal importance. The antimicrobial activity of its EOs against pathogens related to bacterial and fungal infections has not been investigated. Therefore, the objective of the present study was to examine the impact of phenological stage on the chemical composition of *L. callicarpifolia* essential oils and evaluate their antimicrobial activity against bacteria and fungi, including multidrug-resistant microorganisms, since these pathogens have become more significant due to the increasing levels of AMR worldwide. We aimed to develop research that contributes to validating the use of *L. callicarpifolia* as a Mexican medicinal plant and to correlating its chemical composition with the reported activities.

Materials and methods

Plant material

L. callicarpifolia Kunth aerial parts were collected in San José de los Amates, Tonatico, State of Mexico, Mexico at 18°47'4" NL and 99°41'12" WL, at 1676 masl. The materials were collected during three different developmental stages: leaves/stems in the pre-flowering period (September 2022); leaves/stems and flowers in full bloom (November 2022); and leaves/stems and flowers at the end of the flowering stage (February 2023). Taxonomic identification of the plant was carried out by Ma. Edith López-Villafranco of Herbarium IZTA, Facultad de Estudios Superiores Iztacala of Universidad Nacional Autónoma de México, where a voucher specimen was deposited under number 3505.

Extraction of essential oils (EOs)

The aerial parts of *L. callicarpifolia* were classified into five groups based on the specific plant parts and the developmental stage at the time of collection. These categories were LSEP: leaves/stems collected in September 2022 during the pre-flowering period; LNOV and FNOV: leaves/stems and flowers collected in November during full bloom, respectively; and LFEB and FFEB: leaves/stems and flowers collected in February 2023 at the end of the flowering stage, respectively.

Batches of 1.078 Kg of fresh leaves/stems (LSEP), 276.8 g of flowers (FNOV), 658 g of leaves/stems (LNOV), 231 g of flowers (FFEB), and 455 g of leaves/stems (LFEB) were individually extracted by the steam distillation method using a Clevenger apparatus for 2.5 h. The oil layers were separated, and water traces were eliminated by freezing the sample. The essential oils were transferred to a hermetically sealed glass container. The volatile compounds were stored in a freezer until further analysis. Yield determination was achieved by calculating the ratio between the mass of the extracted essential oil and the mass of the leaves/steams or flowers used (w/w) multiplied by a factor of 100.

Analysis of the chemical composition of essential oils by gas chromatography–mass spectrometry (GC–MS)

The GC–MS analysis was performed under the following conditions: 100 µL of HPLC grade hexane was added to each sample, and 1 µL of this solution was injected into the Agilent Technologies 7890B gas chromatograph using an HP-5MS capillary column of 30 m x 0.25 mm x 0.25 µm, with helium as the carrier gas at a flow rate of 1 mL/min with a split ratio of 80:1. The injector temperature was 280°C, and a program was used with an initial temperature of 40°C/min and a final temperature of 310°C for 2.25 min with a ramp of 8°C/min. Each component of the mixture was analysed by mass spectrometry using electron impact ionization at 70 eV on an Agilent Technologies 5977D system over a mass range of 300 to 600 (m/z).

Each component of the oil was identified by computerized comparison with the mass spectral database of the National Standards Institute and Technology (NIST 14) [28], incorporated into the instrument, along with their retention indices (RIs) reported by Adams [29] and Babushok et al. [30]. The RI was determined based on linear interpolation of the retention times of standard n-alkanes (C8 – C20) procured from Sigma–Aldrich, using Pherobase: Kovats Calculator & Match Searches available at the Pherobase 2023 [31]. The

concentrations of compounds, expressed as a percentage of the content, were established by integrating the chromatographic peak areas that corresponded to each compound. Standards such as farnesol and α-pinene were used to ensure the proper functioning of the CG-MS equipment.

Antifungal and antibacterial activities of essential oils

The antifungal activity of the essential oils of *L. callicarpifolia* was assessed on three *Candida* strains acquired from clinical cases: *C. glabrata* and *C. tropicalis* (donated by Hospital Angeles Metropolitano, Mexico) and *C. albicans* 17MR (provided by the Clinical Analysis Laboratory of FES Iztacala, UNAM). Candida stock cultures were maintained at room temperature on potato dextrose agar (PDA, Bioxon).

Antibacterial activity assays were conducted using gram-positive bacteria, such as *Enterococcus faecalis* ATCC 14506, *Staphylococcus aureus* ATCC 29213, and *S. aureus* 23MR (donated by the Clinical Analysis Laboratory of FES-Iztacala), acquired from clinical cases and gram-negative bacteria, including *K. pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922, and *E. coli* 82MR (provided by the Clinical Analysis Laboratory of FES-Iztacala). The bacterial stock strains were preserved at room temperature on Müeller Hinton agar (Bioxon).

The antimicrobial activity of the EOs was evaluated following the Clinical and Laboratory Standard Institute M100 guidelines (2018). To perform the disk diffusion method 4 µL (2.4, 2.6, 2.9, and 3.4 mg/per disk) of the essential oils LSEP, LNOV, FNOV, and FFEB, respectively, were used. The antimicrobial activity of the LFEB essential oil could not be evaluated due to the low yield of oil. Chloramphenicol (25 µg/mL) and nystatin (20.6 µg/mL) were used as positive controls for the experiment [32]. Microdilution tests were conducted to determine the minimal inhibitory concentration (MIC), minimum fungicidal concentration (MFC), and minimum bactericidal concentration (MBC) using serial dilutions ranging from 4 to 0.031 mg/mL. The test was performed in triplicate [33].

The impact of LSEP, LNOV, and FNOV oils on the growth kinetics of *C. glabrata* was investigated using the method described by López et al. [34]. The concentrations used for this purpose were ½ MIC, MIC, and MBC or MFC. The control group was not exposed to any chemical compounds. The colony-forming unit (CFU) was employed to determine the number of survivors, and the resultant data were expressed as log10 CFU/mL.

The antibiofilm activity of *L. callicarpifolia* essential oils was assessed using the crystal violet technique (35-Gómez-Sequeda et al., 2020). The ½ MIC, MIC, and MFC values were implemented in the experiment. The experiment was conducted in triplicate. Quantification of the percentage of biofilm inhibition was performed using a standardized formula.

$$\%Antibiofilm = \frac{ODcontrol - ODexperimental}{ODcontrol} x100$$

OD control: optical density without essential oil;

OD experimental: optical density with essential oil.

Statistical analysis

Statistical analysis of the results was performed using Microsoft Excel software. All experimental procedures were performed three independent times, and the results obtained are presented as the mean ± standard error. The antibiofilm activity results were analysed through one-way analysis of variance (ANOVA). Differences between means were considered statistically significant when $p > 0.05$.

Results

Chemical composition of the essential oils

The essential oils of leaves/stems and flowers collected from *L. callicarpifolia* at three different phenological stages, LSEP (0.23%), LNOV (0.71%), FNOV (1.4%), LFEB (0.48%), and FFEB (0.36%), were extracted by the hydrodistillation method, and a total of 38 compounds were identified (Table 1). The study of the chemical composition of the leaves/stems essential oils showed the LSEP essential oil (pre-flowering stage) was found to contain 17 compounds that accounted for 83.4% of the entire oil composition; the LNOV essential oil (full bloom) yielded 25 compounds, accounting for 88.4% of the total oil; and the LFEB essential oil (end of flowering period) contained identified 32 compounds, comprising 94.8% of the total oil. In turn, analysis of the chemical composition of the flower essential oils showed that in FNOV (full bloom), 29 compounds were identified, constituting 94.5% of the total oil. In contrast, in the FFEB essential oil (end of flowering stage) 33 compounds that accounted for 93.1% of the total oil were identified.

Table 1
Essential oils composition of *L. callicarpifolia* Kunth at three different stages characterized by GC-MS.

Compound	RI_{exp}	RI lit.	SEP	NOV		FEB	
			%Area	%Area	%Area	%Area	%Area
			LSEP	LNOV	FNOV	LFEB	FFEB
α-Thujene	928	930[abc]	-	-	1.2	0.5	1.3
α-Pinene	935	936[abc]	5.1	6.0	11.8	4.4	10.4
Sabinene	975	975[abc]	-	-	10.4	5.0	8.8
β-Thujene	975	978[a]	2.5	4.8	-	-	-
β-Pinene	979	979 [abc]	-	-	6.1	1.6	5.5
β-Myrcene	992	992 [abc]	1.6	1.7	1.9	1.7	2.0
Octanal	1003	1004 [abc]	-	0.8	0.9	1.2	1.1
α-Phellandrene	1006	1006 [abc]	-	0.9	0.7	1.3	1.3
4-Carene	1019	1022[a]	-	-	0.5	0.7	1.1
o-Cymene	1027	1027[acd]	7.6	9.1	7	3.1	1.7
β-Phellandrene	1033	1033[abc]	14.7	15.2	11.7	12.8	10.5
β-Ocimene, (*E*)-	1051	1051[abcd]	-	0.6	0.2	1.2	0.3
γ-Terpinene	1063	1062 [abc]	2.7	4.6	6.8	8.5	9.5
Linalool	1103	1101[abc]	-	1.4	1.9	2.5	1.4
trans-Pinocarveol	1146	1141[abc]	-	-	4.8	-	3.7
cis-Verbenol	1146	1142 [abc]	-	-	-	2.9	-
trans-Verbenol	1150	1150 [ac]	-	1.4	3.9	3.4	2.6
Terpinen-4-ol	1183	1182 [ac]	-	-	0.6	0.2	0.3
Decanal	1207	1204[abc]	-	0.3	0.4	0.8	0.6
δ-Elemene	1346	1342[abc]	-	-	-	0.7	0.6
β-Bourbonene	1395	1388 [abc]	-	0.5	0.2	0.3	0.3
β-Elemene	1399	1394 [abc]	0.9	0.7	-	0.7	0.6
β-Caryophyllene	1433	1428[abcd]	24.4	13.8	6.2	10.1	8.5
Elixene	1442	1445[ad]	0.6	-	-	0.4	-
cis, *cis*, *cis*-1,1,4,8-Tetramethyl-4,7,10-cycloundecatriene	1468	1472[ad]	2.7	1.7	0.9	1.5	1.3
Germacrene D	1495	1496[acd]	7.5	4.8	2.9	5.2	4.6
α-Amorphene	1512	1519[ac]	-	-	-	-	0.8
α-Muurolene	1512	1505[abc]	-	-	0.5	-	-
γ-Cadinene	1527	1530 [abc]	-	-	0.6	-	0.8

Cubebol	1529	1531[ac]	-	0.8	-	0.7	-
δ-Cadinene	1535	1526[abc]	0.7	0.6	1.9	0.8	2.0
Elemol	1560	1550[abc]	-	-	-	0.5	-
β-Germacrene	1573	1569 [ac]	1.4	1.0	0.5	1.2	1.0
Germacrene D-4-ol	1589	1579[abc]	3.1	5.1	3.5	9.0	4.8
Caryophyllene oxide	1598	1595 [abc]	3.4	7.5	1.4	3.8	1.5
Viridiflorol	1607	1608[abc]	-	0.4	-	0.5	0.5
α-Cadinol,epi	1654	1648[abc]	3.5	3.7	2.8	4.6	3.1
α-Cadinol	1669	1667[ac]	1	1.0	2.3	1.3	2.3
Monoterpene			34.2	46.8	70.8	51.8	62.1
Monoterpene hydrocarbons			34.2	42.9	58.3	40.8	52.4
Oxygenated monoterpenoids			0	3.9	12.5	11.0	56.0
Sesquiterpenes			49.2	41.6	23.7	41.33	32.7
Sesquiterpene hydrocarbons			38.2	23.1	13.7	20.9	20.5
Oxygenated sesquiterpenes			11	18.5	10.0	20.43	12.2
Total, identified (%)			83.4	88.4	94.5	93.1	94.8

The list of constituent is arranged in the elution sequence from an HP-5MS capillary column. The experimental retention index (RI exp) was determined by co-injecting a homologous series of n-alkanes C8–C20 and evaluating the corresponding linear retention index. The identification method used was mass spectrometry, which compares the mass spectrum of the unknown compound with the mass spectra stored in the digital mass libraries [a]NIST 14 and [d]NIST 2. The reported retention index (RI lit) is also considered [b]Babushok et al., 2011 and [c]Adams, 2007.

The analysis further established that monoterpenes were the primary elements during the flowering period, accounting for 46.8% in LNOV and 70.8% in FNOV, and at the end of the flowering stage, accounting for 51.8% in LFEB and 62.1% in FFEB. In contrast, sesquiterpenes constituted the primary elements in the pre-flowering period (LSEP), comprising 49.2% of the total oil. The dominant group was the monoterpenes hydrocarbons, which accounted for 42.9%-LNOV and 58.3%-FNOV, 40.8%-LFEB and 52.4%-FFEB, compared with sesquiterpene hydrocarbons, which accounted for 38.2%-LSEP. In contrast, oxygenated derivatives (monoterpenes and sesquiterpenes) were enriched in the essential oils during full bloom as well as at the end of the flowering stage.

Among the main constituents, specimens of leaves and stems collected during the pre-flowering period (LSEP) were predominantly characterized by the presence of sesquiterpenes, with β-caryophyllene comprising 24.4% of the total, followed by the monoterpenes β-phellandrene (14.7%), *o*-cymene (7.6%), germacrene D (7.5%), and α-pinene (5.1%). Conversely, samples collected during full bloom (LNOV and LFEB) exhibited significantly decreased β-caryophyllene values, dropping from 24.4–13.8% and 10.1%, respectively. β-Phellandrene levels remained relatively consistent, whereas *o*-cymene (9.1%), caryophyllene oxide (7.5%), and α-pinene (6.0%) levels experienced increases in proportions in LNOV. In addition, germacrene D-4-ol (9.0%) and γ-terpinene (8.5%) were observed to be the predominant constituents of LFEB. In contrast to the chemical composition of the EOs from leaves/stems, the essential oils extracted from the flowers, FNOV and FFEB, exhibited a higher proportion of α-pinene (11.8% and 10.4%), β-phellandrene (11.7%and 10.5%), sabinene (10.4% and 8.8%), and γ-terpinene (6.8% and 9.5%), respectively. It is noteworthy that *o*-cymene is present as the fifth most prevalent compound with 7.0% in FNOV, whereas in FFEB, the fifth position is occupied by β-caryophyllene with 8.5%.

Regarding the presence of minor constituents, the essential oils from leaves/stems and flowers collected during flowering periods were found to contain α-thujene, sabinene, 4-carene, octanal, α-phellandrene, β-ocimene, (*E*)-, *trans*-verbenol, terpinen-4-ol, β-bourbonene, and viridiflorol, which were not observed in the pre-flowering stage. Additionally, β-thujene was exclusively detected in the leaf/stem oils LSEP and LNOV. Furthermore, *trans*-pinocarveol was observed only in the flower oils FNOV and FFEB. Conversely, cubebol was only present in

the leaf/stem oils LNOV and LFEB during the flowering periods. Similarly, α-muurolene was identified only in the flower oil FNOV, and *cis*-verbenol was present only in the leaf/stem oil LFEB and was not found in the other samples, as shown in Table 1.

Antimicrobial activity of LSEP, LNOV, FNOV and FEB essential oils of L. callicarpifolia.

The pharmacological results indicated that all EOs evaluated from *L. callicarpifolia* exhibited antifungal activity (Table 2). The EO of LSEP displayed inhibitory effects on the growth of *C. albicans* MR, *C. glabrata*, and *C. tropicalis* with inhibition zones of 14.00 ± 1.7, 19.00 ± 0.6, and 22.00 ± 0.6 mm respectively. In contrast, both LNOV and FNOV inhibited the proliferation of C. *albicans* MR, *C. glabrata*, and *C. tropicalis* with inhibition zones of 15.00 ± 1.7, 24.00 ± 1.7 and 13.00 ± 1.7 and 15.00 ± 0.0, 23.00 ± 0.6, and 24.00 ± 0.0 respectively. Moreover, FFEB inhibited the growth of C. *albicans* MR (12.00 ± 0.0), *C. glabrata* (14.00 ± 0.6) and *C. tropicalis* (12.00 ± 0.0). All yeast was donated from clinical cases. Notably, *C. glabrata* displayed the highest sensitivity in the assessment, with the LNOV essential oil showing the highest percentages of inhibition and a minimum inhibitory concentration (MIC) of 0.5 mg/mL to LSEP and FNOV, which had MICs of 1, and FFEB, which had an MIC of ⩾4 mg/mL.

Table 2
Antifungal activity of the essential oils of *L. callicarpifolia*.

Yeast strain	**LSEP**			**LNOV**			**FNOV**			**FFEB**			**Positive control: Nystatin**	
	I. Zone	MIC	MFC	I. Zone	MIC	MFC	I. Zone	MIC	MFC	I. Zone	MIC	MFC	I. zone	MIC
C. albicans 17MR	14.00 ± 1.7	⩾4	⩾4	15.00 ± 1.7	2	⩾4	15.00 ± 0.00	2	4	12.00 ± 0.00	2	4	22.00 ± 0.00	11.00
C. glabrata	19.00 ± 0.6	1	2	24.00 ± 1.7	0.5	1	23.00 ± 0.6	1	2	14.00 ± 0.6	⩾4	⩾4	27.66 ± 2.51	8.00
C. tropicalis	22.00 ± 0.6	⩾4	⩾4	13.00 ± 1.7	⩾4	⩾4	24.00 ± 0.00	2	4	12.00 ± 0.00	4	⩾4	20.33 ± 1.52	9.00

Measurements of the inhibition zone (I. zone) are reported in mm. The results include the mean and standard deviation of the three repetitions. The concentrations per disk were: control (+) nystatin (20.6 µg) and 4 µL (2.4, 2.6, 2.9 and 3.4 mg/per disk) of the essential oils LSEP, LNOV, FNOV, and FFEB, respectively. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) are expressed in mg/mL and the nystatin µg/mL. (-) Does not showed activity.

Furthermore, the antibacterial properties of essential oils were assessed against both gram-positive and gram-negative bacteria, including multidrug-resistant strains isolated from clinical cases. The data reported in Table 3 show that the LSEP oil displayed action against three gram-positive and one gram-negative strains. In contrast, LNOV inhibited the growth of all gram-positive and two gram-negative strains. Most notably, the essential oil of FNOV showed activity against all bacteria examined. Moreover, FFEB exhibited an antibacterial effect against all gram-positive bacteria tested, but among the gram-negative strains, only *K. pneumoniae* ATCC 13883 (17.00 ± 0.6 inhibition zone) and *P. aeruginosa* ATCC 27853 (6.00 ± 0.6 inhibition zone) manifested sensitivity to this oil. The most significant sensitivity was observed in *E. faecalis* ATCC 14506 when exposed to LSEP (10.00 ± 1.0 inhibition zone) and LNOV (12.00 ± 0.0 inhibition zone) with a MIC of 2 mg/mL.

Table 3
Antibacterial activity of the essential oils of *L. callicarpifolia*.

Bacteria strains	LSEP			LNOV			FNOV			FFEB			Positive control: Chloramphenicol	
	I. Zone	MIC	CBM	I. Zone	MIC	CBM	I. Zone	MIC	CBM	I. Zone	MIC	CBM	I. Zone	MIC
E. feacalis ATCC 14506*	10.00 ± 1.00	2	4	12.00 ± 0.00	2	4	11.00 ± 0.6	⩾4	⩾4	17.00 ± 0.00	⩾4	⩾4	21.7 ± 1.5	3.00
E. coli-ATCC 25922	-	-	-	6.00 ± 0.6	⩾4	⩾4	11.00 ± 0.00	⩾4	⩾4	-	-	-	26.7 ± 1.5	-
E. coli 82 MR	-	-	-	-	-	-	10.00 ± 0.6	⩾4	⩾4	-	-	-	22.7 ± 2.08	4.00
K. pneumoniae ATCC 13883	10.00 ± 0.00	⩾4	⩾4	11.00 ± 1.2	⩾4	⩾4	12.00 ± 0.00	⩾4	⩾4	17.00 ± 0.6	4	⩾4	23.7 ± 1.5	1.00
P. aeruginosa ATCC 27853	-	-	-	-	-	-	10.00 ± 0.6	⩾4	⩾4	6.00 ± 0.6	⩾4	⩾4	26.7 ± 2.08	-
S. aureus ATCC 29213	14.00 ± 0.00	⩾4	⩾4	10.00 ± 0.6	⩾4	⩾4	14.00 ± 1.00	⩾4	⩾4	24.00 ± 0.00	⩾4	⩾4	25.00 ± 0.6	8.00
S. aureus 23MR	6.00 ± 0.6	⩾4	⩾4	12.00 ± 0.00	⩾4	⩾4	10.66 ± 0.6	⩾4	⩾4	16.00 ± 0.00	⩾4	⩾4	24.7 ± 2.5	7.00

Measurements of the inhibition zone (I. zone) are reported in mm. The results include the mean and standard deviation of the three repetitions. The concentrations per disk were: control (+) chloramphenicol at 25 µg, and 4 µL (2.4, 2.6, 2.9 and 3.4 mg/per disk) of the essential oils LSEP, LNOV, FNOV, and FFEB, respectively. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) are expressed in mg/mL and chloramphenicol µg/mL. (-) Does not showed activity.

Fungicidal effect on *C. glabrata* was observed when it was exposed to LSEP and LNOV, and this fungal population was eliminated within 2 h. However, it is noteworthy that a lower population density was maintained at the ½ MIC and MIC of the LSEP treatment compared with the LNOV treatment, which showed a growth rate similar to that of the control group but a lower population density (Figs. 1 and 2).

In the case of *C. glabrata*, after being subjected to identical treatments, it was observed that FNOV showed fungicidal behaviour against this microorganism and eliminated the yeast population within 1 h of treatment. Treatment with ½ MIC displayed an inhibitory effect for 2 h following the experiment, after which population growth increased (Fig. 3).

The essential oils LSEP, LNOV, and FNOV demonstrated inhibitory effects on biofilm development in *C. glabrata*. As evidenced by Table 4, the yeast in the untreated control exhibited a substantially higher degree of biofilm growth than that in the essential oil treatments, which yielded respective reductions of 20.1% (LSEP), 18.3% (LNOV), and 24.4% (FNOV).

Table 4
Antibiofilm activity of essential oils of *L. callicarpifolia* on *C. glabrata.*

Essential oil	Concentration (mg/mL)	% Antibiofilm activity
LSEP	½ MIC 0.50	7.8 ± 0.57
	MIC 1.00	16.0 ± 0.57
	CFM 2.00	20.1 ± 0.57
LNOV	½ MIC 0.25	5.5 ± 3.46
	MIC 0.50	17.1 ± 1.52
	CFM 1.00	18.3 ± 2.00
FNOV	½ MIC 0.50	5.9 ± 2.30
	MIC 1.00	16.1 ± 1.00
	CFM 2.00	24.4 ± 1.00

The mean value of three repetitions, accompanied by the standard deviation, is presented. Medium minimum inhibitory concentration (½ MIC), minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC).

Discussion

The genus *Lippia* has gained significant recognition for its aromatic character, with over 86 studies of essential oils described in a review by Pascual and co-workers 2001 [25] and Camilo and co-workers, 2022 [36]. Moreover, the *Lippia* species indigenous to Brazil have demonstrated a vast range of oil compositions, with various chemotypes being described [37]. In the current study, *L. callicarpifolia* was collected within an identical region, although during distinct phenological stages. This comparative analysis using GC–MS revealed that the chemical compositions of EOs derived from *L. callicarpifolia* share qualitative similarities among their main constituents. However, variations in the concentration of such components were detected; moreover, the essential oils exhibited differences in minor compounds across the investigated stages. Several studies have reported significant variations in the chemical composition of essential oils due to the influence of either phenological or seasonal stages [37–42].

According to the literature, geographical, environmental, and ecological factors can impact the chemical configuration and proportional concentration of each element in essential oils [43–44]. Additionally, the production of each element is intricately linked with the physiological processes of the entire plant and is contingent upon the metabolic state and predetermined developmental differentiation program of the synthesizing tissue [45].

The variability observed in the concentrations of different compounds in the vegetative and full bloom stages could be associated with the flowering process, given that shifting from the vegetative stage to the flowering stage is highly complex. Flowering represents a critical developmental stage in the life cycle of a plant, characterized by remarkable transformations, whereby the plant fortifies its defence mechanisms against potential adversaries. This adaptive response may serve the dual function of deterring or luring insects while protecting against pathogenic microorganisms that pose a threat during this pivotal stage [17, 46, 47]. Another explanation for this phenomenon could be attributed to the variance in the age of the plant parts used. The maturity of plant parts and their growth stages are important in the morphological development and physiological mechanisms of numerous plant species [17, 48, 49]. Studies have reported significant variations in the concentrations of the primary constituents of the essential oils of *L. alba* in both different harvests of monthly leaf regrowth within the same areas of study, as well as in harvests conducted in diverse regions [24].

The antifungal activity of *L. callicarpifolia* essential oils was assessed against three yeasts of resistant *Candida* species. The data in Table 2 shows that, overall, the *L. callicarpifolia* LSEP, LNOV, LFEB, FNOV, and FFEB essential oils exhibited effective *Candida* activity. Notably, high activity was presented by LNOV, which was capable of inhibiting the growth *of C. glabrata* with a 0.5 mg/mL MIC value. Moreover, the FNOV oil was found to have a pronounced effect on three *Candida* species, with 1 mg/mL MIC value on *C. glabrata* and 2 mg/mL MIC value on *C. albicans and C tropicalis.* Furthermore, it was observed that utilizing LSEP, LNOV, and FNOV reduced the development of yeast biofilms formed by *C. glabrata* by 18–24%, respectively (Table 3). This is a noteworthy report since the three *Candida* species tested are frequently associated as predominant human pathogens with high levels of resistance to antifungals [50, 3].

During the evaluation of antibacterial activity, all essential oils inhibited the growth of *K. pneumoniae* and all gram-positive bacteria, including multidrug-resistant strains. Moreover, the antibacterial efficacy of *L. callicarpifolia* essential oils against *P. aeruginosa* and *E. coli* was slightly impacted by developmental stage-related changes in the oil composition. Only the LNOV and FNOV essential oils demonstrated inhibitory activity against *E. coli* ATCC 25922. Interestingly, the EO of flowers FNOV expanded the antibacterial spectrum, inhibiting the growth of multidrug-resistant *E. coli* 82MR (provided by the Clinical Analysis Laboratory of FES-Iztacala). Furthermore, the essential oils from the flowering periods, FNOV and FFEB, were solely effective against *P. aeruginosa.* Pharmacological investigations of the genus *Lippia* indicate that essential oils of these plants possess antifungal and antibacterial effects [20, 51–56). Furthermore, methanolic extracts of *L. javanica* and *L. wilmsii* exhibited minimum inhibitory concentrations (MICs) ranging from 0.13 to 0.63 mg/mL against *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa*. This discovery is significant, as natural products with MIC values below 1 mg/mL are typically regarded as promising antibacterial agents [57]. Similarly, ethanolic extracts of *L. palmeri* used in traditional medicine in Baja California Sur, Mexico, have antifungal and antibacterial activity [58]. In addition, pharmacological research has shown the synergistic antibiotic activity of volatile compounds in EOs from *L. sidoides* leaves [59].

To the best of our knowledge, this study represents a first examination of the chemical composition and antimicrobial activity of *L. callicarpifolia* essential oils against bacterial and yeast pathogens. The literature suggests that EOs of *Lippia species* contain a significant amount of limonene, followed by the occurrence of *p*-cymene, α-pinene, camphor, β-caryophyllene, linalool, and thymol [25]. Our study also identified β-caryophyllene and α-pinene as the prevalent components and linalol present in small quantities. In addition, β-phellandrene was identified as the principal component. Limonene, *p*-cymene, camphor, and thymol were absent during all scrutinized periods.

Certain species of *Lippia* produce β-caryophyllene, α-pinene and sabinene as the predominant constituents of essential oils [60–61]. In addition, β-caryophyllene was identified as a significant constituent of *L. alba* essential oil, exhibiting antiviral effects against Zika virus (ZIKV) [62]. β-Caryophyllene is consistently the active constituent of essential oils derived from a diverse selection of spice and food plants and possesses notable anticancer, anti-inflammatory, anticarcinogenic, antimicrobial, and analgesic activities [63]. In contrast to β-caryophyllene, which frequently occurs in *Lippia* species, the presence of β-phellandrene has only been reported in a few species [64–65]. The isomer α-phellandrene is the most common constituent [25, 66]; however, other species of the genus *Monticalia*, such as *M. imbricatifolia* [67] and *M. andicola* [68], mainly produce β-phellandrene and α-pinene as prevalent compounds. Both species exhibited antibacterial effects against gram-positive and gram-negative bacteria. Furthermore, β-phellandrene is present as a primary component of the essential oil of *Schinus molle*. This oil was used in the production of hybrid films made from chitosan, which exhibited fungistatic proprieties against *F. oxysporum* [69].

The main constituents of essential oils are generally considered to be responsible for their antimicrobial properties. This is partly due to the challenge of discerning the biological activities of minor compounds [70]. Synthesis of oxygenated terpenoids, such as aldehydes and alcohols, increased during the flowering period in *L. callicarpifolia* and was correlated with the highest antimicrobial activity and the broader antibacterial spectrum shown. These results agree with reports of antimicrobial activity being associated with the presence of oxygenated compounds [71–72]. However, terpene hydrocarbons were the main constituents in the EOs of *L. callicarpifolia*, and β-caryophyllene, β-phellandrene, α-pinene, sabinene and terpinene were observed in significant concentrations. Previous studies have reported that α-pinene, sabinene, and terpinene possess antimicrobial activity, exhibiting inhibitory effects on the growth of gram-positive bacteria and pathogenic fungi [73–75]. In addition, the essential oil of *M. andicola, which contains* α-pinene and β-phellandrene as principal compounds, showed antibacterial activity against *S. aureus*, *E. faecalis, E. coli*, *K. pneumoniae*, and *P. aeruginosa* with MIC values ranging from 37 to 150 (μg/mL) [68]. Furthermore, the essential oil of *Dalea strobilacea* constituting principal to β-phellandrene along with α-pinene presents antibacterial activity that inhibits the growth of *K. pneumoniae*, *S. aureus*, and *E. faecalis* [76]. In addition, β-phellandrene, a primary component of the EO of *Hyssopus officinalis*, exhibited antifungal activity against *C. albicans* and *C. glabrata* [77]. Similarly, the antibacterial properties of β-caryophyllene against gram-positive bacteria were also reported [78]. Moreover, β-caryophyllene can modify the membrane permeability and integrity of *B. cereus*, resulting in the deterioration of the membrane and leakage of intracellular content, culminating in the eventual demise of the cell [79].

Interactions among these components can result in antagonistic, additive, or synergistic consequences. Several studies have indicated that complete EOs generally exhibit more significant antibacterial properties than their primary components, indicating the crucial role of minor components in synergistic activity. However, antagonistic and additive effects have also been documented [77, 80].

In the case of *L. callicarpifolia* EOs, the antimicrobial activity is likely significantly correlated with the synergistic interactions among the major and minor constituents rather than the presence of a single compound. It is plausible that hydrocarbon type molecules such as β-

caryophyllene, β-phellandrene, α-pinene, and sabinene exhibit synergistic effects with oxygenated terpenoids. Nevertheless, additional research is necessary to confirm this postulation and explain the mode of operation.

Conclusion

The essential oils of *L. callicarpifolia* demonstrated qualitatively similar chemical compositions across three distinct phenological stages. Nevertheless, significant variations were observed in the ratios of primary constituents and in the occurrence of minor components. According to the literature, the variances in the chemical makeup of the five oils arose due to numerous factors that could impact their compositions, including physiology, plant development stage, plant part age, and growing environment. Chemotype variability is essential in selecting favourable chemotypes for commercial products, especially regarding chemical fingerprinting, which is often needed in quality control. In addition, the essential oils of *L. callicarpifolia* display a wide-ranging antimicrobial effect. The EOs from plant parts collected during the flowering period were the most active and successfully contained the proliferation of bacterial and yeast strains. Notably, *Candida* species were found to exhibit a pronounced sensitivity to these oils. This study validates the medicinal use of *L. callicarpifolia* in the management of infections.

Abbreviations

½ MIC: Media Minimal Inhibitory Concentration

µg: Microgram

µL: Microliter

AMR: Antimicrobial Resistance

CFU: Colony-Forming Unit

EOs: Essential Oils

FFEB: **Essential oil of flowers collected** at the end of the flowering stage

FNOV **Essential oil of** flowers collected in full bloom

GC-MS: **Gas Chromatography-Mass Spectrometry**

LFEB: Essential oil of leaves/stems collected at the end of the flowering stage

LNOV: Essential oil of leaves/stems collected in full bloom

LSEP: Essential oil of leaves/stems collected in the pre-flowering period

mg: Milligram

mL: Milliliter

mm: Millimeter

MBC: Minimum Bactericidal Concentration

MFC: Minimum Fungicidal Concentration

MIC: Minimal Inhibitory Concentration

MR: *Multiresistan*

NIST 14: National Standards Institute and Technology version 14.0

RI: Retention Index

Declarations

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Authors' contributions.

Ana Karen Villagómez-Guzmán: Conducted the investigation, formal analysis, and writing of the original draft.

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Ana María García-Bores: The initial idea creation, supervision, management of the project, review.

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Declarations

Competing interests

The authors declare that they don’t possess conflicting financial concerns or personal associations that may have seemed to impact the research presented in this manuscript.

Ethics approval and consent to participate

Not applicable.

Permission to collect

All permissions were obtained to collect *L. callicarpifolia.*

Consent for publication

Not applicable.

Availability of data and materials

The data utilized and examined in the course of this investigation are accessible upon from reasonable request from the author responsible for correspondence.

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Figures

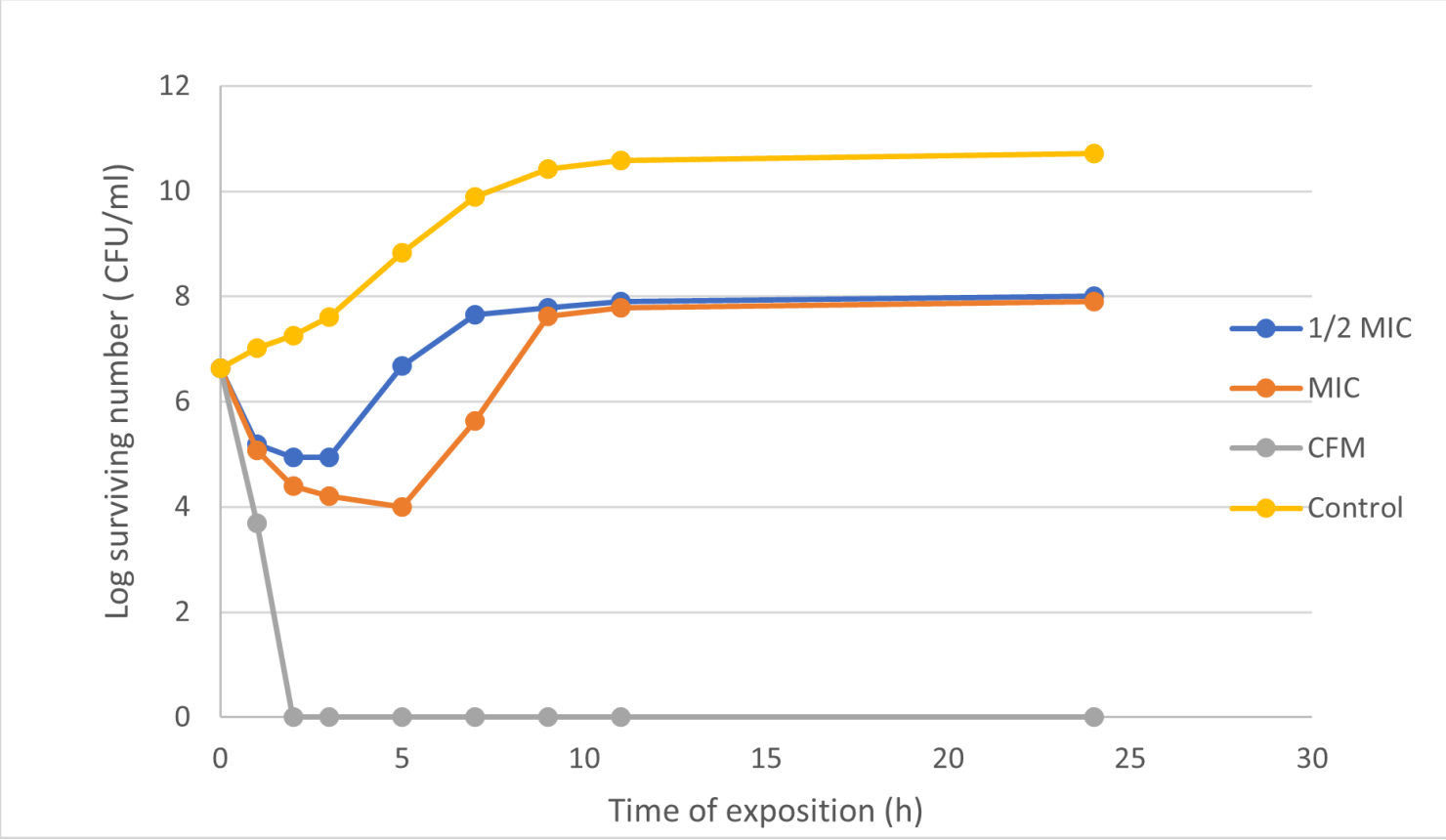

Figure 1

Survival curve of *C. glabrata* exposed to the essential oil of the leaves/stems LSEP. At time zero, the essential oil was added to each experimental culture at concentrations of 0.5 mg/mL (½ MIC), 1 mg/mL (MIC), and 2 mg/mL (MFC). The control group did not receive any essential oil. ½ MIC= Half of the Minimum Inhibitory Concentration MIC= Minimal Inhibitory Concentration and MFC= Minimum Fungicidal Concentration.

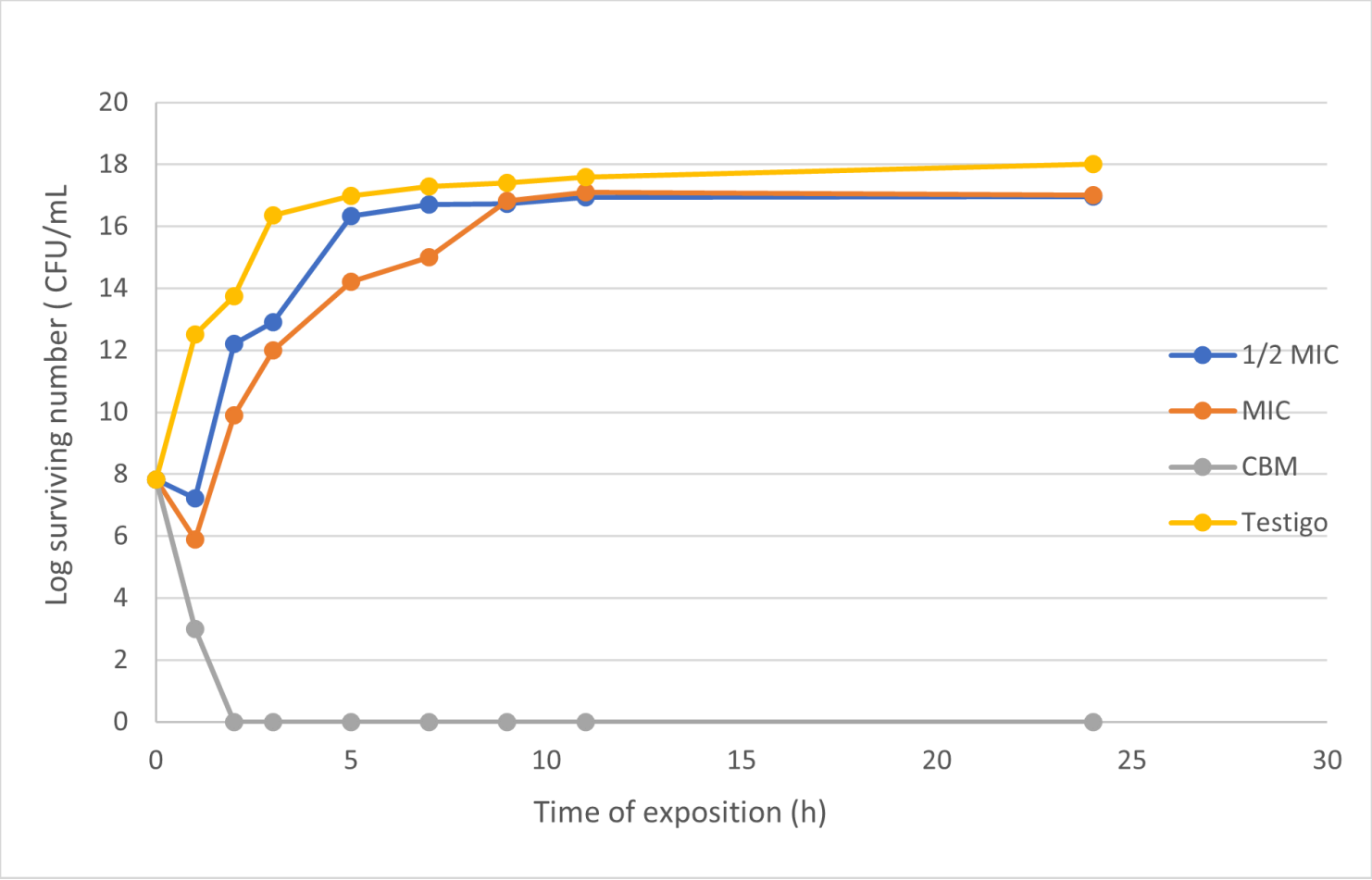

Figure 2

Survival curve of *C. glabrata* exposed to the essential oil of leaves/stems of LNOV. At time zero, the essential oil was added to each experimental culture at concentrations of 0.250 mg/mL (½ MIC), 0.5 mg/mL (MIC), and 1 mg/mL (MFC). The control group did not receive any essential oil. ½ MIC= Half of the Minimum Inhibitory Concentration MIC= Minimal Inhibitory Concentration and MFC= Minimum Fungicidal Concentration.

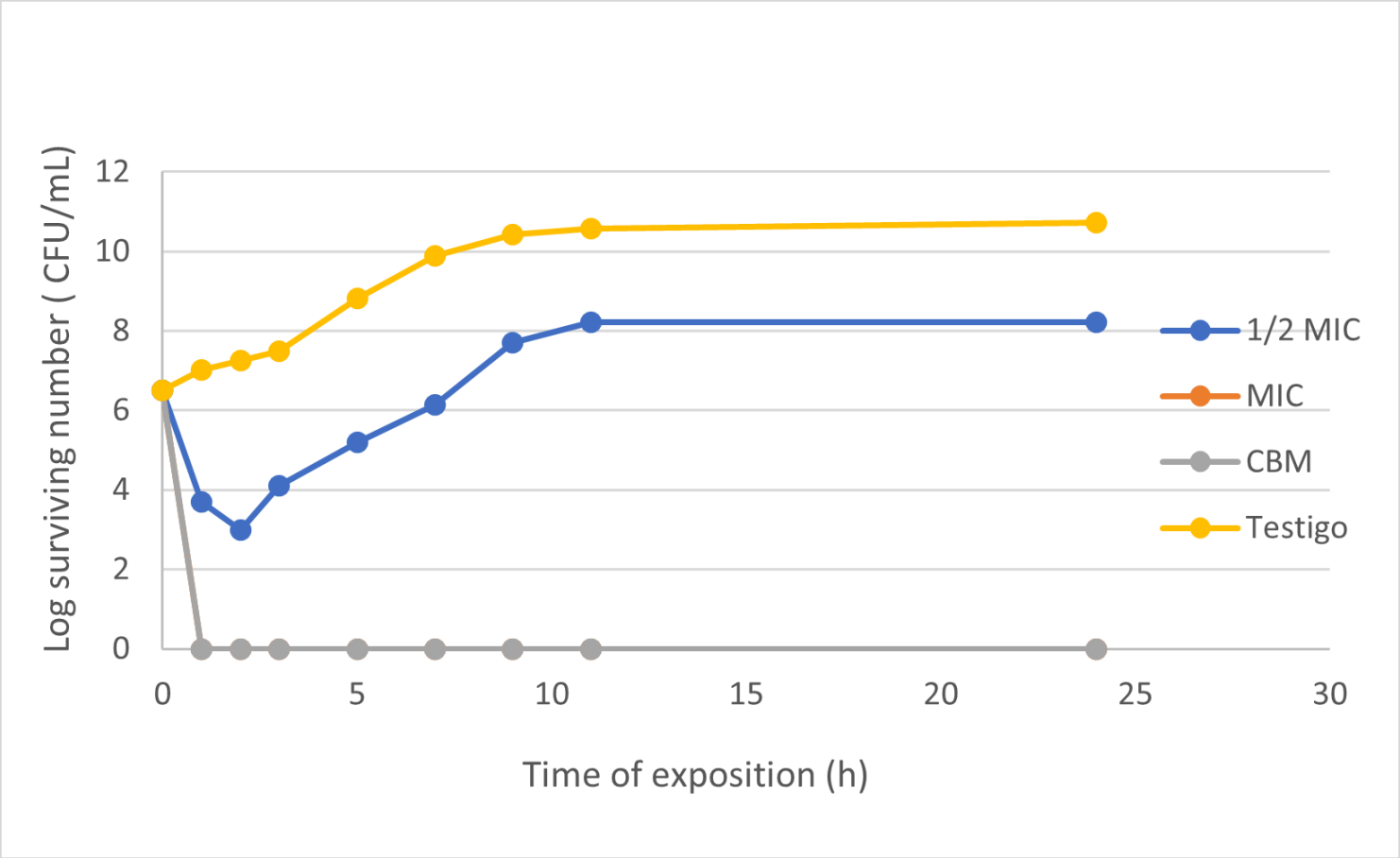

Figure 3

Survival curve of *C. glabrata* exposed to the essential oil of flowers FNOV. At time zero, the essential oil was added to each experimental culture at concentrations of 0.5 mg/mL (½ MIC), 1 mg/mL (MIC), and 2 mg/mL (MFC). The control group did not receive any essential oil. ½ MIC= Half of the minimum inhibitory concentration, MIC= Minimal Inhibitory Concentration and MFC= Minimum Fungicidal Concentration.

Supplementary Files

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