

Antimicrobial and allelopathic effects of leaves extracts of *Myrcia hatschbachii*

Efeitos antimicrobianos e alelopáticos de extratos foliares de *Myrcia hatschbachii*

Efectos antimicrobianos y alelopáticos de los extractos de hojas de *Myrcia hatschbachii*

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Abstract

Plants have been seen as alternatives for researching natural antimicrobials and herbicides. The aim of this work was to evaluate the antibacterial, antifungal, and allelopathic properties of leaves extracts of *Myrcia hatschbachii*. The crude extract and fractions were obtained by Soxhlet apparatus. Antimicrobial activity was determined by the minimum inhibitory concentration (MIC) using the broth microdilution method. The ethyl acetate fraction showed activity against *Candida albicans* (MIC: 62.5 µg/mL), *Staphylococcus aureus* (MIC: 500 µg/mL), and *Pseudomonas aeruginosa* (MIC: 1000 µg/mL). Allelopathic activity evaluated the influence of crude extract and fractions on the germination and growth of *Lactuca sativa*. The crude extract and the chloroform fraction inhibited the growth of the radicle, while the hexane fraction inhibited the growth of the hypocotyl and radicle of lettuce seeds in all concentrations (100-1000 µg/mL). The biological potential of the species motivates further study of new antimicrobial and bioherbicidal agents.

Keywords: Allelopathy; Antifungal; *Candida albicans*; Hypocotyl; Myrtaceae; Radicle.

Resumo

As plantas têm sido vistas como alternativas para a pesquisa de antimicrobianos e herbicidas naturais. O objetivo deste trabalho foi avaliar as propriedades antibacteriana, antifúngica e alelopática de extratos foliares de *Myrcia hatschbachii*. O extrato bruto e frações foram obtidos por Soxhlet. A atividade antimicrobiana foi determinada pela concentração inibitória mínima (CIM) usando o método de microdiluição em caldo. A fração acetato de etila apresentou atividade contra *Candida albicans* (CIM: 62,5 µg/mL), *Staphylococcus aureus* (CIM: 500 µg/mL) e

Pseudomonas aeruginosa (CIM: 1000 µg/mL). A atividade alelopática avaliou a influência do extrato bruto e das frações na germinação e crescimento de *Lactuca sativa*. O extrato bruto e a fração clorofórmica inibiram o crescimento da radícula, enquanto que a fração hexânica inibiu o crescimento do hipocótilo e da radícula das sementes de alface em todas as concentrações (100-1000 µg/mL). O potencial biológico da espécie motiva estudos futuros de novos agentes antimicrobianos e bioherbicidas.

Palavras-chave: Alelopatia; Antifúngica; *Candida albicans*; Hipocótilo; Myrtaceae; Radícula.

Resumen

Las plantas se han considerado alternativas para la investigación de antimicrobianos y herbicidas naturales. El objetivo de este trabajo fue evaluar las propiedades antibacterianas, antifúngicas y alelopáticas de extractos de hojas de *Myrcia hatschbachii*. El extracto crudo y las fracciones se obtuvieron mediante Soxhlet. La actividad antimicrobiana se determinó mediante la concentración mínima inhibitoria (CMI) utilizando el método de microdilución en caldo. La fracción de acetato de etilo mostró actividad contra *Candida albicans* (CMI: 62,5 µg/mL), *Staphylococcus aureus* (CMI: 500 µg/mL) y *Pseudomonas aeruginosa* (CMI: 1000 µg/mL). La actividad alelopática evaluó la influencia del extracto crudo y fracciones en la germinación y crecimiento de *Lactuca sativa*. El extracto crudo y la fracción de cloroformo inhibieron el crecimiento de radícula, mientras que la fracción de hexano inhibió el crecimiento de hipocótilo y radícula de semillas de lechuga en todas las concentraciones (100-1000 µg/mL). El potencial biológico de la especie motiva futuros estudios de nuevos agentes antimicrobianos y bioherbicidas.

Palabras clave: Alelopatía; Antifúngico; *Candida albicans*; Hipocótilo; Myrtaceae; Radícula.

1. Introduction

Plants have been seen as alternatives in the search for potential antimicrobials and natural herbicides. Allelopathy studies the ability of a plant to influence the growth and development of biological and agricultural systems, including positive and negative effects on the germination and development of other plants, through the release of secondary metabolites, especially phenolic compounds, terpenes and alkaloids, which constitute a primary source of potential allelochemicals (Macías et al., 2000; Reigosa et al., 2013). The search for plants with metabolites of allelopathic action has been explored for the production of bioherbicides as a perspective for sustainable agriculture, since they reduce the environmental impact caused by agrochemicals, besides presenting a lower cost compared to the traditional herbicide (Santiago et al., 2017).

In addition, plant species are potential sources of new drugs for infectious diseases (Sa et al., 2017). Infectious diseases are of great interest in the scientific community, as some microorganisms cause severe morbidity and can be lethal. Studies on the antimicrobial properties of natural products have increased in the last decade, as microorganisms have become resistant to various drugs used routinely and this motivates the search in plants for alternative therapies with new agents, and moreover, many species are used as antiseptic drugs in folk medicine. Among them, the Myrtaceae family stands out, which was extensively researched for its antimicrobial effects (Özçelik et al., 2011; Santana et al., 2018; Santos et al., 2018).

Myrcia genus contains more than 300 species distributed in Brazil (Franco et al., 2015). Indigenous tribes and traditional Brazilian communities have been using some of these species as astringents, diuretics, to stop bleeding, against diabetes, diarrhoea, hypertension, and mouth ulcers (Cerqueira et al., 2007). *Myrcia hatschbachii* D. Legrand is a native and endemic tree from the Southern states of Brazil (BFG, 2018). Considering the biological potential of the genus, the aim of this work was to evaluate the antibacterial, antifungal and allelopathic activities in the extracts of species, since these effects were previously demonstrated in its essential oil (Gatto et al., 2020).

2. Methodology

2.1 Plant material

Leaves of *M. hatschbachii* were collected at the Federal University of Parana, Curitiba, Brazil (25°26'52''S and 49°14'25''W). Species identification was performed by comparison of the collection voucher with the specimen held at Municipal Botanical Museum of Curitiba (MBM), under registry number 72379. Access to the plant material was approved and licensed by the Ministry of the Environment and registered under number 02001.001165/2013-47. The leaves were dried at

room temperature and crushed in a knife and hammer mill.

2.2 Thermal analysis

Dried leaves (10 mg) were heated at a constant rate of 10°C/min, being obtained in the temperature range between 20 and 550°C, under argon flow (50 mL/min), in an open alumina calorimetric cell. The STA 6000 instrument (PERKIN ELMER, Waltham, MA, United States) was calibrated using Indium (In; PF: 156.6°C; ΔH fusion: 28.54 J/g) as standard. The study was carried out using the Origin 9.0 program.

2.3 Crude extract and fractions preparation

Dried and crushed leaves (985 g) were extracted with ethanol by using the modified Soxhlet apparatus (Carvalho et al., 2009). This system underwent heating and was left to reflux for 40 hours. The crude extract (EBF) obtained a yield of 12.8%. From EBF concentrated in a rotary evaporator, the fractions were obtained by the liquid-liquid partition system using the Soxhlet apparatus and solvents of increasing polarity: hexane, chloroform, and ethyl acetate. The resulting fractions were named hexane fraction (FFH), chloroform fraction (FFC), and ethyl acetate fraction (FFA).

2.4 Antimicrobial activity

The broth microdilution method was performed according to CLSI, standardized and validated by Veiga et al. (2019). Crude extract and fractions were tested against strains of *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8738, and *Candida albicans* ATCC 10231. EBF, FFC, and FFA were solubilized in 10% methanol and FFH in 20% methanol in sterile water. The microorganisms were inoculated in TSA (bacteria) and sabouraud (yeast) agar and incubated at 33-35°C/18–22 hours. After incubation, part of the isolated colonies was transferred to tubes containing saline solution (0.9%) and the turbidity was adjusted to 0.5 on the McFarland Scale. In microplates, 100 μ L of broth (mueller hinton for bacteria and sabouraud dextrose for yeast) were added in all the orifices. Then, 100 μ L of the samples were added in triplicate in the first line and from this, serial dilutions were performed, maintaining the final volume in all orifices of 100 μ L and obtaining concentrations of 7.8-1000 μ g/mL. Positive controls (100 μ L of broth + 100 μ L of 250 μ g/mL chloramphenicol for bacteria or 500 μ g/mL ketoconazole for yeast), negative control (100 μ L of broth + 100 μ L of diluting solution), and blank (100 μ L broth + 100 μ L of tested sample) were also prepared and 100 μ L were discarded so that all the orifices in the microplate had the same volume. Suspension of microorganisms (10 μ L) for the samples and controls, and saline (10 μ L) for the blank were added to the plates, which were incubated at 33-35°C/20-22 hours. After incubation, 20 μ L of 0.125% 2, 3, 5 triphenyltetrazolium chloride developer solution was added and the plates were reincubated for 2 hours at 33-35°C. The results were interpreted by visual readings, observing the development of pink color for bacteria and the deposit of cells (white to pink button) for yeast. The minimum inhibitory concentration (MIC) is the last concentration that did not develop color and is interpreted as good activity (up to 100 μ g/mL), moderate (100-500 μ g/mL), weak (500-1000 μ g/mL), or inactive (> 1000 μ g/mL) (Tanaka et al., 2005; Ayres et al., 2008; Santos et al., 2008).

The concentrations of samples that inhibited microbial growth were evaluated for their bactericidal/fungicidal or bacteriostatic/fungistatic action. For this, the samples were transferred to TSA (bacteria) and sabouraud (yeast) agar and incubated at 33-35°C/18 hours. The microbiological growth on the agar evidences the bacteriostatic or fungistatic action and the non-growth evidences the bactericidal or fungicidal action (Santurio et al., 2007).

2.5 Allelopathic activity

The method was based on Macías et al. (2000), Chon et al. (2005), and Dias et al. (2005) and evaluated the influence of crude extract and fractions on the germination and growth of *Lactuca sativa* seeds. Extract and fractions solutions were

prepared in methanol to obtain concentrations of 100-1000 µg/mL. Distilled water and methanol were used as controls. Gerbox boxes were sanitized with solutions of hypochlorite and 70% alcohol and two autoclaved Whatman® filter papers were added. In laminar flow, a volume of 6 mL of sample was added to boxes, as well as 6 mL of methanol were added to the solvent control boxes. For each test, two boxes were used: one for germination and one for growth. The boxes were left at room temperature for 24 hours. After total evaporation, the filter papers were moistened with 6 ml of distilled water. In this stage, control boxes containing water were also prepared. Gerbox boxes were divided into four quadrants and in each of them, five lettuce seeds (*L. sativa* variety *Grand rapids*) were deposited, totaling 20 seeds per box. After accommodating the seeds, the boxes were incubated in a BOD at 20 ±5°C. In the germination evaluation, daily readings were taken for 7 days, with the germinated seeds removed. In addition, the germination speed index (GSI) was calculated according to Maguire (1962). The growth verification was performed on the seventh and last day of the test, through the reading in millimeters (mm) of the hypocotyl and radicle. The verification of the differences of the statistically significant means were made by the Scott-Knott test ($p < 0.05$) using the Sisvar software version 5.6.

3. Results and Discussion

Thermal analysis evaluated the physical property of a substance and/or its reaction products. This substance is subjected to controlled temperature programming and under a specific atmosphere, which variations in mass are observed, using a thermobalance of high sensitivity and reproducibility. The data obtained provide information regarding the composition and thermal stability of the sample (Silva et al., 2007) and can be seen in Table 1. The thermal degradation profile of the leaves of the species shows four mass losses in an argon atmosphere. In step two, the decomposition of secondary metabolites such as flavonoids, tannins, and terpenes was evaluated and it was found that the leaves have good thermal stability up to 124°C, which guarantees thermal reliability in the hot extraction processes, as in Soxhlet apparatus, which was used to obtain the extract and fractions.

Table 1. Stages of thermal degradation in an argon atmosphere of leaves of *Myrcia hatschbachii*.

Stage	Temperature	°C	Mass (%)
1 Humidity	Initial	20	6,6
	Final	130	
2 Decomposition of secondary metabolites	Initial	124	46
	Final	402	
3 Slow decomposition of carbonaceous residues and polymers	Initial	405	15,4
	Final	550	
4 Carbonaceous residues	Initial	550	32
	Final	-	

Note: % - Percentage of degraded mass in each stage.
 Source: Authors (2021).

Table 2 shows the MIC results of the samples against the tested microorganisms. Previous studies have also shown activity in the extracts of genus and family (*Syzygium*, *Eucalyptus*, and *Eugenia* genus) and were compared to those obtained by *M. hatschbachii*. The species showed a fungistatic effect against *C. albicans* in all samples, with emphasis on EBF and FFA (MIC: 62.5 µg/mL). Extracts of *Myrcia bella* and *Myrcia fallax* were inactive (Santos et al., 2018), while extract and fractions of *Myrcia tomentosa* were effective against several yeast strains (MIC: 4-256 µg/mL; Sa et al., 2017). In the family, extracts of *Syzygium cumini* (MIC: 125 µg/mL; Pereira et al., 2016), *Eucalyptus intertexta* (MIC: 62.5 µg/mL), *Eucalyptus largiflorens* (MIC: 31.2-125 µg/mL; Safaei-Ghomi & Ahd, 2010), and *Eugenia caryophyllus* (MIC: 17.41 mg/mL; Thirach et al., 2003) also demonstrated activity.

Phenolic compounds and hydrolysable tannins may justify the antimicrobial effect in the *Myrcia* genus. The anti-infective actions of tannins are associated with stimulation of phagocytic cells and host-mediated tumour activity (SANTOS et al., 2018). While phenolic compounds induce damage to the antifungal membrane and increase cell permeability. These characteristics are related to its antimicrobial activity (SA et al., 2017) and corroborate the results obtained for *M. hatschbachii*. FGA showed high antifungal activity (MIC: 62.5 µg/mL) and in previous studies, this same sample showed 75% of phenolics in its composition, antioxidant potential, and the identification of gallic and ellagic acids, which are phenolics and hydrolysable tannin precursors. In addition, phytochemical screening performed by thin layer chromatography on EBF and FFA showed the presence of tannins, flavonoids and coumarins (GATTO et al., 2021).

Hydrolysable tannins (gallotannins and ellagitannins) were also found in *M. guianensis*, *M. bella*, and *M. fallax* (Santos et al., 2018). Acetylated ellagic acid rhamnosides were identified in *Myrcia palustris* (Wubshet et al., 2015). Tannins also have anti-infectious actions, such as stimulation of phagocytic cells and host-mediated tumor activity (Santos et al., 2018) and some of them showed antifungal properties, such as corilagin, pelargoniin B, and phyllantusiin C (MIC: 500 µg/mL; Latté & Kolodziej, 2000).

Table 2. Antimicrobial activity expressed in MIC of crude extract and fractions of *Myrcia hatschbachii*.

Samples	Microorganisms			
	<i>C. albicans</i> MIC (µg/mL)	<i>S. aureus</i> MIC (µg/mL)	<i>P. aeruginosa</i> MIC (µg/mL)	<i>E. coli</i> MIC (µg/mL)
EBF	62.5	500	>1000	>1000
FFH	500	500	>1000	>1000
FFC	250	1000	>1000	>1000
FFA	62.5	500	1000	>1000

Note: Minimum inhibitory concentration (MIC); crude extract (EBF); hexane fraction (FFH); chloroform fraction (FFC); ethyl acetate fraction (FFA).
 Source: Authors (2021).

The bacteriostatic effect was observed against *S. aureus* at a concentration of 500 µg/mL of EBF and FFA, however at the concentration of 1000 µg/mL, the bactericidal effect was seen in both. The other samples tested showed a bacteriostatic effect. In view of the same strain, the *M. tomentosa* species showed activity for the crude extract and fractions of ethyl acetate (MIC: 1000 µg/mL) and hexane (MIC: 250 µg/mL; Sa et al., 2017), as well as aqueous extract of *E. largiflorens* (MIC: 7.8 µg/mL; Safaei-Ghomi & Ahd, 2010).

M. hatschbachii species was inactive for *P. aeruginosa*, except for FFA, which demonstrated a bacteriostatic effect, with a MIC of 1000 µg/mL. Studies from leaves of *M. tomentosa* showed activity for crude extract and ethyl acetate (MIC: 500 µg/mL) and hexane (MIC: 1000 µg/mL) fractions (Sa et al., 2017), as well as in aqueous extracts of *E. intertexta* and *E. largiflorens* (MIC: 31.2 µg/mL; Safaei-Ghomi & Ahd, 2010).

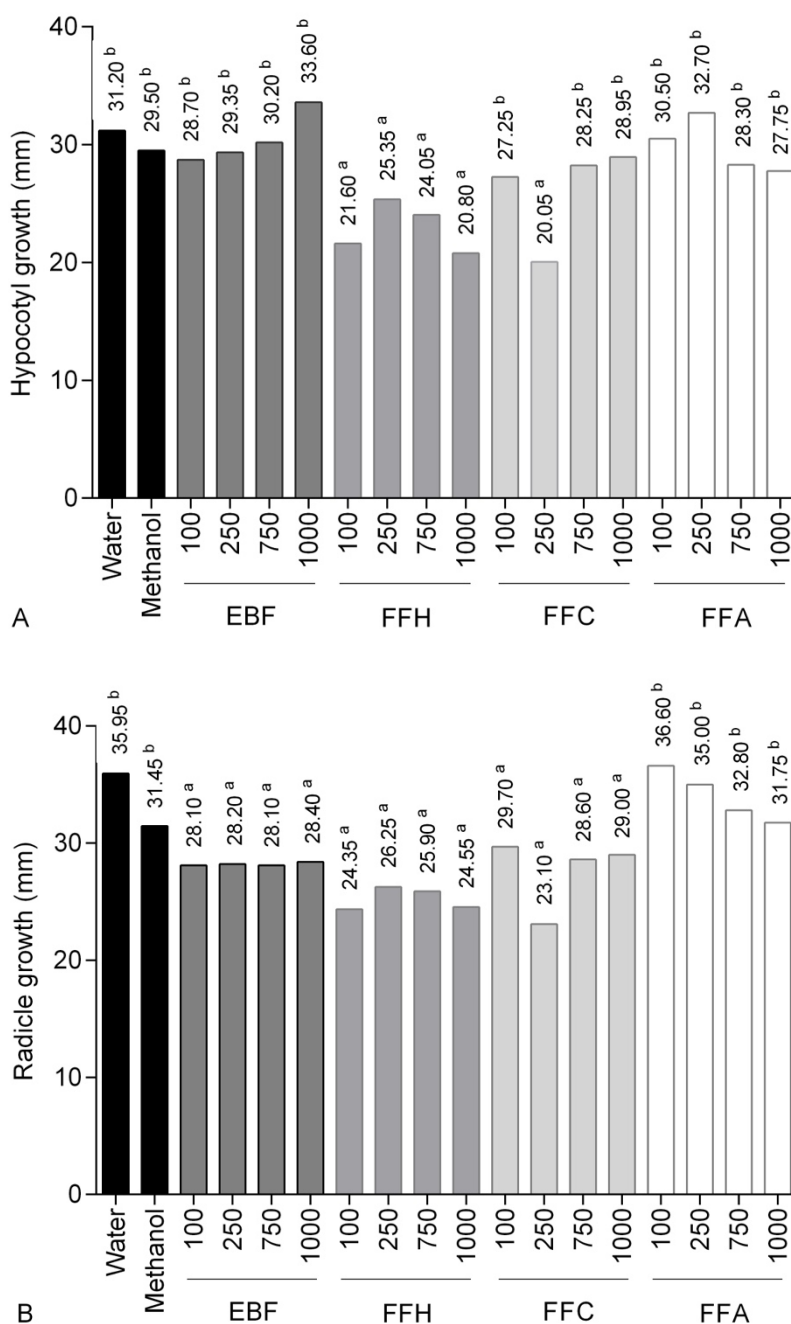
In relation to *E. coli*, the species samples were shown to be inactive, whereas the leaves extracts of *M. bella* and *M. fallax* demonstrated activity (300 and 250 µg/mL, respectively; Santos et al., 2018), as well such as the chloroform fraction of *E. largiflorens* (MIC: 125 µg/mL; Safaei-Ghomi & Ahd, 2010). The presence of an external membrane over the peptidoglycan, presence of capsule and porins may be the reason for the low activity of the extracts in gram-negative bacteria, such as *P. aeruginosa* and *E. coli*. These structural characteristics differ in relation to gram-positive bacteria and can hinder the action of bioactive components (Ayres et al., 2008; Santana et al., 2018).

The crude extract and fractions of *M. hatschbachii* did not influence germination and GSI of *L. sativa* in the tested concentrations. The seeds are considered germinated with the appearance of the radicle protrusion through the tegument (De Feo et al., 2002). The growth verification was performed by reading the length of the hypocotyl and radicle after seven days of storage in a BOD. Allelochemicals accumulate predominantly in the leaves and are released into the environment via root

exudates in the soil or by volatile substances in the air. Its effects on plant growth are related to cell division, organic synthesis, hormonal interactions, nutrient absorption, inhibition of protein synthesis, changes in lipid metabolism, stomatal opening, CO₂ assimilation and photosynthesis (Franco et al., 2015; Silva et al., 2012).

FFH (group a) inhibited the hypocotyl of *L. sativa* (Figure 1A) at all concentrations tested when compared statistically to the water and methanol (group b). While EBF, FFH, and FFC (group a) inhibited the radicle (Figure 1B) at all concentrations compared to the same controls (group b).

Figure 1. Influence of crude extract and fractions of *Myrcia hatschbachii* on the growth of the hypocotyl (A) and radicle (B) of *Lactuca sativa*



Note: Crude extract (EBF); hexane fraction (FFH); chloroform fraction (FFC); ethyl acetate fraction (FFA). Samples classified in the same group do not differ significantly ($p < 0.05$), as determined by the Scott Knott test.
 Source: Authors (2021).

Classic studies of allelopathy have progressed over the years, but molecular advances need further investigation to better understand allelopathic action, especially in relation to root development. The inhibition of root development due to allelopathic activity is initially attributed to changes in gene expression (Franco et al., 2015).

Allelopathic activity has also been described for other species of the *Myrcia* genus and involve studies of inhibition and stimulation of germination and growth of hypocotyl and radicle.

The hydromethanolic extract of leaves of *M. guianensis* drastically inhibited the development of roots of *Sorghum bicolor* (sorghum) and altered the expression of SHR and PHB genes and microRNA 166, which can be used as indicators of allelopathic potential in root development (Franco et al., 2015). Another study with the species showed that extracts of ethyl acetate and methanol from leaves inhibited the germination of seeds of *Mimosa pudica* (malice), whereas the hexane and ethyl acetate fractions showed inhibition of growth of hypocotyl and radicle (Souza Filho et al., 2006).

Extracts of ethyl acetate and acetone from leaves of *M. tomentosa* showed a potent inhibition of coleoptile elongation when tested on *Triticum aestivum* (wheat). The germination rate of seeds of *Lepidium sativum* (watercress) and the growth of hypocotyl of *L. sativa* (lettuce) were also significantly inhibited by dichloromethane, ethyl acetate, acetone and methanol extracts (Imatomi et al., 2013).

The aqueous extracts of leaves of *M. multiflora*, *Myrcia splendens*, and *M. tomentosa* promoted marked delays in seed germination and inhibited the growth of seedlings of *Echinochloa crusgalli*, *Euphorbia heterophylla*, and *Ipomoea grandifolia*, even with more efficient effects than the herbicide oxyfluorfen (Imatomi et al., 2015).

The main biosynthetic pathways that lead to the production of allelochemicals are derived from acetate or chiquimate. Phenolic acids are the compounds most commonly investigated for this purpose, especially p-hydroxybenzoic, vanillic, p-coumaric, syringic and ferulic acids (Chon et al., 2005). Most phenolic compounds are released simultaneously and continuously, perhaps acting synergistically. In addition, chemical interactions between plants are almost never limited to a compound, but to mixtures of various substances, often complex, and these mixtures can have synergistic or antagonistic effects, which can be modified by the chemical, physical and biotic properties of the soil (Reigosa et al., 2013).

4. Conclusion

Extracts of *M. hatschbachii* showed fungistatic and bacteriostatic activities in all samples and concentrations when tested against *C. albicans* and *S aureus*, respectively, except for the crude extract and ethyl acetate fraction, which showed a bactericidal effect at 1000 µg/mL against *S aureus*. The crude extract and the chloroform fraction inhibited the growth of the radicle of *L. sativa*, while the hexane fraction inhibited the growth of the hypocotyl and the radicle of the lettuce seeds at all concentrations (100-1000 µg/mL). The biological potential of the species motivates the in-depth study of new antimicrobial and bioherbicidal agents.

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