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Chemical constituents and bactericidal and fungicide potential of the essential oil of *Pimenta dioica* Lindl against pathogenic microorganisms

Componentes químicos y potencial bactericida y fungicida del aceite esencial de *Pimenta dioica* Lindl contra microorganismos patógenos

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Abstract

This study evaluated the chemical profile and antimicrobial activity of essential oil (EO) of *P. dioica*. The EOs were extracted by hydrodistillation and chemically characterized by gas chromatography coupled to mass spectrometry (GC/MS). The total phenolics were quantified by the Folin Ciocalteu method. For the antimicrobial assay, the Disc Diffusion and Broth Dilution method were applied to obtain the minimum inhibitory concentration and minimum bactericidal concentration. The main constituent of the EO was eugenol. The EO showed bactericidal activity against *E. coli, S. aureus, P. aeruginosa, Salmonella sp., B. cereus, P. mirabilis, K. pneumoniae, S. sonnei, C. albicans, Fusarium sp., Penicillium sp*. and *Aspergilus sp.* The results obtained are encouraged by the potential use of the EO studied in the control and combat of pathogenic microorganisms

Keywords: Antimicrobial; Essential oil; Pimenta.

Resumen

Este estudio evaluó el perfil químico y la actividad antimicrobiana del aceite esencial (AE) de *P. dioica*. Los AE fueron extraídos por hidrodestilación y caracterizados químicamente por cromatografía de gases acoplados a espectrometría de masas (GC/MS). Los fenólicos totales fueron cuantificados por el método Folin-Ciocalteu. Para el ensayo antimicrobiano, se aplicó el método de difusión de discos y dilución de caldo para obtener la concentración inhibitoria mínima y la concentración bactericida mínima. El componente principal de la AE fue el eugenol. La AE mostró actividad bactericida contra *E. coli, S. aureus, P. aeruginosa, Salmonella sp., B. cereus, P. mirabilis, K. pneumoniae, S. sonnei, C. albicans, Fusarium sp., Penicillium sp.* y *Aspergilus sp*. Los resultados obtenidos son estimulados por el uso potencial de la AE estudiada en el control y combate de microorganismos patógenos.

Palabras clave: Antimicrobiana; Aceite esencial; Pimenta.

Introduction

Plants are considered one of the main natural resources for medicinal use, due to their biological potential, either by the action of deadly diseases or diseases that affect living beings; therefore, and according to the World Health Organization, almost 80% of the population in developing countries uses them directly or indirectly for their basic health needs; either because of the cultural tradition or because of the absence of other options, due to the high cost of traditional medicines for this population (Bermúdez et al., 2005; Alitonou et al., 2012; Duarte et al., 2020).

Multidisciplinary efforts have also led to an increase in the number of studies in order to obtain greater knowledge about a medicinal plant (Duarte et al., 2020).) The study of substances extracted from plants has proved indispensable over time; either because of the great biological diversity in Brazil or because of the potential of this extraction. Thus, essential oils represent a viable alternative in several studies involving substances of plant origin (Gomes et al., 2020).

Specific analyses are necessary around the validation process, which include the chemical composition and proof of microbial pharmacological activity that ensure the quality of raw materials of plant origin (Gomes et al., 2020). The toxic potential of essential oils (EOs) and their compounds can significantly vary according to intrinsic

and extrinsic factors (Kim et al., 2016).

The chemical composition of the EOs considerably contributes to the determination of the pharmacological potential attributed to plant species. In the case of EOs, it is regularly recommended to use the mass spectrum-coupled gas chromatography system (GC/MS) in order to identify substances derived from secondary metabolism (Mirzahosseini et al., 2017; Howyzeh et al., 2018). EO extracted from medicinal plants have been widely used successfully in research focused on epidemiological control (Mirzahosseini et al., 2017).

The *Pimenta dioica* (Lindl.) is a perennial tropical species of trees belonging to the family Myrtaceae. Known for its characteristic aroma, therapeutic and culinary qualities that resemble the aroma and flavor of clove and nutmeg (Dexheimer et al., 2017). This plant is known as Jamaica pepper, and its leaves or seeds powder are broadly used in traditional medicine for the treatment of flatulence, diarrhea, neuralgia, rheumatism, and digestive problems (Rao et al., 2012). In addition, it is valued for its secondary metabolites, such as EO with compositions and resin oil, which is highly used in the food, perfumery and cosmetic products industry (Martinez-Velazquez et al., 2011).

To date, there are few publications in relation to compositions and characterization of the EO of *P. dioca* (Martinez-Velazquez et al.; Padmakumari et al., 2011; Rao et al., 2012). Thus, the present work reports the chemical profile and antimicrobial potential of EO *P. dioica*.

Methodology

Essential oil

For extraction of the EO, the hydrodistillation technique was used with a glass Clevenger extractor coupled to a round-bottomed balloon packed in an electric blanket as a heat generating source. 90g of the dried leaves of *P. dioica* were used, adding distilled water (1:10). Hydrodistillation was conducted at 100 °C for 3h collecting the extracted EO. Each EO was dried by percolation with anhydrous sodium sulfate (Na_2SO_4) and centrifugate. These operations were carried out in triplicates and samples stored in amber glass ampoules under 4 °C cooling. Subsequently, the analyses were submitted.

Chemical constituents

The spectra in the infrared region were obtained in the IR PRESTIGE-21 model equipment and were recorded in the 4000-400 cm^{-1} region. All tablets were prepared by using anhydrous potassium bromide (KBr).

The EO constituents were identified by gas chromatography coupled to mass spectrometry (GC-MS). The the analysis conditions were as follows: Method: Adams. M; Injected volume: 0.3 µL; Column: Capillary HP-5MS (5% diphenyl, 95% dimethyl polysiloxane) (Equivalent DB-5MS or CP-Sil 8CB LB/MS), in dimensions (30 m x 0.25 mm x 0.25 µm); Drag gas : He (99.9995); 1.0 mL/min; Injector : 280 ºC, Split mode (1:10); Oven: 40 oC (5.0 min.) up to 240 ºC at a rate of 4 oC .min^{-1}, from 240 oC to 300 oC (7.5 min) at a rate of 8 oC.min^{-1}; tT = 60.0 min; Detector: EM1; EI (70 eV); Scan mode (0.5 sec/scan); Mass range: 40 - 500 daltons (one); Line transfer: 280 oC.; Filament: off 0.0 to 4.0 min; Linear quadrupole mass spectrometer. The AMDIS (Automated Mass spectral Deconvolution Mass & Identification System) program was used to identify the compounds in the sample.

Total phenolics

The determination of total phenolic EO phenolic compounds was performed with adaptation of the Folin-Ciocalteu (Waterhouse, 2002). 5 mg of the essential oil diluted in 1 mL of ethanol was used. To this solution 3 mL of distilled water was added, 500 µL of Folin-Ciocalteu reagent and 2.0 mL of sodium carbonate at 20%. The solution formed was taken to the water bath at 50 ºC for 5 min, removed and left to cool; and then, the reading was performed in a manual spectrophotometer, in a length of 760 nm. The readings were performed in a spectrophotometer at 760 nm, and the standard curve expressed in mg of tannic acid (EAT).

Standardization of microbial inoculum for sensitivity tests

Four strains of bacteria were used: *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 15442), *Bacillus cereus* (ATCC 14579), *Proteus mirabilis* (ATCC 29906), *Klebsiella pneumoniae* (ATCC 700603), *Shigella sonnei* (ATCC 25931) and *Salmonella* sp. (ATCC 700623). The fungi used were: *Penicillium* sp. (ATCC 38498), *Aspergillus* sp. (ATCC 201291), *Fusarium* sp. (ATCC 60289), *Candida albicans* (ATCC 10231). These were previously identified and confirmed by biochemical tests. Pure microbial cultures maintained in TSA Agar were peaked for brain and Heart Infusion Broth (HIB) and incubated at 35 °C until they reached exponential growth phase (4-6 h). After this period, the cultures had their cell density adjusted in 0.85% sterile saline solution, in order to obtain turbidity comparable to that of the standard McFarland solution 0.5, which results in a microbial suspension containing approximately $1.5x10^8$ CFU mL^{-1}, according to (CLSI, 2020).

Disk Diffusion Method (DDM)

The Disc Diffusion technique was performed according to CLSI (CLSI, 2020) and, which standardizes the sensitivity tests of antimicrobials by disc-diffusion. First, the plates were prepared with the Mueller Hinton Agar (MHA) and Sabourad Dextrose Agar (SDA) culture medium after its solidification was distributed to the microbial suspension on the surface of the agar and left at room temperature for 30 min. Soon after the discs containing 50 µL of EOs and discs with defined concentrations of antibiotics,

Gentamicin (GEN, 30 µg) for bacteria and Ketoconazole (CET, 50 µg) for fungi. Using sterile tweezers, the discs were distributed on the surface of the agar. The plates were incubated in a bacteriological greenhouse at 35 °C for 24 hours and 25 °C for 24 hours. The diameters of the inhibition halos were measured, including the diameter of the disc. These trials were done in triplicate. The values of the inhibition halos were the mean measurements of the three results. Tests performed in triplicate.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The Minimum Inhibitory Concentration (MIC) assay was performed by using the broth dilution technique, proposed by CLSI (CLSI, 2020) and by the methodology described by Arendrup et al. (2012). First, 2% solutions were prepared by using dimethylsufoxide (DMSO) at 2%, and serial dilutions were prepared in MH Broth, resulting in concentrations of 10 to 1000 µg mL^{-1}. Microbial suspension containing 1.5 $x10^8$ CFU mL^{-1} of the strains was added to each concentration. The tubes were incubated at 35 ℃ for 24h. Sterility and growth controls were performed for the assay. After the incubation period, the MIC of the EO was verified, being defined as the lowest concentration that visibly inhibited bacterial growth (absence of visible cloudiness). Tests performed in triplicate.

For the Minimum Bactericidal (MBC) e Fungicidal Concentration (MFC) assay, an aliquot of 100 µL of the dilutions from MH broth that visibly inhibited microbial growth was used. The aliquots were inoculated in Mueller Hinton Agar (MHA) for bacteria e Sabourad Dextrose Agar (SDA) for fungi with subsequent incubation at 35°C during 24h e 25 °C for 24h, respectively. The MBC e MFC was determined as the lowest dose that visually, in the MIC assay, showed growth inhibition and that in the culture in AMH also did not present bacterial growth.

Results and Discussion

Table 1 presents the chemical constituents identified in the EO of *P. dioica* L, with eugenol being the majority constituent with 85.673% of the total composition of the EO. Seven constituents were identified in the EO, all belonging to the class of monoterpenes.

Table 1: EO chemical constituents.

Order	RT (min)	Constituent	% Area
1	8,772	Octenol	1,186
2	9,164	Myrcene	2,762
3	10,488	Limonene	1,730
4	13,251	Linalool	0,884
5	16,122	Terpineol	0,973
6	19,026	Chavicol	6,792
7	22,755	Eugenol	85,673

Regarding the chemical profile, similar results were observed by Stewart et al. (2016) when verifying the chromatographic analyses performed by GC-MS of *P. dioica* EO, and observed eugenol (61.36%) as majority constriction followed by β-caryophylenne (4.58%), α-humulene (1.90%), 1.8-cineol (1.89%), and other components in lower concentrations. Monteiro et al. (2015) analyzed the EO of *P. dioica* fruits, and also reported eugenol (76.98%) as the majority component, followed by 5-Idanol (5.88%) and limonen (4.09%).

The results also corroborate the study conducted by Oliveira (2017) and also observed the presence of eugenol (44.9%) *P. dioica* EO*,* followed by β-pineno (21.0%), limonen (10.1%) and chavicol (7.5%). Faria et al. (2017) also analyzed by CG/MS the EO of *P. dioica* and eugenol (60.8%) was the majority constituent of the Constitution of the EO, followed by myrcene (19.3%), limonen (6.48%), and chavicol (4.78%) constituents at lower concentrations. Panawala et al. (2016) verified, in their study, the chemical composition of the EO of the leaves of *P. dioica,* dectify 12 chemical compounds through GC-MS, comprising 97% of the total composition. Eugenol (85.33± 2.0 %) was the major component of EO, followed by β-caryophile (4.36±0.3%) and cineol (4.19%± 0.3%), linalool (0.83 ± 0.11%), α-humuleno (0.76 ± 0.12%).

Analyzing the EO of the fruits of *P. dioica*, Lorenzo-Leal et al. (2018) evaluated the chemical composition of the EO of *P. dioica* dried fruits, using the CG-MS technique, and identified 5 chemical components. The main compounds detected were eugenol (89.55%), α-terpinol (2.04%) and carioene oxide (1.48%). However, different percentages of eugenol were found by some authors such as Mathew (2017) who observed in their analyses that EO of *P. dioica* may contain 80 to 87% of eugenol and Amma et al. (2013) who found the percentage of 68.4% of eugenol.

Voris et al. (2017) observed chromatographic analyses performed by the CG-MS technique of *P. dioica* EO, identified 7 chemical components, methyl-eugenol (55.26%), eugenol (35.72%), β- farnecene (4.54%) and β-pineno (2.94%). The major component of EO was methyl-eugenol, so this result differs from that found in this study. According to Figueiredo et al. (2008), this variation in concentration can be explained among other factors, by soil composition, temperature and climate of cultivation, climatic differences and sun exposure. The chemical composition of the EO of a plant is determined by genetic and physiological factors, but it may undergo significant variations as a result of environmental factors such as climate and the cultivation place (OLIVEIRA et al., 2009). According to Scherer et al. (2009), this variation can directly influence the functional properties of EOs, such as antioxidant and antimicrobial activity.

Total phenolics

In the Folin-Ciocalteu assay, the total phenolic content of 350.14±5.20 mg EAT g^{-1} present in the EO of *P. dioica was quantified.*

For the Folin-Ciocalteu trial, using the same methodology Panawala et al. (2016), compared the total antioxidant capacity, the total content of phenolic and flavonoids, according to different leaf positions of the leaves and parts of *P. dioica*. The total phenolics found were slightly similar to that of this study, with 267.53±5.03 and 192.20±8.00 mg GAE g^{-1} (mg gallic acid equivalent), in the shoots and immature leaves of the plant, respectively.

In other products obtained from *P. dioica*, Manorama (2016) found higher phenolic content in the stem of the aqueous extract, compared to other extracts. The determination that resulted in 16.31±0.5 GAE g^{-1} sample. Kumar et al. (2010), revealed the total phenolic content of *P. dioica aqueous* extract in mg/g of equivalent biting acid, resulting in 0.0524 mg g^{-1}, a value lower than that of this research, further inferring its potential.

Phenolic compounds constitute a large part of the vegetables and have several physiological and morphological properties, mainly linked to antioxidant action in plants (Puupponen-Pimiä et al., 2001; Manach et al., 2005). According to the results of the present study, the EO of P. dioica exhibited a significant value of the compound.

Antimicrobial potential

Table 2 presents the results obtained for antimicrobial activity for EO action.

Table 2: Diameter of inhibition halos (mm), MIC (µg mL-1) and MBC (µg mL-1) of EO.

Microorganisms	CET (50 µg)	GEN (30 µg)	DIH (mm)	MIC (µg mL^{-1})	MBC (µg mL^{-1})	MFC (µg mL^{-1})
E. coli	-	19	15 ± 1,2	100	370	-
S. aureus	-	26	24 ± 2,1	50	200	-
P. aeruginosa	-	13	10 ± 1,5	350	700	-
Salmonella sp.	-	12	11 ± 1,1	220	450	-
B. cereus	-	12	14 ± 1,9	400	800	-
P. mirabilis	-	13	12 ± 1,5	450	870	-
K. pneumoniae	-	21	17 ± 1,7	380	630	-
S. sonnei	-	15	14 ± 1,4	400	690	-
C. albicans	29	-	24 ± 1,3	400	-	620
Fusarium sp.	28	-	25 ± 1,1	450	-	600
Penicillium sp.	30	-	31 ± 0,60	330	-	500
Aspergilus sp.	25	-	20 ± 0,80	520	-	850

Note: CET= ketoconazole; GEN= gentamicin; IHL= diameter of inhibition halos; MIC= minimum inhibitory concentration; MBC= minimum bactericidal concentration; MFC= minimum fungicide concentration.

As observed in Table 2, P. dioica EO was tested against two groups of bacteria (gram-positive and gram-negative) and pathogenic fungi. The results demonstrated that the EO showed significant inhibition against the bacterial and fungal strains tested, presenting inhibition halos between 10 and 31 mm. The best EO results for the bacteria studied were for S. aureus and E. coli with MIC ranging from 50 to 100 µg mL^{-1} and MBC between 200 and 370 µg mL^{-1}. For the other bacterial strains, antimicrobial activity also manifested with strongly inhibited MIC.

In a previous study by Lorenzo-Leal et al. (2019), on the antimicrobial action of *P. dioica* EO, described that the EO presented better activity *on A. baumannii*, *P. aeruginosa*, *S. aureus*, with MIC, ranging between 500 and 2000 µg mL^{-1}. These results revealed the importance of this study, which obtained higher antimicrobial potential of pepper EO. In addition, the bacterial strain of *E. coli* used by Lorenzo-Leal et al. (2019) was resistant to the concentrations tested.

Oussalah et al., (2007), revealed the antibacterial potential of *P. dioica* EO against *E. coli*, *Listeria monocytogenes*, *S. aureus* and *S. typhimurium*, with MIC ranging between 0.1% and 0.2%. When verifying the antimicrobial activity of spices on bacterial development Binatti et al., (2016), they found that the aqueous extract of allspice did not significantly inhibit *the balms B. cereus*, *B. subtilis, S. aureus, S. Enteritidis*, *S. typhimurium*, which exhibited inhibition halos between 6 and 8 mm. Unlike the results found in this study that demonstrated inhibition of 11 mm, 14 mm and 24 mm for *Salmonella* sp., *B. cereus* and *S. aureus,* respectively.

Regarding antifungal activity, *P. dioica* EO was able to inhibit the growth of all four fungal strains tested, especially *Penicillium* sp. which exhibited inhibition halo of 31 mm, a value similar to that of the control (CET=30 mm). However, Martinelli et al. (2017), indicated antifungal action of *P. dioica* EO at a concentration of 0.25 % on *Penicillium* sp.

Lowe et al. (2017), when analyzing the antifungal potential of ethyl acetate extract from *P. dioica leaves,* declared that it was more active to inhibit the growth of *C. albicans,* with MIC of 0.63mg / mL and MFC of 1, 3 mg / mL while in this study, and for this same fungus, the MIC and MFC values were 400 and 620µg mL^{-1}, respectively.

Another study reported that *P. dioica* EO promoted total inhibition of mycelial growth of *Fusarium oxysporum* f species. sp. lycopersici, *Fusarium oxysporum* f. sp. passiflorae, *Fusarium subglutinans* f. sp. ananas, *Fusarium* f. sp. vasinfectum and *Fusarium oxysporum* f. sp. cubense, at concentrations ranging from 86.67 and 97.78 % (Júnior, 2011).

In the study by Kim et al. (2016), the inhibition rates of EO of Allspice were 36.3, 30.5 and 0% against *A. ochraceus*, *A. niger* and *A. parasiticus,* respectively. In this study, Aspergillus sp. exhibited higher inhibition rate with MIC of 520µg mL^{-1} and MFC of 850µg mL^{-1}.

The chemical composition of essential oils is linked to their antimicrobial activity. Thus, eugenol is a component that presents proven antimicrobial activities (Burt, 2004) and it possibly was the cause of antimicrobial activity in

this study. In a work by Devi et al. (2010), on the evaluation of eugenol antimicrobial activity, they observed antibacterial potential on *S. typhi* (MIC=0, 0125%) during 1 hour of eugenol exposure.

The fungal activity of glycosidic derivatives of eugenol was presented by Orlandi et al., (2013), who described the synthesis of four eugenol derivatives, three of which demonstrated biological activity against several species of *Candida.* Therefore, and according to the literary data, this study demonstrated high antimicrobial potential, proving the bactericidal and fungicide effect of the essential oil of *P. dioica*.

Conclusions

The essential oil presented eugenol as the major component, being attributed to it the antimicrobial potential observed, thus encouraging the application of this essential oil in the control and combat of pathogenic microorganisms tested.

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