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Caracterização química, atividade antimicrobiana e toxicidade dos óleos essenciais da *Pimenta dioica* L. e *Citrus sinensis* L. Osbeck

Chemical characterization, antimicrobial activity and toxicity of essential oils of *Pimenta dioica* L. and *Citrus sinensis* L. Osbeck

Caracterización química, actividad antimicrobiana y toxicidad de los aceites esenciales de *Pimenta dioica* L. y *Citrus sinensis* L. Osbeck

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Resumo

Este estudo avaliou a toxicidade e a atividade antimicrobiana frente a *Escherichia coli e Staphylococcus aureus* dos óleos essenciais de *Pimenta dioica* Lindl. e *Citrus sinensis* L. Os óleos essenciais (OE's) foram extraídos por hidrodestilação, com caracterização química através de Cromatografia Gasosa acoplada a espectrometria de massas (CG-EM). Os parâmetros físico-químicos foram determinados de acordo com a Farmacopeia Brasileira. O ensaio de toxicidade seguiu o bioensaio com *Artemia salina Leach*, os OE's aprovados neste ensaio seguiram para avaliação das suas propriedades biológicas. A atividade antimicrobiana seguiu a metodologia descrita pelo Clinical and Laboratory Standards Institute utilizando o Método de Difusão de Disco, Diluição em Caldo para Concentração Inibitória Mínima (CIM) e posterior Concentração Bactericida Mínima para avaliar a ação dos OE's frente a *E. coli* e *S. aureus*. Ambos os OE's apresentaram toxicidade baixa, e assim foram avaliados quanto as propriedades biológicas antimicrobianas. Ambos os OE's apresentaram potenciais bactericidas frente aos microrganismos testados, exibindo resultados satisfatórios para a ação dos mesmos. Os resultados indicam que os OE's avaliados são compostos por substâncias que propiciam e incentivam sua aplicação em virtude de seus potenciais para atividade biológica antimicrobianas.

Palavras-chave: Óleos essenciais; Atividade antimicrobiana; Toxicidade.

Abstract

This study evaluated the toxicity and antimicrobial activity in the face of *Escherichia coli* and *Staphylococcus aureus* of essential oils of *Pimenta dioica* Lindl. and *Citrus sinensis* L. The essential oils (EOs) were extracted by hydrodistillation, with chemical characterization by gas chromatography coupled and mass spectrometry (GC-MS). Physicochemical parameters were determined according to the Brazilian Pharmacopeia. The toxicity test followed the bioassay with *Artemia salina* Leach, the EOs approved in this assay followed to evaluate its biological properties. The antimicrobial activity followed the methodology described by the Clinical and Laboratory Standards Institute using the Disc Diffusion Method, Broth Dilution for Minimum Inhibitory Concentration (MIC) and subsequent minimum bactericide concentration for to evaluate the action of EOs against *E. coli* and *S. aureus*. Both EOs showed low toxicity, and thus were evaluated for the biological antimicrobial properties. Both EOs presented bactericidal potential against the microorganisms tested, showing satisfactory results for their action. The results indicate that the evaluated EOs are composed of substances that provide and encourage their application due to their potential for antimicrobial biological activity.

Keywords: Essential oils; Activity antimicrobial; Toxicity.

Resumen

Este estudio evaluó la toxicidad y la actividad antimicrobiana contra *Escherichia coli* y *Staphylococcus aureus* a partir de los aceites esenciales de *Pimenta dioica* Lindl. y *Citrus sinensis* L. Los aceites esenciales (AEs) fueron extraídos por hidrodestilación, con caracterización química a través de cromatografía de gases acoplado a espectrometría de masas (GC-MS). Los parámetros fisicoquímicos se determinaron de acuerdo con la Farmacopea Brasileña. El ensayo de toxicidad siguió al bioensayo con *Artemia salina* Leach, la AE aprobada en este ensayo siguió para evaluar sus propiedades biológicas. La actividad antimicrobiana siguió la metodología descrita por el Instituto de Normas Clínicas y de Laboratorio utilizando el Método de Difusión de Discos, la Dilución de Caldo para la Concentración Mínima Inhibitoria (MIC) y la posterior Concentración Bactericida Mínima para evaluar la acción de las AE contra *E. coli* y *S. aureus*. Ambos AEs mostraron baja toxicidad, y por lo tanto fueron evaluados para propiedades biológicas antimicrobianas. Ambos AE presentaron posibles bactericidas contra los microorganismos analizados, mostrando resultados satisfactorios para su acción. Los resultados indican que las AE evaluadas están compuestas de sustancias que proporcionan y fomentan su aplicación debido a su potencial para la actividad biológica antimicrobiana.

Palabras clave: Aceites esenciales; Actividad antimicrobiana; Toxicidad.

1. Introduction

Essential oils (EOs) are complex mixtures of low molecular weight and water-insoluble volatile compounds extracted from different extraction techniques, such as distillation that includes steam drag distillation, cold pressing and maceration (Dima & Dima, 2015; Solórzano-Santos & Miranda-Novales,2012). These EOs are one of the most important groups of raw materials for the food, pharmaceutical, perfumery and related industries. In recent years, aromatic plants and their products have been evaluated for their efficacy in relation to food safety. Most of its properties are attributed to essential oils and other components of secondary plant metabolism, which has aroused interest in the food industry due to its antioxidant and antimicrobial activity (Calo et al., 2015, Kfoury et al., 2015).

Among several species of plants composed of EO in which these properties can be found are the *Pimenta dioica* Lindl. and *Citrus sinensis* (L.) Osbeck (sweet orange). *P. dioica*

is given greater prominence as a spice, but it is also widely used for the treatment of certain diseases because it has antihypertensive, anti-inflammatory, analgesic, antimicrobial and antioxidant properties (Paula et al., 2010). The EO of *C. sinensis* can be classified as a mixture of terpenes, hydrocarbons and oxygenated compounds, considered chemically unstable. Sweet orange EO consists of approximately 98% R-limonene and the remaining 2% refers to a mixture of other terpenes and alpheratic aldehydes (Galvão et al. 2015).

Over the last few years natural alternatives to synthetic products have been sought, natural products are an option with less toxicity compared to other synthetic products. Thus, the present study chemically characterized, evaluated the toxicity and antimicrobial activity of The EOs of *P. dioica* and *C. sinensis*, with the perspective of offering a natural alternative to the use of synthetic antimicrobials.

2. Methodology

2.1. Plant material

The sheets of *P. dioica* L. used in this study are recorded in the botanical archives of the Biodynamic Institute (IBD) of Botucatu according to a certificate in CA021205. The barks of *C. sinensis* L were recorded at the Federal Institute of Maranhão by the fruit and vegetable sector, such as D-25 (sweet orange, variation: pear).

2.2. Obtaining essential oils

For the extraction of the EO, the hydrodistillation technique was used with a glass Clevenger extractor coupled to a round bottom balloon packed in an electric blanket as a heat generating source. We used 30g of the dried leaves of *P. dioica* and 120g of the barks *C. sinensis*, adding distilled water (1:10). Hydrodistillation was conducted at 100°C for 5h and the extracted EO was collected. Each EO was dried by percolation with anhydrous sodium sulfate (Na2SO4) and centrifuged. These operations were performed in triplicates and samples stored in amber glass ampoules under 4°C refrigeration. Subsequently submitted the analyses.

The physicochemical parameters of the EOs were determined: density, solubility, color and appearance according to the Farmacopeia Brasileira (Farmacopeia Brasileira, 2019). The EO yield was expressed as a percentage in the mass/volume ratio by density

measurement.

2.3. Análises Químicas

The constituents of The EOs were identified by gas chromatography coupled to mass spectrometry (CG-MS) in the Analytical Center of the Institute of Chemistry of the State University of Campinas.

1.0 mg of the sample was dissolved in 1000 µL of dichloromethane (purity 99.9%). The conditions of analysis were as follows: Method: Adams. M, 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^{-1}; Gun: 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 : EM; EI (70 eV); Scan mode (0.5 sec scan^{-1}); 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.

2.4. Toxicity

For the evaluation of the lethality of *Artemia salina* Leach, a stock saline solution of each EO was prepared at the concentration of 10,000 mg L^{-1} and 0.02 mg of Tween 80 (active tense). Aliquots of 5, 50 and 500 µL of this were transferred to test tubes and completed with saline solution previously prepared up to 5 mL, obtaining concentrations of 10, 100 and 1000 mg L^{-1}, respectively. All tests were performed in triplicates, where ten larvae in the nauplium phase were transferred to each of the test tubes.

For white control, 5 mL of saline solution was used for positive control K2Cr2O7 and for negative control 5 mL of a solution 4 mg L^{-1} of Tween 80. After 24 hours of exposure, the live larvae were counted, considering those that did not move during observation or with the slight agitation of the vial.

The criterion established by Dolabela (1997) was adopted for the classification of the toxicity of The EOs, being considered highly toxic when CL50 ≤ 80 mg L^{-1}, moderately toxic to 80 mg L^{-1} ≤ CL50 ≥ 250 mg L^{-1} and mildly toxic or nontoxic when CL50 ≥ 250 mg L^{-1}.

2.5. Standardization of microbial inoculum for sensitivity tests

Two strains of bacteria were used: *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). These were previously identified and confirmed by biochemical tests.

Pure microbial cultures maintained in TSA agar were peaked for brain and heart infusion broth (BHI) 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 1.5 x 108 CFU mL^{-1} according to the standards of the Clinical and Laboratory Standards Institute (CLSI, 2020).

2.6. Disk Diffusion Method (DDM)

The disc diffusion technique was performed according to the Clinical and Laboratory Standards Institute (CLSI,2020), which standardizes the sensitivity tests of antimicrobials by disc-diffusion. First, the plates were prepared with the Culture Medium Mueller Hinton Agar (AMH) after its solidification was distributed to microbial suspension on the surface of the agar and left at room temperature for 30 min. Soon after the discs containing 50 µL of the EOs and the discs with defined concentrations of antibiotics are prepared. 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. 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 carried out in triplicate.

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

The Minimum Inhibitory Concentration (MIC) assay was performed using the broth dilution technique, proposed by the Clinical and Laboratory Standards Institute (CLSI,2020). First, 2% solutions were prepared using dimethylsulfoxide (DMSO), and serial dilutions were prepared in MH Broth, resulting in concentrations of 10 to 1000 µg mL^{-1}. Microbial suspension containing 1.5 x108 CFU mL^{-1} of the *E. coli* and *S. aureus* strains was added to

each concentration. The tubes were incubated at 35º 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 Concentration (MBC) 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 (AMH) with subsequent incubation at 35°C for 24h. The MBC 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.

3. Results and Discussion

3.1. Physicochemical properties

The physicochemical parameters of The EOs are important not only for quality determination, but also for the control of their purity and these are presented in Table 1. It is observed that the EO of *C. sinensis* obtained a yield of 2.47% higher than the EO of *P. dioica* of 1.80%. By individually comparing the yield of EO of *C. sinensis* to the results obtained by Silva et al., (2016) who extracted the EOs from the peel of dried and fresh fruits, the authors perceived their yield ranging from 1.80 to 2.00%, and this study obtained a yield of +0.47% above the maximum yield obtained by the authors, since the density of the same authors ranged from 0.8480 to 0.8490 g mL^{-1} , density that is similar to this work in a variation of +0.0010 g mL^{-1}.

Table 1: Physicochemical parameters of Eos.

Physicochemical parameters	*P. dioica*	*C. sinensis*
Density (g mL^{-1})	0,9820	0,8500
Refractive index (nD 25°)	1,5185	1,4760
Alcohol solubility at 70% (v/v)	1:3	1:3
Color	Amarelo	Incolor
Appearance	Límpido	Límpido
Yield (%)	1,80	2,47

Source: Authors.

Even the EO yield of *P. dioica* being lower than the yield of *C. sinensis* it is important to emphasize that for EOs yields above 1.5% are of extreme significance. In a study conducted by Voris et al., (2017) when extracting this same EO from the fruit acquired in a retail market in Rio de Janeiro (RJ), the authors employed a period of 4 h in their hydrodistillation, but their maximum yield was 1.60%, compared to the current study that used a shorter hydrodistillation time (3h-100°C) and obtained a yield of 1.80% using a regenerative part of the plant , becomes of utmost importance and significance for visualizing its application potential.

Comparing the values for the EO studied with those of the literature, it can be observed that there was a similarity between them, with regard to the parameters analyzed. The small differences in the values found can be attributed to factors such as collection time, different soil types, storage conditions and time (Costa et al., 2012). It is important to emphasize the yield of 2.47% for the EO of *C. sinensis* that was observed in results higher than the literature, encouraging its production due to the use of barks that are commonly discarded in public fairs or local neighborhoods of São Luís-MA.

3.2. Chemical characterization of essential oils

Chromatographic peaks were identified by comparing the respective mass spectra with data from the wiley 139 spectrothecae (1); (2) NIST107 and (3) NIST21. According to the results obtained, Table 2 presents the compounds identified in the EO extracted from the barks of *C. sinensis* and in Table 3 the compounds identified in the EO extracted from the leaves of *P. dioica*.As can be seen in Table 2, 15 components were identified in the EO sample of *C. sinensis*, being the majority constituent of EO d-limonene with 81.50% of the composition, followed by linalool (6.36%) and β-mircene (2.95%).

The chemical compound d-limonene is confirmed as the main constituent of EO by Araújo et al., (2016) that by extracting it from the fruit peels of *C. sinensis* from the local market of Aracaju, Sergipe performed its chemical characterization through GC/MS and noticed the presence of the constituent in 91.88% of its sample. Results similar to this study are also reported by Martins et al., (2017) that when performing the chemical characterization of commercial EOs of the genus Citrus, observed the presence of d-limonene in 83.33% of the composition of the EO of *C. sinensis*.

Table 2: Chemical constituents in the EO sample of *C. sinensis.*

Order	RT (min.)	Constituents	(%)
1	5,155	α-pinene	0,33
2	6,350	β-myrcene	2,95
3	6,861	octanal	1,93
4	7,610	d-limonene	81,50
5	8,287	1, octanol	0,46
6	8,919	linalool	6,36
7	8,959	nonanal	1,08
8	9,866	cytronelal	0,06
9	10,523	terpineol	0,12
10	10,873	α-terpineol	1,39
11	10,926	decanal	0,25
12	11,352	β-cytronelol	0,08
13	11,643	neral	1,13
14	12,210	cytral	1,17
15	12,496	1, ciclohexene	1,20

Source: Authors.

D-limonene is a relatively stable terpene that has applications in the literature for the development of plant bioproducts (Granja et al., 2015). The EOs of the genus Citrus have this component as the majority in its composition and properties as antimicrobial activity can be proven by Rodrigues (2019), but when we portray *C. sinensis* its bactericidal potential has been little studied, and many studies have been reported in relation to its antimicrobial and larvicidal action (Rodríguez et al., 2017; Araújo et al., 2016). Thus, it is observed that the EO of *C. sinensis* has the potential to explore its bactericidal activity in this study, being of vital importance for the state and for the country a natural product obtained through the part of a vegetable that is commonly discarded or surface applications.

As can be seen in Table 3, 07 components were identified in the sample, with the majority constituent of EO being eugenol with 85.673%, followed by chavicol (6.79%) and myrcene (2.76%).

Eugenol content (85.67%) reported in this study becomes significant when compared with Oliveira et al. (2009) who extracted the EO from the leaves of *P. dioica* collected in Minas Gerais also observed that eugenol as the major constituent, but the observed content was 44.9%. Another fact reported was the presence of limonene in 10.1% of the composition and the chavicol being exhibited in a content of 7.5%. This composition may also be linked to the lower yield of 0.49% obtained by Oliveira (2017), although it is emphasized that the

authors used an extraction time of 4 h.

Table 3: Chemical constituents identified in *P. dioica* EO samples.

Order	RT (min.)	Constituents	(%)
1	8,772	octenol	1,19
2	9,164	myrcene	2,76
3	10,488	limonene	1,73
4	13,251	linalool	0,88
5	16,122	terpineol	0,97
6	19,026	chavicol	6,79
7	22,755	eugenol	85,67

Source: Authors.

Similar results were reported by Oliveira et al. (2009) using plants from southern Bahia state collected in 2006, where they obtained eugenol (75.07%) as the majority consituinte of its EO sample of *P. dioica* leaves, but different from this secondary component study of the authors was myrcene with 8.19% and chavicol was followed by 6.35%.

Eugenol is an extraordinarily versatile molecule and has been included as a spicy aroma in ice cream, bakery products and sweets in restricted concentrations, mouthwashes, pharmaceutical and dental preparations (Oliveira et al., 2009; Padmakumari et al., 2011; Martinez-Velazquez et al., 2011). In addition to having biological properties proven by Kamatou et al., (2012), thus it is vitally important to study the EO extracted from *P. dioica* as a significant natural source of eugenol for both biological applications and industries in general.

3.3. Toxicity

Table 4 presents the Lethal Concentrations 50% referring to the action of the EOs against *Artemia salina* L. and its subsequent classification according to the criterion Dolabela (1997).

Table 4: Lethal Concentration 50% for Action of EOs against *Artemia salina* L.

EO	LC_{50}	Classification
P. dioica	141,3 mg/L	Moderately toxic
C. sinensis	511,6 mg/L	Nontoxic

Source: Authors.

Lethal Concentration 50% (LC50) refers to the point where the number of surviving animals is equal to the number of dead animals, and following the dolabela criterion (1997) it is possible to determine the toxicity of natural products aiming at a specific application of the agent in the target organism, since oils with high toxicity are not recommended for biological applications.

Table 4 shows that none of the oils were classified as toxic, so their applications can be relatively acceptable and encouraged. Thus, antimicrobial activity assays were initiated. It is important to highlight that the EO of *C. sinensis* extracted from fruit bark so far has a significant yield and chemical components of biological importance and in this toxicity assay presents the LC50 of 511.6 mg L^{-1}, well above the criterion that was only 250 mg L^{-1} to be classified as nontoxic. Therefore, this EO has its application potential again encouraged.

It is important to emphasize that studies related to the toxicity of natural products are of vital importance for biological applications and studies in the literature do not yet disclose toxicity of the plants under study in a specific test such as the bioassay against *Artemia salina*.

3.4. Antimicrobial activity

The results regarding the tests to determine antimicrobial activity are presented in Table 5. All oils showed antimicrobial activity against *E. coli* and *S. aureus*.

Tabela 5: Inhibition Halos (IH), Minimum Inhibitory Concentration (µg/mL) and Minimum Bactericidal Concentration (µg/mL) of Eos.

		EO *P. dioica*			EO *C. sinensis*		
	GEN 30 µg (mm)	**IH (mm)**	**MIC (µg mL^{-1})**	**MBC (µg mL^{-1})**	**IH (mm)**	**MIC (µg mL^{-1})**	**MBC (µg mL^{-1})**
E. coli	**25**	**15**	**25**	**50**	**15**	**75**	**100**
S. aureus	**27**	**25**	**10**	**25**	**30**	**10**	**25**

Source: Authors.

When observing Table 5, we noticed that the EO of *P. dioica* was more efficient in inhibiting the bacterium *S. aureus* by the Disc Diffusion Method if we compare its 25 mm halo with the 15 mm halo resulting from the action of the oil against *E. coli*. Both inhibition halos allow classifying bacteria as sensitive by the criterion established by Moreira et al., (2005). The Minimal Inhibitory Concentration assay revealed that *P. dioica* EO inhibits microbial growth of *E. coli* from 25 µg mL^{-1} and *S. aureus* from 10 µg mL^{-1}.

The study by Oliveira (2017), where the author reports the activity of the same oil with a MIC of 5 µg mL^{-1} for *E. coli* and 20 µg mL^{-1} for *S. aureus* portrays the difference in this study where the same oil obtained in two different locations have different properties, since the author's oil was more efficient against E.coli than *S. aureus* , whereas in this study there was the inverse of that observed by the author.

In a recent study by Lorenzo-Leal et al., (2019) in Pueba, Mexico, used the microwell technique to determine the Mic of The EO of *P. dioica* extracted from commercialized fruits and unlike this study did not observe oil activity against E.coli and obtained an extremely high MIC of 2000 µg mL^{-1} for *S. aureus*, which invigorates the satisfactory results obtained in this study , where for *S. aureus* we obtained a MIC of 10 µg mL^{-1}.

By noting Table 5 we can see that the EO of *C. sinensis* was also more efficient in inhibiting the bacterium *S. aureus* by the Disc Diffusion Method if we compare its 30 mm halo with the 15 mm halo resulting from the action of the oil against *E. coli*. Both inhibition halos allow classifying bacteria as sensitive by the criterion established by Moreira et al., (2005). The Minimal Inhibitory Concentration assay revealed that *P. dioica* EO inhibits microbial growth of *E. coli* from 50 µg/mL and *S. aureus* from 10 µg mL^{-1}. It is important to highlight that antimicrobial studies in Brazil with the species *C. sinensis* are relatively new, highlighting the importance of the study to obtain a natural product with biological potential

obtained from a commonly discarded part of a fruit of high local consumption for both the state and the country.

Thus, the results obtained were compared to those of the authors Eldahshan & Halim (2016) who extracted the EO from the leaves of *C. sinensis* collected in Egypt performing a hydrodistillation for 5 h. The authors obtained a halo of 20.1 mm for the action of The EO against *S. aureus* and a similar result of 16.2 mm against *E. coli*, since in this study we obtained a halo of 15 mm using the EO obtained from the bark.

Eldahshan & Halim (2016) still emphasize that this oil had this activity due to the presence of oxygenated compounds in its composition. The authors highlight the potential of EO to be used as antibacterial additives in food and cosmetic products in order to reduce dependence on synthetic food preservation chemicals (Eldahshan & Halim,2016). Finally, we highlight again the biological potential of both species studied in this work as extremely efficient in the control of pathogenic microorganisms, represented by *E. coli* as Gram-negative and *S. aureus* as Gram-positive.

4. Final Considerations

Through the results obtained in the chemical studies, in the evaluation of the toxicity and antimicrobial of The EOs of *P. dioica* and *C. sinensis*, it is concluded that the evaluated EOs are composed of substances that provide and encourage their application due to their potentials for antimicrobial biological activity.

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