Research, Society and Development, v. 12, n. 1, e6212139382, 2023
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v12i1.39382

Fungicidal activity of the nanoemulsion (o/w) of *origanum vulgare* essential oil

Atividade fungicida da nanoemulsão(o/a) do óleo essencial de *origanum vulgare*

Actividad fungicida de la nanoemulsión (o/w) del aceite esencial de *orígánum vulgare*

Recebido: 15/12/2022 | Revisado: 28/12/2022 | Aceitado: 29/12/2022 | Publicado: 01/01/2023

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Abstract
This study aimed to evaluate the fungicidal activity of the nanoemulsion (O/W) of the essential oil of *Origanum vulgare*. *Origanum vulgare* leaf samples were collected in the morning in the city of São Luís (MA). To extract the essential oil, the hydrodistillation technique was used. The essential oil constituents were identified by Gas Chromatography Coupled to Mass Spectrometry (GC-MS). Nanoemulsions were prepared by phase inversion method. The antimicrobial activity was performed by the disk diffusion technique according to the Clinical and Laboratory Standards Institute (2020). According to the GC-MS, 25 chemical constituents were identified, the majority being carvacrol corresponding to 66.36%. The formulated nanoemulsion was characterized as an oil-in-water nanoemulsion, and also evaluated as a stable formulation. The nanoemulsion of *Origanum vulgare* showed fungicidal activity against the fungi tested and its best result was a potent anti-candida efficacy. Finally, the results obtained indicate great potential for the use of essential oils in the formulation of nanoemulsions used in this study as fungicidal agents.
Keywords: Fungi; Nanoemulsion; Essential oil.

Resumo

Este estudo teve por objetivo avaliar a atividade fungicida da nanoemulsão (O/A) do óleo essencial de *Origanum vulgare*. As amostras de folhas de *Origanum vulgare*, foram coletadas no horário matutino no município de São Luís (MA). Para extração do óleo essencial, utilizou-se a técnica de hidrodestilação. Os constituintes do óleo essencial foram identificados por Cromatografia Gasosa Acoplada à Espectrometria de Massas (CG-EM). As nanoemulsões foram preparados por método de inversão de fases. A atividade antimicrobiana foi realizada pela técnica de difusão em disco de acordo com o Clinical and Laboratory Standards Institute (2020). De acordo com a CG-EM foram identificados 25 constituintes químicos, sendo majoritário carvacrol correspondendo a 66,36%. A nanoemulsão formulada foi caracterizada como uma nanoemulsão de óleo em água, e também avaliada como uma formulação estável. A nanoemulsão de *Origanum vulgare* mostrou atividade fungicida frente aos fungos testados e seu melhor resultado foi uma potente eficácia anti-candida. Por fim, os resultados obtidos apontam grande potencial para a utilização dos óleos essenciais na formulação das nanoemulsões utilizadas neste estudo como agentes fungicidas.
Palavras-chave: Fungos; Nanoemulsão; Óleo essencial.

Resumen

Este estudio tuvo como objetivo evaluar la actividad fungicida de la nanoemulsión (O/W) del aceite esencial de Origanum vulgare. Las muestras de hojas de Origanum vulgare fueron colectadas en la mañana en la ciudad de São Luís (MA). Para la extracción del aceite esencial se utilizó la técnica de hidrodestilación. Los componentes del aceite esencial se identificaron mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS). Las nanoemulsiones se prepararon por el método de inversión de fase. La actividad antimicrobiana se realizó mediante la técnica de difusión en disco según el Clinical and Laboratory Standards Institute (2020). Según el GC-MS se identificaron 25 constituyentes químicos, siendo la mayoría carvacrol correspondiente al 66,36%. La nanoemulsión formulada se caracterizó como una nanoemulsión de aceite en agua y también se evaluó como una formulación estable. La nanoemulsión de Origanum vulgare mostró actividad fungicida frente a los hongos ensayados y su mejor resultado fue una potente eficacia anti-cándida. Finalmente, los resultados obtenidos indican un gran potencial para el uso de aceites esenciales en la formulación de nanoemulsiones utilizadas en este estudio como agentes fungicidas.
Palabras clave: Hongos; Nanoemulsión; Aceite esencial.

1. Introduction

Since the most remote times, man has aimed to promote the agreement that facilitates his relationship with the environment to reach and supply all aspects that involve his needs for survival, well-being and cures for possible diseases, having as object and tool, plants with herbal resources (Elias et al., 2022).

The need for plant-based products, with the healing properties of plants, has become increasingly common and relevant, adapting to contemporary medicine as valuable products to combat antimicrobial activity, antioxidants, among others. The knowledge discussed has as its main contribution to the culture passed on from generation to generation, combining the knowledge of the oldest with the rigor of the scientific method (Nogueira et al., 2022).

It is noteworthy that, with the progressive development of large pharmaceutical industries, the role of herbal medicines is still essential, having by definition those that have the ability to synthesize active principles that contribute to the proper functioning of the human organism, restructuring the balance of reactions t(Nogueira et al., 2022).hat govern the activities of the body in cases of illness, and thus are shown to be an instrument for the treatment of various diseases that can cause the imbalance of the human organism (Alves et al., 2021; Da Silva et al., 2021).

In addition, with the continued indiscriminate and intense use of antimicrobials, both in the field of human and animal physiology, high growth rates have been observed in terms of resistance to antibiotics, antifungals and antiparasitics, which make traditional forms of treatment more difficult. in relation to these pathogens, causing problems both in the health and economic spheres (Rodrigues et al., 2022).

In this way, with the increase in the market in terms of the use of natural products, studies for the application of essential oils are becoming increasingly susceptible due to the presence of secondary metabolites, present in plants that guarantee their action against these microorganisms (Villaverde et al., 2016).

Some recent studies point out that essential oils confirm the potential for the action of these chemical constituents in favor of biological activities, with particularity in combating and controlling phytopathogenic fungi (Jing et al., 2018).

Recently, in recent decades there has been greater attention to its antibacterial, antifungal, insecticidal and antioxidant activities, among the OE's stand out: *Origanum vulgare, Cymbopogon citratus, Thymus vulgaris, Pelargonium graveolens, Cinnamomum zeylanicum, Eugenia caryophyllata*, these being attested as antifungal agents to numerous strains tested. The antimicrobial activity is mainly due to a constituent considered to be the majority of EOs, or else the synergistic effect is linked to all constituents that are part of its composition (Carvalho et al., 2021; Xie et al., 2017; De Paula et al., 2021). In this sense, the present study aimed to identify the constituents present and evaluate the antifungal activity of the nanoemulsion (O/W) of the essential oil *Origanum vulgare*.

2. Methodology

2.1 Collection of plant material

Eucalyptus globulus leaf samples were collected in the morning in the city of São Luís. After collection, plant samples were transported to the Laboratory for Research and Application of Essential Oils (LOEPAV/UFMA), where they were weighed, crushed and stored for essential oil extraction.

2.2 Essential oil extraction

For essential oil extraction, the hydrodistillation technique was used with a glass Clevenger extractor coupled to a round bottom flask placed in an electric blanket. 100 g of crushed plant material were used, adding distilled water (1:10). Hydrodistillation was carried out for 3 hours at 100°C and the extracted essential oil was collected and dried by percolation with anhydrous sodium sulfate (Na_2SO_4). These operations were performed in triplicate and the samples stored under 4°C refrigeration. Subsequently, submitted to analysis.

2.3 Gas chromatography coupled to mass spectrometry

The essential oil constituents were identified by Gas Chromatography Coupled to Mass Spectrometry (GC-MS). 1.0 mg of the sample was dissolved in 1000 µL of dichloromethane (99.9% purity).

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 (30m x 0.25 mm x 0.25 µm); Carrier gas: He (99.9995); 1.0 mL.min $^{-1}$; Injector: 280ºC, Split mode (1:10); Oven: 40 ºC (5.0 min.) to 240 ºC at a rate of 4ºC min $^{-1}$, from 240ºC to 300ºC (7.5 min) at a rate of 8ºC.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 type mass spectrometer. For the identification of the compounds in the sample, the program AMDIS (Automated Mass spectral Deconvolution Mass & Identification System).

2.4 *Preparation of nanoemulsions (o/w)*

The preparation of nanoemulsions was carried out according to the adapted methodologies described by Lima et al. (2021), Sugumar et al. (2014), Kubitschek et al. (2014) and Rodrigues et al. (2014). The oil-in-water nanoemulsion was formulated with each oil, nonionic surfactant (tween 20) and water. The required amounts of each oil phase constituent (oil+Tween20) were heated to 65 ± 5 °C. The aqueous phase was separately heated to 65 ± 5 °C, providing a primary formulation, by the phase inversion method.

To prove stability, the formulated emulsion was subjected to different stress tests (Shafiq et al., 2007). Heating-cooling cycle: it was carried out by keeping the formulated nanoemulsion at 40 and 4 °C, alternating each temperature for 48 h. The cycle was repeated three times. Freeze-thaw stress: nanoemulsion alternatively at -21 and 25 °C for 48 h at each temperature. The cycle was repeated three times. Formulations that passed thermodynamic stress tests were taken for further study.

2.5 Disc Broadcast

The antimicrobial activity was performed by the disk diffusion technique according to the *Clinical and Laboratory Standards Institute* (2020), which standardizes antimicrobial susceptibility tests by disk-diffusion, using standardized suspensions of microorganisms distributed on plates containing Mueller Hinton Agar culture medium (AMH, 2% methylene blue) plus disks containing 50 µL of nanoemulsions. As a positive control, Gentamicin (30 µg) was used. The plates were incubated in a bacteriological oven at 27°C/24-48 h, then the reading was performed, measuring the inhibition halos with the aid of a ruler. The entire test was performed in triplicate.

2.6 Inhibitory Concentration (MIC) and Minimum Fungicide (MCM)

MIC and CFM were evaluated in this assay for the action of all nanoemulsion formulations. The Minimum Inhibitory Concentration (MIC) assay was performed using the broth dilution technique, proposed by the Instituto de Normas Clínicas e Laboratoriais (CLSI, 2020). First, solutions were prepared using 2% dimethylsulfoxide (DMSO), and serial dilutions were prepared in RPMI broth for the fungal assay, resulting in concentrations from 10 to 1000 µ g mL $^{-1}$.

Fungal suspensions containing 1.5 x 10 ^{8} CFU mL $^{-1}$ of the strains were added. Tubes were incubated at 27°C for 24-48h for fungal strains. Sterility and growth controls were performed for the test performed. After the incubation period, the MIC was verified, being defined as the lowest concentration that visibly inhibited fungal growth (absence of visible cloudiness). Tests performed in triplicate.

For the Minimum Fungicide Concentration (MFC) assay, a dilution rate of 100 µL of the RPMI broth was used, which visibly inhibited microbial growth. Aliquots were inoculated into AMH (2% methylene blue) with subsequent incubation at 35°C for 24 h. CFMs were determined as the lowest concentration that visually in the CIM assay showed inhibition of growth and that in the cultures for the fungicide assays also did not show microbial growth.

3. Results and Discussion

3.1 Chemical constituents

According to the GC-MS, 25 chemical constituents were identified, the majority being carvacrol corresponding to 66.36%. In view of the certified data, similar values were observed when compared to the study Romero et al. (2012), evaluated by Gas Chromatography coupled to Mass Spectrometry (GC/MS) analysis, in which five major constituents present in the EO of the species *Origanum vulgare* L. were determined, among them are 4-terpineol (8.3%), linalyl acetate (2.0%), thymol (32%), carvacrol (50%) and spathulenol (4.1%), respectively.

It is evident that carvacrol is classified as a phenolic monoterpene, since this constituent present in the chemical composition of essential oils, gives them the characteristic of being a promising antifungal drug (Sousa et al., 2021).

In this sense, results were also obtained by Corrêa et al. (2017) in relation to the EO of the plant *Origanum vulgare L*, determined by analysis Gas Chromatography coupled to Mass Spectrometry (GC/MS), in which 28 chemical components were identified, being named as major secondary compounds 4-terpineol (24, 92%), carvacrol (19.67%), γ-terpinene (11.82%), 4 thujanol (8.31%) and α-terpinene (7.32%) respectively. It is noted that there is significant variation in the quantity of the

oxygenated monoterpene carvacrol, believing that it can be explained by factors such as genetic variation, severe climate changes, geographical and seasonal distribution of the plant or even factors linked to the method of obtaining the EO (Rebey et al., 2012).

Another study that contributes to this essay was obtained by Khan et al. (2018), through Gas Chromatography coupled to Mass Spectrometry (GC/MS) analysis, confirmed the following major constituents: carvacrol (70.2 ± 1.37%), terpinene (5.6 ± 0.11%), p-cymene (4.5 ± 0.42%), trans-sabinene hydrate (3.8 ± 0.07%) and thymol (2.2 ± 0.12%). In this way, it can really be proven that the EO of the species *O. vulgare* has carvacrol as its major constituent.

It is always important to emphasize, even though there are studies that establish the constituent carvacrol as an excellent antifungal agent, as is the case of the essay by Sousa et al. (2021), it is necessary to consider that the constituents found by (GC-MS) through this test, since they help in the synergistic action of the OE *O. vulgare*, otherwise it would be prudent to carry out individual evaluations in favor of each chemical component present Plant EO.

3.2 Fungicidal activity

Table 1 presents the results obtained for the action of nanoemulsions against the tested microorganisms.

Table 1 - Diameter of the inhibition halos (mm), Minimum Inhibitory Concentration (μg mL^{-1}) and Minimum Fungicide Concentration (μg mL^{-1}) of the nanoemulsion.

microorganisms	**CET (50 μg)**	**IHL (mm)**	**MIC (μg mL^{-1})**	**CFM (μg mL^{-1})**
C. albicans	**29**	26.29	315.45	400.68
Fusarium sp.	**28**	19.69	335.97	533.25
***Penicillium* sp.**	**30**	22.88	282.51	444.6
***Aspergillus* sp.**	**25**	16.94	367.11	754.92

Source: Authors.

According to Table 1, *E. globulus essential oil* showed its best result in a potent anti-candida efficacy, indicating that the oil is a potential candidate for mouthwash applications (Emira et al., 2010).

E. globulus essential oil possess antifungal activity against *C. albicans*, which were the main microorganisms responsible for initiating fungal infections globally (Quatrin et al., 2017). These findings were due to the enhanced functionality by nanoencapsulation of the essential oil through protection of the essential oil components, as well as the reduced size of the nanoemulsions which resulted in rapid penetration.

Few studies have been carried out on the impact of *E. globulus leaves* as antifungal agents in the eradication of antibiotic-resistant fungal infections, which is a major challenge worldwide, for example, in the case of *Candida* spp. and other species of fungi. Furthermore, due to the increasing number of cases infected by *Candida* spp. and the increased risk of drug resistance, the discovery of a new therapeutic strategy is considered of fundamental importance (Shala & Gururani, 2021).

The different species of *Candida* are listed as the fourth leading cause of bloodstream infections in the hospital environment, with a mortality rate of approximately 30%-60%, generally presenting a poor prognosis (Flevari et al., 2013; Yapar et al., 2014).

The induced antifungal efficacy has been attributed to the richness of plant parts with diverse phytochemical constituents that represent a cost-effective approach to combating fungal infections (Shala & Gururani, 2021).

Essential oils as pathogen control agents have two main characteristics: first, they are safe for people and the environment and, second, they provide less development of resistance by the pathogen, due to the mixture of essential oil components that, apparently, have different mechanisms of antimicrobial activity (Feng & Zheng, 2007; Derbalah et al., 2011).

Investigations on disease suppression mechanisms by plant products suggested that the active principles present in essential oils can act directly on the pathogen or induce systemic resistance in host plants, resulting in reduced disease development (Sallam, 2011, Nashwa & Abo-Elyousr, 2013).

4. Conclusion

Finally, it is stated that in view of the chromatographic analysis, the major constituent, carvacrol (66.36%), can be attested, which is responsible for the synergism and contributing to antifungal activity, the results obtained point to great potential for the use of essential oils in the formulation of nanoemulsions used in this study as fungicidal agents, since all strains showed a sensitivity profile. In addition, it appears from the observed data that there is a possibility of being applied as a possible alternative to conventional products commercially available, since the highlighted product is shown to have relatively low levels of toxicity and affordable price, leading to larvicidal activities and bacterial, thus qualifying it as promising for biological activities.

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