POTENCIAL ANTIFúngICO E MODULADOR DA RESISTÊNCIA FúngICA DO ÓLEO ESSENCIAL DE Cordia verbenacea DC. EXPOSTO A IRRADIAÇÃO DE MICRO-ONDAS.

RESUMO

Cordia verbenacea, conhecida como “erva-baleera”, é utilizada popularmente no combate a doenças infecciosas e inflamatórias. O presente estudo tem por objetivo verificar o potencial da atividade antifúngica e moduladora do óleo essencial das folhas de Cordia verbenacea DC. (OECv) e sua função biológica após exposição a irradiação micro-ondas. Trata-se de um estudo experimental, de caráter qualitativo e quantitativo, onde os resultados foram submetidos à análise estatística. As linhagens utilizadas foram padrões e resistentes de C. albicans, C. tropicalis e C. krusei e o antifúngico utilizado foi o cetoconazol. Os testes antifúngicos e modulatórios foram realizados pelo método de microdiluição em placas e o efeito da irradiação micro-ondas através da exposição ao forno micro-ondas em diferente período de tempos pré-estabelecidos na potência máxima. Os testes foram realizados em triplicadas e submetidos à análise estatística 2way ANOVA e com testes de significância através de posttests Bonferroni. Após realização dos testes, os resultados em relação à ação antifúngica e efeito modulador da resistência fúngica quando realizado a exposição do óleo essencial de Cordia verbenacea DC. a irradiação micro-ondas, demonstraram que OECv apresentou efeito fungistático nas diferentes combinações do óleo testado. Quanto ao efeito modulador se observou que OECv sem exposição ao micro-ondas apresentou melhor resposta antifúngica. Portanto, os dados obtidos apresentaram informações relevantes sobre o potencial antifúngico e sugerem novos estudos e tests para melhor compreensão dos mecanismos de ação e assim, uma alternativa de utilização associada de produtos naturais.


ABSTRACT

Cordia verbenacea, known as “whiting”, is popularly applied in the fight against infectious and inflammatory diseases. The present study aims to verify the potential of the antifungal and modulatory activity of the essential oil of the leaves of Cordia verbenacea DC. (OECv) and its biological function in exposure to microwave irradiation. This is an experimental, qualitative and quantitative study, where the results were analyzed by statistical analysis. For example, the patterns and strengths of C. albicans, C. tropicalis and C. krusei and the antifungal were ketoconazole. The antifungal and modulable tests were performed by the method of microdilution in plates, and the

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effect of the microwave radiation through exposure to the microwave oven in different time periods pre-established in the maximum condition. The tests were performed in triplicates and submitted to the 2way ANOVA statistical analysis and with significance tests through Bonferroni post-tests. After completion of the tests, the results in relation to the antifungal action and the fungal resistance modulator effect, when held the exhibition the essential oil of *Cordia verbenacea* DC, to the microwave irradiation, showed that OECv presented fungistatic effect in different combinations of tested oil. In place of the modulator effect, noted that without exposure to microwave OECv presented best antifungal response. Therefore, the data presented relevant information about the potential antifungal and suggest new studies and tests to better understand the mechanisms of action and thus an alternative use of natural products.

**Key words:** fungal infections, microwaves, modulation, medicinal plants.
INTRODUCTION

The pharmacological research related to medicinal plants has increased due to the empirical knowledge of populations that use them continuously. A large number of Brazilian populations apply the plants as the only therapeutic measure. Factors such as low level of life of the population and the high cost of drugs influence, keeping the usual practice of consumption of herbal medicines, making valid certain information therapies that have been accumulated over the centuries.

Population considers a phytotherapeutic plant when this proves effective in the relief of a symptom, prevention or treatment of a disease. What is not clear is if its use is suitable for treating the pathology or ill-being desired or if the plant is used as a remedy will have a better effect or will be more powerful than other conventional medicine and if there is sufficient security for general use.

The species Cordia verbenacea DC. (Boraginaceae) is an erect shrub, highly branched, with vigorous growth and spontaneous, presents small and white flowers arranged in terminal inflorescences, born in almost all the regions of Brazil, particularly in the Brazilian coast. Popularly known as whiting, nantgarw-baron, nantgarw-mulatto, salicin and marly-nightshade is widely applied in folk medicine because their healing and anti-inflammatory properties, but also for the treatment of arthritis, rheumatism, tendonitis, muscle aches and bruises.

Studies show that this plant contains anti-inflammatory substances that are classified as a safe and effective alternative in the topical treatment of inflammations due to its effect and reduction of side effects. There is also a high concentration of secondary metabolism in leaves of various plants, such as essential oils and flavonoids, which have antioxidant and anti-inflammatory effects.

A growing and important public health problem are fungal infections that has gradually increased and the immunosuppression or alteration of the anatomical barriers are risk factors for these infections by encouraging the entry of microorganisms in the host.

The fungus Candida albicans is normally found in the mucosa of the gastrointestinal and genitourinary tracts, in 30 to 60% of the population, which is in equilibrium with the bacterial flora and the immune system of the host. Particularly in Brazil, Candida tropicalis presents with frequency, constituting the main publications as one of the main causes of candidemia. Candida krusei is recognized by its potential of multidrug resistance (MDR), because of its relation to resistance to fluconazole associated with reports of suppression of flucytosine and susceptibility to amphothericin B.

Information relating to the fungal resistance to these yeasts makes this genre is increasingly focus of study directed to antimicrobial activity. The high rate of resistance highlights the need for the development of alternative strategies to prevent its spread among the fungi, as has already occurred with the bacteria.

The microwave radiation is a type of electromagnetic energy with a frequency in the range of $10^3$ to $10^6$MHz. Being a non-ionizing radiation, which causes migration of ions and rotation of the dipoles, but did not cause changes in the molecular structure.

The principles involved in heating by microwave, involves chemical concepts, such as temperature, heat capacity, chemical bonding, molecular structure, dipole moment, polarization, dielectric constant among others. In this way, we are faced with the question about the pharmacological potential of Cordia verbenacea DC, specifically regarding the antifungal effect and modulator with drugs, as well as the possibility of indifference the associations of natural products and exposure to microwave irradiation.

Therefore, the present study arose from the need to find new alternatives for the treatment in the reduction or elimination of microorganisms, in addition to the interest in checking the inhibitory potential and specific action to antifungal lineages, showing to be relevant to a possible development of an alternative tool in combating microbial resistance. In addition, the objective of this work was to evaluate the potential of modulating and antifungal activity of the essential oil from the leaves of Cordia verbenacea DC. and its biological function exposed to microwave irradiation.

METHOD

SELECTION AND COLLECTING OF PLANT MATERIAL

The leaves of Cordia verbenacea DC. were collected in August 2017, in the Chapada do Araripe, municipality of Crato-Ceará, Brazil and a specimen of the species was deposited at the Herbarium Prisco Bezerra of the Federal University of Ceará, under the N 044171.

ESSENTIAL OIL EXTRACTION

The extraction of the essential oil of fresh leaves of Cordia verbenacea DC. (OECv) was performed by the method of hydrodistillation using a Clevenger type apparatus, where the leaves were crushed and placed in a glass flask of 5.0 L along with 2.5 L of distilled water, remaining in the boiling water bath for 2 hours. Then add anhydrous sodium sulphate (Na2SO4) to the essential oil obtained and this was stored under refrigeration (±4°C) for conservation until the completion of analyzes, presenting profit of 1.35%.

MICROORGANISMS

The lineages used were Candida albicans INCQS 40006, Candida Tropicalis INCQS 40042, Candida krusei INCQS 40095 and multidrug resistant clinical isolates of Candida albicans URM 5597, Candida tropicalis, Candida krusei URM 4263, where all lineages were provided by the Laboratory of Microbiology and Molecular Biology - LMBM of the Regional University of Cariri - URCA. The strains were kept in agar Brain Heart Infusion (BHI - Acumedia®). The antifungal lineages were activated and inoculated in concentration recommended by the manufacturer and incubated too. Suspensions with growth antifungal drugs were diluted in BHI at a concentration of 10% until obtaining $10^5$ cells/mL.

ANTIFUNGAL TESTS
Preparation of the initial solution and test solutions

In the preparation of the initial solution, the essential oil was solubilized in dimethyl sulfoxide (DMSO - Acumedia®), being observed the following proportions: 10mg of oil dissolved in 1mL DMSO, to obtain an initial concentration of 10 mg/mL. Then, this solution was diluted in distilled water reaching concentration of 1024µg/mL and reducing the concentration of DMSO to 10%. From this, applied 1:2 serial dilutions, during the test micro dilution, obtaining the oil concentrations ranging from 512 to 8 µg/mL and DMSO ranging from 5-0.8% concentration, where DMSO at this concentration there is interference in the antifungal activity evaluated.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The tests for the determination of the MIC of extracts and fractions were performed through the method of broth microdilution, with concentrations ranging from 512 to 8 µg/mL. 18.

Execution and reading the essays

This method uses small medium volumes and solutions prepared from the essential oil, distributed in sterile microplate wells. Testing solutions were prepared at a concentration folded (1024 µg/mL) in relation to the initial concentration defined and volumes of 100 µL and subsequently serially diluted 1:1 in BHI broth 10%. In each cavity with 100 µL of culture medium a sample of antifungal suspension was diluted in the proportion 1:10. Negative controls with the culture medium, positive controls (means + inoculum) and controls of inhibition using solution at a concentration of 512 to 8 µg/mL were included in the tests. Filled plates were incubated at 35ºC for 24 hours18. To highlight the CIM forward solutions to the fungal strains observed the turbidity; in the case of growth through the absorbance was expressed by ELISA.

EVALUATION OF INTERFERENCE OF ESSENTIAL OIL ON THE RESISTANCE TO KETOCONAZOLE

To assess the essential oil as modulators of antifungal action, the CIM to antifungals imidazole derivative (Ketoconazole - Sigma®) was evaluated in the presence and in the absence of oil in sterile microplates. The ketoconazole was evaluated in concentrations ranging from 2500 the 2,5µg/mL.

Running and reading tests

The essential oil was mixed in BHI broth 10% in concentrations sub-inhibitory, obtained and determined after completion of the test for evaluation of CIM, being that for the test of modulation to the concentration of the solution of the essential oil was reduced 8 (eight) times (CIM/8). The preparation of the solution of antifungal drugs was performed with the addition of sterile distilled water in a concentration folded (5000 µg/mL) in relation to the initial concentration defined and volumes of 100 µL serially diluted 1:1 in BHI broth 10%. In each cavity with 100µL of medium culture, containing the fungal suspension diluted (1:10). The same controls used in the evaluation of CIM for the oil were used during the modulation19. The plates filled in alphabetic order and incubated at 35ºC for 24 hours and after this period was done reading to highlight the CIM forward solutions to the fungal strains, observing the turbidity in the case of growth through the absorbance was expressed by ELISA.

MICROWAVE IRRADIATION TESTS

For testing microwave irradiation, there were used initial solution concentration (10mg 1mL oil dissolved in DMSO, diluted in distilled water and reaching a concentration of 1024µg/mL) obtaining oil concentrations ranging from 512 to 8 µg/mL aliquots of this solution were subjected to a microwave oven for periods of different times (20, 40, 60 seconds). At the end of the exposure, there were repeated tests for the microdilution. There is difference of the results of the CIM when compared with the CIM that was not exposed to microwave was also done modulation.

MODEL AND STATISTICAL ANALYSIS

The results of the CIM obtained in triplicate in tests of modulation were tabulated in a spreadsheet using Microsoft Excel 2010 software, and applying the formula of geometric average and arithmetic, in addition to calculating the standard deviation getting parametric data and possible for submission to the statistical analysis and test of significance. For the statistical analysis the data are expressed by the geometric average and arithmetic, in addition to the standard deviations were submitted to analysis of variance (2 way ANOVA), followed by the Bonferroni test of significance, whereas no significant difference when p < 0.001, using the GraphPad Prism 5.0.

RESULTS AND DISCUSSION

In the present study, comparing with the controls, tests for the evaluation of the antifungal activity with the OECv exposed to microwave radiation has been identified an interaction that resulted in the increase of the fungal cell death, as demonstrated by the graphs below.

In Graph 1, when compared to the antifungal activity of OECv exposed and not exposed to irradiation microwave forward to Candida albicans resistant and pattern, with the increase of the concentration- OECv, a decrease in absorbance, whichever one best result for the lineage resistant when exposed to OECv 40s and 60s.
Graph 1: Curve representation of fungal cell death of *Candida albicans*.

Graph 2 shows the antifungal activity of OECv with *Candida tropicalis*, when compared to the fungal cell death between the standard and resistant lineages, in which we observed a better response to resistant lineage, when exposed to the action of the oil to 40s and 60s.

Graph 2: Curve representation of fungal cell death of *Candida tropicalis*.

When done the comparison of OECv from the standard lineages and resistant to *Candida krusei* as shown in graph 3, there was a greater fungal cell death with the increase of the concentration- OECv standard lineages exposed to OECv 20s and 40s.
In Graph 4 comparing the different combinations of exposure of OECv modulation tests before the *Candida albicans*, it is observed antagonist action when exposed the OECv for 20s, 40s and 60s, however the oil without exposure showed an interaction that resulted in synergism.

In relation to the forward modulation to *Candida krusei* shown in graph 5, we observed synergism for the OECv exposed and not exposed by 60s the microwave irradiation, the antagonist action was observed in the OECv exposed by 20s and 40s.

In Graph 6 relating to tests of modulation front of *Candida tropicalis*, there was an antagonist effect for all
combinations with the OECv, demonstrating that the interaction with the microwave irradiation was not enough to promote a synergistic action.

Graph 5: Curve representation of fungal cell death of *Candida krusei*.

![Graph 5](image_url)

Graph 6: Curve representation of fungal cell death of *Candida tropicalis*.

![Graph 6](image_url)

The search for new medicinal products derived from natural products has intensified in recent years by means of exploration chemistry of natural products of plant origin. Through a study using the leaves of *Cordia verbenacea* DC identified the presence of several potentially bioactive compounds extracted from the leaves, such as Asturias, triterpene, tannins, flavonoids, the sesquiterpenes α-humulene and trans-caryophyllene and terpenes those demonstrated its therapeutic potential as antifungal, anti-inflammatory and antimicrobial activity²⁰,²¹.

As a natural defense mechanism against microbial infections, the tannins are derived from secondary metabolism of plants and are defined with water-soluble phenolic polymers that precipitate proteins. The sesquiterpenes α-humulene and
trans-caryophyllene isolates of essential oil of Cordia verbenacea provide molecular and functional information, because it inhibits the activation of NF-κB induced by LPS and the migration of neutrophils, although only the α-humulene has the ability to prevent the production of pro-inflammatory cytokines TNF-α and IL-1β²².

The biological activity of essential oils and their constituents may act as fungistatic agents and/or fungicide called antifungal activity. Depending on the concentrations used, the antifungal activity stems, probably the result of the penetration of citronell in the wall of the hyphae, impairing the cytoplasmic membrane lipoprotein, leading to the extravasation of the cytoplasm, as well as draining and wilting of hyphae, and presence of filaments²³.

Other studies have reported that the antimicrobial property of the essential oils due to their lipophilic characteristic. The hydrophobicity of essential oil allows an interaction between the oil and the lipids of the cell membrane, interfering in its permeability and causing changes in its structure²⁴,²⁵.

The bacteria present greater susceptibility to natural product that the fungi, due to the complexity in eukaryotic cells of fungi and the difficulty in search of an effective chemical constituent that often act as agents fungistatic instead of fungicide²⁶.

The antifungal activity of the essential oil Thapsia villosa against fungal species also tested in this study C. albicans, C. tropicalis and C. krusei, confirming their potential as antifungal agent associated with low toxicity. Similar results with this study with the essential oil of Cordia verbenacea; however when associated with fluconazole not demonstrated synergistic effect as well as modulation tests performed with ketoconazole²⁷. In another study conducted with essential oil of Helichrysum italicum (Roth) G before filamentous fungi and Candida albicans also indicated that the oil is fungistatic, and no growth indicated that the effect is a fungicide as well as demonstrated with the different tested combinations of essential oil of Cordia verbenacea²⁸.

Antibacterial and antifungal activity of the essential oil of Cordia verbenacea DC demonstrating that the essential oil has fungistatic activity against Candida albicans and Candida krusei (CIM 512 μg/ml). Nevertheless, whichever one best oil activity in bacterial lineages, evidencing the need of developing alternative strategies that may potentiate the effect of essential oils leading to reduction or elimination of fungi²⁹.

The microwave irradiation has been used as an alternative method for the inactivation of microorganisms present in food, medical instruments, laboratory, contact lenses, household objects, cosmetics, underwear, hospital waste and biosolids. However, the direct interaction between the power of microwaves and the biological materials, in molecular and cellular bodies, is little known³⁰. Thus, this study investigated how this electromagnetic energy can affect the integrity and the microbiological activity of OECv, promoting the inactivation of C. albicans, C. tropicalis and C. krusei²⁸.

The mechanisms by which the energy of microwave acts on the microorganisms are still not fully understood. The microwave are not powerful enough to directly change the chemical bonds, and, therefore, its effects could be produced via mechanisms such as dielectric relaxation, ionic conductivity and modification of biopolymers³¹.

The microwave ovens appliances use a frequency of 2,450 MHz, which provides rotational wavelength of approximately 12 mm. Consequently, numerous intermolecular collisions occur, and the rotational energy is converted into thermal energy, causing a rapid heating, causing denaturation of proteins and nucleic acids cell phones, since the denaturation of DNA molecules is greater when performed by means of microwave than by another heat source³².

**FINAL CONSIDERATIONS**

In this way, it was established that the antifungal action and the modulating essential oil of Cordia verbenacea exposed to microwave irradiation varied significantly as a function of exposure time and fungal resistance, demonstrating that the OECv fungistatic activity against Candida albicans, Candida tropicalis and Candida krusei. Therefore the combination of natural products of plant origin with exposure to Microwave irradiation and combined with antifungals presents itself as an alternative tool in combating pathogenic fungi.

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