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Chemical composition, antifungal activity and toxicological evaluation of *Lippia sidoides* Cham

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A R T I C L E I N F O

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A B S T R A C T

Candida species are normally commensal yeasts residing in the human body, under certain circumstances, can trigger pathological conditions such as candidiasis, not to mention antifungal resistance. *Lippia sidoides* Cham., or pepper-rosmarinus, is a native plant found in the Northeastern semi-arid region of Brazil, cited in ethnobotanical studies for its bioactive potential. This approach aims not only to expand the understanding of the antifungal efficacy of the ethanol extract of *L. sidoides* (EELs) but also to evaluate synergies that may enhance the available therapeutic arsenal, using the checkerboard method, and to assess potential toxic effects of this extract using model organisms, *Artemia salina* and *Drosophila melanogaster*, to evaluate mortality rates. Antifungal activity was observed at high concentrations (>1024 μg/mL) for both strains of *Candida albicans* and *Candida krusei* tested, with the most significant inhibition of fungal growth when the compound was used in synergy with fluconazole at 8.0 and 128 μg/mL, respectively. This activity likely occurred due to the interaction between the chemical composition of the ethanol extract rich in flavonoids and tannins with the reference drug. Regarding toxicity, both in relation to *D. melanogaster* and *A. salina*, no toxic results were observed at low concentrations. These results suggest that the ethanol extract of EELs may be a potential alternative as an adjuvant to fluconazole in the treatment of fungal infections caused by *Candida* spp. The observed synergy suggests that this combination may help overcome resistance to conventional antifungals and improve treatment efficacy.

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1. Introduction

The World Health Organization (WHO) states that approximately 80 % of the population in developing countries uses plants for basic health care [1]. This is due to its low cost, easy accessibility and minimal side effects [2].

Another reason for studying plants as alternative therapeutic sources is the ineffectiveness of known medications against microorganisms, such as certain pathogenic fungi. Among these, fungi of the genus *Candida* constantly develop resistance mechanisms, thus reducing the effectiveness of medications used to combat them, especially those of the azole and echinocandin classes [3]. This is attributed to several virulence factors, including the presence of degradative enzymes, as well as their ability to adhere to host tissue and form biofilms [4–6].

Infections caused by these fungal strains have been recognized as a significant challenge in terms of public health due to their chronic and opportunistic nature. Although *Candida* species are typically commensal yeasts residing in the human body, under certain circumstances, they can trigger pathological conditions such as candidiasis, with significant mortality rates, especially among hospitalized patients [7,8].

In this scenario, the search for new drugs based on plant-derived products has been growing considerably. The genus *Lippia* is commonly used for the production of natural pharmaceuticals and cosmetics due to its phytotherapeutic potential [9]. Among the species within this genus, *Lippia sidoides* Cham. Verbenaceae (Pepper-rosmarinus) is a shrub used in oral hygiene product formulations due to its antiseptic and antifungal activity [10].

This is because previous studies have demonstrated that the ethanol extract of *L. sidoides* contains high concentrations of polyphenolics, such as flavonoids, for example [11,12]. However, the combination of chemical compounds present in extracts can further enhance the action of the major compound in the sample under study, resulting in significant findings in the search for new drugs.

Pepper-rosmarinus is a commonly cultivated plant and is included in the list of priority plants of the Unified Health System (SUS) in Brazil. However, in addition to tests confirming its efficacy, it is also necessary to evaluate the toxicity of its composition. Despite being a natural remedy, excessive use, like any medication, can lead to adverse effects. Furthermore, most of these natural drugs do not have a well-known toxic profile [13].

To assess its toxicity, one option is mortality tests, commonly using *Drosophila Melanogaster* and *Artemia Salina* Leach due to their rapid reproducibility, easy handling, and affordable cost. Additionally, conducting studies with both organisms does not require an ethics committee [14]. Considering the high use of *L. sidoides* as a therapeutic alternative by society, as described in ethnobotanical studies by Rocha et al. [10], this research aimed to identify the classes of chemical compounds present, evaluate the antifungal action of the ethanol extract of *L. sidoides* against *Candida albicans* and *C. krusei* strains, as well as assess its toxicological profile through mortality tests using the model organisms *A. salina* and *D. melanogaster*.

2. Material and methods

2.1. Botanical material

L. sidoides leaves were collected in February 2021 at 925 m altitude in an Environmental Protection Area (APA) of Chapada do Araripe, belonging to the municipality of Crato – CE, Brazil under coordinates W 39°24′34″ and 7° 14 ′03″ S. Registration numbers were recorded in SISBIO (Sistema de Autorização e informação em Biodiversidade, ID: 91683).

2.2. Extract preparation

The leaves (180g) were washed in running water to remove residue, then dehydrated (45 °C in 24 h) and crushed to increase the contact surface with the solvent. The product of this process was submerged in 1.3 L of 96 % ethanol in amber glass for a period of 72 h. After this exhaustive extraction, the material was filtered and subjected to a rotary evaporator coupled to a water bath, to concentrate the material and obtain the extract.

2.3. Phytochemical prospecting

Phytochemical screening was carried out based on the methodology proposed by Matos [15] to determine the main classes of secondary metabolites, including phenolic compounds, hydrolysable tannins, flababenic tannins, anthocyanins, anthocyanidins, flavones, flavonols, xanthones, chalcones, aurones, flavonoids, leucyanticuanidins, catechins and flavonones, as shown in Table 1. Cross tests were used to evaluate the absence or presence (weak, moderate or strong) of the mentioned phytochemical compounds, where: (0) negative, (+) weak positive, (++) moderate positive and (+++) strong positive [16,17].

2.4. Lethality test with Artemia salina

Toxicity testing was performed as described by Meyer et al. [14], with some modifications. In artificially prepared seawater, *A. salina* cysts were added and subjected to constant aeration for 24 h, the period necessary for incubation of the larvae. After that, the extract was prepared at different concentrations (1000, 500, 250, 100, 50, 25, 10, 5 and 1 μg/mL) with 5 microcrustacean larvae added at each concentration. The reading was performed after 24 h using a stereomicroscope [25].

2.5. Mortality test with Drosophila melanogaster

The flies used in this study were obtained from the Biology and Toxicology Laboratory (BIOTOX) of the Universidade Regional do Cariri (URCA), Ceará-Brazil. The animals were raised in glass containers with controlled temperature (23–25 °C) and relative humidity of 70 %, on a 12h:12h cycle (light/dark). Flies were reared in 340 mL glass containers and cultured in basal diet medium.

To carry out the survival tests, ten flies were placed inside the flasks, where the food was supplemented with the extract (in concentrations of 500, 250, 100, 50 and 25 μg/mL) in the treated groups and the control

Table 1
Qualitative phytochemical determination methods for the ethanolic extract of *Lippia sidoides* (EELs).

Classes	Method	Reference
Phenolics	FeCl3 reaction	BARBOSA et al., 2004; HERNÁNDEZ et al., 2019
Hydrolyzable tannins	FeCl3 reaction	BARBOSA et al., 2004
Flababenic tannins	FeCl3 reaction	BARBOSA et al., 2004
Anthocyanins	Acid and alkaline reaction	BARBOSA et al., 2004
Anthocyanidins	Acid and alkaline reaction	BARBOSA et al., 2004
Flavones	Alkaline reaction	BARBOSA et al., 2004
Flavonols	Alkaline reaction	BARBOSA et al., 2004
Xanthones	Alkaline reaction	BARBOSA et al., 2004
Chalcones	Acid and alkaline reaction	BARBOSA et al., 2004
Auroras	Acid and alkaline reaction	BARBOSA et al., 2004
Flavonoids	Alkaline reaction	BARBOSA et al., 2004
Leucyanticuanidins	Acid and alkaline reaction	BARBOSA et al., 2004
Catechins	Acid and alkaline reaction	BARBOSA et al., 2004
Flavonones	Acid and alkaline reaction	BARBOSA et al., 2004

Source: DE MENEZES FILHO e SANTOS, 2021.

group containing only water. The evaluation of the results was carried out daily during seven days of exposure for the toxicity test, subsequently percentage calculations were applied to determine the rate of live flies over 7 days.

2.6. Antifungal tests

2.6.1. Microorganisms, culture media, inoculum and solution preparation

Two strains of *Candida* were used: *Candida albicans* INCQS 40006 and *Candida krusei* INCQS 40095, acquired from the National Institute for Health Quality Control (INCQS) of the Ostwald Cruz Collection. Sabouraud Dextrose Agar (DAS - KASVI) was the medium used for yeast growth, which were incubated at 37 °C for 24 h. Subsequently, the yeasts were transferred to a saline solution (0.9 %) and standardized at a concentration of 1×10^5 cells/mL on the McFarland scale (0.5) [18]. For determining the minimum fungicidal concentration (CFM) in Sabouraud dextrose broth (SDB, HIMEDIA), double concentrated. Dimethyl sulfoxide (DMSO, Merck, Germany) was used to dilute the *Lippia sidoides* extract, while fluconazole (Capsule; Prati donaduzzi, Brazil) was diluted in sterile distilled water and used as a positive control, with an initial concentration of 16.384 μg/mL [19].

2.6.2. Determination of minimum Fungicide concentration (MFC)

Sterile swabs were inserted into each well of the tested plate to homogenize the medium and transfer an aliquot to the Petri dish containing Sabouraud Dextrose Agar. After 24 h of incubation at 37 °C, the plates were analyzed observing the formation of *Candida* yeast colonies. The concentration at which there was no fungal colony growth was considered as the CMF of the tested product [20].

2.6.3. Assessment of the action-modifying effect of fluconazole

The combination of *L. sidoides* extract with the reference drug (fluconazole) was conducted to determine if the antifungal action was altered by the presence of the extract in the medium. Following the determination of the MIC, the modifying activity of fluconazole was performed as described by Morais-Braga et al. [21].

In flat-bottomed 96-well ELISA plates (Termoplate®), 90 μL of Sabouraud dextrose broth (SDB, Himedia) solutions were added to all wells containing the ethanolic extract of *L. sidoides* at sub-inhibitory concentrations (MIC/16), except the last row, which served as the fungal growth control. This solution was micro diluted with fluconazole (1:1 v/v) up to the penultimate row of wells, with concentrations ranging from 1 to 1.024 μg/mL. Finally, *Candida* spp. was added to achieve a 10 % concentration in each well, following the previously mentioned method.

2.7. Statistical analysis

Statistical analyzes of triplicate means (n = 3) ± standard deviation was performed using the GraphPad Prism 6 software program, using the Two-Way Variance Analysis (ANOVA) statistical formula, followed by Tukey's test at 5 % reliability and Dunnett's for toxicity tests. Additionally, LC50 (50 % Lethal Concentration) was obtained by linear regression using *GraphPad Prism* 6.

3. Results

3.1. Phytochemical analysis

According to the results presented in Table 2, using colorimetric reactions to qualitatively assess the presence of different classes of chemical compounds, it was possible to confirm the presence of phlobatannins and hydrolysable tannins, as well as flavonols, flavones, and catechins as the most representative groups, interpreted by the color change as an indicator of the absence or presence of these substances. It's worth noting, however, that in this method, the presence of a particular constituent can mask the characteristic color of another, which may not allow for a precise analysis of the chemical composition of the extract.

Table 2
Phytochemical prospecting of the ethanolic extract of *Lippia sidoides*.

Classes	Results
Phenols	+
Hydrolyzable tannins	+
Flababenic tannins	+++
Anthocyanins	**0**
Anthocyanidins	**0**
Flavones	+
Flavonols	+
Xanthones	+
Chalcones	+
Auroras	+
Flavonoids	+++
Leucyanticuanidins	**0**
Catechins	++
Flavonones	+++

Absence of change (0); small change (+); intermediate change (++); big change (+++).

3.1.1. Toxic activity using Artemia salina

Toxicological outcome with the model organism *A. salina*, it can be stated that there were no toxic results with the tested model up to a concentration of 500 μg/mL (Fig. 1). However, an effect on the mortali). rate was observed at the last tested concentration of 1000 μg/mL, indicating an LC_{50} (lethal concentration for 50 % of the organisms) > 1000 μg/mL. An LC_{50} lower than 1000 μg/mL is considered bioactive [14].

3.1.2. Toxic activity using Drosophila melanogaster

As shown in Fig. 2, the extract did not present significantly toxic results for D. *melanogaster*, at the concentrations tested when compared to the negative control (>500 μg/mL).

3.2. Antifungal activity

The antifungal effect of the ethanol extract of *L. medoides* can be observed in the reduction of the growth curve of *Candida albicans* and *C. krusei* yeasts, as shown in Figs. 3 and 4, respectively. The extract tested in isolation demonstrated an effective effect on both strains under study but at high concentrations (>1024 μg/mL). However, when combined with the drug fluconazole, its action was intensified. A concentration of 8 μg/mL resulted in a significant effect against *C. albicans* strains (Fig. 3). However, for the *C. krusei* strain, this potentiating effect was only significantly pronounced at a concentration of 128 μg/mL (Fig. 4).

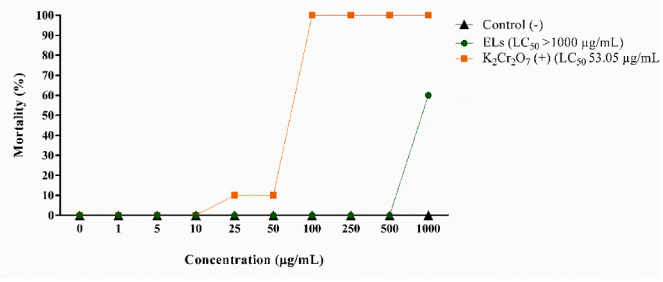

Fig. 1. Percentage of mortality of *Artemia salina* exposed to Ethanolic Extract of *Lippia sidoides* (EELs). Values were calculated as relative percentages of live dead organisms N = 3.

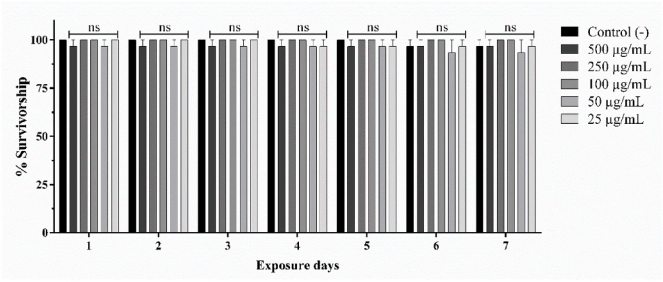

Fig. 2. Survival percentage *of Drosophila melanogaster* exposed to different concentrations of Ethanolic Extract of *Lippia sidoides* (EELs), during 7 days of exposure.

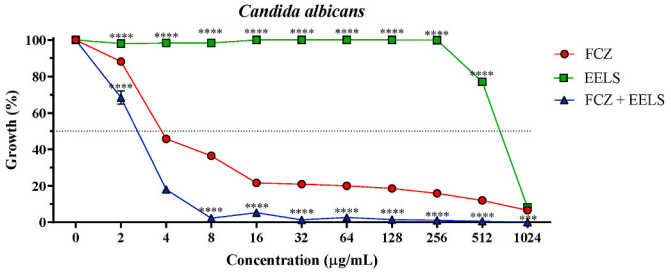

Fig. 3. Fungal growth curve of *Candida albicans* 40006 INCQS exposed to ethanolic extract of *Lippia sidoides* (EELs), fluconazole (FCZ) and combination of fluconazole and natural product (FCZ + EELS). Where: $p < 0.0001$ = **** and $p < 0.001$ = ***.

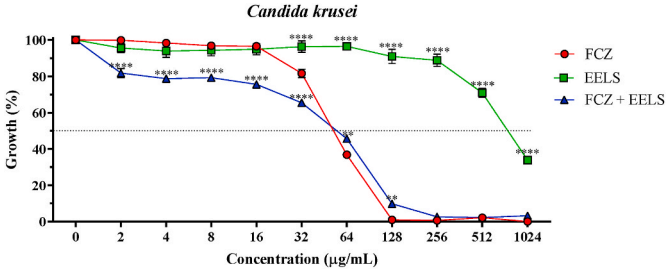

Fig. 4. Fungal growth curve of *Candida krusei* INCQS 40095 exposed to ethanolic extract of *Lippia sidoides* (EELs), fluconazole (FCZ) and combination of fluconazole and natural product (FCZ + EELS). Where: $p < 0.0001$ = **** and $p < 0.01$ = **.

4. Discussion

L. medoides is consistently cited in the literature, especially in ethnobotanical studies, and is used in the production of medicines, including dermatological applications [22,23], indicating its pharmacological properties. Therefore, the significance of studying the constituents found in medicinal plants cannot be overstated, as it contributes to the development of new drugs, dietary supplements, and even the production of aromatic compound-based products by the pharmaceutical industry.

Considering the importance of these studies, it's also essential to emphasize that various factors can alter the chemical composition of plants, from their origin and climatic conditions to the availability of resources and genetic variations that can lead to the formation of these components in these plants [24].

In this context, among the results obtained from the phytochemical prospecting in this study, the presence of phenols, flavones, xanthones, chalcones, aurones, and catechins was observed, which aligns with other findings in the literature, confirming the presence of these compounds. Furthermore, it is worth noting the presence of some classes of flavonoids, flavonones, and certain tannin derivatives, with these being the most frequently identified classes in the ethanol extract of this species [26,27].

This corroborates its use as a natural remedy by the population, as various biological activities related to the previously mentioned classes are documented in the literature. Tannins exhibit antibacterial, antioxidant, antiviral, and cytotoxic activities [28–31]. On the other hand, flavonoids and the highlighted flavonone subclass in the phytochemical analysis conducted possess contested anticancer, anti-inflammatory, antioxidant, antimicrobial, antibacterial, and anti-aging properties [32–36].

With regard to fungal infections, data shows that they are the fifth leading cause of death in the world, manifesting themselves most of the time silently, and, among these cases, approximately 700,000 are directly associated with the genus *Candida* with *C. albicans* responsible for more than half of the cases and the main species associated with invasive nosocomial infections [37–39]. In addition to occurring frequently, it also presents high mortality rates >40 %, mainly associated with immunocompromised patients [40–42].

In the present study, it was possible to observe significant antifungal activity, especially when the ethanolic extract of *L. sidoides* was combined with fluconazole. Concentrations as low as >8 µg/mL were effective against *C. albicans*, while higher concentrations, >128 µg/mL, were required for *C. krusei*. These results indicate that, although *C. krusei* showed greater resistance than *C. albicans*, it is important to note that this strain has considerably reduced virulence capacity, which could be a significant finding [43].

The synergism observed with fluconazole is likely attributable to the chemical composition of the ethanolic extract of *L. sidoides*. Previous studies in the literature suggest that components such as thymol can disrupt the integrity of the fungal cell wall and demonstrate synergistic effects with fluconazole [44,45]. Furthermore, studies indicate that synergism can enhance drug penetration into the fungal cell membrane [46]. The synergy observed between our extract and the reference antifungal medication can thus be explained by the increased permeability of the fungal cell membrane, which facilitates greater drug absorption, the induction of oxidative stress, the inhibition of fungal efflux pumps, and the action on multiple cellular pathways. This results in a more effective and comprehensive therapeutic approach. However, assays evaluating the mechanisms of action are necessary to accurately determine the precise mechanism by which this extract operates.

Although new fungal drugs are constantly emerging, they do not present such favorable results, as fungi continue to acquire resistance, which is just one of the many reasons why there is a reduced quantity of fungal drugs compared to other microorganisms, such as bacteria [41]. Other factors explain this shortage, such as, for example, the delay in diagnosis, since there is a need to perform culture and the symptoms are usually similar to other pathologies, the misuse of antibiotics, high values of fungal therapies, some drugs presenting toxicity and, compromised host immune system [47–50].

Furthermore, the evolutionary proximity of fungi to humans limits the development of new medicines [47]. Finally, it is important to note that more serious infections tend to occur more frequently in developing countries. This, in turn, explains the pharmaceutical industry's lack of interest and the lack of more in-depth research on the subject [49]. All of this can make the treatment of these infections more challenging, consequently leading to a greater number of deaths.

Therefore, it is necessary to carry out more frequent and in-depth research to search for alternative drugs with low toxicity or reduced toxicity, which are economically advantageous for controlling these infections. Therefore, in this study, an assessment of the toxicity of the ethanolic extract (EE) used in two model organisms, *A. salina* and *D. melanogaster*, was carried out.

Among the results obtained, it was observed that the model organism *A. salina* did not present toxic results up to a concentration of 500 µg/

mL, since mortality occurred only at the highest concentration tested, which was 1000 μg/mL. In relation to *D. melanogaster*, toxic results were observed at two concentrations (500 and 50 μg/mL showed significant toxicity). Toxicity tests with the microcrustacean *A. salina* are an excellent option for evaluating the preliminary toxicity of a substance due to its sensitivity to toxic substances and the ease of carrying out these tests [51].

These results are similar to those found in the literature regarding the *Lippia* genus. Fabri et al. [52], for example, related the toxicity of microcrustaceans to human tumor cells and claims to be a good preliminary test. Clement et al. [53], analyzed the toxicity of the aqueous and ethanolic extract of *L. multiflora* with *A. salina* and states that there was no toxicity in their results. Camilo et al. [54], when using the aqueous extract of *L. sidoides* in their tests, obtained similar results, that is, low toxicity at the concentrations tested. Even more, Costa et al. [55], states that there is a weak cytotoxic effect of *L. alba* essential oil tested with the same model organism. Regarding studies with *D. melanogaster*, Camilo et al. [56], evaluated the aqueous extract of *L. sidoides*, and observed that there was considerable mortality at the highest concentration tested (4000 μg/mL). However, the number of studies on the toxicity of plants of the *Lippia* genus using this model organism is limited.

Therefore, this study is important because it contributes to the search for unconventional medicines that have shown promising efficacy, thus filling the gap left by the delay and lack of interest in the production of new drugs, as mentioned previously. Furthermore, this work allowed the evaluation of the ethanolic extract (EE) in relation to two different model organisms, *A. salina* and *D. melanogaster*, both demonstrating low toxicity. However, for complete validation and a deeper understanding of the therapeutic potential of EE, it is essential to carry out more comprehensive toxicity studies, such as large-scale clinical trials, as well as detailed research into the mechanisms of action [57].

Finally, it is important to highlight that there are a high number of people infected with HIV around the world, and this disease is one of the main risk factors for opportunistic infections, especially those of the *Candida* genus. Furthermore, fungi are microorganisms that benefit from higher temperatures; therefore, global warming can significantly contribute to the emergence of increasingly resistant strains and consequently hinder the creation of effective drugs, which is why studies on opportunistic fungi should be an important issue on the global health scene.

5. Conclusions

It is concluded that the ethanolic extract of *L. sidoides* is rich in flabanenic tannins, flavonoids and flavones. As for its antifungal effect, the extract has a considered potentiating effect on the drug fluconazole, being more efficient than when tested alone, mainly for *C. albicans*. In addition to presenting positive results regarding its toxicity both in relation to *Drosophila melanogaster* and also to *Artemia saline*, which did not present significant toxicity to model organisms at relatively low concentrations.

CRediT authorship contribution statement

Maria Aline Oliveira: Methodology, Formal analysis, Conceptualization. **Carlos Alonso Leite dos Santos:** Methodology, Formal analysis. **Bárbara Rayanne da Silva Teles:** Investigation, Formal analysis. **Carlos Vinicius Barros Oliveira:** Investigation, Formal analysis. **Viviane Bezerra da Silva:** Writing – original draft, Investigation. **Ana Letícia Gonçalves Pereira:** Methodology, Formal analysis. **Vanessa Leopoldino Coelho Rodrigues:** Validation, Software. **Victor Juno Alencar Fonseca:** Investigation, Formal analysis. **Mariana dos Santos Santana:** Investigation, Formal analysis. **Clara Mariana Gonçalves Lima:** Writing – review & editing, Visualization, Conceptualization. **Maria Flaviana Bezerra Morais-Braga:** Resources, Methodology, Investigation. **Maria Elizete Machado Generino:** Investigation, Data curation. **Luiz Marivando Barros:** Writing – original draft, Visualization, Validation. **Antonia Eliene Duarte:** Writing – original draft, Investigation. **Maraiza Gregorio de Oliveira:** Writing – original draft, Visualization. **José Weverton Almeida-Bezerra:** Writing – original draft, Investigation. **Adrielle Rodrigues Costa:** Methodology, Formal analysis. **Marcos Aurélio Figueirêdo dos Santos:** Writing – original draft, Investigation. **Saulo Almeida de Menezes:** Visualization, Validation, Software. **Ahmad J. Obaidullah:** Conceptualization, Data curation, Resources. **Talha Bin Emran:** Data curation, Conceptualization. **Henrique Douglas Melo Coutinho:** Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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