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Antifungal Activity of Citral by Disruption of Ergosterol Biosynthesis in Fluconazole Resistant *Candida tropicalis*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JPS, AOCC, MCAL, FQSG, VAS, CPM, FOP and EOL are responsible for drafting the paper and listed below are the individual contributions of each author to the paper. Author JPS participated in the project design, collection and analysis of data, drafting the paper, and critical revision of the intellectual content. Authors AOCC, MCAL, FQSG, VAS, CPM, and FOP participated in data collection, data analysis, and revision of the paper. Author EOL guided all stages of the work and participated in both review and drafting of the project and the paper, including final approval of the version to be published. All authors read and approved the final manuscript.

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ABSTRACT

A limited number of antifungals and the emergence of resistant strains have hindered the treatment of candidiasis, making the search for new antifungals urgent. Citral is a monoterpene with known pharmacological properties, including antimicrobial action.

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Aims: Thus the objective of this study was to investigate the possible mechanism of citral's action against *Candida tropicalis* isolated from human blood, and the effect of its association with antifungals.
Methodology: The minimum inhibitory concentration (MIC), the minimum fungicidal concentration (MFC), the effect of citral on the cell wall (sorbitol assay), and membrane ergosterol binding were evaluated using broth microdilution technique. We also evaluated interference in ergosterol biosynthesis, and the citral-antifungal association effect (checkerboard method).
Results: The MIC_{90} and MFC_{90} of citral were respectively 512 and 1024 µg / mL. The MIC of citral did not increase when sorbitol or ergosterol was added to the medium, suggesting that citral does not act on the cell wall or by membrane ergosterol binding. However, citral inhibited ergosterol biosynthesis, and the citral-fluconazole combination showed synergistic effects for the ATCC strain.
Conclusion: This study contributes to understanding the antifungal mechanism of citral's action, and to the development of new therapies for candidiasis treatment.

Keywords: Mode of action; monoterpene; antifungal activity; candidiasis; ergosterol.

1. INTRODUCTION

Invasive fungal infections with alarming mortality rates have increased significantly in recent decades [1]. More than 70% of invasive fungal infections in hospitalized patients are caused by *Candida spp*., presenting predominantly as candidemia [2]. Although *Candida albicans* is the most common species associated with invasive candidiasis, non - *C. albicans* species are increasingly common [3-5]. This is a concern because resistance to fluconazole and amphotericin B is higher in non-*albicans* species than *C. albicans* [6].

Candida tropicalis is often found in intensive care unit patients, especially those who require prolonged catheterization, receive broad-spectrum antibiotics, or suffer from cancer [7-10]. *C. tropicalis* also seems to exhibit a great potential for dissemination in neutropenic patients [7]. According to some epidemiological studies, *C. tropicalis* is associated with higher mortality than *C. albicans* or other non - *C. albicans* species [3,7,11,12].

The three major classes of antifungal agents used to treat invasive fungal infections are the polyenes, the azoles, and the echinocandins. Polyenes (such as the amphotericin B) bind to ergosterol, the main sterol of fungal cell membranes, forming pores that increase the permeability of the membrane, which leads to cell death. Azoles interrupt ergosterol biosynthesis, inhibiting the enzyme lanosterol 14α-demethylase and consequently inhibiting fungal replication. Echinocandins in turn, inhibit the synthesis of β-1,3-D-glucan, an integral component of the fungal cell wall, causing a weakening of the fungal cell wall, cell lysis, and death [13,14].

Of the few available antifungal agents to treat invasive fungal infections, unfortunately, azoles are usually fungistatic rather than fungicidal and prolonged use contributes to the development of resistance [15]. Polyenes, despite having low resistance rates are very toxic. Lipid formulations of amphotericin B, (less toxic than conventional amphotericin B), and the echinocandins, (a new antifungal class), are both prohibitively expensive [13,16]. Thus, development of new antifungal drugs, and new therapeutic strategies are urgently needed [1]. Plant-derived antifungal compounds are attracting great interest as natural alternatives, due in part to their versatile applications [17,15].

Citral (3,7-dimethyl-2,6-octadienal) is a natural mixture of two acyclic monoterpene aldehyde geometric isomers, geranial (trans-citral or citral A), and neral (cis-citral or citral B). It is present in the essential oil of many plants including lemon and orange species [18,19]. Citral presents different pharmacological properties, such as: anti-tumor [20-22], bronchodilator [23], antiprotozoal [24], and antimicrobial [19,25-34] effects.

Although there are many reports on the antimicrobial properties of citral, there are few studies on its antifungal modes of action, or its combination with antifungals against strains of *Candida tropicalis*. The objective of this study was to investigate the antifungal activity of citral against *C. tropicalis*, its possible mechanism of action, and its effect in combination with antifungal agents.

2. MATERIALS AND METHODS

2.1 Chemicals

Sorbitol, ergosterol, citral, fluconazole, and amphotericin B were purchased from Sigma-Aldrich, Brazil. The drugs were solubilized in 5% dimethylsulfoxide (DMSO), and 2% Tween 80 (Sigma-Aldrich). Next, sterile distilled water was added, and the tubes mixed for 5 min using a Vortex (Fanem), to obtain the desired concentrations.

2.2 Microorganisms

The assays were performed with one standard strain of *Candida tropicalis* ATCC 13803, which is part of the Collection of the Mycology Laboratory, Department of Pharmaceutical Sciences, Center of Health Sciences, Federal University of Paraiba, and 7 clinical strains of *C. tropicalis* (1, 6, 18, 20, 23, 31 and 36) isolated from blood, provided by Professor Everardo Albuquerque Menezes (Department of Clinical Analyses, Ceará Federal University).

Cultures of strains were made in Sabouraud dextrose agar (Difco Lab., USA), and incubated at 35ºC for 24-48 h. Colonies of this culture were suspended in sterile 0.85% NaCl, and the inoculum was standardized to the 0.5 tube of the McFarland scale (1-5 x 10^6 CFU/mL).

2.3 Determination of Minimum Inhibitory Concentration (MIC), and Minimum Fungicidal Concentration (MFC)

The MIC was determined by the microdilution technique in broth medium. RPMI-1640, with L-glutamine (Sigma-Aldrich®, São Paulo, SP, Brazil) without sodium bicarbonate was added to all wells of the 96-well plates. Next, serial dilutions of the citral were made to obtain concentrations varying between 1024 and 0.5 µg/mL. The same procedure was carried out with fluconazole and amphotericin B. DMSO (5%) and Tween 80 (2%), without drugs, served as control. Finally, 10 µL of yeast inoculum was added to all wells, and the plates were incubated at 35ºC for 24 - 48 h. The MIC was defined as the lowest concentration capable of visually inhibiting the fungal growth seen in the wells [35].

To determine the MFC, after reading the MIC aliquots of 20 µL from each of the wells with no fungal growth were seeded in a Sabouraud dextrose agar containing plate, which was then incubated at 37ºC for 24–48 h. The MFC was the lowest drug concentration that showed either no growth, or fewer than three colonies [36]. The assays were performed in triplicates, and the geometric mean values were calculated.

Based on the MIC and MFC results, two representative strains were selected, for the subsequent assays, a clinical strain (*C. tropicalis* 18), and a standard strain (*C. tropicalis* ATCC 13803).

2.4 Sorbitol and Ergosterol Effect Assay

The MIC of citral was determined with *C. tropicalis* 18 and ATCC 13803 by the microdilution method [35], in the absence and presence of 0.8 M of sorbitol, and 400 µg/mL of ergosterol. Amphotericin B was used as a control drug for the ergosterol tests. The plates were read at 48 h and after 5 days [37]. The assays were performed in triplicate and the geometric mean values were calculated.

2.5 Sterol Quantitation Assay

For extraction of total sterol content of the *C. tropicalis* ATCC 13803 cells, 1 mL of inoculum was added to 9 mL of Sabouraud dextrose broth (Difco Lab., USA), containing MIC/2, MIC, and 2 x MIC of citral, the MIC of fluconazole (as positive control), and for negative control (no test compound). The cultures were incubated for 24 h at 35ºC. The fungal cells were then centrifuged at 3,000 rpm for 5 min, washed once with sterile distilled water, and the wet weight of the cell pellet was determined. Three milliliters of 25% alcohol/potassium hydroxide solution (25 g of KOH and 35 mL of sterile distilled water, brought to 100 mL with 100% ethanol), was added to each pellet and vortex-mixed for 1 min. Cell suspensions were incubated in 85ºC water bath for 1 h and then allowed to cool at room temperature. Sterols were then extracted by addition of a mixture of 1 mL of sterile distilled water and 3 mL of n-heptane followed by vigorous vortexing for 3 min. The heptane layer was transferred to Eppendorf tubes and stored under refrigeration for 24 h. Aliquots of the sterol extracts were examined by measuring the absorbance at 281.5 nm and 230 nm with a UV-visible spectrophotometer (Shimadzu). This assay was performed in triplicate. Ergosterol content was calculated as the percentage of the wet weight of cell, as reported by Arthington-Skaggs [38]. The results were expressed in

mean±SE. Statistical analyses were performed with the *t*-test. P ≥0.05 was considered significant.

2.6 Checkerboard Method

Combinations of citral- fluconazole, and citral-amphotericin B were tested in triplicate against *C. tropicalis* 18, and ATCC 13803 using microdilution checkerboard technique [39]. The concentration of each antifungal agent tested ranged from 1/16 to 8 × MIC. The initial inoculum was prepared as described for broth-microdilution susceptibility testing. The inoculated plates were incubated at 35ºC for 24 h. The MIC endpoints were determined as described for the broth-microdilution tests. To determine the activity of the drug combinations, fractional inhibitory concentration (FIC) indices were calculated as $FIC^A + FIC^B$, where FIC^A and FIC^B represent the minimum concentrations that inhibited bacterial growth for the drugs A and B, respectively: $FIC^A = MIC^A$ combination/MIC^A alone, and $FIC^B = MIC^B$ combination/MIC^B alone. A mean FIC index was calculated based on the following equation: FIC index = $FIC^A + FIC^B$, and the interpretation was made as follows: synergistic (≤ 0.5), additive (>0.5 but <1), indifferent (≥1 but <4), or antagonistic (≥ 4.0) [39].

3. RESULTS AND DISCUSSION

The MIC and MFC results against 7 clinical strains of *C. tropicalis* (1, 6, 18, 20, 23, 31, and 36), isolated from blood, and 1 standard strain (*C. tropicalis* ATCC 13803) are shown in Table 1.

The anti-Candida citral potential was previously demonstrated by diffusion in solid medium technique against *C. albicans, C. tropicalis, C. parapsilosis, C. krusei, C. glabrata, C. stellatoidea,* and *C. guilhermondii* [26,28,33]. The essential oil of *Cymbopogon citratus*, whose main constituent is citral, also showed antifungal activity against *C. albicans, C. parapsilosis,* and *C. tropicalis* by disk diffusion method [40]. In *C. albicans*, Lima et al. [29] reported a citral MIC of 512 µg/mL, while Leite et al. [27] obtained an MIC of 64 µg/mL. Mesa-Arango et al. [31] reported an MIC of 125 µg/mL against *C. parapsilosis*,

The MIC of fluconazole was 64 µg/mL for the ATCC strain and > 1024 µg/mL for 6 of the 7 clinical strains, while the MIC of amphotericin B ranged from 0.5 to 2 µg/mL (Table 1).

According to the NCCLS [35], strains exhibiting an MIC for fluconazole of ≥64 µg/mL are considered to be resistant, and strains with an MIC for amphotericin B of ≥2 µg/mL are considered resistant. Thus, according to this classification, resistance to fluconazole was observed for all strains tested, and resistance to amphotericin B for one strain, *C. tropicalis* 1 (Table 1).

Amphotericin B and fluconazole are among the most widely used antifungal agents for the treatment of systemic fungal infections [41]. Fluconazole is widely used in clinical practice due to its efficacy and low toxicity [42], however, with frequent exposure, fluconazole-resistant *Candida* isolates have appeared more often [43-45]. According to Barchiesi et al. [46], and Calvet et al. [47], fluconazole drug resistance in *C. tropicalis* develops much faster than in *C. albicans*.

Four principal mechanisms of azole resistance have been described in *Candida* spp.: Reducing drug penetration and decreased intracellular concentration through activation of drug efflux pumps; change in the target site structure on the enzyme (ERG11 gene); increased concentration of the target enzyme; and development of alternative pathways (ERG3 gene) [48].

The MFC of citral varied between 256 and 1024 µg/mL. Both the MFC_{50} (Minimum Fungicidal Concentration for 50% of strains tested), and the MFC_{90} (Minimum Fungicidal Concentration for 90% of strains tested) for citral were 1024 µg/mL (Table 1).

According Hafidh et al. [49] the MFC/MIC ratio is used to specify the nature of the antimicrobial effect against a particular pathogen. When the MFC/MIC ratio is between 1:1 and 2:1, the chemical is considered fungicidal. On the other hand, if the ratio is > 2:1, it is more likely to be fungistatic. In the present study, the MFC/MIC ratios of citral were 1 or 2; this suggests that citral has a fungicidal effect against the strains tested. In strains of *C. albicans*, Leite et al. [27], and Zore et al. [50] also reported the fungicidal effect of citral, using the kill time method. Fungicidal activity is clinically more important than fungistatic activity. The prophylactic use of fungistatic drugs has been associated with an increased frequency of innate or acquired resistance in clinical isolates [51].

Table 1. MIC of citral, fluconazole, and amphotericin B; and MFC of citral against *C. tropicalis*

Candida tropicalis	Citral (µg/mL)		Fluconazole (µg/mL)	Amphotericin B (µg/mL)	Control strains[a]
	MIC	MFC	MIC	MIC	
1	512	512	> 1024	2	+
6	512	1024	> 1024	1	+
18	512	1024	> 1024	1	+
20	512	1024	> 1024	1	+
23	512	512	> 1024	1	+
31	256	256	> 1024	0.5	+
36	512	1024	512	1	+
ATCC 13803	512	1024	64	1	+

[a]*microorganism growth in RPMI-1640, DMSO (5%), and Tween 80 (2%), without antifungal or monoterpenes*

Two major fungal structures are important targets of antifungal agents: The cell wall and the plasma membrane. To investigate whether citral acts on fungal cell walls, sorbitol testing was performed. Sorbitol, an osmotic protective is used to stabilize the yeast protoplasts. This test compares the MIC values of the antifungal product in the absence and presence of 0.8 M sorbitol. A distinctive feature of drugs that act by inhibiting the synthesis of fungal cell walls is that their antifungal effect is reversed in a medium containing an osmotic stabilizer such as sorbitol [52]. If the product somehow acts on the cell wall, it causes the lysis of yeast cells in the absence of an osmotic stabilizer, but allows growth in the presence of an osmotic medium [37]. And this effect is detected by an increase in the MIC value in media containing sorbitol, as compared to the MIC of media without sorbitol [53].

In this work, when strains of *C. tropicalis* were treated with citral in a medium supplemented with sorbitol, the MIC values did not increase (Table 2), suggesting that citral does not act by inhibiting fungal cell wall synthesis, but probably by affecting another target.

This is the first study to demonstrate the action of citral on the cell wall of *C. tropicalis* using sorbitol tests. The results are in agreement with those reported by Leite et al. [27] and Lima et al. [29] who have shown that citral does not act on the cell wall of *C. albicans*. Since it appears that citral does not act at the level of the fungal cell wall, another possibility investigated was that it might act at the level of the cell membrane. Some antifungal agents interfere with plasma membrane ergosterol by forming complexes, or by inhibiting membrane biosynthesis [54,55].

To investigate whether citral binds ergosterol in fungal membranes of *C. tropicalis,* the MIC of this monoterpene was determined with and without the addition of ergosterol to the medium. If the antifungal activity of citral is caused by binding to ergosterol, the exogenous ergosterol prevents binding to ergosterol in the fungal cell membrane. As a result there is an MIC increase in the presence of exogenous ergosterol compared to the control [37].

As can shown in Table 2, the MIC value of citral was not altered in the presence of exogenous ergosterol, suggesting that citral does not act by binding to membrane ergosterol. Amphotericin B, a positive control having a known interaction with ergosterol [37], showed a MIC value about 100 times greater in the presence of sterol (Table 2). There are few studies on the direct interaction of citral with ergosterol of the fungal cell membrane. However, in *C. albicans*, Leite et al. [27], and Lima et al. [29] did not observe changes in the MIC of citral in the presence of exogenous ergosterol.

Considering ergosterol is an important fungal cell membrane lipid, changes in its biosynthetic pathway may also cause damage to the fungal cell, preventing growth in a way similar to azole compounds, or similar to fluconazole [38]. To examine whether citral interferes with the biosynthesis of ergosterol in *C. tropicalis* cells, it was necessary to quantify the content of sterols produced by the strains in the presence of this monoterpene in different concentrations (MIC/2, MIC, and MIC x 2), and of fluconazole (at its MIC). For this, it was necessary to analyze the absorption of sterols extracted from fungal cultures at the wavelengths of 230, and 281.5 nm. Ergosterol and an intermediate of the metabolic pathway of ergosterol - 24(28) dehydroergosterol (DHE) absorb energy at 281.5 nm, but (DHE) alone shows intense absorption at 230 nm. Changes in this pattern of absorption are indicative of interference in the synthetic pathway of ergosterol [56].

The results were expressed as % ergosterol biosynthesis inhibition in the absence (control), and presence of fluconazole (at MIC), and citral (at MIC/2, MIC, and MIC x 2) (Fig. 1).

As can be seen, fluconazole (used as a positive control), was able to inhibit ergosterol biosynthesis in fungal cells significantly (p<0.05) compared to the control (no drug), while citral at MIC and MIC x 2 inhibited ergosterol biosynthesis (p<0.05 as compared to the control). Both concentrations were as potent as fluconazole (p>0.05).

These results indicate a possible mechanism of action for citral mediated inhibition of ergosterol biosynthesis, similar to the azoles. However, in contrast to the fungistatic nature of fluconazole, citral appears to have fungicidal nature [27,50]. Using this methodology in investigating the mechanism of action of citral on the species *C. tropicalis* has not been reported in the literature, and this study is the first such report. Recent studies have shown that citral inhibits ergosterol biosynthesis in *C. albicans* [57], and *Penicillium italicum* [58]. Ergosterol biosynthesis inhibition has also been observed for citral at 200 µg/mL in *Aspergillus ochraceus* [59].

According Zore et al. [50] citral also causes S phase cell cycle arrest, and induction of apoptosis in strains of *C. albicans*. In a recent study by Zhou et al. [60], citral inhibited mycelial growth in *Geotrichum citri-aurantii,* and its antifungal activity was attributed to a disruption of cell membrane integrity, and loss of cellular components. Combining antifungal drugs may improve therapeutic response. The potential benefits of using therapeutic combinations include a broader spectrum of efficacy, improved cure rates, safety, and tolerability, reduction of resistance to antifungal drugs, dose reduction, and thus reduced toxicity [61].

Thus, after investigating the mode of action, we evaluated the effect of citral in association with the antifungal fluconazole, and amphotericin B against *C. tropicalis* strains, using the checkerboard technique. The results are shown in Table 3.

Table 2. MIC (µg/mL) of drugs in the absence and presence of sorbitol and ergosterol against *C. tropicalis*

Drugs	*C. tropicalis* 18			*C. tropicalis* ATCC 13803		
	Without sterols[a]	With sorbitol	With ergosterol	Without sterols[a]	With sorbitol	With ergosterol
Citral	512	512	512	512	512	512
Ampho B[b]	1	-	128	1	-	128

[a]*The results are expressed as geometric mean of three experiments.* [b]*Positive control - Not tested*

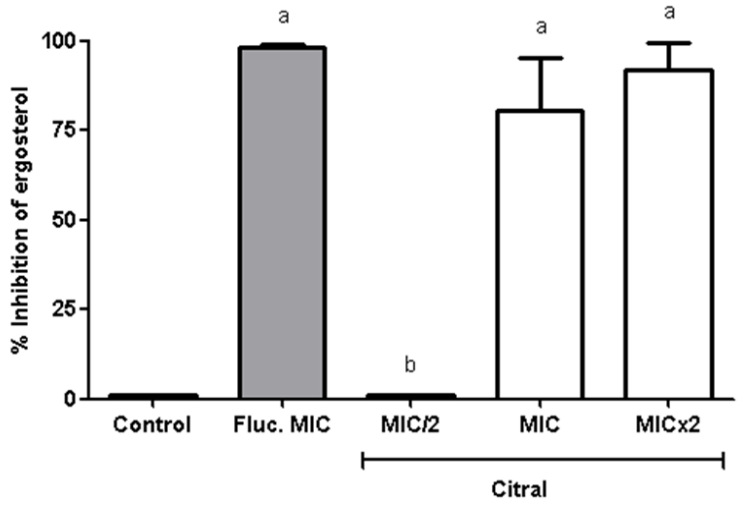

Fig. 1. Percentage of ergosterol biosynthesis inhibition in the absence (control), and presence of citral at MIC/2, MIC, and MIC x 2, and fluconazole at MIC against *C. tropicalis* ATCC (a) p<0.05 compared to control; (b) p<0.05 compared to fluconazole

Table 3. MIC* of citral and effect of combination with antifungals against *C. tropicalis*

Citral + Antifungal	*C. tropicalis* 18		*C. tropicalis* ATCC 13803	
	MIC (µg/mL)	FIC* index (type of interaction)	MIC (µg/mL)	FIC* index (type of interaction)
Citral	512		512	
Amphotericin B	1		1	
Fluconazole	> 1024		64	
Citral/Amphotericin B	128/0.5	0.75 (additive)	256/0.5	1.0 (indifferent)
Citral/Fluconazole	-	-	128/16	0.5 (synergic)

**MIC, minimal inhibitory concentration; *FIC, fractional inhibitory concentration*

As can be seen, the citral-amphotericin B combination was additive (FICI = 0.75) for the clinical strain, and indifferent (FICI = 1.0) for the ATCC strain, while the citral- fluconazole combination was synergistic (FICI = 0.5) against the ATCC strain. The clinical strain of *C. tropicalis* was not evaluated with the citral-fluconazole combination because the MIC of fluconazole was greater than 1024 µg/mL. Fortunately, no antagonistic actions were observed for the combinations studied.

The fact that the citral-amphotericin B combination present different results for the ATCC and clinical strains of the same species (*C. tropicalis*) (Table 3) can be explained in part, by the fact that each strain displays a variety of types of resistance according to the environment and substances to which it has already been exposed. According to published reports, the effect of combining amphotericin B and flucytosine, for example, has varied between synergism and antagonism, and also changes according to the species, and even which strain is tested [62,63].

The focus of this evaluation is of the efficacy of combination antifungal drugs with respect to the extent or rate of death of the fungal pathogen, although other potential interactions (such as pharmacokinetic drug interactions), may well impact efficiency when agents are used together [63].

There are few studies on the citral-antifungal associations against *C. tropicalis* strains. However, in *C. albicans* strains, previous studies have shown synergistic effects for citral-fluconazole [64,50], and for citral-amphotericin B; effects ranging from indifferent to synergistic [64].

There are several synergistic activity mechanisms involved in antifungal combinations: inhibition of the fungal intracellular pathways essential for cell survival in different stages; increased penetration of the antifungal agent provided by the action of the another cell membrane antifungal; inhibition of protein carriers; simultaneous inhibition of different cellular targets [63].

4. CONCLUSION

The results of this study demonstrate that citral shows antifungal activity against strains of fluconazole resistant *C. tropicalis* isolated from blood, increasing the arsenal of products with anti-Candida activity. The citral-fluconazole combination showed synergistic effects. The likely mechanism of citral's action appears not to involve cell walls, or binding to membrane ergosterol, but is likely mediated through inhibition of ergosterol biosynthesis. This study represents an advance in our understanding of citral's antifungal mechanisms of action. However, more studies are needed to investigate whether citral also acts on other targets in the fungal cell.

CONSENT

All the authors declare that no consent was obtained for this study.

ETHICAL APPROVAL

For this study was considered the ethical and legal aspects of research involving human subjects in this study (including human material or human data) is in compliance with the Helsinki Declaration(http://www.wma.net/en/30publications/10policies/b3/index.html). Patient data involved in the research are confidential and sensitive, ensuring respect for privacy and anonymity of the subjects. It was not possible to obtain written informed consent of each patient, since this is a retrospective study. The research was conducted with the approval of the Research Ethics Committee of University Hospital Lauro

Wanderley of the Federal University of Paraíba, Brazil, reference number 0295/11.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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