EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF *Cinnamomum zeylanicum* ESSENTIAL OIL AND *trans*-CINNAMALDEHYDE AGAINST RESISTANT *Mycobacterium tuberculosis*

AVALIAÇÃO DA ATIVIDADE ANTIMICROBIANA DO ÓLEO ESSENCIAL DO Cinnamomum zeylanicum ESSENTIAL OIL E DO trans-CINAMALDEÍDO CONTRA Mycobacterium tuberculosis RESISTENTE

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ABSTRACT: The essential oil (EO) extracted from the bark of *Cinnamomum zeylanicum* (Czey; also known as cinnamon), mostly derives its properties from its major compound *trans*-cinnamaldehyde (TCin). The present study evaluated the antimycobacterial activity of the essential oil from Czey (CzeyEO) and TCin against sensitive and resistant clinical isolates of *Mycobacterium tuberculosis*, as well as the combinatorial effects of CzeyEO and TCin with the anti-tuberculosis (TB) drugs rifampicin (RIF) and isoniazid (INH). The resazurin microtiter assay method was used to determine the minimum inhibitory concentration (MIC) of the components tested on the clinical isolates of *M. tuberculosis*. The effects of the CzeyEO/RIF, CzeyEO/INH, TCin/RIF, and TCin/INH combinations on the *M. tuberculosis* H37Rv reference strain were evaluated using the checkerboard method to determine the fractional inhibitory concentration index (FICI). CzeyEO and TCin inhibited all bacterial clinical isolates. In the interactive experiment, CzeyEO and TCin were found to be highly effective in reducing the resistance of resistant *M. tuberculosis* to RIF and INH. All four tested combinations demonstrated synergistic and additive effects, with no antagonistic effects. The synergistic combinations of CzeyEO/RIF and CzeyEO/INH exhibited FICI values of 0.375 and 0.5, respectively, while the TCin/RIF and TCin/INH combinations exhibited FICI values of 0.31 and 0.5, respectively. These results indicate that CzeyEO and TCin are potential candidates for the treatment of drug-resistant tuberculosis in combination therapy with INH and RIF.

KEYWORDS: *Mycobacterium tuberculosis*. *Cinnamomum zeylanicum*. *trans*- Cinnamaldehyde. Tuberculosis. Anti-bacterial agent.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by the bacilli *Mycobacterium tuberculosis*. In 2016, an estimated 6.3 million new cases occurred worldwide, with approximately 1.4 million deaths from TB alone and 476,000 deaths from HIV-TB coinfection (WHO, 2017). Only 22% of the estimated global incidence in 2015 started with successfully completed the treatment.

The continued increase in mycobacterial resistance to drugs, often associated with treatment withdrawal, has hindered the effective control of TB globally. Disease control is especially difficult in the cases of multidrug-resistant TB, which consists of cases resistant to isoniazid (INH) and rifampicin (RIF) which are the two main anti-TB drugs in current use (WHO, 2017). Considering the increase in mycobacterial resistance to drugs, the development of new drugs is necessary, especially against multidrug-resistant strains (FONSECA et al., 2015). Consequently, medicinal plants through their essential oils (EOs) are considered promising alternatives for the development of new drugs. It is estimated that 25% of the currently used medications are derived directly or indirectly from medicinal plants (WALLIS et al., 2016). One of these medicinal plants that exhibit several biological activities is the *Cinnamomum zeylanicum* (Czey) cinnamon" or "Ceylon ehyde (TCin) is the major component of the EO that is extracted from the bark of *C. zeylanicum* (CzeyEO).

Combination antimicrobial therapy is one of the alternatives for the treatment of infectious diseases caused by multidrug-resistant bacteria. These combinations of antimicrobial agents have been used for the treatment of TB and leprosy (PERRICONE et al., 2015). The analysis of antimicrobial activity of potential active substances and the use of double or triple combinations in *in*

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Received: 15/04/18
Accepted: 05/10/18

vitro studies demonstrated positive interactions capable of microorganism growth inhibition (ODDS, 2003).

Bioactivity against *M. tuberculosis* has already been demonstrated by the Egyptian *Cinnamomum glanduliferum* bark oil (TAHA; ELDAHSHAN, 2017), compounds of *Cinnamomum kotoense* (CHEN et al., 2005), and *Cinnamomum verum* essential oil and extract (ANDRADE-OCHOA et al., 2013; VAIDYA et al., 2016). Extracts and EOs of *C. zeylanicum* exhibit significant antimicrobial activities against several organisms, including the fungus *Aspergillus* (GOMEZ et al., 2018), *M. tuberculosis* (MOTA et al., 2018), gram-positive bacteria, (WIWATTANARATTANABUT et al., 2017), and gram-negative bacteria (LIU et al., 2017). The antibacterial activity of TCin has also been evaluated against several gram-negative bacteria (UTCHARIYAKIAT et al., 2016), gram-positive bacteria, (FERRO et al., 2016), and H37Rv strain of *M. tuberculosis* (ANDRADE-OCHOA et al., 2015; POLAQUINI et al., 2017). However, the antimycobacterial activities of CzeyEO and TCin have not been assessed against sensitive and resistant clinical isolates of *M. tuberculosis*. The antimycobacterial potential of these compounds also has not been studied. Furthermore, the interactions of CzeyEO and TCin with anti-TB drugs have not been determined. In this context, we evaluated the antibacterial properties of CzeyEO and TCin against clinical isolates of *M. tuberculosis* with different resistance profiles, as well as the effects of combinations of CzeyEO and TCin with INH and RIF.

MATERIAL AND METHODS

Chemicals

Isoniazid (purity ≥ 99%), rifampicin (purity ≥ 97%), *trans*-cinnamaldehyde (purity = 99%), Tween® 80 (purity ~ 70%), resazurin sodium salt (purity ~ 80%), and Middlebrook ADC growth supplement were acquired from Sigma-Aldrich (São Paulo, SP, Brazil). Difco™ Middlebrook 7H9 broth base was procured from Becton-Dickinson Biosciences (Franklin Lakes, USA). Lowenstein-Jensen (LJ) medium was purchased from Newprov (Pinhais, Brazil). All chemicals and reagents used were of analytical grade.

Collection and processing of materials for study

The bark samples of *C. zeylanicum* were obtained from the local herb market of Fortaleza, Ceará, Brazil. The samples were stored at the Federal University of Ceará Herbarium. A modified Clevenger apparatus was employed to extract the EOs, as previously described (OKOH et al., 2011). Essential oil yield (wt/wt %) from the bark was then calculated.

Characterization of essential oil by gas chromatography-mass spectrometry (GC-MS)

GC-MS was performed to analyze and identify the EO components. The GC-MS conditions were programmed as previously described (ADAMS, 2004), using a GCMS-QP2010 Ultra mass spectrometer (SHIMADZU, Shimadzu do Brasil, Barueri, Brazil). Parameters specific to the GC-MS were as follows: 5% phenyl 95% dimethylpolysiloxane column: capillary, 30 m × 0.25 mm internal diameter (i.d.), purge flow 30 mL/min, purge time 0.20 min, helium gas; temperature gradient of 4°C/min (50°C to 280°C), inlet: splitless, initial temperature 250ºC. Electron energy of 70 eV was used. The identification of each component was carried out through the agreement of their mass spectral data with the reference from the National Institute of Standards and Technology Mass Spectrometry database.

Mycobacterial isolates and growth conditions

A sensitive reference H37Rv strain and nine clinical isolates from the laboratory stock of Dr. Carlos Alberto Studart Gomes Hospital, Fortaleza, Ceará, Brazil, were used for the antibacterial test.

All mycobacterial clinical isolates previously confirmed using the BACTEC 460 TB System (Becton-Dickinson Biosciences) were tested against CzeyEO, TCin, RIF, and INH following Clinical & Laboratory Standards Institute (CLSI) guidelines (CLSI, 2011). The mycobacterial strains included three drug-sensitive and six multidrug resistant clinical isolates (four strains were RIF- and INH-resistant and two were RIF-, INH-, ethambutol-, and streptomycin-resistant). The minimum inhibitory concentration (MIC) of the essential oils and controls was determined.

The bacterial isolates were grown on LJ medium for 21 days at 37°C. Inocula were prepared from the LJ medium through suspension in saline solution containing 0.04% (vol/vol) Tween® 80 and 0.2% bovine albumin. The bacterial suspension was further diluted into 7H9 broth to a concentration of approximately 3×10^8 colony-forming units (CFU)/mL (1.0 McFarland). The final inoculum concentration of 3×10^7 CFU/mL was then achieved by 1:10 dilution into 7H9 broth, using 100 μL inoculum.

MIC evaluation

The resazurin microtiter assay method was performed to evaluate the MICs as described previously (FRANZBLAU et al., 1998; PALOMINO et al., 2002). All antimycobacterial assays with the reference strain and clinical isolates were performed in triplicates.

The CzeyEO and TCin solutions were diluted in distilled water containing 1% Tween® 80 to a concentration of 1,250 µg/mL. INH and RIF solutions were prepared at concentrations of 4.0 µg/mL in distilled water and 8.0 µg/mL in methanol, respectively, and were filter-sterilized and kept frozen until use. Resazurin sodium salt powder was prepared at 0.02% (wt/vol) in distilled water and filter-sterilized. It could be stored at 4°C for one week.

Aliquots (100 µL) of CzeyEO, TCin, INH, and RIF solutions were added to each well containing 7H9 media. Serial two-fold dilutions of each oil and drug in 100 µL of 7H9 medium were prepared directly in 96-well plates at concentrations ranging from 312 µg/mL to 2.44 µg/mL for CzeyEO and TCin, respectively, 1.0 to 0.07 µg/mL for INH, and 2.0 to 0.0015 µg/mL for RIF. Growth controls without antibacterial agents and sterility controls without bacterial inoculation were also included. The plates were then covered, sealed, and incubated at 37°C. After 7 days of incubation, 30 µL of resazurin solution was added to each well, incubated at 37°C for 24 to 48 h, and assessed for color development. The blue dye resazurin is irreversibly reduced to a pink-colored resorufin in the presence of viable bacterial cells. Where bacterial growth was inhibited, the solution in the well remained bluish after incubation with the dye. The lowest concentration that exhibited blue color was defined as the MIC.

The checkerboard method

The study of the interaction of CzeyEO and TCin with RIF and INH was conducted using the checkerboard method (ROSATO et al., 2007; LECHARTIER et al., 2012). Two-fold serial dilutions of each drug prepared in horizontal rows of a 96-well microtiter plate were subsequently cross-diluted vertically by two-fold serial dilutions of EO and TCin. Microtiter plates were inoculated with the *M. tuberculosis* H37Rv reference strain and incubated for 7 days at 37°C as described above. MIC values were also determined as described above. Interaction between the EO and each drug was then determined by calculating the fractional inhibitory concentration index (FICI). The FICI is defined as follows: (MIC of substance "A" tested in combination/MIC of substance "A" tested alone) + (MIC of substance "B" tested in combination/MIC of substance "B" tested alone). The FICI is interpreted as follows: FICI $\leq$ 0.5, synergistic effect; 0.5 < FIC $\leq$ 1, additive effect; 1 < FICI $\leq$ 4, indifferent effect; and FIC > 4, antagonistic effect. The synergistic effect is shown graphically by applying the isobole method.

Statistical analysis

The experimental data are expressed as mean ± standard deviation (± SD) from three independent experiments. GraphPad Prim 7 software was used to build the isobolograms.

Ethics

This study was approved by the institutional review board of the Ethical Committee of Federal University of Ceará, Fortaleza, CE, Brazil (Approval number: 1292579). Guidelines of the Ethical Committee were followed during the research.

RESULTS

The EO yield (w/w%) of *C. zeylanicum* was 0.13%. CzeyEO was analyzed by gas chromatography-mass spectrometry (GC-MS) and 12 components were identified, representing 100% of the EO. The main components were Tcin (86%), cinnamic acetate (5.46%), α-pinene (1.76%), and eucalyptol (1.65%).

The *in vitro* antibacterial activity of CzeyEO, TCin, RIF, and INH against the tested mycobacteria was determined by the presence or absence of growth and by MIC values. As shown in Table 1, INH and RIF exhibited lower MICs (0.125 and 0.06 µg/mL, respectively) than CzeyEO and TCin (19.5 µg/mL) against the reference *M. tuberculosis* H37Rv strain. In the clinical isolates, the mean MIC of CzeyEO was 21.7 µg/mL (± 6.5 µg/mL), whereas the mean MIC of TCin was 15.2 µg/mL (± 5.1 µg/mL) (Table 1 and Figure 1).

In this study, we evaluated the synergistic potential of CzeyEO and its main component, TCin, when used in combination with INH and RIF against the reference *M. tuberculosis* H37Rv strain using the checkerboard method (Figure 2). Two dilutions of the CzeyEO and INH combination against the reference strain resulted in synergism and they exhibited FICI of 0.375 and 0.5. Moreover, this observation was similar to that of the CzeyEO and RIF combination. The additive effects were observed in five dilutions (FICI of 0.56 to 1.0). The lower FICI value represented an eight-fold reduction

in the MIC of the RIF and INH combination and s
four-fold reduction in the MIC of CzeyEO.

Table 1. Antibacterial activity of isoniazid (INH), rifampicin (RIF), streptomycin (SM), and ethambutol (EMB) drugs against *M. tuberculosis* isolates and minimum inhibitory concentrations (MICs) of CzeyEO and TCin (μg/mL).

***M. tuberculosis* Strains**	**Sensitivity profile***				**MIC (μg/mL)**			
	INH	**RIF**	**SM**	**EMB**	**INH**	**RIF**	**CzeyEO**	**TCin**
Reference H37Rv	S	S	S	S	0.125	0.06	19.5	19.5
Clinical isolates:								
1	S	S	S	S			19.5	19.5
2	S	S	S	S			19.5	9.75
3	S	S	S	S			39.0	19.5
4	R	R	S	S			19.5	9.75
5	R	R	S	S			19.5	19.5
6	R	R	S	S			19.5	9.75
7	R	R	S	S			19.5	19.5
8	R	R	R	R			19.5	9.75
9	R	R	R	R			19.5	19.5
Mean							21.7	15.2
SD							± 6.5	± 5.1

S: susceptible; R: resistant; CzeyEO: essential oil of *C. zeylanicum*; TCin: *trans*-Cinnamaldehyde; SD: standard deviation. *antibacterial activity determined using CLSI guidelines (CLSI, 2011).

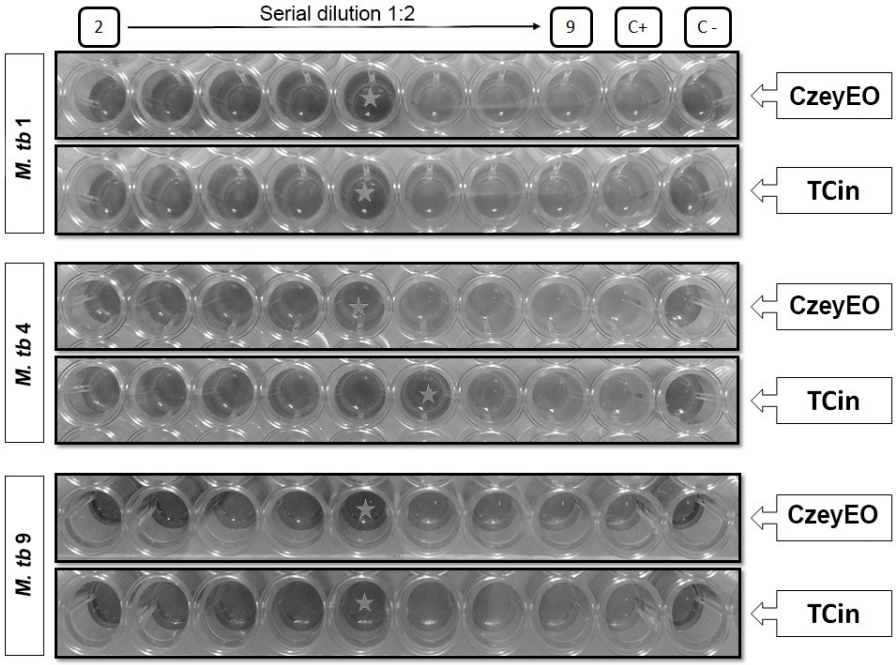

Figure 1. Antibacterial activity by resazurin microtiter assay of CzeyEO and TCin against three clinical isolates of *M. tuberculosis* and minimum inhibitory concentrations (MICs) expressed in μg/mL. The darker wells indicate bacterial growth. The white stars indicate the MIC, the lowest concentration with bacterial growth. The C+ column indicates the positive growth control. The C- column indicates no growth of the negative control. The 2 and 9 indicate the numbers of the plate column. *Mtb* 1, *Mtb* 4, and *Mtb* 9 are *M. tuberculosis* clinical isolates. CzeyEO: *C. zeylanicum* essential oil, TCin: *trans*-Cinnamaldehyde.

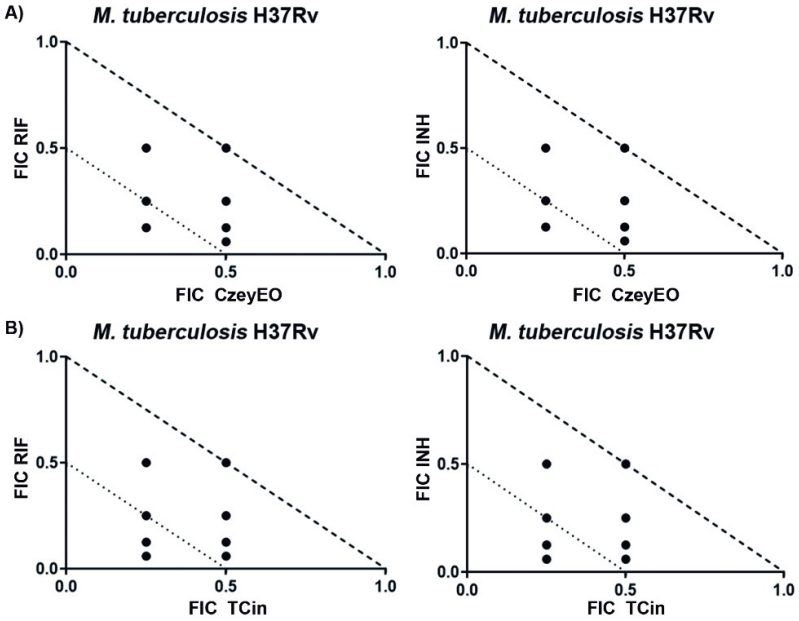

Figure 2. Isobologram showing the synergistic effect of *C. zeylanicum* EO and Tcin with isoniazid and rifampicin drugs against the reference strain *M. tuberculosis* H37Rv. Fractional inhibitory concentration index (FICI) is indicated by black dots. Additive effect is indicated by dots between the dotted line (FICI > 0.5) and the dashed line (FICI ≤ 1.0). Synergistic effect is indicated by dots situated below the dotted line (FICI ≤ 0.5). A) Isobolograms for *C. zeylanicum* essential oil; B) Isobolograms for *trans*-Cinnamaldehyde. RIF: rifampicin, INH: isoniazid, CzeyEO: *C. zeylanicum* essential oil, TCin: *trans*-Cinnamaldehyde.

Similar to what was observed with CzeyEO, TCin interacted with RIF and INH, resulting in a synergistic effect in three dilutions against *M. tuberculosis* H37Rv strain (FICI ranging from 0.31 to 0.5) and an additive effect in five dilutions (FICI ranging from 0.56 to 1.0). Of all the observed FICI values, the lowest value represented a sixteen-fold reduction in the MIC of the RIF and INH combination and a four-fold reduction in the MIC of TCin.

DISCUSSION

The results obtained for the chemical characterization of CzeyEO by GC-MS were similar to those found by other authors, who reported the characteristic presence of TCin as the main component, although there were differences in the observed concentrations (RAVINDRAN et al., 2003; ANDRADE et al., 2012). In addition, we found synergistic association of TCin and CzeyEO with RIF and INH against sensitive and resistant *M. tuberculosis* clinical isolates.

The differences in the observed concentrations among different studies can be attributed to differences in extraction methodology, environmental variations (soil, humidity, temperature, and climate), physiological variations of the plant (evolutionary cycle, growth phase, and stress conditions), and genetic factors (BAKKALI et al., 2008).

CzeyEO and TCin exhibited high antimycobacterial activity and were able to inhibit the growth of all clinical isolates of *M. tuberculosis*. Similar results were found in another study with CzeyEO inhibiting the growth of clinical isolates of *M. tuberculosis* with different resistance profiles, with MICs ranging from 12.5 to 25 μg/mL (ANDRADE-OCHOA et al., 2013). The same authors, in a subsequent study (ANDRADE-OCHOA et al., 2015), observed that TCin was also able to inhibit the growth of *M. tuberculosis* at a concentration of 3.12 μg/mL and of *M. bovis* at a concentration of 12.5 μg/mL.

It has been observed that the strains of gram-negative bacteria are more resistant to EOs *in vitro* compared to strains of gram-positive bacteria (RANA et al., 2011). This is similar to the characteristics of mycobacteria, which have high lipid content in their cell walls, conferring low permeability and resistance to hydrophilic agents (HOFFMANN et al., 2008; FORRELLAD et al., 2013).

The results obtained for the sensitivity of MICs of INH and RIF against the reference *M. tuberculosis* H37Rv strain were similar to those described in the literature, as the breakpoint concentrations for resistance to INH and RIF are 0.25 μg/mL and 0.5 μg/mL, respectively (SINGH et al., 2012). Our MIC results demonstrated lower breakpoint concentrations for resistance to INH and RIF (0.125 and 0.06 μg/mL, respectively). Other authors reported an MIC of 0.06 μg/mL for INH and RIF against *M. tuberculosis* H37Rv strain (CAMACHO-CORONA et al., 2008). Similarly, low MICs of 0.05 μg/mL for INH and 0.03 μg/mL for RIF have been reported (KASULE et al., 2016).

Studies of synergistic and additive activities of EOs and their active components with drugs against several microorganisms have been reported, but there are a few reports of these interactions on *M. tuberculosis* (RASTOGI et al., 1998; CHEN et al., 2011; JIMENEZ-ARELLANES et al., 2013; NAIK et al., 2014; ARO et al., 2016).

Previous studies demonstrated a synergistic activity of *trans*-cinnamic acid with antimycobacterial drugs against sensitive and resistant *M. tuberculosis* clinical isolates (RASTOGI et al., 1998; CHEN et al., 2011). In rats and mice, TCin has been shown to be metabolize into cinnamic acid, an active metabolite against *M. tuberculosis* (NATIONAL TOXICOLOGY, 2004).

We compared the MIC values of the components tested in our study on the clinical isolates of *M. tuberculosis* and observed that TCin exhibited higher inhibitory activity as compared to CzeyEO. Likewise, we observed that in the test for synergism, the interactions were more effective with TCin compared to those with CzeyEO. Our findings corroborate the study carried out in Mexico that found no difference in the MIC values of CzeyEO between the different resistant clinical isolates of *M. tuberculosis* and the standard H37Rv strain (ANDRADE-OCHOA et al., 2015).

Our results suggest that the inhibitory activity of CzeyEO on *M. tuberculosis* is attributed mainly, but not exclusively, to its major component, TCin. Other components, including some terpenes—which exhibit antibacterial activity against *M. tuberculosis* (ANDRADE-OCHOA et al., 2015)—present in CzeyEO also possess antibacterial activity that has been previously reported in the literature (RANA et al., 2011). The synergistic effect has also been reported in the association of α-pinene with limonene in *S. cerevisiae*, which are also identified in CzeyEO (BASSOLE et al., 2011).

The MIC values found in our study for CzeyEO and TCin against the sensitive clinical isolates were similar to those of the clinical isolates resistant to the tested drugs [INH, RIF, streptomycin (SM), and ethambutol (EMB)]. Therefore, we propose that the mechanism of action of CzeyEO and TCin components is distinct from that described for the above mentioned anti-TB drugs. Furthermore, it has been suggested that the CzeyEO and Tcin combination exhibits a synergistic effect to enhance the activities of INH and RIF by inhibiting synthesis of mycolic acids and protein, respectively (PALOMINO; MARTIN, 2014).

The mechanism of action of CzeyEO and TCin, specifically related to the hydrophobicity of their components, has already been investigated in several bacteria, but there have been no studies on their mechanism of action in *M. tuberculosis*. These components of CzeyEO and TCin act primarily on the cytoplasmic membrane, resulting in the altered selective permeability for some ions, altered membrane potential, reduced internal pH, causing loss of proton motive force, membrane rupture, inhibition of the activity of membrane-bound ATPs, and anti-quorum sensing properties (BRACKMAN et al., 2011; YAP et al., 2015).

It is important to mention that all the sensitive and resistant clinical isolates were inhibited by both tested components, demonstrating the absence of intrinsic resistance of *M. tuberculosis* to CzeyEO and TCin, as well as the fact that they were not affected by the mechanisms of resistance to INH, RIF, SM, and EMB. Due to its multiple mechanisms of action in the bacterial cell, studies of resistance or adaptation to CzeyEO have not been described. Therefore, CzeyEO might be used as a potent antibacterial agent.

CONCLUSION

The antimycobacterial activity of CzeyEO and TCin, as well as the synergistic activities observed with INH and RIF, disclose their potential as candidates for combination therapy against drug-resistant *M. tuberculosis*, as well as a new antimicrobial agent for TB treatment. We suggest studies to evaluate time of exposure through the time kill method, *in vitro* studies in macrophages and *in vivo* studies in mice infected with *M. tuberculosis*. We also propose to conduct *in vitro* cytotoxicity studies in different cell lines and investigate mechanisms of action.

ACKNOWLEDGEMENTS

Authors are grateful to the staff of the Dr. Carlos Alberto Studart Gomes Hospital, to the Herbarium, and to the Laboratory of Mycobacteria of the Federal University of Ceará, for their help and guidance.

RESUMO: O óleo essencial (EO) extraído da casca do *Cinnamomum zeylanicum* (CzeyEO), conhecido como canela, tem como seu principal composto o *trans*-cinamaldeído (TCin). O presente estudo avaliou a atividade antimicobacteriana de CzeyEO e do TCin contra isolados clínicos sensíveis e resistentes de *Mycobacterium tuberculosis*, bem como os efeitos das associações de CzeyEO e do TCin com os fármacos anti-TB, rifampicina (RIF) e isoniazida (INH). A técnica de ensaio de microtitulação da resazurina foi utilizada para determinar a concentração inibitória mínima (CIM) dos componentes testados nos isolados clínicos de *M. tuberculosis*. Os efeitos das associações CzeyEO/RIF, CzeyEO/INH, TCin/RIF e TCin/INH contra a cepa de referência H37Rv de *M. tuberculosis* foram avaliados pelo método Checkerboard, determinando o índice de concentração inibitória fracionária (ICIF). Todos os isolados clínicos bacterianos foram inibidos por CzeyEO e TCin. As interações de CzeyEO e TCin foram altamente eficazes na redução da resistência do *M. tuberculosis* resistente a RIF e INH. Todas as quatro combinações testadas resultaram em efeitos sinérgicos e aditivos, sem efeito antagônico. Ambas as associações de sinergismo de CzeyEO/RIF e CzeyEO/INH mostraram valores de ICIF de 0,375 e 0,5, enquanto as associações de TCin/RIF e TCin/INH apresentaram valores de ICIF de 0,31 e 0,5. CzeyEO e TCin são potenciais candidatos em terapia combinada com INH e RIF para o tratamento da tuberculose resistente.

PALAVRAS-CHAVE: *Mycobacterium tuberculosis*. *Cinnamomum zeylanicum*. *trans*-Cinamaldeído. Tuberculose. Agente antibacteriano**.**

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