Biological activity of the essential oils from *Cinnamodendron dinisii* and *Siparuna guianensis*

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Abstract

This study had analyzed the antibacterial, antifungal and trypanocidal activity of the essential oils from *Cinnamodendron dinisii* Schwacke (Canellaceae) and *Siparuna guianensis* Aublet (Siparunaceae). The essential oils were obtained from fresh leaves by hydrodistillation, using a modified Clevenger apparatus. Chemical analysis by gas-liquid chromatography coupled to mass spectrometry (GC-MS) showed that these essential oils are rich in monoterpene and sesquiterpene hydrocarbons. Activity against the pathogenic bacteria *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella choleraesuis* and *Staphylococcus aureus* was evaluated with the agar cavity diffusion method, while activity on the filamentous fungi *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus carbonarius* and *Penicillium commune* was evaluated by the disk diffusion technique. Trypanocidal activity was tested against *Trypanosoma cruzi* epimastigotes, using the Tetrazolium salt (MTT) colorimetric assay. Both essential oils exhibited low inhibitory effect towards bacteria, showing high MIC values (125-500 μg mL⁻¹), with Gram positive bacteria being more susceptible. Better inhibitory effect was obtained for the evaluated fungi, with lower MIC values (7.81-250 μg mL⁻¹), being *A. flavus* the most susceptible species. Both essential oils presented low trypanocidal activity, with IC₅₀/24 h values of 209.30 μg mL⁻¹ for *S. guianensis* and 282.93 μg mL⁻¹ for *C. dinisii*. Thus, the high values observed for the MIC of evaluated bacteria and for IC₅₀/24 h of *T. cruzi*, suggest that the essential oils have a low inhibitory activity against these microorganisms. In addition, the low MIC values observed for the tested fungi species indicate good inhibitory activity on these microorganisms’s growth.

Key words: volatile oils, fungi, bacteria, *Trypanosoma cruzi*.

Introduction

There is increasing evidence showing the potential of plant constituents as antimicrobial agents. They appear as a source of alternative, efficient and feasible compounds to control growth and survival of microorganisms in diverse areas, such as agriculture, human health, food and cosmetology (Bakkali et al., 2008; Borges et al., 2012). Among the natural compounds with antimicrobial potential we may highlight the essential oils. These compounds are volatile products synthesized from glucose through the secondary metabolism of plants. They are characterized by strong odor and complex composition and can be obtained from different plant structures through hydrodistillation or steam distillation (Dewick, 2002).
Bacteria are the main microorganisms involved in food contamination processes because they act at different substrate types, wide temperature and pH ranges, as well as diverse environmental conditions. These microorganisms account for a high number of outbreaks and deaths, causing infections, toxinoises or toxinfections. In smaller proportions, fungi are also responsible for foodborne illnesses, exploiting food subjected to inadequate storage conditions, producing mycotoxins and/or causing deterioration (Sousa, 2003; Jay, 2005).

The indiscriminate use of antibiotics to control bacteria and fungi led to the emergence of drug-resistant strains. The difficulties to discover and launch new antimicrobials have stimulated research on new synthetic and/or natural antimicrobial compounds, mainly of plant origin (Cowan, 1999).

The protozoan *Trypanosoma cruzi* is the causer of Chagas disease, an endemic parasitic disease in tropical and subtropical countries of Latin America. This neglected disease affects approximately 10 million people around the world and about 25-100 million people live in areas at risk of infection (WHO, 2010; Salomon, 2012). No vaccine has been discovered up to now for Chagas disease and current treatment with nifurtimox or benznidazole is ineffective due to differences in susceptibility to *T. cruzi*. Furthermore, these drugs are often poorly tolerated, have serious side effects and efficient only in the acute phase of the disease (Saúde-Guimarães and Faria, 2007; Campos et al., 2009), fact that makes it extremely relevant research aimed at finding effective drugs to combat Chagas disease.

Thus, essential oils appear as an alternative source of antimicrobial agents against spoilage microorganisms, pathogens that cause foodborne illnesses and also the pathogenic protozoan *Trypanosoma cruzi*, opening new perspectives for the discovery of more effective drugs of plant origin. Essential oils can interact with the microbial membrane and cause drastic physiological changes leading to loss in membrane permeability, ultimately resulting in cell death (Borges et al., 2012). However, due to the large number of components and synergistic or antagonistic interactions among them, it is possible that essential oils have cellular targets other than cell membranes.

The aim of this study was to determine the antibacterial, antifungal and trypanocidal activity of the essential oils from *Cinnamodendron dinisii* Schwacke and *Siparuna guianensis* Aublet. *Cinnamodendron dinisii* (Canellaceae) is an endemic tree from the Brazilian Atlantic Forest, popularly known as “pimenteira” (Peixoto and Barros, 2010), while *Siparuna guianensis* (Siparunaceae) is a typical bush of the Brazilian cerrado, popularly known as “negramina”. Decoction of its leaves is traditionally used to relieve fever and body pain (Valentini et al., 2008).

### Material and Methods

#### Plant material

*Cinnamodendron dinisii* and *Siparuna guianensis* leaves were collected in February 2011, in the morning, at the Horto de Plantas Medicinais-UFLA (Federal University of Lavras, MG, Brazil) and the UFLA campus, respectively. The collection sites have the following coordinates: *C. dinisii*: -21°13′49.0476″ latitude, -44°58′27.4764″ longitude and 933 m altitude; *S. guianensis*: -21°13′41.9952″ latitude, -44°58′9.0048″ and 951 m altitude.

The collected material was sent to the Laboratory of Organic Chemistry - Essential Oils of the Chemistry Department of UFLA, where mature and healthy leaves with no injuries caused by pathogens, insects or heat stroke were selected. The plant material was properly packed and kept under refrigeration (7 °C) until extraction of essential oils. Species were properly identified and a voucher specimen was deposited at the Herbário ESAU, located in the Biology Department of UFLA, with the following registration numbers: 26,285 (*Cinnamodendron dinisii* Schwacke) and 26,623 (*Siparuna guianensis* Aublet).

#### Essential oils

Essential oil extraction was performed at the Laboratory of Organic Chemistry - Essential Oils, UFLA, using fresh leaves and a 2 h distillation period. The extraction method was hydrodistillation, using a modified Clevenger apparatus (Brasil, 2010). The oil was then separated from the hydrolate by centrifugation at 1,100 g for 5 min, using a horizontal crosspiece benchtop centrifuge. The essential oil was collected, packed in glass bottle and stored under refrigeration (4 °C), protected from light (Guimarães et al., 2008).

#### Identification and quantification of the essential oil constituents

The identification of the constituents of the essential oils was performed by gas-liquid chromatography coupled to mass spectrometry (GC-MS), using an Autosystem XL equipped with a DB-1 fused silica column (30 m x 0.25 mm ID, film thickness 0.25 μm; J & W Scientific Inc.) connected to a Perkin-Elmer Turbomass. The oven temperature was programmed from 45 to 175 °C in increments of 3 °C min⁻¹ and subsequently at 15 °C min⁻¹ to 300 °C. On reaching 300 °C, the temperature was kept isothermal for 10 min, temperature of the transfer line, 280 °C; temperature of the ionization chamber 220 °C, helium carrier gas, adjusted to a linear velocity of 30 cm s⁻¹; split-flow ratio 1:40, ionization energy 70 eV, ionization current, 60 μA; mass range, 40-300 U, scan time 1 s. The compounds were identified by comparison of their retention indices, relative to those of *n*-alkanes C₁₀-C₂₁ and by comparison with a library of mass spectra developed in the laboratory of the Centre for Plant
Biotechnology, Faculdade de Ciências da Universidade de Lisboa-Portugal (Mendes et al., 2011).

The content of each constituent was determined by gas chromatography (GC-FID) in a Perkin Elmer 8700 gas chromatograph equipped with two Flame Ionization Detectors (FID), a data processing system and an injector, in which two columns of different polarity were installed: DB-1 fused silica, with immobilized methylsilicone phase (30 m x 0.25 mm ID, film thickness 0.25 m; J & W Scientific Inc.) and DB-17HT fused silica (30 m x 0.25 mm ID, film thickness 0.25 mm; J & W Scientific Inc.). The oven temperature was programmed from 45 °C to 175 °C in increments of 3 °C min⁻¹ and subsequently at 15 °C min⁻¹ to 300 °C. On reaching 300 °C, the temperature was kept isothermal for 10 min. Injector and detector temperature, 290 °C and 280 °C, respectively. Hydrogen was used as carrier gas adjusted to a linear velocity of 30 cm s⁻¹. Split-flow ratio of 1:50. The percentage of oil constituents was determined by integration of peak areas without using correction factors. The values shown correspond to the average value of two injections (Mendes et al., 2011).

Antibacterial activity

Evaluation of the antibacterial activity of the essential oils was performed at the Laboratory of Food Mycology, in the Food Science Department of UFLA. Bacteria used were Staphylococcus aureus ATCC 13565, Listeria monocytogenes ATCC 19117, Escherichia coli ATCC 11229, Salmonella cholerasuis ATCC 6539 and Pseudomonas aeruginosa ATCC 15442.

The bacteria were subcultured in BHI (Brain Heart Infusion) medium and incubated at 37 °C for 24 h. Aliquots were then transferred to a tube containing 5 mL of tryptic soy broth (TSB). The tubes were incubated at 37 °C until reaching turbidity of a 0.5 McFarland standard solution, equivalent to a suspension with 10⁸ cfu mL⁻¹. Turbidity reading was performed using a spectrophotometer (Shimadzu UV-1601 PC) at a wavelength of 625 nm (NCCLS, 2003).

Concentration inoculum of 10⁸ CFU mL⁻¹ was determined by the 0.5 McFarland scale, this trial was diluted in the experimental culture medium (Tryptic Soy Agar for Listeria monocytogenes and Mueller-Hinton agar for the other bacteria) until the final concentration of 10⁶ CFU mL⁻¹. Agar inoculated with the bacterial culture was layered on the same agar, previously solidified, where essential oil deposition wells (4 mm diameter) were made with the aid of glass beads. These wells were filled with 10 μL of the essential oil (at 500, 250, 125, 62.5, 31.25, 15.62, 7.81 and 3.90 μg mL⁻¹) diluted in DMSO. The plates were incubated at 37 °C for 24 hours and the diameter of the inhibition halos was measured. Three replicates were performed for each treatment, with one control well containing 10 μL DMSO (Lima et al., 2012). A solution of 100 μg mL⁻¹ chloramphenicol (CL) was used as positive standard to evaluate bacterial sensitivity.

The growth inhibition halo was measured from the circumferencce of the well to the edge with microorganism growth. From the obtained measurement, which evidenced microorganism sensitivity, it was possible to evaluate bacterial sensitivity at different essential oil concentrations. The minimum inhibitory concentration (MIC) was defined as the lowest essential oil concentration that produced an inhibition halo.

Antifungal activity

Evaluation of the antifungal activity was performed at the Laboratory of Food Mycology in the Food Science Department, UFLA. The following fungi were used: Aspergillus flavus, Aspergillus carbonarius, Aspergillus niger and Penicillium commune. The fungi were kept at 25 °C in Malt Extract Agar (MEA) medium.

For the inhibitory effect on filamentous fungi, a disk diffusion test was used, with an inoculum concentration of 10⁶ spores mL⁻¹. This inoculum was transferred to a plate containing solidified Malt Extract Agar (MEA) medium, using the surface scattering technique. Filter paper discs (4 mm in diameter) soaked with 10 μL of different concentrations of the essential oils (500, 250, 125, 62.5, 31.25, 15.62, 7.81 or 3.90 μg mL⁻¹) were placed on the solidified culture medium. A control was made using discs impregnated with 10 μL DMSO. A 20 μg mL⁻¹ sodium hypochlorite solution was used as positive standard to evaluate fungi sensitivity. The plates were incubated at 25 °C for 72 h (Barry and Thornsberry, 1991).

The growth inhibition halo was measured starting from the paper disc circumference to the edge with microorganism growth. Orthogonal measurements were obtained, each one corresponding to the mean of two diametrically opposed measurements. MIC was defined as the lowest essential oil concentration that allowed identification of an inhibition halo.

Trypanocidal activity

Activity of the essential oils against Trypanosoma cruzi epimastigotes, strain DM28c, was analyzed at the Instituto Carlos Chagas/Fiocruz-PR, Curitiba/PR. Culture epimastigotes were kept at 28 °C in LIT (Liver Infusion-Tryptose) medium containing 10% fetal calf serum (Camargo, 1964). Three-day-old epimastigotes, at the mid-logarithmic phase of growth, were used in the experiments. The essential oils were first solubilized in dimethyl sulfoxide (DMSO) and then diluted to 1 mg mL⁻¹ in LIT medium (stock solution), so that the DMSO concentration never exceeded 1% in the experiments. Screening was made by serial dilution of the stock solution with LIT in a 96-well ELISA (Enzyme-Linked Immunosorbent Assay) microtiter plate, in a final volume of 20 μL for each well. Concentrations used were: 500, 250, 125, 62.5, 31.25,
15.62, 7.81 and 3.90 μg mL⁻¹. After essential oil dilution, 180 μL of culture medium with epimastigotes (2.10^9 cells mL⁻¹) was added to each well. Control wells contained 200 μL of culture medium with parasites, without the compounds. The plates were incubated for 24 h at 28 °C and then 50 μL of Tetrazolium salt (MTT) solubilized at 10 μg mL⁻¹ in PBS (phosphate buffered saline, pH 7.2) were added to all wells (final concentration: 2 μg mL⁻¹ per well). The plates, wrapped in aluminum foil, were incubated at 37 °C for 3 h (Mosmann, 1983).

After the MTT incubation, 50 μL of 4% paraformaldehyde in PBS (pH 7.2) was added to each well. The plate was centrifuged at 2,100 g for 10 min, the medium was removed by sudden inversion of the plate, the parasites were resuspended in 20 μL of 0.01 M HCl containing 10% sodium dodecylsulphate (SDS) and the plate was incubated at 37 °C for 1 h, or until lysis of all parasites. After adding 80 μL DMSO to all wells the plates were incubated at 37 °C for 30 min, followed by stirring until complete solubilization of the formazan crystals. Reading was performed at 550 nm in an ELISA reader. The percentage of unviable epimastigotes (%UE) cells was determined by the equation %UE = [100 (A_c-A_t)]/A_c, where A_c represents the absorbance of the control and A_t the absorbance of treated cultures. The experiments were performed in triplicate.

The statistical program used was SISVAR (Ferreira, 2008). Data were subjected to analysis of variance. Means were subjected to regression and were also compared by the Scott-Knott test, both at 5% probability. The adjusted equations were used to calculate the IC_{50} value and graphs were plotted with values of %UE versus concentrations analyzed, using the software Origin 6.0.

Results and Discussion

The essential oil from C. dinisii fresh leaves is composed mainly by monoterpene hydrocarbons (76.20%), followed by sesquiterpene hydrocarbons (10.80%), with main components α-pinene (35.41%), β-pinene (17.81%), sabinene (12.01%) and bicyclogermacrene (7.59%). The essential oil from S. guianensis fresh leaves contains sesquiterpene hydrocarbons (41.50%), oxygenated sesquiterpenes (19.40%) and monoterpene hydrocarbons (17.90%). The main compounds are β-myrcene (13.14%), germacrene-D (8.68%) and bicyclogermacrene (16.71%).

Minimum inhibitory concentration values (MICs) of the essential oils for some Gram-positive and Gram-negative bacteria are expressed in Table 1. Both essential oils showed weak inhibitory effect with high MIC values (range: 125.00 to 500.00 μg mL⁻¹) for Gram-positive bacteria, with no inhibitory effect on Gram-negative bacteria. The Gram-positive bacteria L. monocytogenes and S. aureus were more susceptible to the S. guianensis essential oil at the evaluated concentrations. All tested bacteria were sensitive to 100 μg mL⁻¹ chloramphenicol.

A higher susceptibility of Gram-positive bacteria, as compared to Gram-negative bacteria, towards essential oils has been already observed (Burt, 2004). Gram-negative bacteria have an outer membrane with lipopolysaccharide molecules, resulting in a hydrophilic surface (Burt, 2004). Thus, the outer membrane acts as a barrier to penetration of macromolecules and hydrophobic compounds, making Gram-negative bacteria relatively resistant to antibiotics and other hydrophobic drugs, such as essential oils.

The essential oils from C. dinisii and S. guianensis are rich in monoterpene and sesquiterpene hydrocarbons. Since these molecules are hydrophobic (Burt, 2004), they probably move from the aqueous phase into cell membranes, causing toxic effects to the bacterial membrane structure and function. Furthermore, essential oils make cell membranes permeable to compounds that usually are unable to cross intact membranes, thus promoting changes in cytoplasmic density and coagulation of some cytoplasmic components. This damage may ultimately lead to bacterial cell death (Cox et al., 2001).

Evaluation of the bacteriostatic and bactericidal activity of the monoterpenes α- and β-pinene, (R and S)-limonene, 1,8-cineole and borneol towards Listeria monocytogenes showed that α-pinene was the most active component, with MIC = 0.019% for this microorganism (Mourey and Canillac, 2002). Thus, the antibacterial activity of the essential oil from C. dinisii towards L. monocytogenes here observed may be related to the high content (35.41%) of α-pinene in this essential oil.

MIC values with the tested fungi are expressed in Table 2. Both essential oils were active against all evaluated fungi, with inhibitory concentration ranging from 7.81 to 250 μg mL⁻¹. The essential oil from S. guianensis was more effective, with lower MIC values. The species A. flavus showed a higher sensitivity for the S. guianensis essential oil and P. commune for the C. dinisii essential oil, while

| Bacteria              | Gram | MIC (μg mL⁻¹) | Inhibition halo (mm) |
|-----------------------|------|--------------|----------------------|
|                        |      | SG           | GD       | CD       | CL       |
| S. aureus             |      | 125.00       | 250.00   | 6.00     | 14.80    |
| L. monocytogenes      | +    | 250.00       | 500.00   | 6.00     | 3.20     |
| E. coli               | -    | 500.00       | NI*      | 5.30     | 14.80    |
| P. aeruginosa         | -    | 500.00       | -        | 6.00     | 20.00    |
| S. choleris           | -    | NI*          | NI*      | -        | 18.90    |

*NI: no inhibition.

Table 1 - Minimum inhibitory concentration (MIC) and length of inhibition halos caused by the essential oils from S. guianensis (SG) and C. dinisii (CD), and by chloramphenicol (CL) at 100 μg mL⁻¹ for the evaluated bacteria.
**Table 2**
Minimum inhibitory concentration (MIC) and length of inhibition halos caused by the essential oils from *S. guianensis* (SG) and *C. dinisii* (CD), and by sodium hypochlorite (SH) at 20 μg mL⁻¹ for the evaluated fungi.

| Fungi                | MIC (μg mL⁻¹) | Inhibition halo (mm) |
|----------------------|---------------|----------------------|
|                      | SG    | CD    | SG    | CD    | SH    |
| *A. flavus*          | 7.81  | 125.00 | 6.70  | 6.30  | 25.70 |
| *A. carbonarius*     | 125.00| 250.00 | 7.00  | 6.30  | 17.20 |
| *A. niger*           | 31.25 | 62.50  | 6.00  | 6.00  | 19.20 |
| *P. comune*          | 31.25 | 31.25  | 7.00  | 6.70  | 31.00 |

*A. carbonarius* was the most resistant fungal species, when compared to both evaluated essential oils. All microorganisms were sensitive to 20 μg mL⁻¹ sodium hypochlorite.

Montanari *et al.* (2010) showed that *S. guianensis* essential oil inhibited growth of *C. albicans*, *Cryptococcus neoformans*, *Trychophyton rubrum* and *A. fumigatus* at low concentrations, especially the fungus *C. neoformans* (16 μg mL⁻¹). The antifungal properties of the essential oil from *Pleodendron costaricense* (Canellaceae), which presents α- and β-pinene, β-myrcene, β-thujene, and β-caryophyllene as major compounds, was also evaluated, with high activity against *Alternaria alternata*, *Candida albicans* and *Wangiella dermatitidis* (Amiguet *et al.*, 2006). Our data corroborate these studies regarding the antifungal potential for *S. guianensis* and in the case of *C. dinisii*, for plants belonging to the Canellaceae family.

As for bacteria, the mechanism of inhibitory action of essential oils on fungi is based on their lipophilicity. Their interference on composition or tridimensional arrangement of the microbial plasma membrane will render it devoid of the basic structure for homeostatic balance and optimal physiological functioning. Furthermore, ergosterol is present in fungal membranes, acting as a membrane fluidity modulator. Chemically it is classified as a highly lipophilic membrane steroid and any action of essential oils on this molecule may trigger an imbalance in fluidity of the fungal plasma membrane, leading to changes in intracellular homeostasis (Souza *et al.*, 2003).

Both essential oils showed weak inhibitory effect on the protozoan *T. cruzi*, with increased unviability of epimastigotes observed with increasing concentrations of both essential oils after 24 h of treatment (Table 3). Best activity was obtained for *S. guianensis* (IC₅₀/24 h = 209.30 μg mL⁻¹), followed by *C. dinisii* (IC₅₀/24 h = 282.93 mg mL⁻¹). These values are high, when compared to that obtained with the reference compound benznidazol (IC₅₀/24 h = 12.8 μg mL⁻¹) (Lamas *et al.*, 2006).

This is the first report on the trypanocidal activity of the essential oil extracted from *C. dinisii* and *S. guianensis* leaves. There is increasing evidence that essential oils, as well as their constituents, present biological activity against pathogenic trypanosomatids (Anthony *et al.*, 2005; Alvino *et al.*, 2012). It has been proposed that the activity of essential oils on trypanosomatids is mainly due to terpene composition (Borges *et al.*, 2012). Terpenes are responsible for the hydrophobic character of essential oils, thus allowing their diffusion through the parasite cell membrane, affecting intracellular metabolic pathways and organelles. Accordingly, it has been shown that citral, an oxygenated terpene found in the volatile oil of *Cymbopogon citratus*, presents activity against *T. cruzi* (Santoro *et al.*, 2007), possibly by inducing cell membrane lysis, with leakage of cytoplasm. Although oxygenated terpenes are found in small proportions in the essential oil from *C. dinisii* (12% monoterpenes and oxygenated sesquiterpenes) and *S. guianensis* (19.40% oxygenated sesquiterpenes), it is possible that these compounds are associated to the trypanocidal activity of these essential oils in a synergistic behavior.

**Table 3** - Activity of essential oils from *S. guianensis* and *C. dinisii* on *Trypanosoma cruzi* epimastigote forms.

| Concentration (μg mL⁻¹) | *S. guianensis* | *C. dinisii* |
|-------------------------|-----------------|--------------|
| 3.90                    | 0.36 a**        | 3.37 a       |
| 7.81                    | 2.10 a          | 8.39 b       |
| 15.62                   | 9.50 b          | 10.57 c      |
| 31.25                   | 21.73 c         | 13.22 c      |
| 62.50                   | 25.34 c         | 15.04 c      |
| 125.00                  | 45.80 d         | 24.78 d      |
| 250.00                  | 84.28 e         | 36.89 e      |
| 500.00                  | 86.81 e         | 87.96 f      |

IC₅₀/24 h (μg mL⁻¹)*

193.30 282.93

*IC₅₀ = Concentration that causes lysis or death of 50% of the population of *T. cruzi* epimastigotes in 24 h. ** Mean values in a column followed by the same letter do not differ by the Scott-Knott test at 5% significance.

**Conclusion**

The essential oils of *Cinnamomendron dinisii* and *Siparuna guianensis* presented small inhibitory effect on bacteria, showing high MIC values (125-500 μg mL⁻¹) or even no inhibition, especially for Gram-negative bacteria. The tested microorganisms were less sensitive to the essential oil from *C. dinisii* in the evaluated concentrations.

The essential oils presented inhibitory effect for the evaluated fungi, with low MIC values (7.81-250 μg mL⁻¹). In the evaluated concentrations, the tested microorganisms were less sensitive to the essential oil from *C. dinisii*.

Growth inhibition of *Trypanosoma cruzi* epimastigotes allowed to determine an IC₅₀/24 h value of 282.93 μg mL⁻¹ for *C. dinisii* and 209.30 μg mL⁻¹ for *S. guianensis*. These values are high, when compared to that obtained with the reference compound benznidazol (IC₅₀/24 h = 12.8 μg mL⁻¹).
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