Chemical composition of essential oil from ripe fruit of *Schinus terebinthifolius* Raddi and evaluation of its activity against wild strains of hospital origin

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Abstract

The essential oil (EO) composition of ripe fruit of *S. terebinthifolius* Raddi was analyzed by GC-MS. The oil extraction yielded 6.54 ± 1.06% (w/w). Seventeen compounds were identified, accounting for 91.15% of the total oil, where monoterpenes constituted the main chemical class (85.81%), followed by sesquiterpenes (5.34%). The major monoterpene identified was δ-3-carene (30.37%), followed by limonene (17.44%), α-phellandrene (12.60%) and α-pinene (12.59%). Trans-caryophyllene (1.77%) was the major sesquiterpene identified. The antibacterial activity of the essential oil was evaluated against wild strains of hospital origin (*Escherichia coli*, *Pseudomonas* sp., *Klebsiella oxytoca*, *Corynebacterium* sp., *Staphylococcus aureus*, *Enterobacter* sp., *Enterobacter agglomerans*, *Bacillus* sp., *Nocardia* sp. and *Streptococcus* group D). The essential oil of the ripe fruit of *S. terebinthifolius* Raddi has shown to be active against all tested wild strains, with minimum inhibitory concentration ranging from 3.55 µg/mL to 56.86 µg/mL. However, it has revealed some differences in susceptibility: the general, Gram-positive species showed greater sensitivity to the action of EO, which is probably due to the lower structural complexity of their cell walls.

Key words: essential oil, *Schinus terebinthifolius* Raddi, GC-MS, antibacterial activity.

Introduction

Currently, the problem of drug resistance in human pathogens and animals poses a serious challenge to both developed and developing countries. The consumption of more than one daily ton of antibiotics in some European countries has resulted in resistance of bacterial populations, thus causing a serious public health problem (Duarte, 2006) and becoming one of the biggest causes of failure in the treatment of infectious diseases (Hirakata *et al.*, 1998).

The search for natural methods that are less aggressive to humans has increased considerably in recent years. Medicinal plants have been an important therapeutic resource since the dawn of time. The herbal medicine is growing, especially in recent years (Yunes *et al.*, 2001). However, despite the increase of studies in this area, available data show that only 15 to 17% of plants were studied for their medicinal potential (Turolla and Nascimento, 2006).

*Schinus terebinthifolius* Raddi, popularly known as mastic, mastic red-pepper, pepper tree, Brazilian pepper or Christmas Berry, from the family Anacardiaceae, is a species originated in South America, mainly from Brazil, Paraguay, and Argentina. In Brazil, it is found from Ceará (northeast) to Rio Grande do Sul (south) (Jones, 1997).

Its bark has action against fever, hemoptysis and uterine disorders in general. Bark extract and oil are used against tumors and corneal diseases (Degáspari *et al.*, 2004; Moustafa *et al.*, 2007). The fruit and their essential oil (EO) are assigned antimicrobial activity on Gram-positive and anti-inflammatory by inhibiting the enzyme phospholipase A₂ (Pires *et al.*, 2004).

The species has been studied in relation to chemical composition and biological activities, because of its medic-
inal and phytochemical (Lenzi and Orth, 2004a; Lenzi and Orth, 2004b) potential. Essential oils are volatile substances that usually have pleasant odors and are found in virtually all living tissue of plants and usually extracted by hydrodistillation (Isman, 2000). These oils play an important role in protecting against microorganisms and are bound to plant survival due to their different functions. Studies estimate that approximately 60% of EO have antifungal activity and 35% have antibacterial activity (Lima et al., 2006a), and they are active against viruses and protozoa (Cowan, 1999).

EO consists of a mixture of hydrocarbons (terpenes) and oxygenates derived from an isoprenic unit, which in turn originates from mevalonic acid, or phenylpropanoids, stemming from shikimic acid (Cowan, 1999; Guenther, 1977; Rates, 2001).

The EO extracted from ripe fruit of S. terebinthifolius provides a predominantly monoterpene composition (Malik et al., 1994; Pieribattesti et al., 1981).

Based on these considerations, this paper aims to characterize chemically and physicochemically the EO extracted from fruits of S. terebinthifolius Raddi, from the State of Espírito Santo, and evaluate its antibacterial activity against wild strains of hospital origin.

Material and Methods

Plant material

Ripe fruit from S. terebinthifolius Raddi were collected on the campus of Federal University of Espirito Santo (UFES), Goiabeiras, Vitória, Espírito Santo, Brazil (S 20°16.8696', W 040°18.1194').

Plant origin was identified by Solange Zanotti Schneider from the Biology Department of Vila Velha University (UVV-ES). A voucher specimen was deposited at the Herbarium of UFES (VIES 14711).

The material was subjected to drying in open air for a week at room temperature, so that there was no loss of volatile components. Subsequently, the fruits were peeled and subjected to extraction by hydrodistillation.

Bacterial strains

The bacterial cultures used in the tests were provided by a public hospital in the metropolitan region. Gram-negative bacteria: Escherichia coli; Klebsiella oxytoca; Pseudomonas sp.; Enterobacter sp.; Enterobacter agglomerans and Gram-positive bacteria: Streptococcus group D; Staphylococcus aureus; Corynebacterium sp.; Bacillus sp. and Nocardia sp.

Extraction of essential oil

The EO was extracted by hydrodistillation method using modified Clevenger apparatus coupled to a round bottom flask of 3000 mL.

Ripe fruit of S. terebinthifolius (200 g) were peeled and ground in a blender to achieve uniform particle size, and along with 1500 mL of deionized water were extracted for 6 h counted from the start of reflux.

The hidrolact obtained was partitioned with three portions of 30 mL dichloromethane in a separatory funnel, and dried with anhydrous sodium sulfate, and then filtered and the solvent removed under reduced pressure (40 mm Hg). The EO obtained was weighed and stored in amber bottle in the refrigerator. The yield of the extraction procedure was determined in triplicate.

Gas chromatography with mass spectrometry (GC-MS)

The EO was then analyzed by GC-MS using a Shimadzu QP-5000 mass spectrophotometer equipped with fused silica DB-5 [30 m x 0.25 mm (inside diameter), 0.25 μm film thickness], using helium as carrier gas at a split ratio of 20:1. The injector and ion detector temperatures were set at 220 °C and 230 °C, respectively. The furnace temperature was programmed from 60 °C to 240 °C at 3 °C/min. The mass spectra were scanned in the range of 40 m/z-450 m/z. Different constituents were identified on the basis of: a) Computer matching of mass spectra with NIST library (Nist 62 MS Library); b) Comparison of their retention indices relative to homologous series of n-alkanes (C9-C24) (Adams, 2001).

Specific density

This parameter was determined through digital densimeter (Anton Paar, model Stabinger number SVM 3000), calibrated at 20 °C, in compliance with the ASTM D5002 standard methodology (ASTM, 1999).

Refractive index

This determination was made through Abbe refractometer (Carl-Zeiss Jena, Model G), at 20 °C, in compliance with the AOAC methodology (AOAC, 1995).

Optical rotation

Measurements of optical rotation of the EO, undiluted, were performed in digital polarimeter (Perkin Elmer-Polarimeter 241) that uses sodium D ray (λ = 589.3 nm) with optical path of 1 dm and a bucket with a 0.8 mL capacity, at 23.5 °C.

Evaluation of antibacterial activity

Sample preparation

The EO of S. terebinthifolius Raddi was initially diluted in dimethylsulfoxide (DMSO) in order to obtain a stock solution concentration of 454.85 μg/mL. Intermediate concentrations were prepared by diluting the stock solution in an appropriate medium so as to result in final
concentrations 227.43, 113.71, 56.86, 28.43, 14.21, 7.11, 3.55 and 1.78 μg/mL.

Both EO and DMSO were previously sterilized using membrane filter of 0.22 mm in pore size.

**Preparation of culture medium**

We used the Micromed® nutritive broth, prepared from dehydrated medium with the addition of distilled water according to the manufacturer’s recommendation. Then it underwent autoclaving process and was deposited in test tubes.

**Inoculum preparation**

Before the tests, the bacterial cultures were activated by subculture on Mueller-Hinton agar for 24 h at 37 °C. After activation, the inoculum was standardized to 10^8 cells/mL, which consisted in preparing a bacterial suspension in sterile saline with a turbidity tube similar to Mac Farland 0.5 Scale (0.05 mL barium chloride 1% and 9.95 mL sulfuric acid 1%).

**Proof of sensitivity by broth dilution: dilution assay in tubes**

In this assay, aliquots of 100 μL of each EO dilution, 940 μL of culture medium and 10 μL of each microbial suspension were sampled. Then the tubes were incubated at 37 °C for 24 h. The test was performed in triplicate, where the Minimum Inhibitory Concentration (MIC) was defined as the lowest test concentration that inhibited visible growth of the microorganism tested (turbidity of tube).

**Positive control with gentamicin**

Positive control antibiotic Gentamicin (10 μg/disk) was used. When impregnated in paper discs, it is diffused into the culture medium and, in case of inhibitory activity over the microorganism tested, it forms a non-growth halo around the disc impregnated. After the incubation period the plates had undergone (24 h, 37 °C), the inhibition zones around the disc impregnated. After the incubation period, the tubes were incubated at 37 °C for 24 h. The test was performed in triplicate. Interpretation of the results was carried out by checking the turbidity of the contents of the tube.

**Negative control with DMSO**

Along with MIC test, the feasibility of the microorganism was also carried out, in which an equivalent volume of DMSO was used as a negative control. To sterile tubes containing 940 μL of nutrient broth were added 100 μL of DMSO and 10 μL of each of the microbial suspensions used. Then the tubes were incubated at 37 °C for 24 h. The test was performed in triplicate. Interpretation of the results was carried out by checking the turbidity of the contents of the tube.

**Results and Discussion**

**Essential oil extraction**

The essential oils showed strong odor, pungent flavor and yellow coloring.

The percentage of essential oil extracted from the ripe fruit of *S. terebinthifolius* Raddi was 6.54 ± 1.06% (w/w). However, this content is still below the 10.00% (w/w) reported by Lloyd *et al.* (1977). The average value found is four times higher than that reported by Pieribattesti *et al.* (1981) - 1.50%. In all these studies, it was used the same extraction protocol.

The nature and amount of essential oils produced by plant species along its development can be significantly affected by factors such as light intensity, temperature, level of nutrition and water availability (called abiotic factors) (Lima *et al.*, 2003).

**Physicochemical properties of the essential oil**

The results obtained in the physicochemical and chemical characterization of the essential oil of ripe fruit of *S. terebinthifolius* Raddi are presented in Table 1.

The EO of *S. terebinthifolius* showed predominance of monoterpenes (85.81%), presenting as major constituents δ-3-carene (30.37%), limonene (17.44%), α-phellandrene (12.60%), α-pinene (12.59%), myrcene (5.82%) and α-cymene (3.46%); sesquiterpenes appeared as minor proportion (5.34%).

This result shows a slight qualitative similarity to those reported for samples of essential oil made from the fruit of *S. terebinthifolius* from the USA (Lloyd *et al.*, 1977; Pieribattesti *et al.*, 1981) and leaves collected in India (Jamal and Agusta, 2001; Singh *et al.*, 1998). Ibrahim *et al.* (2004) in their study on the fruits of the plant detected monoterpenes α-pinene (15.01%) and germacrene D (14.31%) and sesquiterpene eilxene (15.18%) as major constituents of the EO. Pieribattesti *et al.* (1981) obtained α-pinene (26.50%), α-phellandrene (22.30%), limonene (16.00%) and β-phellandrene (15.00%) as the monoterpene predominant species. In the study carried out by Barbosa *et al.* (2007) on the analysis of the variation in volatile composition of the EO from the fruits of *S. terebinthifolius* vs. time of extraction, three of the four main chemical constituents obtained after one hour of extraction were identified as: α-pinene (6.48%), α-phellandrene (7.45%) and δ-3-carene (17.15%). Nascimento *et al.* (2011), in their work with the EO of ripe fruit of *Schinus* obtained limonene (31.8%), thujene (21.7%), sabinene (15.8%) and α-phellandrene (11.9%) as major compounds.

Table 2 shows the chemical composition of the essential oils from different parts of the plant *S. terebinthifolius* Raddi collected in different regions of the world. When comparing the chemical composition of the EO from fruits
to the results shown in Table 1, there is significant variation in the composition and quantity of chemical constituents.

This observed variation in the chemical composition of the essential oil from fruits of *S. terebinthifolius* Raddi, using the same extraction protocol, holds a direct relationship with the environment in which the plant develops, the type of crop to which it is submitted and the part of the plant submitted to the extraction (Lima et al., 2006a).

**Antibacterial activity**

Assays of antibacterial activity performed by the broth dilution method showed that EO of *S. terebinthifolius* fruits was active against the microorganisms tested. Table 3 (data expressed as a function of MIC).

The EO showed to be particularly active against gram-positive bacteria *Corynebacterium* sp. (3.55 μg/mL), *Bacillus* sp. (7.11 μg/mL) and *Nocardia* sp. (7.11 μg/mL), whose MIC values were the lowest among the tested bacteria, while Gram-negative species *Enterobacter* sp. (56.86 μg/mL), *E. agglomerans* (28.43 μg/mL), *E. coli* (28.43 μg/mL) and *K. oxytoca* (28.43 μg/mL) showed less sensitivity to oil (evidenced by the higher MIC values). The high frequency in some of these bacteria is detected in hospitals, especially *E. coli*, *Enterobacter* sp. and *S. aureus* (proven fact according to survey data recorded in the book of the hospital supplying the strains).

The marked differences among Gram-negative and Gram-positive bacteria are related to the structure of their cell walls: Gram-negative bacteria have more complex cell wall composed of a thin peptidoglycan layer, and an outer membrane containing lipopolysaccharides, which are responsible for an additional hydrophobic barrier. On the other hand, the cell wall of Gram-positive bacteria, even though thicker, shows predominantly one type of macromolecule (90% peptidoglycan) (Murray et al., 2002). As showed in results section, the Gram-positive species are more sensitive to the EO, which is very likely to be ex-
Table 2 - Compounds identified in the EO, using different plant parts collected from different regions of the world.

| Compounds | Plant part | Place of Collection | Reference |
|-----------|------------|---------------------|-----------|
| α-pinene (26.5%), α-phellandrene (22.3%), limonene (16.00%), carene (traces) | Fruits | USA | (Pieribattesti et al., 1981) |
| α-cadinol (16.26%), elemol (13.62%), δ-cadinene (6.33%), δ-3-carene (5.82%), germacrene D-4-ol (5.33%), epi-α-cadinol (4.56%), β-phellandrene (4.49%), germacrene D (4.39%) | Fruits | Brazil | (Barbosa et al., 2007) |
| Limonene, δ-3-carene, sabinein, p-cymene | Fruits | USA | (Lloyd et al., 1977) |
| Elixene (15.18%), α-pinene (15.01%), germacrene D (14.31%) | Fruits | Egypt | (Ibrahim et al., 2004) |
| cis-β-terpinolene (17.87%), (E)-caryophyllene (17.56%), β-cedrene (9.76%), citronelal (7.03%) | Leaves | Egypt | (El-Massy et al., 2009) |
| 3-carene, α-pinene, β-pinene, α-phellandrene, d-limonene, sabinein, p-cymene, β-cymene, β-elemene, isocaryophyllene, α-cubene, etc. (68.63% of monoterpenes) | Leaves | India | (Jamal and Agusta, 2001) |
| α-pinene (24.4%), limonene (11.9%), p-cymene (14.3%) | Leaves and inflorescences | India | (Singh et al., 1998) |
| α-pinene (43.20%), camphene (0.42%), β-pinene (2.29%), sabinein (1.91%), α-phellandrene (18.85%), 3-carene (0.27%), p-cymene (0.84%), γ-terpinene (0.76%), terpinolene (1.07%), β-caryophyllene (0.41%) | Part unspecified | India | (Malik et al., 1994) |
| α-phellandrene (34.38%), β-phellandrene (10.61%), α-terpinol (5.60%), α-pinene (6.49%), β-pinene (3.09%) and p-cymene (7.34%); marked quantity of γ-cadinine (18.04%) | Berries | Tunisia | (Bendaoud et al., 2010) |
| high percentage of sesquiterpenes and monoterpenic hydrocarbons | Leaves and fruits | Brazil | (Santos et al., 2009) |
| p-meth-1-en-9-ol (8.32%), β-pinene (1.43%), α-thujene (1.30%), camphene (4.78%), α-fenchene (8.46%), terpinen-4-ol acetate (0.62%), bornyl acetate (1.80%), caryophyllene (2.19%), terpinen-4-ol (1.31%), α-terpinol (1.38%), germacrene-D (7.91%), δ-cadinene (1.09%), hedycaryol (18.73%), α-gurjunene (12.03%), α-eudesmol (9.18%), β-eudesmol (11.15%) | Seeds | Brazil | (Oliveira Junior et al., 2013) |
| germacrene D (23.7%), bicyclogermacrene (15.0%), β-pinene (9.1%) and β-longipinene (8.1%) as the main compounds | Leveles | Brazil | (Santana et al., 2012) |
| α-pinene (22.56%), sabinein (15.78%), Z-salvane (10.69%), β-pinene (10.52%), α-fenchene (8.82%) and limonene (5.52%) | Fruits | Brazil | (Carvalho et al., 2013) |
| α-pinene (30.27%), camphene (0.58%), β-myrcene (6.60%), β-pinene (7.96%), myrcene (1.63%), α-phellandrene (9.86%), α-terpinene (0.77%), sabinein (40.66%), trans-β-ocimene (0.30%), γ -terpinene (0.77%), 3-cyclohexen-1-ol (0.61%) | Fresh leaves | Zimbabwe | (Gundidza et al., 2009) |

plained by the lower structural complexity of their cell walls.

The results obtained were consistent with the results of Lima et al. (2006b), who showed in their study on crude extracts obtained from the stem bark of S. terebinthifolius that there is great potential inhibitory effect on S. aureus, with MIC values below 100 mg/mL.

Martínez et al. (1996) and Guerra et al. (2000) reported the capacity of the ethanol extract of leaves of S. terebinthifolius to inhibit the growth of S. aureus and Pseudomonas aeruginosa. Pereira et al. (2011), in their work with 3 different extracts of S. terebinthifolius (ethanol, n-butanol and n-hexane), against S. aureus, found the results 16.33 ± 1.00 mm, 21.11 ± 1.17 mm e 15.33 ± 0.81 mm, respectively.

Degaspari et al. (2005), tested the alcoholic and aqueous extracts obtained from fruits of S. terebinthifolius Raddi, checking inhibitory effect of alcoholic extract on S. aureus ATCC 6538 and Bacillus cereus ATCC 11778, but not over other bacterial strains tested: E. coli ATCC 25922, P. aeruginosa ATCC 10145 and Salmonella choleraesuis ATCC 10708; aqueous extract showed no inhibitory effect for any of the microorganisms tested.

The EO from the fresh leaves of S. terebinthifolius from Zimbabwe exhibited potent antibacterial activity against Yersinia enterocolitica, P. aeruginosa, E. coli, Acinetobacter calcoaceticus, Bacillus subtilis, Klebsiella pneumoniae and Bacillus subtilis with at least 58% inhibition compared to the positive control (Gundidza et al., 2009).
According to Koyama et al. (1997), many components of the essential oils have the ability to disrupt or penetrate the lipid structure present in Gram-negative bacteria.

The toxic effects of monoterpenes in bacterial cell membrane results in expansion of the membrane with increased fluidity and permeability, disturbance in membrane proteins, inhibition of respiration and changes in ion transport process (Bisignano et al., 2005). However, considering that the EO is comprised of variety of chemical constituents, it is not possible to assign oil a specific mechanism of action, since each component can act at different sites in the microbial cell (Carson et al., 2002).

The monoterpenes are most likely responsible for the activities presented by EO tested, either by acting alone or acting synergistically with other constituents.

**Experimental evaluation of controls**

As positive control was used gentamicin, being this one aminoglycoside antibiotic of broad spectrum. The test results demonstrated susceptibility of strains Gram-positive and Gram-negative nosocomial opposite antimicrobial, therefore comparatively evaluated the efficacy of EO: gentamicin has proven bactericidal action, while the proposed test to evaluate the bacteriostatic activity EO, through MIC (Murray et al., 2002). Negative control (DMSO) showed no antibacterial activity against any of the microorganisms tested. The choice of dispersing and emulsifying agent used in the oil-water emulsion to be one of the factors observed to not cause interference with the MIC values obtained by dilution methods. At high concentrations, interference emulsifying agent in the susceptibility of bacteria to EO can be explained by the possible influence this has on bacterial growth and/or on the cell membrane permeability. The emulsifiers may act synergistically or antagonistically to active components of the EO. To minimize these effects, some authors have proposed the use of emulsifiers, including DMSO, at concentrations ranging from 0.5 to 20% solution in oil (Nascimento et al., 2007). However, in this study, the concentrations of DMSO used did not exceed 10%.

**Conclusion**

The extraction process of the EO from the ripe fruit of *S. terebinthifolius* Raddi by hydrodistillation showed yield compatible with literature data, as well as values of specific density and refractive index. On the other hand, this did not occur for specific rotation values (this difference is due to variations in the chemical composition of the EO, which can also be related to conditions of analysis: specific rotation generally decreases linearly with increasing temperature, and varies with the concentration).

The study of antibacterial properties proved the sensitivity of all wild strains tested to EO: *E. coli*; *Bacillus* sp.; *Pseudomonas* sp.; *K. oxytoca*; *Corynebacterium* sp.; *Nocardia* sp.; *S. aureus*; *Enterobacter* sp.; *E. agglomerans* and *Streptococcus* group D. It showed, however, some differences in sensitivity profile, and Gram-positive species are more sensitive to the EO, which is very likely to be explained by the lower structural complexity of their cell walls. Traditional antibiotics act on a single cell site, and thus can develop bacterial resistance, there is then the EO as an alternative to the use of conventional antibiotics. The results open perspectives for future use in hospital settings.

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**Table 3 - MIC of EO from *S. terebinthifolius* Raddi (µg/mL) in different nosocomial bacteria.**

| Microorganism                  | MIC (µg/mL) |
|-------------------------------|-------------|
| Enterobacter sp.¹              | 56.86 ± 0.84|
| *E. agglomerans*¹             | 28.43 ± 0.46|
| *E. coli*¹                    | 28.43 ± 0.41|
| *K. oxytoca*¹                 | 28.43 ± 0.44|
| *Streptococcus* grupo D²      | 14.21 ± 0.65|
| *S. aureus*²                  | 14.21 ± 0.60|
| *Pseudomonas* sp.¹            | 7.11 ± 0.63  |
| *Bacillus* sp.²               | 7.11 ± 0.84  |
| *Nocardia* sp.²               | 7.11 ± 0.63  |
| *Corynebacterium* sp.²        | 3.55 ± 0.44  |
| Positive control (gentamicin)  | 10 µg/disk   |
| Negative control (DMSO)        | -           |

¹Gram-negative bacteria; ²Gram-positive bacteria.
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