Antimicrobial activity of *Melaleuca sp.* oil against clinical isolates of antibiotics resistant *Staphylococcus aureus*[1]

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ABSTRACT

PURPOSE: To extract the *Melaleuca* sp. oil and to assess its *in vitro* inhibitory effect against *Staphylococcus aureus* isolates obtained from lower limb wounds and resistant to several antibiotics.

METHODS: A total of 14 test-tubes containing Mueller-Hinton broth were used to determine the Minimum Inhibitory Concentration (MIC). The following concentrations of the *Melaleuca* sp. oil were added to the first 11 tubes: 8; 4; 2; 1; 0.5; 0.2; 0.1; 0.05; 0.025; 0.0125 and 0.00625%. The 12th and 13th tubes, with and without oil, were used as the positive and negative controls, respectively. The experimental study was carried out in triplicate at 37ºC for 18 hours. The Minimum Bactericidal Concentration (MBC), able of killing all the microorganisms, was also determined. Two *S. aureus* isolates were obtained from lower limb wounds of female patients and the identification of the microorganisms (*Staphylococcus aureus*) and the test for susceptibility to the antimicrobial agents were carried out by automation using the apparatus MicroScan®. After identification, the isolates were preserved in liquid Trypticase Soy medium, and inoculated for determination of the MIC and MBC.

RESULTS: The MIC was 0.2% and the MBC was 0.4%.

CONCLUSION: The *Melaleuca* sp. oil showed antimicrobial properties *in vitro* against strains isolated from lower limb wounds which were resistant to multiple antibiotics.

Key words: Phytotherapy. Tea Tree oil. *Staphylococcus aureus*. *In Vitro* Techniques. Injuries.

Introduction

Staphylococcus aureus are pathogenic microorganisms responsible for a mortality superior to that caused by AIDS, tuberculosis and viral hepatitis together in the United States of America[1]. They are commonly found associated with surgical site infections and several other clinical implications[2]. The presence of infectious microorganisms is a major cause of fail on wound healing[3].

The indiscriminate use of antibiotics against staphylococcal infections has been a practice used throughout the world, which has resulted in an increase in methicillin-resistant *Staphylococcus aureus* (MRSA) and other antimicrobial resistance profiles[4].

Research into the medicinal action of essential oils from some plants is becoming more popular, since many synthetic drugs are related to undesirable collateral effects, such as nephrotoxicity and ototoxicity[5]. Among the studied herbal substances, the plant known as the "tea tree" (*Melaleuca alternifólia* Cheel) is one of the most important, since it has already shown action against MRSA, coagulase-negative streptococcus and staphylococcus and coliforms[6-8].

Australia was pioneer in the use of *Melaleuca alternifólia* oil (TTO), but many other species can be found around the world[9]. Species of *Melaleuca armillaris*, *M. acuminata* and *M. stypheloiodes* are cultivated in Tunisia[10], and oils from species of *M. ericifolia*, *M. leucadendron*, *M. armillaris* and *M. styphelioides* have been extracted in Egypt[11]. The use of *M. leucadendron* has been described in western Malaysia and Vietnam and *Melaleuca quinquenervia* in the USA[12,13]. Although the oil of *M. alternifólia* is the most commercialized one throughout the world, the water distillation of other species can produce oils with similar constitutions, such as *M. linariifolia* and *M. dissitifloria*[14].

The action of *Melaleuca alternifólia* oil was studied using transcription profiles for a better understanding of the activity of TTO on *S. aureus* strains[15]. This research showed that the action could be constituted not only of a mechanism, but also by an action with multi-component characteristics mainly affecting the cell wall[15]. These results corroborated other authors findings, which described the bactericidal activity of TTO as being attributed to its ability to denature proteins and alter the structure and function of the cell wall membrane[9,16-18].

Studies have been carried out with the species *M. hypericifolia* and *M. thymifolia* in Brazil, at the Federal University of Viçosa[17]. The *Melaleuca* sp. are cultivated as ornamental plants in the city of Pouso Alegre, in the south of the state of Minas Gerais, and the oil extracted from these plants could have properties equal or similar to that extracted from *M. alternifólia*, but at a lower cost, since it is cultivated in Brazil. Thus, the aim of the present study was to extract the oil from *Melaleuca* sp. and to assess the *in vitro* inhibitory effect of this oil against *Staphylococcus aureus* obtained from lower limb wounds, which were resistant to several antibiotics.

Methods

Extraction and identification of the chemical properties of the essential oil from Melaleuca sp

The oil was extracted from the plant leaves by water distillation using Clevenger type equipment (Hermex Glassware – Brazil). After extraction, it was stored in dark flasks in a refrigerator at 5ºC. The chemical properties were identified by gas chromatography using a model 7890 gas chromatograph (Agilent, USA) coupled to a model 5975 linear quadrupole type mass spectrophotometer (Agilent, USA). The oil components were identified based on the NIST11 mass spectra data (Agilent, USA) and on the Automated Mass Spectral Deconvolution Mass and Identification System (Agilent - USA). The concentration of the compounds was expressed as a percentage of the normalized peak area (each separated substance appeared as a peak on the chromatogram). The index adopted for quality was above 70%.

Isolation and tests with Staphyloccus aureus: bacterial samples

Two *S. aureus* isolates were obtained from lower limb wounds of female patients aged 36 and 57 years-old, located on the foot and knee, respectively. The isolate from the foot wound was resistant to the antibiotics Amoxicillin/Clavulanic acid, Ampicillin/Sulbactam, Ceftriaxone, Erythromycin, Oxacillin, Penicillin and Tetracyclin. The resistance was intermediary for the antibiotic Clindamycin. The isolate from the knee wound was resistant to the antibiotics Amoxicillin/Clavulanic acid, Ampicillin/Sulbactam, Ceftriaxone, Ciprofloxacin, Clindamycin, Erythromycin, Levofloxacin, Moxifloxacin, Oxacillin, Penicillin and Synercid.

Identification of the microorganisms (*Staphylococcus*

aureus) and the test for susceptibility to the antimicrobial agents were carried out by automation using the apparatus MicroScan® auto SCAN-4 System (Siemens, Germany) with the use of the com Pos Combo Type 41 panel (Siemens, Germany), and seeding was carried out using various slopes as the stock cultures, from which they were reactivated and inoculated for determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Determination of the Minimum Inhibitory Concentration (MIC)

From the stock cultures, the *Staphylococci* were transferred to the Thioglycollate medium (Himedia®), incubated at 37°C for 24 hours and then spread on a mannitol salt agar medium and then again incubated at 37ºC for 24 hours. The concentration for the inoculation of each species of *S. aureus* was $1x10^6$ CFU (colony forming units)/mL and it was obtained using a sterile swab, which was touched on the surface of 4 to 5 great colonies isolated in the mannitol salt agar plate and emulsified in 3 mL of water for the inoculum (autoclaved deionized water). The final turbidity was equivalent to a 0.5 McFarland turbidity standard. The turbidity was confirmed by the use of the MicroScan® turbidity meter with an interval of 0.08 ± 0.02 (Procedure manual for Gram-Positive Dehydrated Panels-MicroSan®-SIEMENS Panels).

The microorganisms were transferred to Thioglycolate medium (Himedia®) and incubated at 37ºC. Fourteen test tubes, each one containing 5 mL of Mueller-Hinton broth (MHB - Difco Co), were used to determine the MIC of each isolate. Progressive concentrations of the extracted oil were added to 12 tubes, the 12th tube being used as the control (smallest oil concentration, no inoculum). The 13th tube was used as the positive growth control (no oil, with inoculum) and the 14th tube was used as the negative control (no oil and no inoculum, just the culture medium). The MIC was established for the bacteria isolated from the two lower limb wounds and for the strain *Staphylococcus aureus subsp*. *aureus* (ATCC® 25923™), which is a standard American strain.

The oil concentrations used were: 8; 4; 2; 1; 0.4; 0.2; 0.1; 0.05; 0.025; 0.0125 and 0.00625% and for each tube the assay was carried out in triplicate. After adding the oil, the first 11 tubes (including the positive control) received 250µL of inoculum at a concentration of $1x10^6$ CFU/mL. All the tubes were incubated at 37ºC for 18 hours and then each tube examined visually for the presence of turbidity. The first tube showing turbidity was considered to be that containing the MIC. To enable the solubility of the oils tested in the medium culture, a Tween 80 was used, because this kept the oil soluble for a longer period of time required to perform the tests thus making it efficient. The concentration that was used in the experiments was 5 µL/mL of the final concentration in the culture medium.

Determination of the Minimum Bactericidal Concentration (MBC)

Aliquots of 5 µL were removed from the tubes showing no turbidity and used to seed Petri dishes containing mannitol salt agar (Difco Co. - USA). The lower concentration which not allowed the growth of any colonies on the agar surface was considered to be the MBC, that is, the concentration able to kill all the microorganisms.

Results

A total of 27 peaks were detected in the *Melaleuca* sp. oil, of which 21 are presented in Table 1. The six unidentified peaks could be related to new structures that were not recognized by the data library of the program, or could be peaks that do not separate from the others. There was a slight prevalence of monoterpenes (57.2%) in relation to sesquiterpenes (42.8%). The main monoterpenes were eucalyptol or 1.8 cineol with 70.8%, followed by terpineol with 8.95% and limonene with 8.25%. Of the sesquiterpenes encountered the most abundant was globulol followed by octahydrotetramethyl with 0.82% and 0.62%, respectively.

The values obtained for the MIC and MBC were respectively 0.1% and 0.4% for the *S. aureus* isolate obtained from the foot wound, and 0.2% and 0.4% for the isolate from the knee

TABLE 1 - Compounds detected in the *Melaleuca* sp. oil sample.

Peak	RT	Name of Compound	% A	Quality
1	8.88	Trimethyl Bicyclic Heptene	3.39	97
2	10.20	Dimethyl Methylene Bicyclic Heptane	1.09	97
3	10.67	Myrcene	1.99	AMDIS
4	11.04	Methylene Methylethenyl Cyclohexane	0.15	76
5	11.44	Methyl Methylethylidene Cyclohexane	0.20	95
6	11.69	Tetramethyl Benzene	0.13	81
7	11.82	Limonene	8.25	99
8	11.89	Eucalyptol	70.08	99
9	12.76	Terpinene	0.64	94
10	13.67	Isopropylidene Methyl Bicyclic Hexane	0.13	89
11	16.03	Terpineol (isomer)	0.22	AMDIS
12	16.33	Terpineol (isomer)	0.84	93
13	16.72	Terpineol (isomer)	7.89	87
14	22.76	Not determined	0.15	-
15	23.02	Not determined	0.13	-
16	23.51	Decahydro Trimethyl Methylene Cyclopropazulene	0.50	95
17	24.06	Aloaromadendrene	0.27	99
18	24.91	Octahydro Tetramethyl Cyclopropazulene	0.62	96
19	25.56	Hexahydro Dimethyl Methyl Ethyl Naphthalene	0.43	90
20	26.45	Not determined	0.12	-
21	26.65	Not determined	0.21	-
22	27.03	Globulol	0.82	98
23	27.23	Octahydro Dimethyl Methyl Ethenyl Azulene	0.45	87
24	27.26	Not determined	0.34	-
25	27.45	Eudesmol	0.29	AMDIS
26	27.92	Not determined	0.32	-
27	28.03	Hexahydro Dimethyl Methylethyl Naphthalene	0.23	87

Peak: Peak number according to elution order from the column.
RT: Retention time from the column in minutes.
Name of compound: Most common name of compound identified.
%A: Percentage of normalized area which indicates the relative distribution of the compounds in the sample.
Quality: Thethis is the search index in the database that reflects the similarity of the mass spectrum obtained with those registered in the libraries used. Quality indexes > 70 were adopted.
AMDIS: Automated Mass Spectral Deconvolution Mass and identification System.

wound. For the strain ATCC® 25923™ the MIC and MBC were respectively 0.2% and 0.4%.

Discussion

Carson *et al*.[9] reported that the oil isolated from the tea tree (*M. alternifolia*) was constituted of terpene hydrocarbons, mainly monoterpenes, sesquiterpenes and other associated alcohols. The same was observed in the present study with very similar percentages. Nevertheless, comparing the compounds of the two plants, *Melaleuca* sp. and *M. alternifolia*, differences can be found in the constitutions of the oils, such as a prevalence of eucalyptol (1.8 cineol) in the Brazilian sample and terpinen-4-ol as the main constituent described in the Australian samples[9].

One of the difficulties of establishing phytotherapy as a medical practice is the lack of standardization of the chemical constituents of the plants. This has frequently been reported in the literature, and other authors have described the variations in the constituents of *Melaleuca alternifolia* oil, even when using the same varieties or chemotypes or even from different samples[9,19]. The oil extracted from *Melaleuca* sp. is rich in eucalyptol (1.8 cineol) and, in recent years, research has attributed antimicrobial

action to this molecule, which acts by facilitating permeability of the membranes of microorganisms such as *S. aureus*[9]. The eucalyptol found in the Brazilian sample opens the possibility for future studies on the action of this substance against pathogenic bacteria, particularly those of the genus *S. aureus*. In fact, *in vitro*, its action was just as efficient as the oils cultivated in Australia, which have terpinen-4-ol as the main antimicrobial agent.

Data related to the MIC and MBC were also obtained by Cox *et al*.[16] when they studied the action of the tea tree against *E. coli* (AG100) and *S. aureus* (NCTC 8325). The MIC obtained by these authors for the isolate of *Staphylococcus aureus* (ATCC® 25923), an oral pathogen, was 0.1% and the MBC 0.4%[17]. Other studies have shown that oils rich in monoterpenes and sesquiterpenes have bactericidal and bacteriostatic characteristics[20,21]. The *Melaleuca* sp. oil controlled the bacterial strains in a highly efficient way at a very low concentration, showing itself to be a good antimicrobial agent.

Possibly the *S. aureus* isolates are not always inhibited by such low doses of the *Melaleuca* sp. oil, since results for the susceptibility observed in 20 clinical MRSA samples, obtained from South Korean patients, showed values for MIC and MBC much higher than those observed in the present study[21]. In this same line of research, the MICs of 13 essential oils were evaluated in relation to 65 bacterial strains, amongst which *S. aureus*, including two metacycline susceptible strains and five methicillin susceptible strains[22]. These authors classified the oils based on their antimicrobial activities in relation to the genus *Staphylococcus*. The oil from the *Melaleuca alternifolia* was included in the group showing bactericidal activity at concentrations above 2% after 24 hours[22]. They also showed that other types of essential oil showed greater bactericidal activity, in which the action occurred at concentrations equal or below 2% and in a time not exceeding 30 minutes[22].

Since 1990s, studies with plants from the genus *Melaleuca* have foccused on their bactericidal action against microorganisms that had acquired antibiotic resistance, principally the methicillin-resistant *S. aureus*[9]. The search for active principles with the capacity to substitute common antibiotics, to which many strains of microorganisms already show resistance, has been one of the priorities on the current world scenario.

Conclusion

Melaleuca sp. oil extracted in Brazil showed bactericidal potential *in vitro* against the bacterial isolates obtained from lower limb wounds and also to the standard *S. aureus* strain (ATCC® 25923™).

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