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SUSCEPTIBILITY OF THREE CLINICAL ISOLATES OF *ACTINOMODURA MADURAE* TO α-PINENE, THE BIOACTIVE AGENT OF *PINUS PINASTER* TURPENTINE OIL

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Abstract — In vitro susceptibility of the turpentine oil obtained from *Pinus pinaster* oleoresin was evaluated against three Sudanese clinical isolates of *Actinomadura madurae*, which is the main causative agent of actinomycetoma. The minimum inhibitory concentrations (MICs) of the oil ranged from 100.3 to 124.8 μL/mL, and the minimum microbicidal concentrations (MMCs) were between 100.3 and 150.0 μL/mL. α-Pinene exhibited prominent bioactivity with MICs ranging between 3.3 and 5.0 μL/mL, while its MMC was 10.0 μL/mL against the same clinical isolates. *Pinus pinaster* turpentine oil and α-pinene might be useful agents in the treatment of mycetoma caused by *A. madurae*.

Key words: Actionomycetoma, *Pinus pinaster, Actinomadura madurae,* turpentine oil, α-pinene, antimicrobial activity

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INTRODUCTION

Actinomycetoma is one of the most neglected of diseases. It is caused by aerobic actinomycete bacteria via a localized trauma of the skin. *Actinomadura madurae* is best known worldwide as the cause of actinomycotic mycetomas (Fahal, 2004). Mycetoma is a chronic specific granulomatous subcutaneous inflammatory disease that is produced by various causative agents that can be either true fungi or aerobic actinomycetes of the genera *Actinomadura*, *Nocardia,* and *Streptomyces* (Ahmed et al., 2004). *Actinomadura* spp. commonly occur in some countries and can produce as much as 50% of the total cases of mycetoma (Welsh, 1991). The majority of reports of infections by this species are from tropical and subtropical countries. It has been reported that *A. madurae* can be treated with several drugs: amikacin, amoxicillin-clavulanate, cefotaxime, ceftriaxone, doxycycline, imipenem, minocycline, sulfamethoxazole, trimethoprim-sulfamethoxazole, ampicillin, ciprofloxacin, erythromycin, and streptomycin (Welsh, 1991; McNeil et al., 1990). Effective chemotherapy may be complicated by adverse drug reactions, antimicrobial resistance, and a lack of effective antimicrobial agents. Some of these drugs have to be taken for a long period, and in some patients they can produce serious side effects, such as ototoxicity or nephrotoxicity in treatment with amikacin; and hemolytic and aplastic anemia, urticaria, and photosensitivity in treatment with sulfonamides (McNeil et al., 1990). The discovery of lead compounds with the potential to become usable drugs in treating diseases caused by *A. madurae* is therefore of prime importance.

Pinus pinaster Aiton (Maritime Pine) is a member of the family Pinaceae. Its oleoresin, known as

turpentine, consists of the resin colophonium and turpentine oil. Turpentine oil is mainly composed of mono-and sesquiterpene hydrocarbons and has been of very limited pharmaceutical use in recent years. A substantial part of its world production is mainly used in the perfume and fragrance industry. Nevertheless, traditional therapeutic and pharmaceutical uses of its oils are recorded all around the world (Schauenberg and Paris, 1977). The objectives of this study were to determine the chemical composition of *Pinus pinaster* turpentine oil from Greece and investigate the antimicrobial effects of the obtained oil. Special consideration was given to the antimicrobial action of its main bicyclic monterpene, α-pinene, on three clinical isolates of *Actinomadura madurae* using an *in vitro* method.

MATERIAL AND METHODS

Isolation of the turpentine oil

Turpentine oil was isolated from crude oleoresin of *Pinus pinaster* Aiton (originating from the Greek peninsula of Halkidiki) by hydrodistillation in a Clevenger-type apparatus for 3 h according to procedure I as described in Yugoslav Pharmacopoeia IV (1984). The obtained turpentine oil was stored at +4°C.

Chemical analyses

Qualitative and quantitative analyses of the turpentine oil were performed using GC and GC/MS. The GC analysis of the oil was carried out on a GC HP-5890 II apparatus equipped with a split-splitless injector attached to a HP-5 column (25 m × 0.32 mm, 0.52 μm film thickness) and fitted to FID. The carrier gas was H2 (1 mL/min). A measured volume (1 μL) of sample solution in ethanol (0.2%) was injected in split mode (1:30) at 250°C.

The detector temperature was 300°C (FID), while column temperature was linearly programmed from 40 to 260°C at a rate of 4°C/min. For GC/MS analysis, a Hewlett-Packard G 1800C Series II GCD instrument and a HP-5MS column (30 m × 0.25 mm × 0.25 μm) were used. Mass spectra were recorded in an EI regime (70 eV) in a m/z range 40-400. The transfer line was heated at 260°C. Identification of the individual turpentine oil components was accomplished by comparison of retention times with standard substances and by matching mass spectral data with MS libraries (Adams, NIST and Wiley 275.l) using a computer search and literature (Adams, 2007). For this purpose, quantitative analysis areapercents obtained by FID were used as the base. Quantitative analysis for area percent obtained by FID was achieved by computer search and literature review.

In order to investigate the potential activity of α-pinene, we used a commercial sample of the mentioned compound from Sigma-Aldrich, Belgium (Catalog number 26, 807-0).

Bioassays

Three Sudanese clinical isolates of *Actinomadura madurae* were used for bioassays. *Actinomadura madura* strains were directly isolated from patients at the Mycetoma Research Center, University of Khartoum, Khartoum, Sudan. The cultures were grown on Mueller-Hinton agar (MH), stored at +4°C, and subcultured once a month.

Microdilution method

In order to investigate the antimicrobial activity of the isolated turpentine oil and α-pinene, a modified version of the microdilution technique was used (Hanel and Raether, 1988; Daouk et al., 1995; Soković et al., 2000). *Actinomadura madurae* isolates were cultured at 37°C in TSB medium for 5 days. The cell suspension was adjusted with sterile saline to a concentration of approximately 1.0 x 10^5 in a final volume of 100 μL per well. The inocula were stored at +4°C for further use. Dilutions of the inocula were cultured on solid MH to verify the absence of contamination and check the validity of the inoculum. Determination of minimum inhibitory concentrations (MICs) was performed by a serial dilution technique using 96-well microtiter plates. The investigated turpentine oil was added to tryptic soy broth (TSB) medium with the inoculum. Microplates were incubated for 72 h at 37°C. The lowest concentrations without visible growth (under a binocular microscope) were defined as the MICs. The minimum microbicidal concentrations (MMCs) were determined by serial subcultivation

Table 1. Chemical composition of Pinus pinaster turpentine oil. Abbreviations: *KIE: RRI experimentally determined (calibrated AMDIS), **KIL: RRI literature values (Adams, 2007).

Compound	Content (%)	KIE*	KIL**
trycyclene	0.27	914.0	921
α-pinene	**90.82**	**931.9**	**932**
camphene	0.84	947.0	946
thuja-2,4(10)-diene	0.05	953.3	953
β-pinene	0.64	974.4	974
myrcene	0.40	990.6	988
δ^3-carene	0.04	1007.5	1008
p-cymene	0.03	1021.5	1020
limonene	1.03	1025.4	1024
α-pinene oxide	0.59	1092.5	1099
α-fenchocamphoron	0.28	1101.5	1104
trans-pinocarveol	0.14	1132.4	1135
trans-verbenol	0.38	1140.2	1140
pinocarvone	0.12	1155.7	1160
α-terpineol	0.22	1184.9	1186
estragol	0.19	1191.5	1195
verbenone	0.15	1202.9	1204
trans-verbenyl acetate	0.03	1287.5	1291
trans-pinocarvyl acetate	1.18	1293.5	1298
cis-pinocarvyl acetate	0.80	1309.6	1311
myrtenyl acetate	0.15	1316.6	1324
cyclosativene	0.07	1357.2	1369
α-copaene	0.16	1366.4	1374
trans-caryophyllene	0.69	1409.0	1417
α-humulene	0.12	1443.0	1452
α-muurolene	0.25	1493.2	1500
caryophyllene oxide	0.37	1571.1	1582
Total	**100**		

of a 2 μL on microtiter plates containing 100 μL of broth per well and further incubation for 72 h at 37°C. The lowest concentration with no visible growth was defined as the MMC, indicating 99.5% killing of the original inoculum. Each experiment was repeated in triplicate. Streptomycin was used as a positive control (0.1-2 $mg \cdot mL^{-1}$).

Statistical analyses

The results of antimicrobial activity were analyzed by two-factorial analysis of variance (ANOVA). The Statistica Package program (version 4.5, Copyright StatSoft, Inc., 1993) was used for statistical evaluation. Experiments were replicated twice under the same conditions. All analyses were run in triplicate for each replicate ($n = 2 \times 3$).

RESULTS AND DISCUSSION

The yield of turpentine oil in *P. pinaster* oleoresin was 23.6% (*v/m*). Gas chromatographic analysis (GC and GC/MS) showed a total of 27 mono- and sesquiterpenes, representing 100.0% of the oil. The principal compound was the bicyclic monoterpene, α-pinene (90.82%). Other compounds were present in less than 1%, except limonene with 1.03% and trans-pinocarvyl acetate with 1.18% (Table 1).

Table 2. Activity of *Pinus pinaster* turpentine oil, α-pinene, and streptomycin (MICs and MMCs in μL/mL) against *Actinomadura madurae* clinical isolates.

Actinomycetes	*Pinus pinaster* turpentine oil		α-pinene		Streptomycin	
	MIC	MMC	MIC	MMC	MIC	MMC
A. madurae I	124.8±0.3	150.0±0.5	5.0±0.5	10.0±0.5	50.0±0.5	75±0.5
A. madurae II	99.8±**0.3**	100.3±**0.3**	3.3±**0.3**	10.0±**0.5**	29.8±**0.3**	50±**0.5**
A. madurae III	100.3±0.3	124.8±0.3	5.0±0.5	10.0±0.5	50.0±**0.5**	50±**0.5**

Effects of *P. pinaster* turpentine oil against *A. madura* clinical isolates are presented in Table 2. Minimum inhibitory concentrations (MICs) of the oil were in range of 100.3 μL/mL – 124.8 μL/mL, and minimum microbicidal concentrations (MMCs) ranged between 100.3 μL/mL – 150.0 μL/mL. As for activity of the compound α-pinene, MICs (from 3.3 μL/mL to 5.0 μL/mL) and MMCs (10.0 μL/mL) were much lower than values obtained for turpentine oil and streptomycin. Although *P. pinaster* turpentine oil contains a high concentration of α-pinene, there are considerable differences in activity, which could be attributable to antagonistic effects of the compounds present in the turpentine oil and/or to cell membrane selectivity of this microorganism.

Voluminous literature exists regarding the antimicrobial activity of volatile oils in general (Dorman and Deans, 2000) and volatile oils obtained from coniferous species such as *Pinus densiflora* and *P. koraiensis* in particular (Hong et al., 2004). Systematic research has been conducted to correlate the effects of different chemical structures, functional groups, and stereochemistry associated with various antimicrobially active low-molecular-weight terpenoids, and it seems that a certain degree of structure-activity correlation exists (Dorman and Deans, 2000). It is of particular interest to note that Dorman and Deans (2000) established that α–pinene is antibacterially inactive relative to its isomer β–pinene when tested *in vitro* against 25 genera of Gram-positive and Gram-negative bacteria. α–pinene is a monoterpene usually present in most essential oils (Soković et al., 2000; Knežević-Vukčević et al., 2005). However, the present result indicates that α–pinene has high selective toxicity against the three tested strains of *A. madura*. This enables us to speculate about the possible mechanism of α–pinene action on the given microorganism in light of the fact that the bacterial metabolism of α–pinene, which usually involves oxidation to its respective α–pinene oxide followed by conversion of the latter to acyclic metabolites, has been demonstrated in *Nocardia* (Griffiths et al., 1987), a genus very closely related to *Actinomadura*. Further studies are required in order to gain an insight into the metabolic fate of α–pinene in *A. madura*.

The established high selective toxicity of α-pinene towards *A. madura* strains provides compelling evidence of its efficacy *in vitro*. *In vivo* and possibly clinical studies are unquestionably needed to substantiate these findings.

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ОСЕТЉИВОСТ ТРИ КЛИНИЧКА ИЗОЛАТА *ACTINOMODURA MADURAE* НА α-ПИНЕН, БИОАКТИВНИ АГЕНС ТЕРПЕНТИНСКОГ УЉА *PINUS PINASTER*

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Осетљивост три клиничка изолата *Actinomadura madurae*, најчешћег изазивача актиномицетоме, тестирана је *in vitro* на терпентинско уље добијено из смоле *Pinus pinaster*. Минималне инхибиторне концентрације (МИК) уља биле су 100.3 - 124.8 μL/mL, а минималне микробицидалне концентрације (ММК) 100.3 - 150.0 μL/mL. α-пинен је показао снажну биоактивност, са вредностима МИК 3.3 - 5.0 μL/mL, док је ММК износила 10.0 μL/m. Терпентинско уље *Pinus pinaster* и α-пинен могу бити корисни агенси у третману мицетоме изазване таксоном *A. madurae*.