

Supporting Information

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Chemical Composition and Antimicrobial Activity of *Thymus praecox* Opiz ssp. *polytrichus* Essential Oil from Serbia

Nada V. Petrović^{*1}, Slobodan S. Petrović¹,
Mihailo S. Ristić², Ana M. Džamić³ and Ana D. Ćirić⁴

¹*Development and Production Center BIOS – PS and others, Bulevar oslobođenja 401 i, 11000 Belgrade, Serbia*

²*Institute for Medicinal Plant Research “Dr Josif Pančić”, Tadeuša Košćuška 1, 11000 Belgrade, Serbia*

³*University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden “Jevremovac”, Studentski trg 16, 11000 Belgrade, Serbia*

⁴*Institute for Biological Research “Siniša Stanković”, University of Belgrade, Despota Stefana 142, 11000 Belgrade, Serbia*

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S1: Experimental

Isolation of Essential Oil

The air-dried aerial parts of *T. praecox* ssp. *polytrichus* were milled to a particle size of around 0.5 mm. The plant material (50 g) was subjected to hydrodistillation using Clevenger-type apparatus until there was no significant increase in the volume of oil collected (2.5 h). The obtained essential oils were dried with anhydrous sodium sulphate and stored at +4°C. The experiment was performed in triplicate. Yield of essential oil expressed as mean \pm standard deviation.

Essential oil analysis

GC/FID analysis was carried out on a HP-5890 Series II GC apparatus [Hewlett-Packard, Waldbronn (Germany)], equipped with split-splitless injector and automatic liquid sampler (ALS), attached to HP-5 column (25 m x 0.32 mm, 0.52 μ m film thickness) and fitted to flame ionisation detector (FID). Carrier gas flow rate (H_2) was 1 ml/min, split ratio 1:30, injector temperature was 250°C, detector temperature 300°C, while column temperature was linearly programmed from 40-260°C (at rate of 2°/min), and kept at 260°C additional 15 minutes. Solutions of tested samples in ethanol (~1%) were consecutively injected by ALS (1 μ l, split mode). Area percent reports, obtained as result of standard processing of chromatograms, were used as base for the quantification purposes. The same analytical conditions as those mentioned for GC/FID were employed for GC/MS analysis, along with column HP-5MS (30 m x 0.25 mm, 0.25 μ m film thickness), using HP G 1800C Series II GCD system [Hewlett-Packard, Palo Alto, CA (USA)]. Vacuum system of the MS detector consisted of the external rough pump Edwards (UK), model Edwards 1.5, and small air-cooled diffusion pump (DP) Edwards, model EO50/60 (built in). Instead of hydrogen, helium was used as carrier gas. Transfer line was heated at 260°C. Mass spectra were acquired in EI mode (70 eV), in m/z range 40-450. Sample solutions (~1 %) were injected by ALS (200 nl, split mode). Constituents of tested isolates were identified by comparison of their mass spectra to those from Wiley275 and NIST/NBS libraries, using different search engines. The experimental values for retention indices were determined by the use of Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1.), freshly calibrated with hexane solutions of n-alkanes from nonane to untriacontane, compared to those from available literature [1] and used as additional tool to verify MS findings.

Microorganisms

For the bioassays eight bacterial strains were used: Gram-positive *Bacillus cereus* (human isolate), *Micrococcus flavus* (ATCC 10240), *Staphylococcus aureus* (ATCC 6538) and *Listeria monocytogenes* (NCTC 7973), and Gram-negative *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter cloacae* (human isolate) and *Salmonella typhimurium* (ATCC 13311). In antifungal assay eight fungi were used: *Aspergillus fumigatus* (ATCC 9197), *A. versicolor* (ATCC 11730), *A. ochraceus* (ATCC 12066), *A. niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 10509), *P. ochrochloron* (ATCC 9112) and *P. verrucosum* var. *cyclopium* (food isolate). All of the tested microorganisms were from the Laboratory of Mycology, Institute for Biological Research “Siniša Stanković”, University of Belgrade, Serbia. The micromycetes were maintained on malt agar (MA), bacteria on Mueller–Hinton agar (MH); cultures were stored at +4 °C and subcultured once a month [2].

Antimicrobial activity

In order to investigate minimum inhibitory (MIC) and minimum bactericidal/fungicidal (MBC/MFC) concentrations of the essential oil and pure thymol, the modified microdilution method in 96 well microtitre plates was used [3,4]. Bacterial species were cultured overnight at 37 °C in Tryptic soy broth (TSB) medium. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline. Cultures of bacteria were adjusted with sterile 0.85% saline to a concentration of 1.0×10^8 CFU per well, and 1.0×10^6 CFU per well for fungi.

Minimum inhibitory concentrations (MICs) determination was performed by a serial dilution technique using 96-well microtitre plates. The extracts (10 mg/mL in 5% solution of DMSO), essential oil and thymol were added in broth medium with inoculum. The microplates were incubated for 24 h at 37 °C for bacteria, and 72 h at 28 °C for micromycetes. The MIC was defined as the lowest concentration of essential oil/extract inhibiting the visible growth of the test strain. The MBCs/MFCs were determined by serial subcultivation of a 2 µL into microtitre plates containing 100 µL of broth per well and further incubation for 24 h at 37 °C and 72 h at 28 °C, respectively. The lowest concentration with no visible growth was defined as the MBC/MFC, indicating 99.5% killing of the original inoculum. However, the MIC/MBC values for bacteria and yeasts were detected following the addition of 2 ml of p-iodonitrotetrazoliumviolet (INT) 0.2 mg/mL (Sigma I8377) and incubation at 37 °C for 30 min (Tsukatani et al., 2012). Streptomycin (Sigma P 7794) and ampicillin (Panfarma, Belgrade, Serbia) (0.05–3 mg/mL) were used as a positive control for bacterial growth, and bifonazole (Srbolek, Belgrade, Serbia) and ketoconazole (Zorkapharma, Šabac, Serbia) (0.1–3 mg/mL) were used as positive controls for fungi. For each species, three samples were used and all the assays were carried out in triplicate. The results were expressed as mean values with standard deviation.

Statistical analysis

For each species, three samples were used and all the assays were carried out in triplicate. The results were expressed as mean values and standard errors, and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This analysis was carried out using SPSS v. 20.0 program.

S2: References

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