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Comparative study of the essential oils of four *Pinus* species: Chemical composition, antimicrobial and insect larvicidal activity

Zorica S. Mitić[a,*], Boris Jovanović[b], Snežana Č. Jovanović[c], Tatjana Mihajilov-Krstev[a], Zorica Z. Stojanović-Radić[a], Vladimir J. Cvetković[a], Tatjana Lj. Mitrović[a], Petar D. Marin[d], Bojan K. Zlatković[a], Gordana S. Stojanović[c]

[a] Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia
[b] Chair for Fish Diseases and Fisheries Biology, Faculty of Veterinary Medicine, Ludwig Maximilian University of Munich, Munich, Germany
[c] Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia
[d] Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia

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ABSTRACT

Comparative analysis of the chemical composition and biological activity of the essential oils from the needles of four *Pinus* species (*P. mugo* subsp. *mugo*, *P. nigra* subsp. *nigra*, *P. sylvestris* and *P. peuce*) from the central Balkans was performed. Although different chemical profiles of the dominant terpenes for each species were determined, essential oils were, generally, dominated by monoterpenes with α-pinene as one of the first two major volatiles. Tested oils showed inhibitory action against respiratory pathogenic bacterial strains, isolated from human swabs, in the range of 1.25–20.00 mg/mL (MICs) and bactericidal effect (MBCs) at concentrations from 2.50 to 40.00 mg/mL. Considering cell wall composition, there were no significant differences in sensitivity between Gram-positive and Gram-negative strains. In addition, the place of isolation did not provide evidences for higher susceptibility/resistance of the strains obtained from the particular infection site. On the other hand, toxicity of essential oils towards developing *Drosophila melanogaster* larvae was low, except *P. mugo* subsp. *mugo* oil, which did not show any signs of toxicity at concentrations up to 3%. Highest mortality of larvae and adults of *D. melanogaster* was noted for *P. sylvestris* essential oil with an estimated LC50 at the end of experiment (14 days) of 2.78% with a 95% confidence interval of 2.15–3.42%. Short-term 96 h LC50 values due to low mortality at larval stage could not be calculated for any oil and are expected to be much higher than 3%. According to the obtained antimicrobial activity and toxicity towards *D. melanogaster*, the oils can be arranged in the following order: *P. sylvestris* > *P. peuce* > *P. nigra* subsp. *nigra* > *P. mugo* subsp. *mugo*.

1. Introduction

The genus *Pinus* L. comprises 130 extant species (The Plant List, 2013), of evergreen, resiniferous trees or shrubs, widely distributed mainly in the Northern Hemisphere (Farjon, 2001). Various parts of the *Pinus* species (buds, young needles and female cones) as well as their essential oils and resins are used in traditional medicine for the treatment of several diseases and many respiratory infections accompanied by common colds, cough, bronchitis, bronchial asthma, emphysema, tracheitis, sinusitis, laryngitis, pharyngitis, tonsillitis and influenza (Ari et al., 2014; Dervendzi, 1992; Menković et al., 2011). On the other hand, increasing incidence of multiresistant microbial strains represents the major issue in medical microbiology, which raises the need for new, efficient and safe antimicrobial agents. Therefore, there are many studies focused on finding new molecules with antimicrobial properties, which are, in many cases, inspired by traditional utilization of medicinal plants. Furthermore, insecticides of herbal origin have received considerable attention because they contain rich sources and various bioactive compounds, many of which are selective and have little or no harmful effect on non-target organisms and the environment (Govindarajan et al., 2016).

Over the past two decades, there has been an increased interest in studying chemical composition as well as biological activities of the essential oils isolated from different pine species. Many studies have shown that pine's essential oils exhibit significant biological and pharmacological activity, such as antimicrobial (Hammer et al., 1999; Karapandzova et al., 2011a; Karapandzova et al., 2011b; Šarac et al., 2014), molluscicidal (Lahlou, 2003), acaricidal (Macchioni et al.,

* Corresponding author.
E-mail addresses: saraczorica@gmail.com (Z.S. Mitić), nanoaquatox@gmail.com (B. Jovanović), pdmarin@bio.bg.ac.rs (P.D. Marin), stgocaus@yahoo.com (G.S. Stojanović).

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2002), larvicidal activity and repellency against some Culicidae (Govindarajan et al., 2016; Koutsaviti et al., 2015), herbicidal (Amri et al., 2014), antioxidant (Ustun et al., 2012; Xie et al., 2015), antiplatelet (Tognolini et al., 2006) and anticancer activity (Hoai et al., 2015). *Pinus* essential oils have also been proven to play an important role in the defense system of conifers against numerous herbivorous insects and pathogens (Gijzen et al., 1993).

The territory of central Balkans is inhabited by many conifers, including four autochthonous pines: *P. mugo* Turra (dwarf mountain pine), *P. nigra* Arnold (black pine), *P. sylvestris* L. (Scots pine), which have wide European or Eurasian distribution, and *P. peuce* (Macedonian pine), a Balkan endemic of the high mountains of Bulgaria, Macedonia, Serbia, Montenegro, Albania and Greece (Gaussen et al., 1993). The aim of this study was to assess the potential of ethnopharmacological uses of the needle essential oils of these four pines in the treatment of respiratory infections, as well as a natural insecticide. The essential oils were tested against respiratory pathogenic bacterial strains, isolated from human swabs. To the best of authors' knowledge, these are the first results obtained against clinical strains, isolated from patient's respiratory specimens. Finally, a subchronic test on *Drosophila melanogaster*, frequently used animal model in toxicology studies (Jovanović et al., 2016; Mihajilov-Krstev et al., 2014; Nazir et al., 2003), was performed in order to evaluate insecticidal activity of the studied essential oils against dipteran insects.

2. Materials and methods

2.1. Plant material

Plant material (fresh needles) of four *Pinus* species was collected from their wild-growing populations from the central Balkans: *P. mugo* subsp. *mugo* (Šar Mountains, 42°14′12.80″N, 20°55′13.53″E; 1734 masl; August 2016), *P. nigra* subsp. *nigra* (Mileševka Canyon, 43°21′23.72″ N, 19°45′13.62″ E; 853 masl; May 2013), *P. sylvestris* (Tara Mountain, 43°52′40.30″N, 19°24′55.56″E; 912 masl; June 2013), and *P. peuce* (Šar Mountains, 42°9′57.46″N, 21°0′57.20″E; 1890 masl; August 2016). The needles from lower third of crown of ca. 30 randomly selected trees in each of the populations were collected. Plant material was deposited in polyethylene bags (labeled with data on sample plot, date of collection, locality), transferred to a freezer and stored at −20 °C prior to further analysis. Voucher specimens of each taxon were deposited in the "Herbarium Moesiacum Niš" (HMN) of the Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Niš under the acquisition numbers 12810, 6920, 6938 and 12812, respectively.

2.2. Isolation of essential oils

The fresh needles of each species were cut into small pieces and separately subjected to hydrodistillation using a Clevenger-type apparatus for 2 h. The obtained essential oil was extracted with diethyl ether, and dried over anhydrous sodium sulfate. After filtration, the solvent was removed under a gentle stream of nitrogen at room temperature, in order to exclude any loss of the essential oil.

2.3. Gas chromatography (GC) and gas chromatography – mass spectrometry (GC–MS) analyses

Quantitative and qualitative data of essential oil were obtained by GC and GC–MS analyses. Details about GC and GC–MS analyses, identification and calculation of relative amounts of essential oil compounds have been reported previously (Mitić et al., 2017).

2.4. Antimicrobial assays

Bacterial strains: *in vitro* antimicrobial studies were carried out against 9 bacterial multiresistant strains isolated from human material: *Klebsiella pneumoniae* (from nasal and throat swabs), *Escherichia coli* (from nasal and throat swabs and sputum), *Morganella morganii* (from nasal swab), *Staphylococcus aureus* (from nasal and throat swabs and sputum), were isolated in the laboratory of polyclinic "Human" in Niš, Serbia.

Micro-well Dilution Assay: Determination of the minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) was carried out using microdilution method (CLSI, 2012). Inoculums were prepared by suspending cells from overnight cultures of the tested isolates. The suspensions were adjusted to 0.5 McFarland standard turbidity using densitometer (DEN-1, Biosan). A serial doubling dilutions of the essential oils (in 10% aqueous DMSO) were prepared at concentrations ranging from 0.02 to 40.00 mg/mL in a 96/well microtiter plate with inoculated nutrient broth (each well contained the final bacterial concentration of 10^6 CFU/mL). The plate was incubated for 24 h at 37 °C. Chloramphenicol served as a positive control (Sigma Aldrich Chemical Co., St Louis, MO, USA), whose tested concentrations ranged 0.001–0.010 mg/mL. Experiments were performed in triplicate against each strain. MIC was defined as the lowest concentration of essential oils at which microorganisms showed no visible growth after addition of the redox indicator triphenyl tetrazolium chloride (TTC, aq. 0.5%). Determination of MBC was performed by inoculation of broth taken from all clear wells on Mueller Hinton agar (MHA), which were further incubated for 24 h at 37 °C. The MBC is defined as the lowest concentrations of the pine essential oils at which 99.9% of inoculated microorganisms were killed.

2.5. Sub-chronic toxicity and larvicidal test

Larvicidal test of essential oils of four *Pinus* species against three-day-old *Drosophila melanogaster* larvae was conducted. Canton-S (Stock number 1) wild-type strain was used (Bloomington *Drosophila* Stock center at Indiana University, USA). Flies were maintained during experiment under constant conditions of 25 ± 1 °C, 12/12 h day/night regime and 60% humidity. Adults were grown on, and laid eggs on a standard cornmeal-based *Drosophila* feeding medium consisting of 9% sugar, 10% corn meal, 2% agar, 2% yeast, with addition of fungicide Nipagin® (2.50 mg/mL). Young adults were allowed to mate for 3 days and then were transferred onto fresh medium to lay eggs for 8 h period. After 8 h, adults were removed from vials and medium, while laid eggs remained for incubation. After three days of incubation time, hatched larvae, age 72 ± 4 h, were washed with distilled water and transferred to the treatment mediums as it was described earlier (Castañeda et al., 2001). Treatments included five different concentrations of pure essential oils that were homogeneously mixed with 3 mL feeding medium in order to obtain final concentration. Essential oil concentrations in feeding medium were 3%, 1.5%, 0.75%, 0.38% and 0.19% v/v. For each concentration, three replicates consisting of 20 larvae each were set. A negative control, medium with ddH_2O addition instead of essential oil, was set in three replicates consisting of 20 larvae each. After larvae were transferred to the treatment medium in 50 mL vials, they started to feed immediately. Number of pupae and number of hatched adults were recorded every day over incubation time until all flies finished their development. Incubation time lasted 15 days, when all flies reached imago stadium.

Risk Assessment Tool Analysis Software – RA V1.0 was employed in order to estimate LC50 (the lethal concentration required to kill 50% of the experimental population in a given time). Development time (larva to pupa; larva to adult) was calculated for each replica of 20 eggs (N = 3) according to the following formula DT = Σnd*d/nt, where nd is the number of pupating larvae/emerging flies d days after the eggs were laid, and nt is the total number of individuals pupating/emerging at the end of experiment. Results were analyzed with one-way ANOVA followed by Dunnett's procedure if significant.

In addition, frequency analysis were performed by pooling together three replicas (total N = 60 per concentration) and results were