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Phenolic profiles and antimicrobial activity of various plant resins as potential botanical sources of Serbian propolis

Ivica Dimkić[a,1], Petar Ristivojević[b,1], Tamara Janakiev[a], Tanja Berić[a], Jelena Trifković[c], Dušanka Milojković-Opsenica (PhD)[c,**], Slaviša Stanković (PhD)[a,*]

[a] Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia
[b] Innovation Centre of the Faculty of Chemistry Ltd., Studentski trg 12-16, 11000 Belgrade, Serbia
[c] Department of Analytical Chemistry and Centre of Excellence for Molecular Food Sciences, Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, P.O. Box 51, 11158 Belgrade, Serbia

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ABSTRACT

Extensive employment of plant resins and propolis, as an antiseptic agents dating from ancient times in numerous cultures indicating that it may have antimicrobial and other biological properties. Resins from deciduous trees from the *Populus* and *Salix* genera, several fruit trees from the genus *Prunus* and a few other species were analyzed for their phenolic composition and antibacterial activity. Phenolic profiling of the plant resins was performed by high-performance thin-layer chromatography (HPTLC) and ultra-high-performance liquid chromatography (UHPLC) coupled with hybrid mass spectrometry. Antimicrobial activity against seven bacterial species was determined by minimum inhibitory concentration (MIC) assays and bioautography. The synergism, additivism, and antagonism of phenolic compounds were used to define the nature and type of interactions. *Populus* spp. showed higher amounts of *p*-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid, chrysin, apigenin, quercetin, pinocembrin, pinobanksin and galangin, which confirmed the botanical origin of the orange and blue types of propolis. In addition, Gram-positive bacteria exhibited high susceptibility to poplar samples while being resistant to samples from other origins. Cherry bud samples had high amounts of naringenin and showed strong activity against *Bacillus subtilis* and *Listeria monocytogenes*. The combinations of tested phenolics showed mainly additive or indifferent effects.

1. Introduction

Plant resins have been used in folk medicine for thousands of years to treat diseases, and their use by the pharmaceutical industry predates the introduction of modern antibiotics (Khan et al., 2009). Plant resins contain a diverse array of secondary metabolites whose main function is to protect plants against various predators and microbial pathogens (Bassolé and Juliani, 2012). The antimicrobial activity of plant resins can be attributed to various organic compounds such as alkaloids, phenols and terpenes. According to the World Health Organization (WHO), medicinal plants and their resins could be the best source for obtaining a variety of drugs (Khan et al., 2009). Among the natural sources of antimicrobial agents used to treat infectious diseases, propolis and essential oils from aromatic plants have been studied the most (Probst et al., 2011).

Propolis is a resinous natural substance collected by honeybees (*Apis mellifera* L.) from the buds, leaves, and bark of trees and other plants. During the production of propolis, honeybees mix resinous plant material with their own wax and enzymes. Propolis resin is mainly composed of flavonoids, phenolic acids and their esters, which often form up to 50% of the total ingredients (Bankova et al., 2000; Ristivojević et al., 2015a; Sforcin and Bankova, 2011). Many biological effects (antimicrobial, antioxidative, and antifungal effects) and health benefits have been associated with the phenolic compounds contained in propolis. Numerous reports describe the antimicrobial properties of plant resins and propolis and identify their active components (Probst et al., 2011). Bioautography, a method of microbiological screening hyphenated with planar chromatography techniques, is commonly used for the identification of bioactive compounds present in crude extracts (Choma and Grzelak, 2011) The chemical composition of a given propolis sample varies considerably depending on its geographic origin, the plant of origin, climate factors and collection season, as well as the

* Corresponding author.
** Corresponding author at: Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, 11158 Belgrade, Serbia.
E-mail addresses: ivicad@bio.bg.ac.rs (I. Dimkić), ristivojevic@chem.bg.ac.rs (P. Ristivojević), tamara.janakiev@bio.bg.ac.rs (T. Janakiev), tanjab@bio.bg.ac.rs (T. Berić), jvelicko@chem.bg.ac.rs (J. Trifković), dusankam@chem.bg.ac.rs (D. Milojković-Opsenica), slavisas@bio.bg.ac.rs (S. Stanković).
[1] These authors contributed equally to this work.

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species of bee that collected it. A significant number of papers have focused on connecting propolis samples to their botanical origin, depending on the plant sources present in the area of resin collection (Rai et al., 2012). The best indicator for the botanical origin of propolis is its chemical composition, which can be compared with the composition of the proposed plant source (Bankova et al., 2000). Propolis is often collected from the resins of trees such as poplars and conifers. The bud resin from trees such as *Populus* spp. (*P. alba* L., *P. tremula* L. and *P. nigra* L.) have been reported as the primary source of propolis in temperate zones that include Europe, Asia, North America, and the continental part of Australia. It is rich in flavonoids, phenolic acids and their derivatives (Huang et al., 2014). Secondarily important sources of this so-called "poplar type" of propolis are *Betula pendula* L., *Acacia* sp., *Aesculus hippocastanum* L., *Alnus glutinosa* L., *Pinus* sp. and *Salix alba* L. (Bertrams et al., 2013). According to Crane (1999), *Prunus* spp. (*P. cerasifera* Ehrh., *P. armeniaca* L., *P. avium* L. and *P. cerasus* L.) were also recognized as botanical sources of propolis.

Several publications have confirmed the existence of two main subtypes of European propolis, orange and blue (Morlock et al., 2014; Sârbu and Moţ, 2011). Our previous articles confirmed the affiliation of Serbian propolis to the European poplar type of propolis and the existence of both of it's subtypes; chromatograms of samples corresponding to the predominant orange subtype were characterized by an almost identical pattern, while profiles of blue subtype samples were quite different from one another (Ristivojević et al., 2014, 2015b).

Among the numerous publications related to the botanical origin of propolis there are a group of articles which have assumed the plant sources of propolis samples only by observing the bees' foraging behaviors, without comparing the chemical identity of secondary plant metabolites in the propolis and in the plant source. In order to provide a theoretical basis for studying the chemical composition and pharmacological activity of Serbian propolis, 50 samples from 14 different plant sources were analyzed for their phenolic composition and antibacterial activity. They contained resins from deciduous trees from the Salicaceae family (*Populus* spp. and *S. alba*), fruit trees from Rosaceae family (*Prunus* spp.) and a few other species. Comprehensive phenolic profiling of the plant resins was performed using high-performance thin-layer chromatography and ultra-high-performance liquid chromatography (UHPLC) coupled with hybrid mass spectrometry, which combines a Linear Trap Quadrupole (LTQ) with an OrbiTrap MS/MS mass analyzer. Antimicrobial activity against various bacterial strains was determined by minimum inhibitory concentration (MIC) assays and bioautography. The main goal of this study was to accurately define the most prominent compounds relevant to the botanical origin of both subtypes of propolis by simultaneously analyzing the main plant sources within the territory of Serbia. To our knowledge this is also the first report that comprises such a high number of different plant species and determination of the botanical origin of propolis. Furthermore, the current study was designed to assess the antibacterial activity of plant resins as potential botanical sources of propolis. In addition, the contribution to antimicrobial activity of phenolic acids and flavonoids as propolis' main constituents, as well as the synergistic/antagonistic effects between them, were investigated.

2. Materials and methods

2.1. Chemicals and materials

2-Aminoethyl diphenylborinate (NTS) was purchased from Fluka (Steinheim, Germany); dichloromethane, toluene and ethyl acetate from Merck (KGaA, Darmstadt, Germany); polyethyleneglycol (PEG), methanol and ethanol from Sigma-Aldrich (Steinheim, Germany), and formic acid from Kemika (Zagreb, Croatia). Phenolic standards such as quercetin, apigenin, kaempferol, rutin, myricetin, chrysin, luteolin, pinostrombin, pinobanksin, and naringenin were purchased from Fluka AG (Buch, Switzerland), while *p*-coumaric, caffeic, ferulic, chlorogenic, cinnamic and gallic acids were supplied by Sigma-Aldrich (Steinheim, Germany). Syringe filters (13 mm, PTFE membrane 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA). Two solutions were used: an aqueous resazurin solution (0.675 mg/mL final concentration) of Resazurin Sodium Salt $C_{12}H_6NNaO_4$ (TCI, Zwijndrecht, Belgium); and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution of 0.2% Thiazolyl Blue Tetrazolium Bromide $C_{18}H_{16}BrN_5S$ (Sigma-Aldrich, Carlsbad, CA, USA) with 0.1% Triton X-100 ($C_{14}H_{22}O$ $(C_2H_4O)_n$) (Sigma-Aldrich, USA).

2.2. Sample preparation and analysis

All fifty resin samples were collected from different regions of Serbia during the spring of 2014. Approximately 2 g of plant resin or raw propolis was cut and extracted with 30 mL of a mixture of ethanol and water (16:4, *v*/*v*) in an ultrasound bath (MRC Lab Equipment, London, UK) for 45 min. Subsequently, the extracts were filtered, evaporated to dryness (Büchi Rotavapor R-215, Flawil, Switzerland), and diluted with 5 mL of methanol. The methanolic solutions were refrigerated prior to analysis. Basic information about the plant resin samples, their geographical origins, masses and the insoluble portion of each sample is presented in Supplementary Table S1. We confirmed that no specific permissions were required by authorities for the locations or activities involved. We also confirmed that the scope of our study did not involve endangered or protected species.

2.3. Bacterial strains and growth conditions

Antibacterial activity was tested using five Gram-positive strains: (i) *Listeria monocytogenes* ATCC 19111, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923 and Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 33591. Two Gram-negative strains were also used: (ii) *Shigella flexneri* ATCC 9199 and *Yersinia enterocolitica* ATCC 23715. The bacterial strains were cultured in MHB and MHA (Müller-Hinton broth and agar, HiMedia, Mumbai, India), with the exception of *L. monocytogenes* which was cultured in BHI broth (Brain – Heart Infusion, Biomedics, Madrid, Spain) for 24 h at a temperature of 37 °C. Suspensions were adjusted to McFarland standard turbidity (0.5) (BioMérieux, Marcy-l'Étoile, France), which corresponds to 10^7–10^8 CFU/mL.

2.4. Well diffusion test

A modified well diffusion test (Harris et al., 1989) was used as a preliminary screening method for the antimicrobial potential of the resins. Sterile molds for the wells (made from the bottom parts of 200 μL pipette tips, 5 mm in diameter) were placed on the MHA which was used as the solid medium. MHB/BHI soft agar previously inoculated with 60 μL of the appropriate strain was uniformly spread on the MHA. After the soft agar solidified, the molds were removed and 20 μL of each test substance was added to the wells. As a negative control, 20 μL of methanol was used. The petri dishes were incubated for 24 h at 37 °C. The level of bacterial susceptibility according to zone diameter was determined. Zones of inhibition were expressed in mm. Antibiotics clotrimazole, rifampicin, amphotericin B, methicillin and cefpodoxime (HiMedia, Mumbai, India) were used as positive controls.