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HPLC/MS-TOF Analysis of Surface Resins from Three Poplar Clones Grown in Serbia

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ABSTRACT

Background and Purpose: Poplar clones grown in Serbia are fast growing tree species important for many different purposes in forestry and industry. In this study chemical content of the surface resins of three poplar clones grown in Serbia - M1, B229 and PE 19/66 was analyzed, aiming at their potential usage as a source of natural products important for pharmacy and chemotaxonomy.

Materials and Methods: Using HPLC/MS-TOF we gained the first information on chemical compounds which comprise of resins on terminal twigs cuttings of commonly grown poplar clones. Provided from the nursery of the Institute of Lowland Forestry and Environment (Serbia), terminal twigs cuttings with leaves of different development stage from two year old seedlings of M1 poplar clone (*Populus euramericana* L.), PE 19/66 clone and B229 clone (both belonging to *Populus deltoides*) were sampled. The washing of the surface resins from terminal twigs cuttings of every sample was done with methylene-chloride until the samples were prepared for HPLC/MS-TOF analysis.

Results: Out of 38 different compounds which were identified, M1 clone qualitatively differed for 14 compounds as compared to two other clones. Generally, the results showed that the composition of the resins consisted of different phenolic acids, phenolic esters, flavonoids and other contents.

Conclusion: These three poplar clones are potent producers of pharmacologically and chemotaxonomically potent compounds in forest ecosystems, especially M1 clone.

Keywords: phenols, poplars, resins, terminal twigs

INTRODUCTION

The genus *Populus* from *Salicaceae* family consists of about 30 species, growing in southern and central Europe, in central Asia, Siberia, and North America, characterized and differentiated by the presence of different flavonoids, phenolic derivatives, and terpenoids in particular in young leaves, buds, and bud exudates [1]. Several species of *Populus* have been traditionally used in medicine, especially for their anti-inflammatory properties [2]. Nowadays, large-scale poplar production is based on clone culture production of controlled hybrids. The complex chemistry of poplar bud exudates includes about a hundred different phenolic and terpenoid compounds [3], several of which are involved in biological processes [1]. Poplar biomass is a potential resource for natural production of bioactive molecules which could be substituted for some synthetic compounds associated with human and animal medicine [1]. Buds from Populus species are the main source of resins in propolis in Europe and North America ("poplar type" propolis) [4]. Resins comprising flavonoids and related phenolic acids represent approximately half of the propolis constituents [5]. Still, the chemical composition of the bee glue is extremely dependent on the plants found around the hive, as well on the geographic and climatic characteristics of the site.

Less commonly, species such as *Betula, Ulmus, Pinus, Quercus, Salix* and *Acacia* are also used in the production of propolis by bees [6, 7]. For the first time, the chemical composition of the cuticular waxes of poplar clones from Serbia was done and the results of GC/MS analysis showed that there are no significant differences in presence of alkanes: *n*-nonacosane, *n*-hexacosane, *n*-untriacosane and *n*-octacosane within leaf wax of these three clones [8].

Chemical content of the surface resins from three poplar clones was in the focus of this research. This type of chemical analysis of the surface resins from any fast-growing tree species from Serbia has previously not been undertaken.

MATERIALS AND METHODS

The Sampling of Plant Material

Five terminal twigs cuttings with healthy leaves of different development stage from two year old seedlings of M1 (Panonnia) poplar clone (*Populus euramericana* L.), cl. PE 19/66 and cl. B229 (both belonging to *Populus deltoides*) were sampled at the same time from the nursery collection of the Institute of Lowland Forestry and Environment, University of Novi Sad. The collections were grown as separate plantations, consisting of seedlings of the same age and origin, and influenced by the same climatic and weather conditions since they have been grown in the open. The two *Populus deltoides* clones B-229 and PE 19/66, and the hybrid *P. x canadensis* clone M-1 have previously been reported to be suitable for high biomass plantations [9-11]. *Populus deltoides* clones such as PE 19/66 were showed greater net photosynthesis than hybrid poplars (e.g. M-1), but hybrids were superior in water use efficiency [12]. The collections are part of the experimental Kać Forest estate located north-east from the city of Novi Sad, near the Kać village (N45° 17' 41" E19° 53' 30"). The genetic structure of the M1 and B229 clones has previously been determined through SSR and AFLP marker system and published, confirming the specific DNA profile of two different species originating from this nursery collection [13]. As the objective of the study was to examine the chemical composition and not the variability between single plants, the results were obtained for one pooled sample of terminal twigs cuttings with leaves from five plants per cultivar.

Preparation of Resin Extracts

Terminal twigs cuttings were immediately transported in sterile plastic bottles (5 mL volumes) on ice to laboratories of the Centre for Instrumental Analysis in Belgrade. Poplar clones' surface resins were washed out from the terminal shoots with 10 mL dichlormethane. Afterwards, the extracts were evaporated under a stream of N_2 for 23 minutes and dissolved in methanol at a concentration of 10 mg/mL, respectively.

HPLC/MS-TOF Analysis

High-resolution Liquid Chromatography/Photo-Diode Array/Electro Spray/Time of Flight mass spectra (HRLC/PDA/ESI/TOF MS) were measured on a HPLC instrument (Agilent 1200 Series) equipped with an autosampler, using a Zorbax Eclipse Plus C18 analytical column (1.8 µm particle size, 4.6 × 150 mm i.d., Agilent Technologies), and a PDA detector (DAD) coupled with a 6210 TOF LC/MS system (Agilent Technologies).

The mobile phase for HRLC/PDA/ESI/TOF MS was 0.2% formic acid in water (A) and acetonitrile (B), and analyses were carried out under the following conditions: (0-3 min) 10% B, (3-8 min) 10-25% B, (8-11 min) 25% B, (11-18 min) 25-30% B, (18-48 min) 30-40% B, (48-68 min) 40-60% B, (68-88 min) 60-90% B, (88-100 min) 90% B, (100-101 min) 90-10% B, and (101-120 min) 10% B. The flow rate was 1.20 $mL \cdot min^{-1}$, the injection volume was 5 µL, while the temperature of the column oven was set at 40°C. UV Spectral data from all peaks were accumulated in the range of 190-450 nm and chromatograms were recorded at 280 nm. MS data have been collected by applying the following parameters: ionization, negative ESI capillary voltage 4000 V, gas temperature 350°C, drying gas 12 $L \cdot min^{-1}$, nebulizer pressure 45 psi, fragmentor voltage 140 V, mass range 100-2000 *m/z*. A personal computer system running MassHunter Workstation software was used for data acquisition and processing.

Exact mass measurements of pseudomolecular ions of analytes performed with a time-of-flight (TOF) mass spectrometer in negative polarity mode enabled the determination of molecular formula of most of the constituents. All identified compounds exhibited quasi-molecular ion $[M-H]^-$ in the negative mode, confirming the molecular mass. Peak identification was mainly tentative, by comparison of their retention time, mass, and UV spectra with the literature data. For nine compounds we had standards for additional conformation of identification.

RESULTS AND DISCUSSION

Thirty-eight different compounds in resin extract of M1 clone and 24 common compounds within resin extract of B229 and PE 19/66 clones (Table 1, Figure 1) were identified. Fourteen compounds were unique for M1 clone, giving possibility to use them as potential species specific chemotaxonomic markers. Those are the compounds belonging to the caffeic, coumaryl and pinobaksin groups of compounds.

Our HPLC/MS-TOF analysis confirmed that Serbian poplar clones mainly consists of three groups of phenolic compounds: phenolic acids (caffeic acid, *p*-coumaric acid, cynammic acid), phenolic esters, and flavonoid aglycons of flavanones (pinocembrin, pinobaksin), flavonols (galangin, quercaetin and kaempferol), flavones (chrysin and its derivates, apigenin). We have also identified salycilate like populin. The results obtained in the experiment were compared with literature data on *P. nigra* HPLC bud and propolis analysis in the following text. Black poplar buds are coated with a viscous substance, an exudate which contains different varieties of phenolic compounds: flavonoid aglycons and their chalcones and phenolic acids and their esters [2]. Also, the chemical characterization of bud exudate has allowed the identification among the flavonoid aglycons of some flavanones such as pinocembrin and pinostrobin, some flavonols such as galangin, quercetin and kaempferol, some flavones such as chrysin and apigenin [14-16] and some esters of phenolic acids, similar to our data when it comes to the phenolic content. Such compounds have also been reported in propolis [17]. Bud extract of *P. nigra* was mainly composed of phenolic acids (caffeic, *p*-coumaric, ferulic,

TABLE 1. A list of identified compounds within surface resins of three poplar clones. Abbreviation NI means Not Identified. Compounds marked with * were previously reported in Trudić *et al.* [20]. Bolded compounds are compounds identified by internal standards.

No.	UV max	Quasi-molecular ion $[M-H]^-$	Exact mass	Molecular formula	Compound name	M1 Clone	B229 Clone	PE19/66 Clone
1	198; 216;274	109,0287	110,0360	$C_6H_6O_2$	Benzenediol	+	+	+
2	198; 216; 274	123,0442	124,0515	$C_7H_8O_2$	Methyl benzenediol	+	+	+
3	230; sh296; 324	179,0341	180,0414	$C_9H_8O_4$	**Caffeic acid ***	+	+	+
4	sh290, 310	163,0372	164,1445	$C_9H_8O_3$	***p*-Coumaric acid ***	+	+	+
5	244; sh296; 322	423,1294	424,1364	$C_{20}H_{24}O_{10}$	Furanocoumarin	+	+	+
6	234; 288	285,0762	286,0835	$C_{16}H_{14}O_5$	Pinobaksin-5-methyl-ethar	+	+	+
7	242; 268; 300	389,1226	390,1309	$C_{20}H_{22}O_8$	Populin *	+	+	+
8	292	271,0604	272,0677	$C_{15}H_{12}O_5$	**Naringenin ***	+	+	+
9	264; 310	267,0654	268,0727	$C_{16}H_{12}O_4$	Chrysin-5-methyl-eter	+	+	+
10	264; 338	269,0452	270,0525	$C_{15}H_{10}O_5$	**Apigenin ***	+	+	+
11	292	271,0604	272,0676	$C_{15}H_{12}O_5$	Pinobaksin *	+	+	+
12	266; 364	285,0396	272,0676	$C_{15}H_{10}O_6$	**Kaempferol ***	+	+	+
13	236; 288	269,0812	270,0885	$C_{16}H_{14}O_4$	NI	+	+	+
14	254; 372	315,0504	316,0576	$C_{16}H_{12}O_7$	**Isorhamnetin**	+	+	+
15	264; 348	299,0554	300,0626	$C_{16}H_{12}O_6$	Luteolin-3'methyl-ethar	+	+	+
16	254; 356	329,0660	330,0733	$C_{17}H_{14}O_7$	Quercetin-dimethyl-ethar	+	+	+
17	260; 302; 352	283,0603	284,0676	$C_{16}H_{12}O_5$	Galangin-methyl-ethar	+	+	+
18	232; 286; 324	327,0869	328,0942	$C_{18}H_{16}O_6$	Pinobaksin-5-methyl-ethar-3-acetate	+	+	+
19	244; 294sh; 326	315,0844	316,0919	$C_{17}H_{16}O_6$	NI	+		
20	268; 312	253,0497	254,0570	$C_{15}H_{10}O_4$	**Chrysin**	+	+	+
21	296; 326	247,0967	248,1040	$C_{14}H_{16}O_4$	Caffeic acid prenyl ester	+		
22	294sh; 328	269,0810	270,0883	$C_{16}H_{14}O_4$	Caffeic acid benzyl ester	+		
23	290	255,0655	256,0728	$C_{15}H_{12}O_4$	**Pinocembrin**	+	+	+
24	266; 360	269,0445	270,0518	$C_{15}H_{10}O_5$	**Galangin**	+	+	+
25	290	285,0760	286,0833	$C_{16}H_{14}O_5$	Pinobaksin-7-O-methyl eter	+		
26	294	313,0712	314,0784	$C_{17}H_{14}O_4$	Pinobaksin-3-O-acetate	+	+	+
27	298; 328	283,0968	284,1041	$C_{17}H_{16}O_4$	Caffeic acid Phenylethyl eter	+		
28	264; 312	283,0603	284,0676	$C_{16}H_{12}O_5$	Methoxy-Chrysin	+	+	+
29	230; 290sh; 310	231,1015	232,1088	$C_{14}H_{16}O_3$	*p*-Coumaryl prenyl ester	+		
30	220; 314	253,0861	254,0935	$C_{16}H_{14}O_3$	*p*-Coumaryl benzyl ester	+		
31	220; 288sh; 312	231,1017	232,1089	$C_{14}H_{16}O_3$	*p*-Coumaryl prenyl ester	+		
32	248; 316	295,0967	296,1040	$C_{18}H_{16}O_4$	Caffeic acid cinnamyl ester	+		
33	238; 232sh; 324	311,2222	312,2294	$C_{18}H_{32}O_4$	NI	+		
34	294	327,0868	328,0941	$C_{18}H_{16}O_6$	Pinobaksin-3-O-propionate	+		
35	314	267,1017	268,1090	$C_{17}H_{16}O_3$	*p*-Coumaryl phenylethyl ester	+		
36	268; 312	279,1016	280,1097	$C_{18}H_{16}O_3$	*p*-Coumaryc cinnamyl ester	+		
37	294	341,1020; 377,0788	342,1093	$C_{19}H_{18}O_6$	Pinobaksin-3-O- butyrate	+		
38	292	355,1172; 391,0958	356,1245	$C_{20}H_{20}O_6$	Pinobaksin -3-O pentanoate	+		

isoferulic, *di*-O-methyl caffeic and cinnamic acids) (5.2%), followed by salicylates (salicin) (1.8%) and flavonoid aglycons (pinobaksin 5-methyl ethar, pinobaksin and pinocembrin) (1.5%) [18].

Data comparison of poplar bud absolute markers after true quantitation by derivatization-GC-MS and HPLC-PDA was also reported in study of Rubiolo *et al.* [18]: benzoic acid, cinnamic acid, *p*-methoxycinnamic acid, *p*-coumaric acid, dimethoxycinnamic acid, isoferulic acid, ferulic acid, caffeic acid, 1,1-dimethylallyl caffeate, pinostrobin, pinocembrin, tectochrysin, chrysin, galangin. Salicyl aldehyde in fresh 0.2% and in dried 1.4% plant material of *P. nigra* L. was also identified in study of Jerković and Mastelić [15]. This compound may originate from salicin and/or populin by hydrolysis and oxidation and as mentioned before, we identified populin in our resins extract. Jerković and Mastelić [15] have also

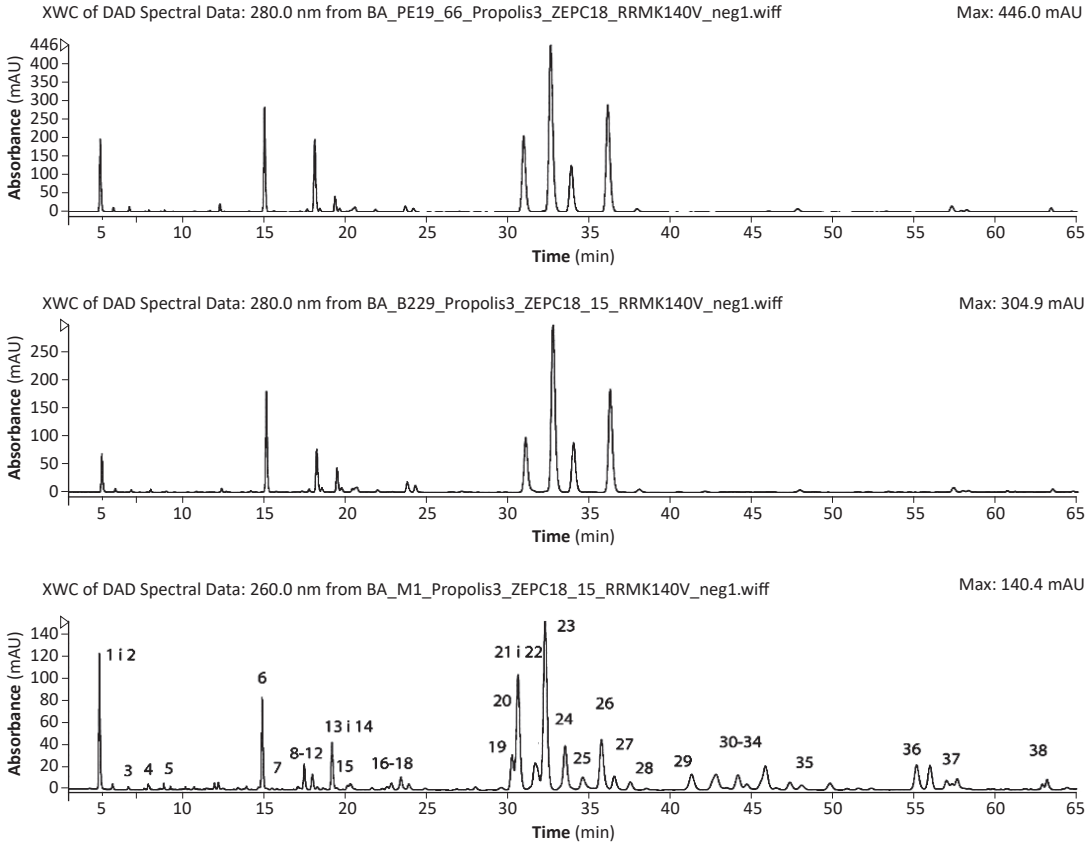

FIGURE 1. DAD chromatograms of identified resin compounds measured on 280 nm, 65 minutes. Peak numbers refer to those reported in Table 1.

reported prenyl alcohols with higher percentage (7.6%) in dried buds. Non-terpenes presented 9.8-13.5% of the total isolate. The gross compositions of non-terpenes were aliphatic and aromatic alcohols, carbonyl compounds and aliphatic acids. Benzyl alcohol and 2-phenylethanol were previously identified (ca. 0.2%) in the bud extract of *P. candicans* [19]. The analysis of the phenolic extract of Portuguese propolis allowed the detection of dihydroflavonols, flavones, flavanones and flavonols, either as a free form or their methylated/esterified forms [7]. In particular, it was possible to identify the aglycones forms of apigenin, pinobaksin, pinocembrin and chrysin, the esterified derivatives pinobaksin-3-O-acetate, pinobaksin-3-O-propionate, pinobaksin-2-O-butyrate or isobutyrate and pinobaksin-3-O-pentanoate or 2-methylbutyrate and the methylated derivatives pinobaksin-5-methyl-ethar, pinocembrin-5-methyl-ethar, chrysin-5-methyl-ethar, and chrysin-6-methyl-ethar. In propolis from temperate zones, the most frequently reported phenolic acids are caffeic acid, ferulic acid and the cinnamic acid [4]. We reported the following esters and ethar derivates: *p*-cinamyc cinamyl ester, pinobaksin-3-O-butyrate, pinobaksin-3-O pentanoate, pinobaksin-5-methyl-ethar, luteolin-3-methyl-ethar, quercetin-dimethyl-ethar, galangin-methyl-ethar, pinobaksin-5-methyl-ethar-3-acetate, caffeic acid prenyl ester, caffeic acid benzyl ester, pinobaksin-7-methyl-eter, pinobaksin-3-O-acetate, 2-phenylethyl caffeate, coumaril prenil ester, coumaril benzyl ester, coumaril prenil ester, caffeic acid cinnamyl ester, pinobaksin-3-O-propionate. Similarity with the chemical profile of *P. nigra* might indicate that some of those phenolic compounds and its ester might be common for the genus *Populus*, but further analysis within more *Populus* species is needed to confirm such hypothesis.

CONCLUSIONS

Direct assessment of antioxidant activity of resins is required through FRAP, DPPH, ORAC, ABTS, lipid peroxidation and other tests to screen its radical scavenging capacity and correlate it with quantity analysis of its bioactive compounds. In this case, 70% ethanol should be used as a common and nontoxic solvent for extraction. It is also possible that isomers can be present, although further NMR analysis must be performed.

The results presented in this study shows application in:

- Chemical compounds identified so far can be applied in pharmaceutical research and thus involve more nursery production of poplar clones' biomass as a resource of those molecules;
- To understand the chemical profile of propolis, since resins are the starting material for its production by bees;
- Chemotaxonomy, since we confirmed that there is a quite different chemical profile of surface resins between two species of poplars from our collection. In our study, 14 compounds were specifically characteristic for M1 clone (*P. euramericana* L) surface resin extract, indicating their species-specific significance. However, further comparative study with other poplar species is needed to determine the presence/absence of those compounds within genus.
- To monitor and predict poplar's biomass pharmacological potential and label them as a nursery with ecosystem service significance.

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