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Research article

UPLC-PDA-Q Exactive Orbitrap-MS profiling of the lipophilic compounds product isolated from *Eucalyptus viminalis* plants

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ABSTRACT

The lipophilic compounds product (LCP), which was isolated and purified from *Eucalyptus viminalis* plants, has shown earlier broad antimicrobial and anti-inflammatory activities. To study secondary compounds responsible for the pharmacological activities, chemical composition of the LCP was studied with application of ultra-performance liquid chromatography combined with photodiode array detector and high-resolution Q Exactive Orbitrap mass spectrometer (UPLC-PDA-HRMS/MS). There were found thirty two compounds: twenty phloroglucinol derivatives (isopentyl diformyl phloroglucinol, macrocarpals, sideroxylonals, etc.), eight ursane type triterpenoids (loxanic acid, dehydroursolic acid lactone, dehydroursolic acid lactone acetate, two isomers of p-coumaroyl-dehydroursolic acid lactone and two isomers of feruloyl-dehydroursolic acid lactone), sequiterpenoid (S)-β-macrocarpene and three unknown phenolics. The major compounds of the LCP were pharmacologically active macrocarpals A and B, dehydroursolic acid lactone and its derivatives. It is supposed that previously discovered antimicrobial and anti-inflammatory activities of the LCP is due to the high contents of these secondary compounds.

1. Introduction

Eucalyptus plants (Myrtaceae) synthesize and accumulate large quantities of various secondary compounds such as hydroxycinnamic acids, flavonoids, hydrolyzable and condensed tannins, essential oils, triterpenoids, phloroglucinol derivatives, etc (Ghisalberti, 1996; Domingues et al., 2010, 2012; Brezáni and Šmejkal, 2013; Okba et al., 2017; Santos et al., 2019; Celaj et al., 2020). These compounds are important components to plants, because of their ecological functions such as defense against microorganisms, insects and herbivorous vertebrates, protection from UV radiation and other environmental stressors (Moore et al., 2005; Jensen et al., 2015; Liu et al., 2019).

Eucalyptus extracts, rich in secondary compounds, possessed many pharmacological properties: anti-inflammatory, anticancer, antibacterial, antiviral, antispasmodic, antioxidant, antileishmanial, etc (Murata et al., 1990; Nishizawa et al., 1992; Yamakoshi et al., 1992; Takazaki et al., 1995; Singh and Bharate, 2006; Yang et al., 2007; Dixit et al., 2012; Brezáni and Šmejkal, 2013; Kaur et al., 2017; Celaj et al., 2020). To understand which secondary compounds determine these pharmacological activities, different metabolites or fractions of the Eucalyptus plants have been isolated and purified, and their properties have been studied. At the same time, lipophilic compounds, such as triterpenoids and phloroglucinol derivatives, attracted particular attention of researchers, since they had the most pronounced and diverse pharmacological activities.

The main triterpenoids of Eucalyptus plants have the structures of lupane (betulinic and betulonic acid), oleanane (oleanolic acid, 3-acetyloleanolic acid, and β-amyrin), and ursane (ursolic acid, 3-acetylursolic acid, and dehydroursolic acid lactone) (Horn and Lamberton, 1964; Savina et al., 1988; Domingues et al., 2012; Sidana et al., 2012). Plants of *E. tereticornis* and *E. globulus* were found to contain *p*-coumaroyl ester of dehydroursolic acid lactone and terethicornate A or feruloyl ester of dehydroursolic acid lactone (Wang and Fujimoto, 1993; Brezáni et al., 2018). Most triterpenoids exhibited a wide range of pharmacological activities, including anti-inflammatory, anticancer, antioxidant, antibacterial, hypoglycemic, etc (Ali et al., 2007; Laszczyk, 2009; Patlolla and Rao, 2012; Silva et al., 2012; Ku and Lin, 2013; Shanmungam et al., 2013; Nascimento et al., 2014; Wozniak et al., 2015; Hussain et al., 2017; Cör et al., 2018; Brezáni et al., 2018). Ursolic acid derivatives are considered as promising neuroprotective compounds in the treatment of Parkinson's disease (Ali et al., 2007; Macdonald et al., 2018).

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Phloroglucinol derivatives, such as macrocarpals, euglobals, eucalyptone, sideroxylonals, etc., is a group of secondary metabolites typical to the genus Eucalyptus (Myrtaceae) (Singh and Etoh, 1995; Singh and Bharate, 2006; Santos et al., 2019). These compounds consist of formylated phloroglucinols with an attached various monoterpenes and sesquiterpenes moieties. For example, macrocarpal A, which is the main phloroglucinol derivative in some Eucalyptus species, consist of sesquiterpene alcohol globulol linked to an isopentyl diformyl phloroglucinol (Murata et al., 1990; Maghsoodlou et al., 2015; Santos et al., 2019). The macrocarpals class includes many individual compounds and isomers, all of which are adducts of the same isopentyl diformyl phloroglucinol with different mono- and sesquiterpene moieties (Tian et al., 2014; Shang et al., 2016). In addition to phloroglucinol monomers, there were found different sideroxylonals that are simple dimers of isopentyl diformyl phloroglucinol without any attached terpene group (Eyles et al., 2003).

The study of phloroglucinol derivatives have been a hot research topic of the Eucalyptus natural products for many years due to their numerous pharmacological activities (Shang et al., 2016). They possess a wide range of anti-inflammatory, anticancer, antimicrobial, antiallergic, enzyme inhibitory, angiotensinase inhibitory and neuroregenerative activities (Singh et al., 2009; Yang et al., 2007; Hussein and El-Anssary, 2018). It has also been shown that phloroglucinol derivatives can be used to modulate the central nervous system, for example, in the treatment of depression, to elevate mood, to increase behavioral initiative, and the like (Roemer and Grothe, 2012). Therefore, lipophilic secondary compounds of Eucalyptus plants have multiple biological and pharmacological activities, but their mechanism of action and the relationship between structure and activity are under study.

Earlier, preparative method isolation and purification of the lipophilic compounds product (LCP) from *Eucalyptus viminalis* plants was developed (Savina et al., 1995). This product showed broad antimicrobial activity against a number of pathogenic microorganisms (*Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Bacillus anthracoides, Corynebacterium diphtheria*), including the antibiotic resistant strains of *Streptococcus pneumonia* (Semkina et al., 2006; Vichkanova, 2012). High anti-inflammatory activity of the LCP was found also (Tarasova et al., 1998). Further, the LCP was used for development of many medicinal preparations that widely using now in dermatology, stomatology, genecology, urology, proctology, and for the treatment of inflammatory diseases of the upper respiratory tract (Semkina et al., 2006; Vichkanova, 2012). The study of pharmacologically active compounds in the LCP with application of thin layer chromatography showed that the product contain phloroglucinols and some triterpenoids (Savina et al., 1995; Vichkanova, 2012). However, their detailed composition in the LCP was not investigated.

Ultra-performance liquid chromatography coupled with photodiode array detector and electrospray ionization high resolution tandem mass spectrometry (UPLC-PDA-HRMS/MS) is the most suited method for the analysis of complex mixtures of secondary compounds in the medicinal and food plants and their products (Cendrowski et al., 2017a, b; Okba et al., 2017; Santos et al., 2019; Kalisz et al., 2020). Therefore, to the comprehensive study composition of secondary compounds in the LCP, which determine its pharmacological activities, we applied the UPLC-PDA system combined with high-resolution Thermo Scientific Q Exactive Orbitrap mass spectrometer. This technique is generated very accurate and reliable MS and MS/MS information that extremely valuable for identification of the plant metabolites. As a result, we detected and identified in the LCP thirty-two secondary compounds that belong to biologically active phloroglucinol derivatives and triterpenoids.

2. Materials and methods

2.1. Plant object

The study object was lipophilic compounds product (LCP) that was isolated from the leaves of *Eucalyptus viminalis* Labill trees (Myrtaceae family). The Eucalyptus trees were grown in a greenhouse of the Botanical Garden of the All-Russian Institute of Medicinal and Aromatic Plants, Moscow (55°33′52″N, 37°35′30″E), to where they were introduced from the North Caucasus, Russia. Shoots (about 1 m long and a base diameter of up to 0.5 cm) with leaves were harvested in June 2016. For the rational use of plant raw material, a two-stage process of LCP isolation was applied. First, the leaves with shoots were used for isolation of the essential oils by hydrodistillation method. On the second step, the remaining raw material was dried, the leaves detached from shoots, ground and used for LCP isolation and purification in according to preparative methods described in Savina et al. (1995).

2.2. LCP isolation and purification

Sample of the dry leaves (100 g) was ground with ball mill MM 301 (Retsch GmbH & Co. KG, Germany), suspended in 700 ml of ethanol and allowed to stand for 3 h at room temperature with continuous stirring. The extract was separated by decantation and the pellet was re-extracted twice. The combined ethanol extract was concentrated under reduced pressure, and the residue (10–12 ml) was dissolved in 50 ml of water. Then, the water solution was extracted with 50 ml of dichloromethane in separatory funnel. The dichloromethane fraction was separated and the water phase was re-extracted twice with 25 and 12 ml. The combined dichloromethane solution was extracted three times with 2% solution of NaOH in water (38, 38 and 18 ml). The combined alkaline extract was acidified with 15% HCl to pH 1, and the forming precipitate was filtered off, washed with distilled water to neutral pH and dried at 80 °C. The dry product was dissolved in 150 ml of ethanol and purified on an aluminium oxide column (21 g). The column was washed with ethanol and the combined solution was evaporated under reduced pressure to a small volume (about 20 ml). Then a 9-fold volume of 0.1% HCl added and the forming LCP precipitate was filtered off, washed with distilled water until neutral pH and dried at 60 °C. Dry LCP preparation was a light-grey powder, insoluble in water, and soluble in organic solvents. Quality control of the LCP was carried out in accordance with requirements that were published (Savina et al., 1995) and registered in the State Pharmacopoeia of Russian Federation (Pharmacopeia article # 42-3605-98, 2003). The yield of the LCP was 3.6–3.8% of the raw plant material (Savina et al., 1995).

2.3. UPLC–PDA–HRMS/MS analysis

Samples of the LCP, 8–9 mg of dry mass, dissolved in 1 ml of methanol-toluene mixture (9/1, v/v) 60 min at room temperature with constant stirring (VORTEX Genie 2, Scientific Industries) and filtered through a syringe filter (4 mm, 0.2 μm PTFE, Thermo Fisher Scientific Inc., Waltham, USA). Preliminary, the syringe washed 4 times with methanol to remove traces of lubricant.

The UPLC system (Acquity UPLC® 2.9.0, Waters Corporation, Milford, USA) consisted of a sample manager, a binary solvent manager, and a photodiode array detector (PDA). The Acquity UPLC® BEH Phenyl column (2.1 × 100 mm, 1.7 μm, Waters Corporation, Wexford, Ireland) was used. Two eluents, 0.1% formic acid (A) and acetonitrile (B) were used in a gradient program: 0–0.5 min, 0.1 % B in A; 0.5–10.0 min, 0.1–95.0 % B in A (linear gradient); 10.0–13.0 min, 95.0 % B in A (isocratic); 13–15 min, washing and stabilization of the column. The flow rate was 0.5 ml/min and the injection volume was 5 μl. The PDA detector operated in the range 190–500 nm.

The UPLC system was combined with a Thermo Fisher Scientific high resolution Q Exactive Orbitrap mass spectrometer 2.5 equipped with heated electrospray ionization (HESI) source. The mass spectrometer operated in negative or in positive ionization mode and ions scanned in the range m/z 150–2000. The HESI conditions were as follows: sheath gas flow rate was set at 60, the auxiliary gas flow rate at 20, spray voltage at 3 kV, capillary temperature at 380 °C, and S-lens RF level at 60.0. The settings for full scan mode were: microscans 1, resolution 140,000

FWHM (full MS) and 34,599 FWHM (data dependent MS2). AGC target 3 × 106 and maximum IT 200 ms. Pierce™ ESI negative ion and Pierce™ ESI positive ion calibration solutions (Thermo Fischer Scientific Inc., Waltham, MA, USA) were used to the mass-spectrometer calibration. The data was processed with Thermo Xcalibur Qual Browser software (Version 3.0.63, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.4. Structure metabolite analysis

Based on the high-resolution measurement of ions with Q Exactive Orbitrap-MS in the negative and positive scan mode, the elemental compositions of the monoisotopic masses of detected compounds were calculated with the maximum tolerance of mass error at 2 ppm. The UV and MS spectral data (measured $[M\text{-}H]^-$ or $[M+H]^+$ and MS/MS fragments of the parent ion) were applied for identification or tentative characterization of the compounds with application of mass-spectrometry databases "Metlin" (Guijas et al., 2018) and "The Human Metabolome Database" (Wishart et al., 2018), and data published in the literature.

2.5. Quantitation of phenolic compounds

Total content of phenolic compounds was determined in the samples of the three independently isolated LCP with application of Folin–Ciocalteu method with gallic acid as a standard (Ossipov et al., 2001).

2.6. Chemicals

The acetonitrile used was LiChrosolv® hypergrade for LC-MS (Merck KGaA, Darmstadt, Germany). Analytical grade formic acid, methanol, dichloromethane, hydrochloric acid (HCl, 37%), NaOH and toluene purchased from Sigma-Aldrich (Steinheim, Germany) and ethanol (99.5%, v/v) was from Primalco (Rajamäki, Finland). Pure water obtained by an Elgastat UHQ-PS purification system (Elga, Kaarst, Germany). Gallic acid was from Sigma-Aldrich.

3. Results and discussion

The chemical composition of the LCP was analysed with UPLC-PDA-HRMS/MS both, in negative and positive modes to maximize the obtained information (Figure 1). Based on the UV and MS data, the detected thirty two compounds were tentatively classified as phenolic compounds and terpenoids. Phenolic compounds had the best ionisation at the negative mode, and terpenoids – at the positive mode. Retention times, UV absorption maxima, the main m/z values of mass-spectra, parent ion MS/MS fragmentation, monoisotopic masses and compound characterizations presented in Table 1.

3.1. Phenolic compounds

Twenty phenolic compounds were classified as phloroglucinols with an attached monoterpene or sesquiterpene moiety. Their identification was based upon the combination of UV absorbance at 277 nm, comparison of measured and theoretical monoisotopic mass within $< \pm 2$ ppm error and the presence of the diagnostic MS/MS fragments (Table 1) (Eschler et al., 2000; Singh and Bharate, 2006; Santos et al., 2019).

Fourteen phloroglucinol derivatives were tentatively identified as macrocarpals (Table 1). Their mass-spectra contained major m/z value that corresponded to the $[M-H]^-$ ion and intensive MS/MS fragment of parent ion with m/z 207.03 $[C10H7O5]^-$, which is a diagnostic ion for this group of phloroglucinol derivatives (Eyles et al., 2003; Okba et al., 2017; Santos et al., 2019). The presence of the diagnostic fragment indicates that all detected macrocarpals have the same phenolic unit isopentyl diformyl phloroglucinol and differ only in the mono- or sesquiterpene part of the molecules (Santos et al., 2019).

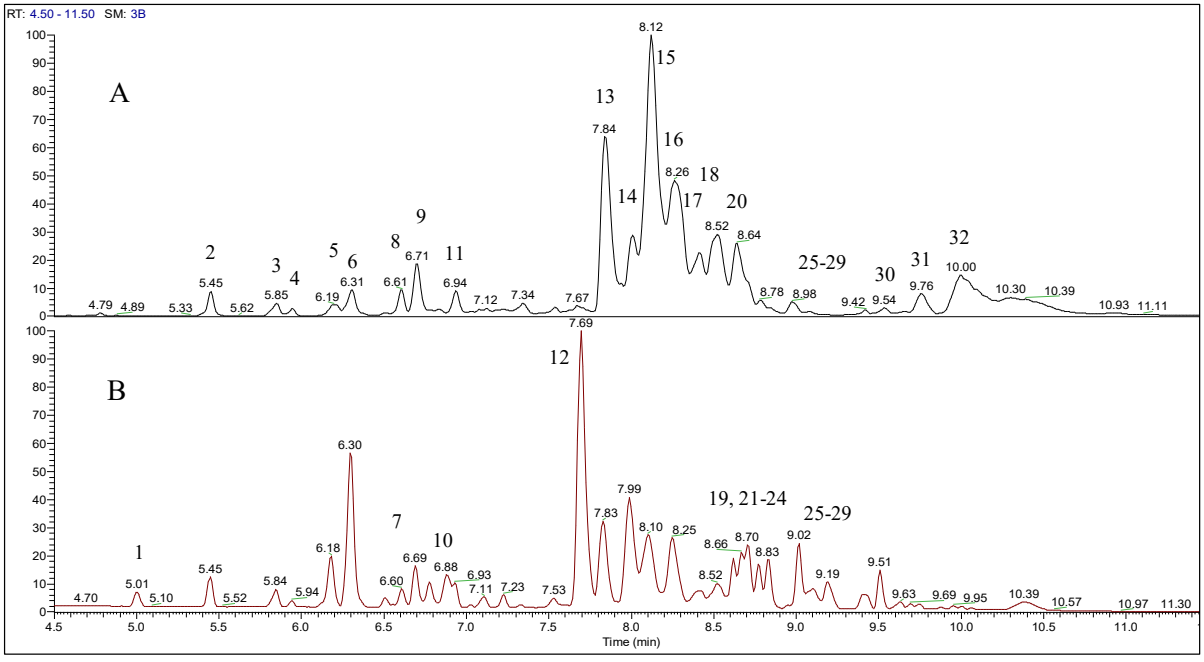

Figure 1. UPLC-HRMS profiles of lipophilic compounds product isolated from *Eucalyptus viminalis* plant extract in both, negative (A) and positive (B) modes. Number compounds from Table 1: 1. (S)-β-Macrocarpene; 2. Unknown phenolic compound 1; 3. Unknown phenolic compound 2; 4. Unknown phenolic compound 3; 5, 6. Callistenone K or isomer; 7. Loxanic acid; 8. Isopentyl diformyl phloroglucinol; 9, 11. Macrocarpal I or J; 10. 3β,12α-dihydroxyurs-12-en-28-oic acid; 12. Dehydroursolic acid lactone; 13. Macrocarpal A; 14. Macrocarpal am1 (eucalyptone); 15. Macrocarpal B; 16. Macrocarpal unknown 1; 17. Macrocarpal unknown 2; 18. Macrocarpal unknown 3; 19. Acetyl-dehydroursolic acid lactone; 20. Macrocarpal unknown 4; 21, 22. *p*-Coumaroyl-dehydroursolic acid lactone isomers (tereticornate B isomers); 23, 24. Feruloyl-dehydroursolic acid lactone isomers (tereticornate A isomers); 25, 27, 30. Sideroxylonal A, B or C; 26. Macrocarpal unknown 5; 28. Macrocarpal unknown 6; 29. Macrocarpal unknown 7; 31, 32. Macrocarpal G or its isomer.

Table 1. UPLC-PDA-Q Exactive Orbitrap-HRMS/MS characterization of lipophilic compounds product isolated from *Eucalyptus viminalis* leaves.

Number	Retention time (min)	UV maxima (nm)	$[M-H]^-$ or $[M+H]^+$(m/z)	Values of parent ion MS/MS fragments m/z (intensity, %), [formula]	Observed monoisotopic mass (Da)	Molecular formula	Calculated monoisotopic mass (Da)	Error (ppm)	Compound characterisation
Negative mode									
2	5.45	288	209.0810	194.06 (51), $[C10H10O4]^-$, 152.01 (100), $[C7H4O4]^-$	210.0888	C11H14O4	210.0892	-1.95	Unknown phenolic compound 1
3	5.85	292	223.0968	208.07 (26) $[C11H12O4]^-$, 152.01 (100), $[C7H4O4]^-$	224.1046	C12H16O4	224.1049	-1.16	Unknown phenolic compound 2
4	5.95	292	223.0967	208.07 (32), $[C11H12O4]^-$, 152.01 (100), $[C7H4O4]^-$	224.1045	C12H16O4	224.1049	-1.60	Unknown phenolic compound 3
5	6.19	268, 333	251.1285	207.14 (75), $[C13H19O2]^-$, 123.08 (23), $[C8H11O]^-$	252.1363	C14H20O4	252.1362	0.58	Callistenone K or isomer (Liu et al., 2016)
6	6.31	279, 328	251.1286	207.14 (73), $[C13H19O2]^-$, 123.08 (23), $[C8H11O]^-$	252.1364	C14H20O4	252.1362	0.98	Callistenone K or isomer (Liu et al., 2016)
8	6.61	277	251.0922	249.08 (39), $[C13H13O5]^-$, 125.06 (38), $[C7H9O2]^-$	252.1000	C13H16O5	252.0998	0.79	Isopentyl diformyl phloroglucinol (Santos et al., 2019)
9	6.71	277	489.2853	207.03 (100), $[C10H7O5]^-$, 205.01 (65), $[C10H5O5]^-$	490.2931	C28H42O7	490.2931	0.09	Macrocarpal I or J (Moore et al., 2004)
11	6.94	277	489.2853	207.03 (100), $[C10H7O5]^-$, 205.01 (65), $[C10H5O5]^-$	490.2931	C28H42O7	490.2931	0.09	Macrocarpal I or J (Moore et al., 2004)
13	7.84	277	471.2753	207.03 (100), $[C10H7O5]^-$, 205.01 (49), $[C10H5O5]^-$	472.2831	C28H40O6	472.2825	1.27	Macrocarpal A (Amakura et al., 2002)
14	8.01	277	485.2542	207.03 (100), $[C10H7O5]^-$, 205.01 (51), $[C10H5O5]^-$	486.2620	C28H38O7	486.2617	0.52	Macrocarpal am1 (eucalyptone) (Okba et al., 2017)
15	8.12	277	471.2752	207.03 (100), $[C10H7O5]^-$, 205.01 (63), $[C10H5O5]^-$	472.2830	C28H40O6	472.2825	1.08	Macrocarpal B (Amakura et al., 2002)
16	8.26	277	471.2753	207.03 (100), $[C10H7O5]^-$, 205.01 (68), $[C10H5O5]^-$	472.2831	C28H40O6	472.2825	1.27	Macrocarpal unknown 1 (Santos et al., 2019)
17	8.42	277	471.2754	207.03 (100), $[C10H7O5]^-$, 205.01 (62), $[C10H5O5]^-$	472.2832	C28H40O6	472.2825	1.48	Macrocarpal unknown 2 (Santos et al., 2019)
18	8.52	277	471.2753	207.03 (100), $[C10H7O5]^-$, 205.01 (55), $[C10H5O5]^-$	472.2831	C28H40O6	472.2825	1.27	Macrocarpal unknown 3 (Santos et al., 2019)
20	8.64	277	471.2752	207.03 (100), $[C10H7O5]^-$, 205.01 (53), $[C10H5O5]^-$	472.2830	C28H40O6	472.2825	1.06	Macrocarpal unknown 4 (Santos et al., 2019)
25	9.01	277	499.1607	249.08 (100), $[C13H13O5]^-$, 247.06 (52), $[C13H11O5]^-$, 133.06 (30), $[C9H9O]^-$	500.1685	C26H28O10	500.1682	0.60	Sideroxylonal A, B or C (Wallis et al., 2003)
26	9.02	277	385.2018	207.03 (100), $[C10H7O5]^-$205.01 (51), $[C10H5O5]^-$	386.2096	C23H30O5	386.2093	0.78	Macrocarpal unknown 5 (Santos et al., 2019)
27	9.08	277	499.1607	249.08 (100), $[C13H13O5]^-$, 247.06 (49), $[C13H11O5]^-$, 133.06 (37), $[C9H9O]^-$	500.1685	C26H28O10	500.1682	0.60	Sideroxylonal A, B or C (Wallis et al., 2003)
28	9.15	277	385.2020	207.03 (100), $[C10H7O5]^-$, 205.01 (63), $[C10H5O5]^-$	386.2098	C23H30O5	386.2093	1.29	Macrocarpal unknown 6 (Santos et al., 2019)
29	9.18	277	385.2020	207.03 (100), $[C10H7O5]^-$, 205.01 (52), $[C10H5O5]^-$	386.2098	C23H30O5	386.2093	1.29	Macrocarpal unknown 7 (Santos et al., 2019)

(*continued on next page*)

Table 1 (*continued*)

Number	Retention time (min)	UV maxima (nm)	$[M-H]^-$ or $[M+H]^+$(m/z)	Values of parent ion MS/MS fragments m/z (intensity, %), [formula]	Observed monoisotopic mass (Da)	Molecular formula	Calculated monoisotopic mass (Da)	Error (ppm)	Compound characterisation
30	9.54	277	499.1610	249.08 (100), $[C13H13O5]^-$, 247.06 (47), $[C13H11O5]^-$, 133.06 (35), $[C9H9O]^-$	500.1688	C26H28O10	500.1682	1.20	Sideroxylonal A, B or C (Wallis et al., 2003)
31	9.76	277	453.2643	207.03 (100), $[C10H7O5]^-$, 205.01 (55), $[C10H5O5]^-$	454.2721	C28H38O5	454.2719	0.39	Macrocarpal G or its isomer (Yamakoshi et al., 1992)
32	10.00	277	453.2642	207.03 (100), $[C10H7O5]^-$, 205.01 (54), $[C10H5O5]^-$	454.2720	C28H38O5	454.2719	0.17	Macrocarpal G or its isomer (Yamakoshi et al., 1992)
Positive mode									
1	5.01		205.1953	135.12 (50), $[C10H15]^+$, 123.12 (100), $[C9H15]^+$, 81.07 (40), $[C6H9]^+$	204.1875	C15H24	204.1878	-1.49	(S)-β-Macrocarpene (Bett et al., 2016)
7	6.59		455.3517	437.34 (100), $[C30H45O2]^+$	454.3438	C30H46O3	454.3447	-1.97	Loxanic acid (Sidana et al., 2012)
10	6.88		471.3467	425.34 (3), $[C29H45O2]^+$, 235.17 (4), $[C15H23O2]^+$	470.3391	C30H46O4	470.3396	-1.05	3β,12α-dihydroxyurs-12-en-28-oic acid (Sidana et al., 2012)
12	7.69		455.3517	437.34 (63), $[C30H45O2]^+$, 119.09 (100), $[C6H11]^+$	454.3438	C30H46O3	454.3447	-1.97	Dehydroursolic acid lactone (Brezáni et al., 2018)
19	8.62		497.3623	437.34 (100), $[C30H45O2]^+$	496.3547	C32H48O4	496.3553	-1.21	Acetyl-dehydroursolic acid lactone (Katai et al., 1983)
21	8.66	275–310	601.3884	437.34 (100), $[C30H45O2]^+$	600.3808	C39H52O5	600.3815	-1.17	p-Coumaroyl-dehydroursolic acid lactone isomer 1 (Wang and Fujimoto, 1993)
22	8.70	275–310	601.3883	437.34 (100), $[C30H45O2]^+$	600.3810	C39H52O5	600.3815	-0.83	p-Coumaroyl-dehydroursolic acid lactone isomer 2 (Wang and Fujimoto, 1993)
23	8.77	280–320	631.3989	489.28 (32), $[C34H33O3]^+$, 437.34 (100), $[C30H45O2]^+$	630.3916	C40H54O6	630.3920	-0.63	Feruloyl-dehydroursolic acid lactone or tereticornate A isomer 1 (Wang and Fujimoto, 1993)
24	8.83	280–320	631.3984	437.34 (100), $[C30H45O2]^+$	630.3911	C40H54O6	630.3920	-1.43	Feruloyl-dehydroursolic acid lactone or tereticornate A isomer 2 (Wang and Fujimoto, 1993)

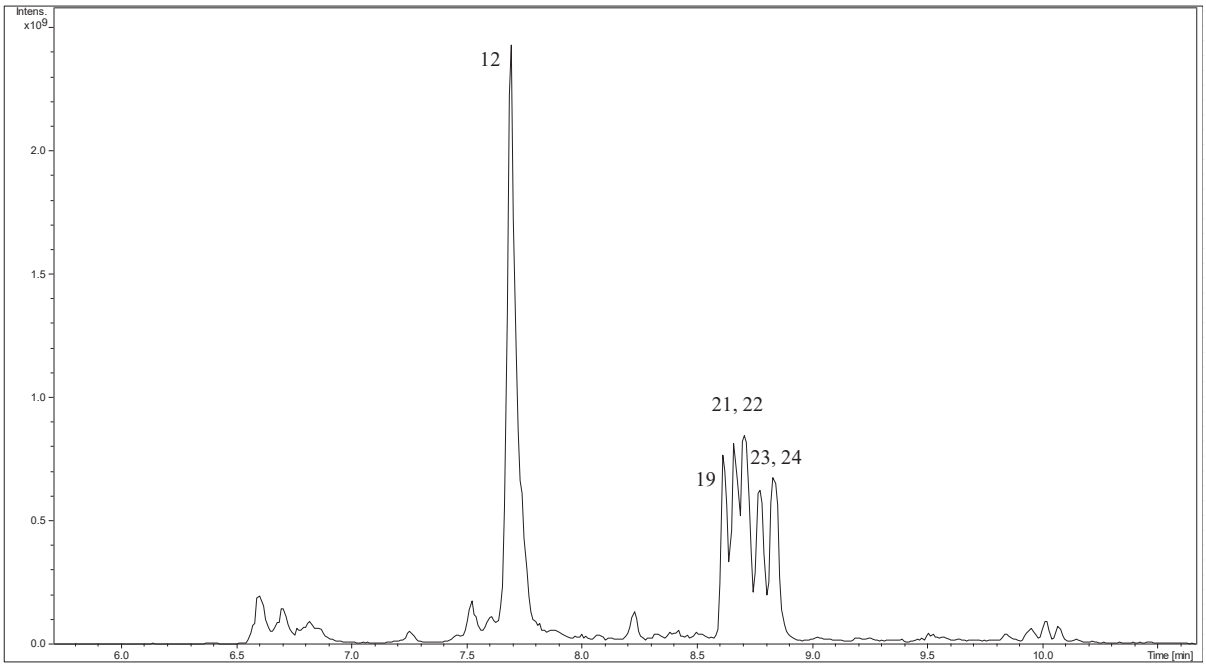

Figure 2. Extracted ion chromatogram (EIC) in positive mode of the m/z 437.34 that is diagnostic ion of MS/MS fragmentation of dehydroursolic acid lactone and its esters. Number compounds from Table 1: 12. Dehydroursolic acid lactone, 19. Acetyl-dehydroursolic acid lactone, 21, 22. *p*-Coumaroyl-dehydroursolic acid lactone isomers (tereticornate B isomers), 23, 24. Feruloyl-dehydroursolic acid lactone isomers (tereticornate A isomers).

Macrocarpals (12) and (14) had monoisotopic mass 472.2825 Da and molecular formula C28H40O6, and were identified as well-known macrocarpals A and B (Table 1) (Singh and Bharate, 2006). These macrocarpals are major phloroglucinol derivatives in many Eucalyptus species. They are stereoisomers, which structures have a isopentyl diformyl phloroglucinol moiety joined to the sesquiterpene globulol that contained one oxygen in the molecule (monoisotopic mass 222.1984 Da; molecular formula C15H26O) (Bolte et al., 1984; Murata et al., 1990; Santos et al., 2019; Maghsoodlou et al., 2015). Position of the macrocarpal A and B on the chromatogram were determined in according to the published HPLC data of isolated and purified standard macrocarpals (Amakura et al., 2002; Moore et al., 2004; Santos et al., 2019) (Figure 1). The others four phloroglucinol derivatives with monoisotopic mass 472.2825 Da (15, 16, 17, 19) were tentatively identified as unknown macrocarpals (Table 1).

Macrocarpals (8) and (10) with monoisotopic mass 490.2931 Da and molecular formula C28H40O7 were identified as macrocarpals I and J (Table 1) (Singh and Bharate, 2006; Chenavas et al., 2015). Earlier, these stereoisomers were isolated from the *E. globulus* leaves (Osawa et al., 1996, 1997). Positions of the macrocarpals I and J on the chromatogram were determined in according with HPLC data of their standards (Moore et al., 2004). In contrast to macrocarpals with monoisotopic mass 472.2825 Da, the sesquiterpene part of the macrocarpal I and J contained two oxygens (Moore et al., 2004; Singh and Bharate, 2006).

Macrocarpals (30) and (31) with monoisotopic mass 454.2719 Da and molecular formula (C28H38O5) were identified as macrocarpal G and its isomer (Yamakoshi et al., 1992) (Table 1). Earlier, NMR analysis of macrocarpal G structure showed an absence of oxygen in the sesquiterpene part of the molecule and supposed that biosynthetically macrocarpal G is a product of dehydration of macrocarpal A or its isomers (Yamakoshi et al., 1992).

The next three macrocarpals (25), (27) and (28) had monoisotopic mass 386.2093 Da and molecular formula C23H29O5 (Table 1). Earlier, compounds with the same masses were identified in the Eucalyptus plants as euglobales (Umehara et al., 1998; Eschler et al., 2000; Singh and Bharate, 2006). However, the presence in the MS/MS spectrum of intensive diagnostic fragment with m/z 207.03 indicates that these are apparently some unknown macrocarpals (Table 1).

One more macrocarpal (13) had monoisotopic mass 486.2617 Da and molecular formula C28H38O7 (Table 1). Phloroglucinol derivative with these chemical properties was identified earlier as macrocarpal am1 or eucalyptone (Osawa et al., 1995, 1996, 1997). It was shown that this compound has a unique sesquiterpene moiety with two oxygens and both a five-membered ring and a cyclopropane ring system (Osawa et al., 1995; Singh and Bharate, 2006).

Three phloroglucinol derivatives (24), (26) and (29) had monoisotopic mass 500.1685 Da and molecular formula C26H28O10 (Table 1). The presence diagnostic fragment ion m/z 249 allow identifying these compounds as dimers of isopentyl diformyl phloroglucinol, probably, sideroxylonals A, B and C (Table 1) (Eyles et al., 2003; Sidana et al., 2012; Okba et al., 2017; Santos et al., 2019). However, due to the differences of parent ion fragmentation at the Orbitrap mass-spectrometer compared with the earlier used Bruker qTOF-MS (Okba et al., 2017; Santos et al., 2019), the second diagnostic fragment for sideroxylonals with m/z 181 was not found (Table 1).

Phloroglucinol (7) had monoisotopic mass 252.0995 and formula C13H16O5 (Table 1). Compounds with the same monoisotopic mass and formula were found earlier in the Myrtaceae plants (*Eucalyptus pulverulenta* and *Callistemon viminalis*) and identified on the basis of NMR and MS/MS data as grandinol (2,4,6-trihydroxy-3-methyl-5-(3-methylbutanoyl)benzaldehyde) (Bolte et al., 1984) or 2,4-dihydroxy-6-methoxy-3-(3-methylbutanoyl)benzaldehyde (Liu et al., 2016). However, the presence in the MS/MS spectrum of parent ion compound (7) the diagnostic fragment m/z 249.08 $[C13H13O5]^-$ (Table 1) allow to identify this compound as isopentyl diformyl phloroglucinol that was considered as phenolic moiety of macrocarpals and sideroxylonals (Eyles et al., 2003; Sidana et al., 2012; Okba et al., 2017; Santos et al., 2019).

There are many speculations regarding of the initial compound in the biosynthesis pathway of various phloroglucinol derivatives, but the experimental biochemical evidences were not obtained yet. It was assumed that their precursor can be 4,6-diformyl-2-isopentanoyl-phloroglucinol (Tian et al., 2014; Shang et al., 2016), jensenone and grandinol,

or both compounds together (Singh et al., 1997; Brezáni and Šmejkal, 2013). Since most macrocarpals are adducts of isopentyl diformyl phloroglucinol with various moieties of mono- and sesquiterpenes, it seems that the more likely precursor of phloroglucinol derivatives is precisely isopentyl diformyl phloroglucinol (Table 1). At least this assumption is justified enough for the biosynthesis of various macrocarpals and sideroxylonals in *E. viminalis*. To the same conclusion was reached earlier in the study of Santos et al. (2019), where most phloroglucinol derivatives of different Eucalyptus species were identified as various macrocarpals and sideroxylonal A.

In the LCP were found also five phenolic compounds (2), (3), (4), (5) and (6), which had UV spectra different from those of phloroglucinol derivatives (Table 1). The molecular formula of compound (5) and (6) was established to be C14H20O4 in according to the monoisotopic masses 252.1363 (calculated mass 252.1362). Earlier, compound with the same mass and formula was isolated from *Callistemon viminalis* (Myrtaceae) and identified as 3-hydroxy-5-ethoxy-6,6-dimethyl-2-(2-ethylbutanoyl)cyclohexa-2,4-dienone (trivial name - callistenone K) (Liu et al., 2016). However, the lack of information about MS/MS fragmentation in the literature and databases does not allow us to confidently identify compounds (5) and (6) as isomers of callistenone K (Table 1).

Compounds with monoisotopic masses 210.0890 Da (2) and 224.1046 Da (3 and 4) were tentatively identified as unknown phenolics (Table 1). Other classes of phenolic compounds like flavonoids, galloylglucoses or ellagitannins that are characteristic to Eucalyptus plants (Marsh et al., 2017) were not found in the LCP.

Total content of phenolic compounds in gallic acid equivalent was 59 ± 3 (Mean value ± STD) mg per 100 mg of the LCP. It was shown earlier that the total phenolic content in the Eucalyptus extract obtained by the same method roughly corresponds to the sum of content of individual phloroglucinol derivatives (Amakura et al., 2002). Therefore, the phloroglucinol derivatives (macrocarpals and sideroxylonals) are the major components of the LCP isolated from *E. viminalis* plants.

3.2. Terpenoids

Nine compounds of the LCP had much more efficient ionisation in positive mode of mass-spectrometer (Table 1). Tentatively they were identified as terpenoids. Since most of the terpenoids were poorly separated on the chromatogram from phloroglucinol derivatives, UV spectra were determined for some of them only.

UPLC-HRMS/MS data revealed that the major terpenoid (12) was dehydroursolic acid lactone (3β-hydroxyurs-11-en-13β(28)-olide), which belong to ursane type of pentacyclic triterpenoids and identified earlier in *E. viminalis* leaves (Horn and Lamberton, 1964; Savina et al., 1988). Monoisotopic mass 454.3438 Da, formula C30H46O3, the diagnostic ion 437.34 $[M\text{-}OH+H]^+$ and others MS/MS data supported its identification (Sidana et al., 2012; Brezáni et al., 2018) (Table 1, Figure 2).

The LCP also contains small amounts of triterpenoid (7) that had identical monoisotopic mass 454.3438 Da, formula C30H46O3, and diagnostic ion with m/z 437.34 (Table 1). This compound was found in the leaf extracts of *E. loxophleba* and *E. globulus* along with dehydroursolic acid lactone and identified as 3β-hydroxyursa-9(11), 12-dien-28-oic acid (trivial name is loxanic acid) (Sidana et al., 2012; Brezáni et al., 2018).

Triterpenoid (10) had monoisotopic mass 470.3391 Da and molecular formula C30H46O (Table 1). Compound with these characteristics was isolated from epicuticular wax of *E. globulus* fruits and identified as 3β,12α-dihydroxyurs-12-en-28-oic acid (Sidana et al., 2012). Comparison of m/z fragments of MS/MS spectra confirmed identification of this compound in the LCP from *E. viminalis* (Table 1).

Along with dehydroursolic acid lactone, five triterpenoids more (19, 21–24) also contained in the MS/MS spectrum the diagnostic ion 437.34 as a main m/z fragment (Table 1, Figure 2). It suggests, that these triterpenoids are derivatives of dehydroursolic acid lactone. Triterpenoid (19) had monoisotopic mass 495.3391 Da and molecular formula C30H46O. Analysis of MS/MS fragmentation showed that ion 437.34 is belong to fragment $[M\text{-Acetyl}+H]^+$ (Table 1). Therefore, triterpenoids (19) was identified as acetyl-dehydroursolic acid lactone. Earlier, this triterpenoid was isolated and identified in the bark of *Pieris japonica* (Katai et al., 1983) and in the leaves of *E. loxophleba* (Sidana et al., 2012).

Triterpenoids (21, 22) and (23, 24) had monoisotopic masses respectively 600.3810 and 630.3916 Da, and molecular formulas - C39H52O5 and C40H54O6 (Table 1). The study of parent ions fragmentation showed that major m/z fragment 437.34 of MS/MS spectra belong to ion $[M\text{-p-coumaroyl}+H]^+$ compounds (21) and (22), and ion $[M\text{-feruloyl}+H]^+$ compounds (23) and (24) (Table 1, Figure 2). Therefore, triterpenoids (21) and (22) were identified as p-coumaroyl-dehydroursolic acid lactone isomers (tereticornate B isomers), and triterpenoids (23) and (24) – as feruloyl-dehydroursolic acid lactone isomers (tereticornate A isomers) (Table 1). Earlier, these p-coumaroyl- and feruloyl-esters of dehydroursolic acid lactone were found in plants of *E. tereticornis* and *E. globulus* (Wang and Fujimoto, 1993; Brezáni et al., 2018).

In addition to triterpenoids, in the LCP was identified also the only sesquiterpenoid (S)-β-macrocarpene (Table 1).

3.3. Biologically active compounds of LCP

Plant secondary compounds are an essential part of the human diet and are of considerable interest for their potential health benefits and as a resource for new drug development. For example, secondary compounds, such as digitalis, vincristine, Taxol and morphine isolated from foxglove, periwinkle, yew, and opium poppy, have a profound and long-lasting impact on human health and successfully used for decades (Brown and Murch, 2012). Eucalyptus plants (Myrtaceae) also synthesize and accumulate a large number of different secondary compounds that have various pharmacological activities (Murata et al., 1990; Nishizawa et al., 1992; Yamakoshi et al., 1992; Takazaki et al., 1995; Singh and Bharate, 2006; Yang et al., 2007; Singh et al., 2009; Dixit et al., 2012; Brezáni and Šmejkal, 2013; Okba et al., 2017).

The LCP isolated from *Eucalyptus viminalis* plants showed anti-inflammatory and broad antimicrobial activity against of pathogenic microorganisms (Savina et al., 1995; Tarasova et al., 1998; Semkina et al., 2006; Vichkanova, 2012). To study the secondary compounds responsible for these pharmacological activities, chemical composition of the LCP was studied with using of UPLC-PDA-HRMS/MS. As a result, thirty-two compounds were discovered that belong to phloroglucinol derivatives and triterpenoids.

The major compounds of the LCP are macrocarpals A and B, dehydroursolic acid lactone and its derivatives (acetyl-dehydroursolic acid lactone, two isomers of *p*-coumaroyl-dehydroursolic acid lactone, and two isomers of feruloyl-dehydroursolic acid lactone or tereticornate A). Pharmacological activities of these compounds are well known. For example, dehydroursolic acid lactone and ursocholanic acid were selected among 2000 tested compounds as the most promising neuroprotective compounds for trials in Parkinson's disease (Mortiboys et al., 2013). Both compounds markedly increased activity of the mitochondrial respiratory chain and rescue mitochondrial function in Parkinson's mutant fibroblasts (Macdonald et al., 2018). In addition, ursolic acid and its analogues have excellent anticancer, antidiabetic, antiarrhythmic, antihyperlipidemic, antimicrobial, antihypercholesterolemic, and anti-cardiovascular properties (Patlolla and Rao, 2012; Nascimento et al., 2014; Wozniak et al., 2015; Hussain et al., 2017; Brezáni et al., 2018). Tereticornate A demonstrated the strongest inhibitory potential against herpes simplex virus (HSV-1) (Brezáni and Šmejkal, 2013). Its antiviral effect was approximately twice greater than that of acyclovir, the standard drug used in clinical practice. In addition, tereticornate A also showed statistically significant anti-inflammatory activity, higher than that of prednisone, a routinely used drug (Brezáni et al., 2018).

Phloroglucinol derivatives identified in the LCP also have a range of pharmacological activities. For example, macrocarpals A and B are known to have strong antibacterial activity against cariogenic and

periodontopathic bacteria (Murata et al., 1990; Yang et al., 2007). Moreover, these phloroglucinol derivatives, especially macrocarpal B, have high anti-HIV activity (Nishizawa et al., 1992). Macrocarpal A and sideroxylonal B were showed activity against the three-tested human cancer cell lines (HEP2, CaCo and MCF7) and relatively low cytotoxicity against normal cell lines that indicating their selectivity (Soliman et al., 2014). Macrocarpal A, eucalyptone and their derivatives, can be useful for modulation of central nervous system, for example, in the treatment of depression, to elevate mood and to enhance behavioral initiative (Roemer and Grothe, 2012).

The phloroglucinol derivatives identified in the LCP are lipophilic phenolic compounds. There is growing evidence that the consumption of the phenolic compounds with plant foods or dietary supplements can reduce the risk of health problems due to their antioxidant activity (Shahidi and Ambigaipalan, 2015; Roleira et al., 2015; Panche et al., 2016; Kalisz et al., 2020). In this regard, the extract of *E. globulus* leaves, containing a large amount of phenolic compounds, was included as an antioxidant in the list of existing food supplements in Japan (Amakura et al., 2002, 2009). A comparative study of the antioxidant activity of phenolic compounds isolated from Eucalyptus extract showed that this property is largely due to hydrolysable tannins. The antioxidant activities of isolated macrocarpals A, B, C, D, E and eucalypton were much lower than that of tannins (Amakura et al., 2002). However, it has been suggested that lipophilic macrocarpals, like terpenoids (Ali et al., 2007; Cör et al., 2018), may have significant antioxidant activity in the lipid phase of the membrane due to their high permeability (Liu et al., 2019).

Therefore, the high antimicrobial and anti-inflammatory activities of the LCP (Tarasova et al., 1998; Semkina et al., 2006; Vichkanova, 2012) may be associated with the presence of macrocarpals A and B and ursane type triterpenoids. However, composition of the LCP metabolites and their known properties suggest that the range of pharmacological activities of the LCP may be much wider than previously shown.

4. Conclusions

An application of UPLC-PDA-HRMS/MS allowed to detect in the LCP isolated from *E. viminalis* plants thirty two secondary compounds: twenty phloroglucinol derivatives (isopentyl diformyl phloroglucinol, macrocarpals and sideroxylonals), three other phenolics and nine terpenoids (dehydroursolic acid lactone and its acetyl-, *p*-coumaroyl- and feruloyl-esters, loxanic acid, 3β,12α-dihydroxyurs-12-en-28-oic acid and (S)-β-macrocarpene). The major compounds of the LCP were pharmacologically active macrocarpals A and B, dehydroursolic acid lactone and its derivatives. It was supposed that previously discovered antimicrobial and anti-inflammatory activities of the LCP could be due to the presence of these secondary compounds. However, chemical composition of the LCP allow suggesting that its pharmacological activities could be significantly wider than it was shown before. Therefore, further the major macrocarpals and triterpenoids will be isolated and purified in preparative amounts and used as standards for the quantitative analysis of the LCP metabolites, and for determination of their more fine structure and individual pharmacological activities. In addition, the applied technology to obtain the LCP will be optimized taking into account the requirements of green chemistry and to the increasing efficiency and rationality the using of Eucalyptus raw material.

Declarations

Author contribution statement

Vladimir Ossipov: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Anne Koivuniemi: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Praskovia Mizina: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Juha-Pekka Salminen: Analyzed and interpreted the data; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

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