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Cotinus coggygria Wood: Novel Flavanone Dimer and Development of an HPLC/UV/MS Method for the Simultaneous Determination of Fourteen Phenolic Constituents

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Abstract

Phytochemical investigations of *Cotinus coggygria* Scop. wood, a medicinal and tinctorial plant used since antiquity, resulted in the isolation and structure elucidation of the novel C-3/C-3″ dimer of butin (3′,4′,7-trihydroxyflavanone) and other known compounds: gallic acid and its methyl ester; catechin; profisetinidins: fisetinidol-(4α→8)-(+)-catechin and epifisetinidol-(4β→8)-(+)-catechin; flavanonols: fustin and dihydroquercetagetin; flavanones: butin and eriodictyol; flavonols: fisetin and quercetin; the chalcone butein and the aurone sulfuretin. The isolated compounds were used for the development and validation of a HPLC-method which enables the determination of these bioactive substances in *C. coggygria* extracts. Separation was possible on an ether-linked phenyl column material, using as mobile phase mixtures of water, methanol, and acetonitrile with 0.02% trifluoroacetic acid. Sensitivity, selectivity, linearity, precision, accuracy, and repeatability of the method were verified and assured suitability for its intended use. LC-MS experiments performed in positive and negative electrospray ionization mode confirmed the identity of analytes and allowed unambiguous assignment of all peaks of interest. The analysis of different *C. coggygria* samples revealed that sulfuretin (0.38–0.69%) and fustin (0.33–0.59%) dominated, followed by dihydroquercetagetin (0.12–0.35%), a rare flavanonol derivative with a 5,6,7-trihydroxysubstituted A-ring. The new natural compound C-3/C-3″ flavanone dimer occurred in concentrations of 0.03–0.06%; the two latter compounds could represent valuable markers for the identification and quality control of *C. coggygria* wood.

Keywords

Cotinus coggygria; Anacardiaceae; HPLC-DAD; LC-MS; validation; biflavonoids

Introduction

Cotinus coggygria Scop. (Anacardiaceae), commonly known as fustic or sumac, is a deciduous shrub widespread in Southern Europe, the Balkans, and South-Western and Central Asia. Leaves, twigs, wood, and inflorescences of fustic are used in the

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ethnomedicine of Eastern and Southeastern Europe as well as China [1,2]. The plant is mainly employed to treat injuries of the skin and mucosal tissues (buccal, gastric, intestinal); other uses concern hepatobiliary disorders, hepatitis, and fever reduction. These indications are supported by the occurrence of tannins, essential oils and various flavonoids. Tanning compounds from fustic are represented by gallic acid (**1**) (Fig. 1) and its derivatives [methyl gallate (**2**), pentagalloyl glucose] with antioxidative effects [3], as well as by catechin (**3**) and procyanidins. Essential oils from sumac mostly consist of monoterpenes and display potent antibacterial and antifungal activities [4]. Research on fustic phytochemistry has especially focused on flavonoids. Sulfuretin (3′,4′,6-trihydroxyaurone, **11**), fisetin (3′,4′,7-trihydroxyflavonol, **9**), and fustin (3′,4′,7-trihydroxyflavanonol, **5**) were the first known compounds from sumac [5]. Other compounds obtained more recently are sulfurein (glycosil-7-O-sulfuretin) and a dimer of sulfuretin [3], taxifolin (3′,4′,5,7-tetrahydroxyflavanonol), 4′,7-dihydroxyflavanonol, butin (3′,4′,7-trihydroxyflavanone) (**8**), liquiritigenin (4′,7-dihydroxyflavanone), quercetin (3′,4′,5,7-tetrahydroxyflavonol, **12**), butein (*trans*-2′,3,4,4′-tetrahydroxychalcone, **13**), 4′,5,7-trihydroxyflavanone, and isoliquiritigenin (*trans*-2′,4,4′-trihydroxychalcone) [6]. Among the flavonoids, sulfuretin displays several pharmacological properties relevant for the traditional indications: it has antinociceptive, anti-inflammatory, and antioxidative effects [3,7,8]. Total flavonoids from leaves revealed anti-inflammatory effects after oral administration to mice [9]. Patents of sumac preparations include: mouthwash [10], topical treatment of skin and mucosal injuries [11] and of hemorrhoids [12].

Given the interesting pharmacological properties of fustic and its effectiveness reflected by long-term traditional use, adequate analytical assays are necessary in order to assure quality and efficiency of the plant material and its preparations. To our knowledge, no analytical methodology has yet been published on the simultaneous determination of all major nonvolatile secondary metabolites from fustic. The analysis of eleven flavonoids in *C. coggygria* wood and historical garments by HPLC has recently been published [6]. However, the employed method was originally validated for another mixture of standard compounds containing only two marker-compounds for *C. coggygria* (sulfuretin and fisetin) besides ten other substances specific to various tinctorial plants [13]; this method did not allow good separation of all subsequently investigated flavonoids from fustic. It is also noteworthy that data on the chemical structure of several compounds relevant for the pharmacological activity are incomplete. Furthermore, quantifications of biological-active compounds from plant material are missing. This situation encouraged us to elucidate the structure of new compounds from fustic and to develop and validate an assay suitable for the separation and quantification of fourteen major *C. coggygria* compounds by means of HPLC and LC-MS. These substances comprised, besides the already known ones (**1–3, 5, 8, 9, 12, 13**), five compounds described for the first time for the plant: profisetinidins **4** and **6**; 3′,4′,5,6,7-trihydroxyflavanonol (dihydroquercetagetin, **7**); 3′,4′,5,7-dihydroxyflavanone (eriodictyol,**10**), and a new flavanone dimer (**14**). Subsequently, the method was applied to assess the content of polyphenolic compounds **1–14** in several samples of sumac heartwood and branches.

Materials and Methods

Plant material

Cotinus coggygria Scop. plant material (stems and branches) was collected and identified by Dr. D.S. Antal in Romania (Banat region) in June 2007. A voucher specimen (001 CC-AD) is deposited at the herbarium of the Institute of Pharmacy/Pharmacognosy, Leopold-Franzens-University, Innsbruck.

Standards

Compounds **1–14** were separated and purified from a crude extract of *C. coggygria* heartwood by chromatographic methods (using Sephadex LH-20, RP-18 material and HSCCC), as described in Supporting Information. Purity of the isolated compounds was assured by TLC and HPLC. Structures of **1–3, 5**, and **7–13** were confirmed by comparing their spectroscopic data with those reported in literature: for **1** (gallic acid) and **2** (methyl gallate) see [14], for **3** (catechin) see [15], for **5** (fustin) and **12** (quercetin) see [16], for **7** (dihydroquercetagetin) see [17], for **8** (butin) and **13** (butein) see [18], for **9** (fisetin) see [19], for **10** (eriodictyol) see [20], for **11** (sulfuretin) see [21]. Full assignment of ^{13}C and ^{1}H-NMR signals of compounds **4** (including rotamers) and **6** are presented in Supporting Information, as existing references only concern derivatized profisetinidins [22,23].

C-3/C-3″dimer of 3′,4′,7-trihydroxyflavanone [2,2′-bis-(3,4-dihydroxy-phenyl]-7,7′-dihydroxy-2,3,2′,3′-tatrahydro-[3,3′]bichro menyl-4,4′-dione, new compound **14** was isolated as a yellowish powder (4.82 mg); $C_{30}H_{22}O_{10}$; m.p.: 234°C uncorrected; LC-online UV; λ_{max} = 200, 230, 275, 315 nm; FT-IR: ν_{max} = 3356, 1651, 1603, 1526, 1465, 1336, 1284, 1252, 1166, 1118, 1056 cm^{-1}; optical rotation $[\alpha]_D^{20}$: + 91.85 (CH_3OH, *c* 0.0958); ESI-MS (positive mode): *m/z* = 543.1 $[M + H]^+$, ESI-MS (negative): *m/z* = 269.1 $[M/2–2H]^-$, 541.2 $[M – H]^-$, 1083.1 $[2M – H]^-$; HR ESI-MS (negative mode): **m/z** = 541.1022 $[M – H]^-$ (calculated for $C_{30}H_{21}O_{10}^-$ 541.1140).

Equipment and HPLC conditions

HPLC—Analyses were carried out on a HP 1090 system (Agilent) equipped with auto sampler, PAD, and column thermostat; a Phenomenex Synergi Polar RP 80A column (250 × 4.6 mm i.d., 4 μm particle size) was employed. The mobile phase comprised a mixture of 0.02% (v/v) trifluoroacetic acid in water (solvent A) and 0.02% (v/v) trifluoroacetic acid in CH_3OH/CH_3CN 3:1 (solvent B). The elution program started with 95:5 (A:B), with solvent B progressively increasing to reach 28.4% at 31.2 min, 30.4% at 31.4 min, 34.7% at 38 min, 45.0% at 51 min, 54% at 56 min, 82% at 69 min, and 98% at 73 min; stop time 85 min. A post time of 20 min followed each run. Other parameters: detection wavelength 230 nm; column temperature, 45°C; injection volume, 10.0 μL; flow rate, 1.0 mL/min.

LC-MS data were obtained with an Esquire 3000plus (Bruker Daltonics) mass spectrometer coupled to an Agilent HPLC system type HP 1100. MS parameters: split, 1:5; ESI, alternative ion polarity mode; spray voltage, −4.5 kV; interface temperature, 350° C; drying gas flow rate, 10.00 L/min; nebulizer gas, 40 psi; mode, full scan: *m/z* range, 100–1500. Solvent A was changed to a mixture of water with 0.9% formic acid and 0.1% acetic acid (all v/v); gradient, stationary phase, column temperature, and detection wavelenghts were as above.

HR-ESIMS data were recorded on a Bruker MicrOTOF QII by injecting a methanolic solution of the compounds directly via syringe pump (180 μL/h) into the MS.

NMR—2D and 1D measured on a Bruker (Bruker Biospin) at 300 MHz (^{1}H) and 75 MHz (^{13}C); TMS as an internal standard.

Melting point—Kofler hot-stage, uncorrected.

Optical rotation—Perkin-Elmer 341 polarimeter.

Ft-IR spectra—ZnSe disc (2 mm thickness) Bruker IFS 25 FTIR-spectrometer, transmission mode within 4000 to 600 cm^{-1}.

HPLC sample preparation

Three samples of air-dried plant material were prepared by grinding decorticated stems with a diameter of 7–10 cm (CC-1), decorticated branches with a diameter of 2–3 cm (CC-2), and undecorticated branches with a diameter of 1.5–3 cm (CC-3). The finely powdered plant material (1.000 g) was extracted five times with 40 mL of CH_3OH by sonication (15 min each, at ambient temperature), and then centrifuged at 3300 rpm for 7 min. The extracts were combined, evaporated under reduced pressure and subsequently redissolved in CH_3OH, quantitatively transferred to a volumetric flask and adjusted to the final volume with CH_3OH. Extract from sample CC-1 was adjusted to 10 mL, whereas extracts from samples CC-2 and CC-3 were adjusted to 5 mL, in order to suit HPLC calibration range. Prior to injection all solutions were filtered through a 0.45 μm nylon membrane filter (Phenomenex). Each sample solution was assayed in triplicate.

Validation of assay

A standard stock solution of **1–14** was prepared by dissolving all standard compounds in 5.00 mL CH_3OH (1.00 mg of **12, 14**; 2.00 mg of **1, 2, 4, 6, 8, 10, 13**; 3.00 mg of **3, 7, 9**; 8.00 mg of **5, 11**). Five additional calibration levels were prepared by diluting this solution 1:1 with CH_3OH, and each level was assayed in triplicate. The standard solutions were stable for at least 2 weeks if stored at 4°C (confirmed by reassaying). Extraction efficacy was investigated by comparing peak areas of the same sample extracted with different solvents, volumes of CH_3OH and sonication times. Limit of detection is defined as concentration showing peak heights of three times baseline noise and the limit of quantitation as 10 times baseline noise; respective values were determined by consequently diluting standard solutions to appropriate concentrations. Peak purity was assured by evaluating available DAD data using the respective “peak purity” option within the Chemstation software, as well as LC-MS results. Peaks of interest were found to be pure and free of co-eluting compounds. Accuracy was determined by spiking sample CC-1 with three concentrations of standard compounds (low, medium, and high spike). Known amounts of **1–14** were added to the dry, powdered material, followed by extraction and analysis of the spiked material as described before. Repeatability of method was assured by observing relative standard deviations of multiple injections of the same sample solution.

Precision of method was evaluated by running five replicate samples of CC-1 prepared independently on day 1; the same procedure was repeated on two more days. By comparing variations within the same days, intra-day precision was determined; by observing differences within the three days inter-day precision was deduced.

Supporting information

^{1}H NMR, ^{13}C NMR, and HMBC spectra of compound **14**, isolation of compounds **1–14**, physical characterization of **1–13**, full assignment of ^{13}C and ^{1}H-NMR signals, and structure elucidation of compounds **4** and **6** are available as Supporting Information.

Results and Discussion

Isolation and structural elucidation of compounds **1–13** are described in Supporting Information. Structure of compound **14**, a novel C-3/C-3″ dimer of 3′,4′,7-trihydroxyflavanone, was determined as follows. High resolution FAB (*m/z*: 541.1022 $[M - H]^-$ for $C_{30}H_{21}O_{10}$; calculated: 541.1140) and ESI mass spectra (negative mode, *m/z*: 541.2 $[M - H]^-$, positive mode, *m/z*: 543.1 $[M + H]^+$) displayed a molecular weight of 542.

The ^{1}H NMR and ^{13}C NMR spectra displayed common (identical) signals for corresponding atoms belonging to each monomer, probably due to high symmetry of the molecule. Structure of monomers was elucidated in analogy to the previously isolated and available compound **8**, butin. Well-resolved signals in the ^{1}H NMR spectrum with typical multiplicities and coupling constants afforded the identification of two *AMX* systems, as follows: proton signals at δ = 6.26 (d, *J* = 2.2 Hz, H-8/H-8″), δ = 7.67 (d, *J* = 8.7 Hz, H-5/H-5″), and δ = 6.48 (dd, *J* = 2.2 Hz, H-6/H-6″) were ascribable to rings A/A′, whereas proton signals at δ = 6.56 (d, *J* = 2.1 Hz, H-2′/H-2‴), δ = 6.75 (d, *J* = 8.1 Hz, H-5/H-5‴), and δ = 6.40 (dd, *J* = 2.1 Hz, H-6/H-6‴) were ascribable to rings B/B′.

A low-field signal at 193.7 ppm in the ^{13}C NMR spectrum for C=O (C-4/C-4″) along with the signals at 85.80 (C-2/C-2″) and 52.57 (C-3/C-3″) ppm are typical of a flavanone structure with a C-5 hydroxyl (signals for the C=O in C-5 unsubstituted compounds appear at a significantly higher field [24]). The flavanone skeleton of compound **14** was further defined by the presence of two characteristic doublets for H-2/H-2″ at δ 5.78 (*J* = 11.8 Hz) and for H-3/ H-3″ at 2.65 (*J* = 11.8 Hz), respectively, in the ^{1}H NMR spectrum. The magnitude of the coupling constants of H-2 and H-3 ($J_{2,3}$ = 11.8 Hz) suggests a 2,3-*trans* geometry and the diaxial orientation of H-2 and H-3 (the same observation applies for H-2″ and H-3″). The HSQC spectrum established all of the correlations between protons and carbons of the compound, whereas the connectivities from the HMBC spectrum (detailed in Table 1) enabled unequivocal assignment of all protons and carbons within each monomer but also pointed to the linkage position between the two moieties. Cross-peaks of protons H-2/2″ with C-3″, and of H-3 with C-3″ offered firm proof for the 3 → 3″ connection between monomers. Remarkable differences of δ-values and multiplicity of the signals of carbons C-3 (52.57 ppm for the dimer, 44.08 ppm in the monomer butin, both measured under the same conditions) further confirmed that dimerization occurred at position C-3/C-3″. Several nonstandard long-range correlations across four bonds were clearly observable in the HMBC spectrum, even at regular thresholds in the contour plot (Fig. 3S, Supporting Information). This phenomenon is most probably related to the symmetry of the dimer molecule **14**, giving rise to cross-peaks which originate from two identical sets of C–H correlations. The example of compound **14** enforces the opinion that in HMBC experiments, very long-range correlations ($^{n}J_{C,H}$ n > 3) are less exceptional than believed, and complements the list of substances for which such observations have already been recorded [25].

The complex composition of *C. coggygria* extracts with a wide polarity range of analyte classes raised several problems when developing a HPLC method for the simultaneous separation of the major constituents. To achieve a satisfactory separation, all parameters therefore had to be carefully assessed. The disadvantages of the HPLC analytical method reported so far for *C. coggygria* [6] are: its limitation for flavonoids, the merger of some peaks, as well as its validation for a mixture of substances from *C. coggygria* and other plants.

First attempts to obtain good separation of peaks made it obvious that the mobile phase had to be acidic (0.020% TFA was added to solvent A and B), and that using CH_3CN alone as solvent B could not lead to satisfactory results, regardless of which stationary phase (C-8, C-12, C-18 or phenyl-hexyl) was employed. Screens revealed best peak resolution with a Synergi Polar RP column (Phenomenex) having longer dimensions as conventionally (250 × 4.6 mm vs. 150 × 4.6 mm); Synergi Polar RP columns have also been cited in the literature for proper HPLC-separation of polycarboxylic acids and polyphenol compounds [26]. Good separation was obtained by adding CH_3OH to CH_3CN in solvent B; different proportions were assayed (CH_3OH:CH_3CN = 1:1, 2:1, 3:1, 4:1, pure CH_3OH) with 3:1 showing best results. Replacing CH_3OH with 1-propanol, 2-propanol in various proportions, or using

these alcohols in small amounts as additives to the mobile phase did not show better results; neither did the use of tetrahydrofuran.

The separation of compounds **5–6** and **11–12** in the mixture of standard reagents, but also the separation of the assayed substances from minor components occurring in the *C. coggygria* extracts were difficult to achieve. The peak shapes of compound **3**, (±)-catechin, and to a lesser extent of compound **2**, methyl gallate, remained unsatisfactory in the conditions of the developed method, despite the purity of the analytes (documented by HPLC and NMR) and optimization efforts (gradient, temperature, additives to mobile phase, different stationary phase). If good peak shapes were obtained for **2** and **3** (especially with pure CH_3CN as solvent B), separation of several other analytes was compromised (merger of **4–5**, or **5–6**, as well as of **11–12** or **10–12**). Composition and pH of the mobile phase, as well as the type of stationary phase are known to have a decisive influence on peak shapes and separation of phenolic acids and polyphenols [26,27]. Different optimum conditions for the analysis of these substances, on one hand, and for the assay of flavonoids, on the other hand, could explain the observed phenomenon.

For LC-MS experiments, the trifluoroacetic acid was replaced by 0.90% of formic acid and 0.10% of acetic acid (v/v/v) in order to improve ionization of the analytes. The wavelength of 230 nm proved to be suitable for the sensitive detection of all compounds of interest. An elevated column temperature of 45°C was beneficiary in order to reduce the required separation time, yet maintaining good resolution between the compounds of interest.

Several analytical parameters indicate the suitability of the developed method for the assessment of *C. coggygria* constituents and extract. Calibration data for compounds **1–14** presented in Table 2 show that the detector signal was linear in the tested range, with correlation coefficients of 0.9990–0.9999. Limits of detection (S/N ratio of three, based on a 10 μL injection) ranging from 0.58 μg/mL to 3.07 μg/mL, and limits of quantitation (S/N ratio of ten) from 2.34 μg/mL to 12.29 μg/mL indicated the sensitivity of the method. All compounds, even closely structurally related ones, were baseline resolved, underlining the selectivity of the assay. Precision of the method was determined by repeated extractions and analysis of one sample (CC-1) over a period of 3 days, intra- and inter-day [28]. These results showed maximum deviations of 5.3% (intraday precision for compound **2**, on day 3) and 5.8% (inter-day precision for **12**) and indicate the method's precision (see Supporting Information, Table 4S).

Accuracy was determined in recovery experiments in which three different concentrations of standard compounds were added to dry *C. coggygria* sample CC-1 (low, medium, and high spike). After extraction and analysis, the obtained values were compared with theoretical amounts (natively present plus added amount). As shown in Table 3, all results were within the usually required recovery range of 100 ± 5%; maximum deviations were reached for compound **14** (recovery at medium spike: 95.2%) and compound **10** (recovery at high spike: 104.9%). Repeatability of the method was indicated by maximum relative standard deviation of 4.99% for multiple injections (Table 4) and by very stable retention times over the whole study period. Prior to sample analysis, optimum extraction conditions were established. Different solvents (CH_3OH, CH_3CN, water, and mixtures thereof) and extraction procedures (sonication, shaking) were evaluated. Repeated sonication of the plant material was most efficient; due to the compact nature of the plant material (heartwood, branches), sonication had to be repeated five times for 15 minutes each. After the last repetition, no marker compounds were left in the matrix (results not shown in detail). Good recovery rates of 100 ± 5% additionally indicated an exhaustive extraction procedure.

Chromatograms of the standard mixture and of a plant extract under optimized HPLC conditions are displayed in Fig. 2. All fourteen compounds are well separated and could be assigned by comparison of their retention times and UV-spectra with respective standards, and by LC-MS experiments (using the alternating mode). In order to present the results in a clear form, the extracted ion chromatograms (EIC) were selected for presentation (Fig. 3). The MS signals of compounds **1–6** and **8–14** were assigned as $[M - H]^-$ ions. For derivative **7**, signal assignment was based on the adduct $[2\ M + Na]^+$; elimination of one water molecule during LC-MS experiments of **7** has previously been reported [17]. For compounds **1–13**, deduced molecular masses are in agreement to values in the literature and allowed unambiguous identification.

Three samples of *C. coggygria* stem wood were analyzed by the newly developed HPLC-assay. Large branches and trunks (sample CC-1) were nearly twice as rich in flavonoid derivatives as branches with a small diameter (CC-2, CC-3); these data have practical relevance for the collection of plant material for medicinal purposes (Table 4). Flavan precursors are initially produced by the cambium, deposited in the sapwood and converted in the heartwood to condensed tannins, flavanols, flavonols, chalcones, and aurones [29]. In fustic, it can be assumed that precursor leucofisetinidin is converted to the main 5-dehydroxiderivatives fustin, fisetin, butein, and sulfuretin which are deposited in the heartwood, explaining the high occurrence of these compounds as shown in the present study.

All investigated compounds could be detected in the analyzed samples with sulfuretin (0.38–0.69%) and fustin (0.33–0.59%) as major components. Generally, the relative proportion of compounds remains constant between the samples, inversions only occur between **8** and **9** (**8** in higher concentration than **9** in CC-1, but lower than **9** in CC-2 and CC-3), and between **13** and **14** (more **13** than **14** in CC-1, but less in CC-2 and CC-3).

Dihydroquercetagetin (**7**) is a rare flavanonol derivative that has previously been isolated from *Wendita calysina* (Geraniaceae) [17]. With contents of 0.35% in the heartwood of > 7 cm diameter stems and 0.12% in the wood of smaller branches, this compound may become an interesting marker for the analyzed species. An additional unexpected finding was the isolation of racemic (±)-catechin from the heartwood; this form is only occasionally found [30]. Reports on other Anacardiaceae species like *Schinopsis* spp. mention the isolation of (+)-catechin.

Flavonoid dimers are considered to be a common feature of Anacardiaceae [31]. The current study afforded the isolation and quantification of a new flavanone dimer (compound **14**), along with two stereoisomer leucofisetinidins (Table 4). Others dimers between flavonoids of various classes were pointed out by LC-MS in fustic extract; their purification and structural elucidation is an ongoing project. These latter minor compounds with low polarity elute, in the conditions of the given HPLC method, at higher retention times (60–80 min, see Fig. 4S in Supporting Information). The described method was as well optimized for the separation of late-eluting derivatives and should be suitable for their assessment, once the isolation and characterization are achieved.

Supplementary Material

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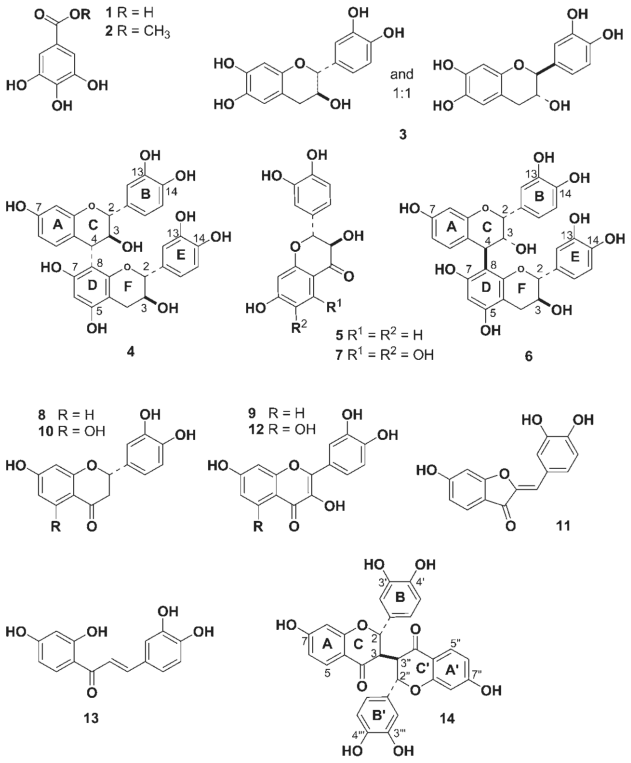

Fig. 1.

Chemical structures of compounds isolated from *C. coggygria* in the presented study: gallic acid (**1**), methyl gallate (**2**), (±)-catechin (**3**), fisetinidol-(4α→8)-(+)-catechin (**4**), fustin (**5**), epifisetinidol-(4β→8)-(+)-catechin (**6**), dihydroquercetagetin (**7**), butin (**8**), fisetin (**9**), eriodictyol (**10**), sulfuretin (**11**), quercetin (**12**), butein (**13**), and C-3/C-3″ dimer of butin (**14**).

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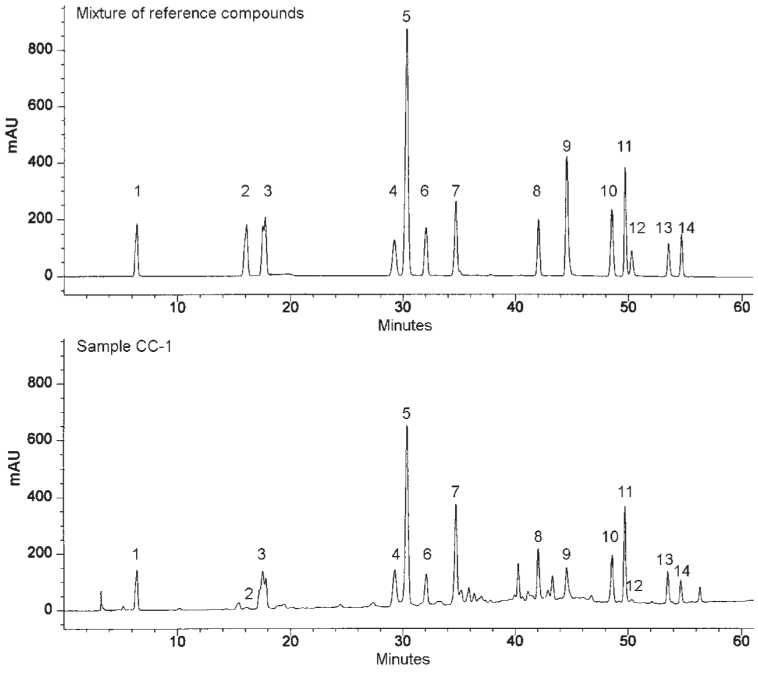

Fig. 2.

HPLC chromatograms of a standard mixture of compounds **1–14**, and sample solution CC-1, obtained under optimized separation conditions. Column: Synergi Polar RP 80A, 250 × 4.6 mm, 4 μm; mobile phase: 0.02% (v/v) trifluoroacetic acid in water (solvent A) and 0.02% (v/v) trifluoroacetic acid in CH_3OH/CH_3CN 3:1 (solvent B). Gradient started with 95:5 (A:B), with solvent B progressively increasing to 28.4% at 31.2 min, 30.4% at 31.4 min, 34.7% at 38 min, 45.0% at 51 min, 54% at 56 min, 82% at 69 min, and 98% at 73 min; stop time at 85 min; post time: 20 min; detection: 230 nm; temperature: 45°C; injection volume: 10.0 μL; flow rate: 1.0 mL/min.

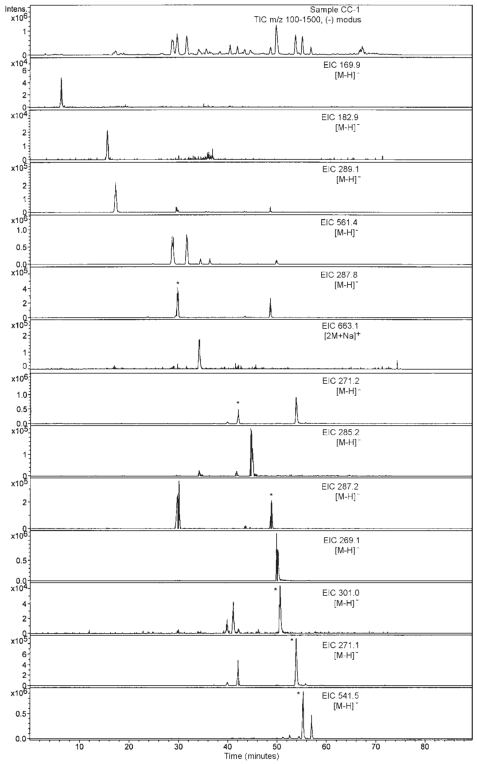

Fig. 3.

LC-MS analysis of sample CC-1. LC-conditions according to Fig. 2, except the replacement of trifluoroacetic acid by 0.9% formic acid and 0.1% acetic acid (v/v/v). TIC: Total ion chromatogram, EIC: Extracted ion chromatogram.

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Table 1

^{1}H- and ^{13}C-NMR assignments, and HMBC correlations of compound **14** (in CD_3OD, 300 MHz for ^{1}H-NMR, 75 MHz for ^{13}C-NMR, δ in ppm). *J* (Hz) in parentheses.

Carbon		δ_C	δ_H (*J*)	HMBC correlation H→C
2	2″	85.80	5.78, d (11.85), 2H	C-3, C-3″, C-4, C-1′, C-2′, C-6′, C-9
3	3″	52.57	2.65, d (11.80), 2H	C-2, C-3″, C-4, C-1′
4	4″	193.74	–	–
5	5″	130.01	7.67, d (8.75), 2H	C-4, C-7, C-8, C-9
6	6″	111.71	6.48, dd (2.27; 8.73), 2H	C-7, C-8, C-9, C-10
7	7″	166.65	–	–
8	8″	103.63	6.26, d (2.23), 2H	C-4, C-6, C-7, C-9, C-10
9	9″	165.03	–	–
10	10″	115.16	–	–
1′	1‴′	130.11	–	–
2′	2‴′	115.87	6.56, d (2.04), 2H	C-2, C-1′, C-3′, C-4′, C-6′
3′	3‴′	146.47	–	–
4′	4‴′	147.27	–	–
5′	5‴′	116.37	6.75, d (8.09), 2H	C-2, C-1′, C-2′, C-3′, C-4′
6′	6‴′	120.33	6.40, dd (2.09; 8.16), 2H	C-2, C-1′, C-2′, C-4′, C-5′

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Table 2

Calibration data of compounds **1–14**, including regression equation (y reflects peak area, x the amount in μg/mL), correlation coefficient (R^2), linear range (μg/mL), limit of detection (μg/mL), and limit of quantitation (μg/mL).

Compound	Regression equation	R^2	Linear range (μg/mL)	Limit of detection (μg/mL)	Limit of quantitation (μg/mL)
1 Gallic acid	y = 0.0648 x − 2.7823	0.9999	11.25–360	0.703	2.812
2 Methyl gallate	y = 0.0620 x − 3.2022	0.9999	7.03–450	0.879	3.516
3 Catechin	y = 0.0673 x − 3.7981	0.9999	21.12–676	1.320	5.281
4 Fisetinidol-(4*α*→8)-(+)-catechin	y = 0.0589 x − 1.2843	0.9999	10.93–350	0.683	5.469
5 Fustin	y = 0.0539 x − 10.570	0.9999	53.8–1722	0.841	3.363
6 Epifisetinidol-(4*β*→8)-(+)-catechin	y = 0.0645 x − 2.0720	0.9999	12.4–396	0.773	3.094
7 Dihydroquercetagetin	y = 0.0601 x − 4.9549	0.9996	16.1–514	1.004	4.016
8 Butin	y = 0.0518 x − 1.5002	0.9999	9.37–300	0.586	2.344
9 Fisetin	y = 0.0483 x + 4.1126	0.9999	20.87–668	1.305	5.219
10 Eriodictyol	y = 0.0423 x − 1.4596	0.9999	9.87–316	0.617	2.469
11 Sulfuretin	y = 0.1264 x + 161.34	0.9990	394–1574	3.074	12.297
12 Quercetin	y = 0.0641 x + 2.9354	0.9998	6.00–192	1.500	3.000
13 Butein	y = 0.1184 x + 2.8736	0.9996	12.6–402	0.785	6.28
14 Butin dimer	y = 0.0619 x − 0.9573	0.9999	7.25–232	0.453	1.812

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Table 3

Accuracy of the HPLC assay based on recovery experiments (low, medium, and high spike) utilizing sample CC-1; quantitative values in μg/mL.

Com-pound	Amount in sample	Spike 1			Spike 2			Spike 3		
		Added	Found	Recovery (%)	Added	Found	Recovery (%)	Added	Found	Recovery (%)
1	131.4	36.0	171.8	102.63	18.0	146.1	97.79	13.5	142.4	98.27
2	7.8	45.0	52.8	100.00	22.5	29.2	96.37	16.9	25.7	104.05
3	250.2	67.6	309.7	97.45	33.8	290.9	102.43	25.3	273.8	99.39
4	178.9	35.0	217.2	101.54	17.5	187.9	95.67	13.1	187.9	97.86
5	662.9	172.2	860.5	103.04	86.1	738.3	98.57	64.6	714.4	98.20
6	101.6	39.6	141.4	100.14	19.8	115.7	95.30	14.8	112.8	96.91
7	345.9	51.4	401.5	101.06	25.7	357.2	96.12	19.3	352.7	96.58
8	163.5	30.0	201.2	103.98	15.0	173.3	97.09	11.2	168.4	96.39
9	159.0	66.8	230.5	102.08	33.4	183.6	95.43	25.0	180.3	97.99
10	112.0	31.6	150.7	104.94	15.8	125.1	97.89	11.8	118.6	95.80
11	696.2	157.4	861.8	100.96	78.7	744.7	96.10	59.0	728.1	96.41
12	22.3	19.2	41.5	99.93	9.6	31.2	97.71	7.2	28.7	97.29
13	187.8	40.2	232.6	102.03	20.1	203.5	97.90	15.1	196.7	96.96
14	64.2	23.2	86.9	99.45	11.6	72.2	95.28	8.7	70.2	96.32

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Table 4

Content of analyzed compounds in different samples of *Cotinus coggygria* wood (RSD: relative standard deviation).

Compound	CC-1		CC-2		CC-3	
	Content (%)	RSD	Content (%)	RSD	Content (%)	RSD
1 Gallic acid	0.131	0.24	0.063	2.55	0.058	0.36
2 Methyl gallate	0.011	3.56	0.067	1.58	0.039	2.57
3 Catechin	0.248	0.89	0.317	2.66	0.325	0.21
4 Fisetinidol-(4α→8)-(+)-catechin	0.183	0.79	0.046	2.69	0.048	2.69
5 Fustin	0.589	0.61	0.341	2.44	0.335	0.97
6 Epifisetinidol-(4β→8)-(+)-catechin	0.136	0.23	0.046	2.73	0.052	1.88
7 Dihydroquercetagetin	0.349	0.22	0.119	2.29	0.119	0.75
8 Butin	0.163	0.48	0.060	2.01	0.060	0.49
9 Fisetin	0.159	0.59	0.153	0.38	0.147	0.37
10 Eriodictyol	0.112	0.78	0.062	0.85	0.086	8.90
11 Sulfuretin	0.696	0.48	0.386	1.61	0.402	0.34
12 Quercetin	0.022	1.49	0.041	4.99	0.047	2.66
13 Butein	0.186	0.05	0.033	4.53	0.034	4.83
14 Butin dimer	0.063	0.66	0.030	3.45	0.028	4.27