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Research Article

Isolation and characterization of 3-*O*-caffeoyloleanolic acid from *Robinia pseudoacacia* stem bark

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Abstract

Robinia pseudoacacia, a deciduous tree native to North America, has various medicinal properties, including antioxidant, antitumour, diuretic and antispasmodic effects. The plant contains various bioactive compounds, such as alkaloids, flavonoids, tannins, and phenols. However, caution is advised as all parts of the plant, except the flowers, are poisonous due to the phytotoxin robinin and its glycoside. The bark, on the other hand, shows resistance to rot due to the antifungal compounds dihydrorobinetin and robinetin. This study focuses on the stem bark of *R. pseudoacacia* from Bulgaria, a widely distributed wild species. Using advanced chromatographic techniques, we isolated and identified 3-*O*-caffeoyloleanolic acid, a new compound in the genus *Robinia* and *R. pseudoacacia*. Structural characterization was performed by state-of-the-art spectroscopic methods, including ^{1}H NMR, ^{13}C NMR and 2D NMR (COSY, HSQC, HMBC), as well as by LC-HRESI-MS analysis.

Keywords

Robinia pseudoacacia, genus *Robinia*, 3-*O*-caffeoyloleanolic acid, stem bark

Introduction

R. pseudoacacia is native to Atlantic North America but it withstands well the climatic conditions of Northern and Southern Europe, which makes it possible to cultivate large areas. The genus *Robinia* contains about 20 species. *R. pseudoacacia* is a tree with a height of 12 to 30 meters and a diameter of 0.61 to 1.2 m and may reach in age up to 100 years. The wood rots hard, lasting up to 80 years outdoors. The stem is covered with deeply embossed, gray-brown bark (Kaloo et al. 2018). In general, the whole plant is toxic, and toxicity is attributed to the presence of the phytotoxin robinin and the glycoside robitin, which are found in all parts of the plant, including minimal amounts in the flowers. The leaves, particularly wilted ones, seeds, and inner bark are the main toxic parts. Adverse effects on humans and animals are believed to be caused by toxic glycoproteins known as lectins (Tian et al. 2001). Clinical symptoms of *R. pseudoacacia* poisoning include the following: diarrhea, anorexia, weakness, mydriasis, loss of appetite, arrhythmia, difficulty breathing. Intestinal problems can lead to bloody diarrhea. Symptoms may appear up to 1–2 hours after ingestion, and death is not common (Anadón et al. 2018). Treatment of suspected toxicosis is primarily symptomatic and should include removal of the source (Talcott 2018).

The variety of biologically active substances, characteristic of the different parts of the plant, predetermine its important role and its potential for inclusion in the treatment of various diseases. Currently, *R. pseudoacacia* is used as an antispasmodic, antioxidant, diuretic, emollient, antitumor

agent. It is characterized by significant antibacterial and antitumor activity, which allows the extract of this plant to be considered as a resource for potential antimicrobial and antitumor agents (Patra et al. 2015). In addition to these actions, antimycotic, antiviral and antioxidant properties of the plant have also been evaluated. Precisely since it possesses these diverse pharmacological effects due to the rich set of active compounds, the plant is of interest for research and reporting of new substances, hiding within itself a huge potential for future use as herbal medicinal products in the therapy of various diseases (Guo et al. 2019). While the resistance of the bark against decay is mainly due to the two flavonoids: dihydrorobinetin and robinetin, known for their antifungal activity (Hosseinihashemi et al. 2016). The homomonoterpene robinlin was also isolated from the species (Kaloo et al. 2018). Other compounds isolated from wood are aliphatic compounds such as fats and waxes i.e., terpenes and terpenoids, and phenolic compounds, among which lignans, oligolignans and stilbenes dominate softwood (Sergent et al. 2014). In the present work the isolation and structure elucidation of rare terpenoid obtained from stem bark of *R. pseudoacacia* is reported.

Materials and methods

General experimental procedures

Solvents EtOAc, MeOH, hexane, CH_2Cl_2, MeCN and NH_4OH were obtained from Fischer Chemicals (Loughborough, UK) and were of least of analytical grade. The water used for assays was obtained from a Millipore Milli-Q system (Bedford, MA, USA) dispenser and filtered through a 0.22 µm membrane before utilization. TLC Silica gel 60 F_{254} (20 × 20) were used. The chromatographic plates were examined using UV light at wavelengths of 254 nm and 366 nm both before and after applying the iodoplatinate reagent for visualization (Waksmundzka-Hajnos et al. 2008). Rapidly vanishing white spots were observed during this process. A sequence of column chromatographic (CC) separations was conducted using Polyamide CC 6 (0.05–0.16 mm) (Sigma-Aldrich, Bornem, Belgium), as well as Silica gel (40–60 µm, 60 Å, 230–400 mash) and Diaion HP-20 (Mitsubishi Chemical Co., Japan). To isolate pure compounds, we utilized PLC Silica gel 60 F_{254} plates with a thickness of 0.5 mm (20 × 20 cm). The bands containing the desired substances were carefully scraped off the plates and separated from the sorbent through repeated percolation with MeOH. ^{1}H and ^{13}C NMR spectra as well as two-dimensional NMR (COSY, HSQC, HMBC) experiments were recorded on a Bruker AVII+ 600 spectrometer (Bruker, Karlsruhe, Germany), operating at a proton NMR frequency of 600.13 MHz in C_5D_5N (99.5%, Deutero GmbH). LC-HRESI-MS analysis of pure compounds were performed at Thermo Scientific Q Exactive Plus - Quadrupole - Orbitrap mass spectrometer used in ultra-high resolution mode (70 000, at m/z 200) coupled with a UPLC Dionex Ultimate 3 000 RSLC system equipped with a RP-18 Kinetex column (2.10 mm × 100 mm, 2.6 µm, Phenomenex (Corporation, Torrence, CA, USA). A gradient elution was performed using filtered and degassed MS grade solvents A (0.1% FA in H_2O) and B (0.1% FA in MeCN) as follow: 20% B at 1', reaching 95% B at 12.5', and maintaining the same composition until 14.5'. The operating conditions of the HR-ESI source ionization device were: - 3.5 kV voltage and 320 °C capillary temperature, 25 units of carrier gas flow and 5 units of dry gas flow. All other detector parameters were set in such a way as to obtain the most intense ions detected in negative polarity. Nitrogen was used to atomize the samples.

Plant material

The stem bark of *R. pseudoacacia* L. (Fabaceae) was collected in August 2018 from a southern slope at the foot of the Karandila mountain locality near Sliven.

Chemistry

Spectral data for 3-*O*-caffeoyloleanolic acid: ^{1}H NMR (C_5D_5N) δ, ppm: 0.83 (s, 3H, $-CH_3$), 0.86 (m, 1H, –CH=), 0.92, 0.94 (m, 2H, $-CH_2-$), 0.93 (s, 3H, $-CH_3$), 0.94 (s, 3H, $-CH_3$), 0.94 (s, 3H, $-CH_3$), 0.99 (s, 3H, $-CH_3$), 1.00 (s, 3H, $-CH_3$), 1.18, 2.18 (m, 2H, $-CH_2-$), 1.20, 1.46 (m, 2H, $-CH_2-$), 1.27, 1.44 (m, 2H, $-CH_2-$), 1.28 (m, 2H, $-CH_2-$), 1.28, 1.80 (m, 2H, $-CH_2-$), 1.28 (s, 3H, $-CH_3$), 1.30, 1.44 (m, 2H, $-CH_2-$), 1.64 (m, 1H, –CH=), 1.69, 1.77 (m, 2H, $-CH_2-$), 1.83, 2.05 (m, 2H, $-CH_2-$), 1.88 (m, 2H, $-CH_2-$), 3.30 (dd, 1H, J = 4.48, 14.03, –CH=), 4.86 (dd, 1H, J = 4.47, 11.92, –CH=), 5.47 (m, 1H, –CH=), 6.71 (d, 1H, J = 15.78, –CH=, H-7'), 7.23 (dd, 1H, Ar, J = 1.73, 8.27), 7.24 (d, 1H, Ar, J = 6.96), 7.70 (s, 1H, Ar,), 8.05 (d, 1H, J = 15.92, –CH=, H-8'). ^{13}C NMR (C_5D_5N) δ, ppm: 15.2 (C^{25}, $-CH_3$), 16.9 (C^{24}, $-CH_3$), 17.08 (C^{26}, $-CH_3$), 18.3 (C^6, $-CH_2-$), 23.5 (C^{11}, $-CH_2-$), 23.6 (C^{30}, $-CH_3$), 23.9 (C^2, $-CH_2-$), 26.0 (C^{27}, $-CH_3$), 28.0 (C^{15}, $-CH_2-$), 28.1 (C^{23}, $-CH_3$), 30.0 (C^{16}, $-CH_2-$), 30.6 (C^{20}), 32.6 (C^7, $-CH_2-$), 33.0 (C^{22}, $-CH_2-$), 33.2 (C^{29}, $-CH_3$), 34.0 (C^{21}, $-CH_2-$), 36.8 (C^{10}), 37.9 (C^1, $-CH_2-$), 38.0 (C^4), 39.4 (C^8), 41.7 (C^{18}, –CH=), 42.0 (C^{14}), 46.2 (C^{19}, $-CH_2-$), 46.5 (C^{17}), 47.7 (C^9, –CH=), 55.4 (C^5, –CH=), 80.1 (C^3, –CH=), 115.3 ($C^{8'}$=C), 115.6 ($C^6{}_{arom}$), 116.6 ($C^3{}_{arom}$), 122.1 ($C^2{}_{arom}$), 122.2 (C^{12}, –CH=), 126.7 ($C^1{}_{arom}$), 144.7 (C^{13}), 145.5 ($C^{7'}$=C), 147.7 ($C^4{}_{arom}$), 150.3 ($C^5{}_{arom}$), 167.2 (O–C=O), 180.0 (C^{28}, –COOH). HR-ESI-MS protonated molecular ion at m/z 617.4780 $[M-H]^-$ (Suppl. material 1: fig. S5).

Results and discussion

Extraction and isolation

The pre-dried and ground plant material, the stem bark of *R. pseudoacacia* (1.99 kg), was subjected to extraction by percolation using 80% MeOH until complete exhaustion. The collected extracts were concentrated using a vacuum evaporator, yielding a total extract, which was further defatted using cyclohexane (3 × 1 L). The total defatted extract (458.00

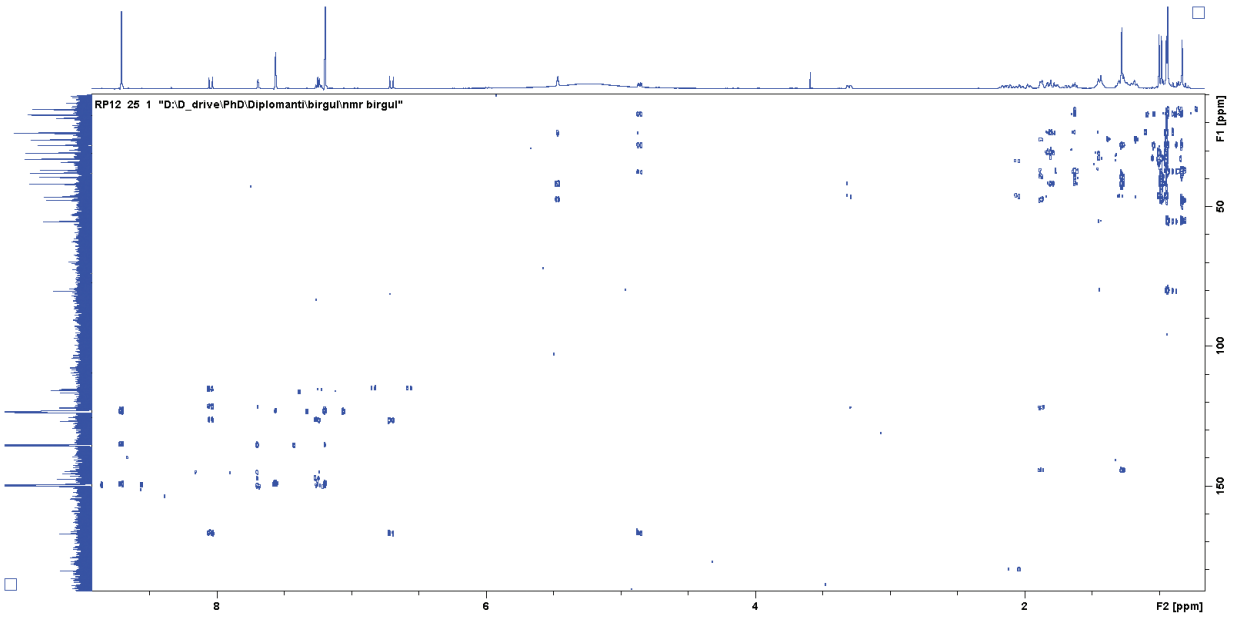

Figure 1. HMBC spectrum (C_5D_5N) of 3-*O*-Caffeoyloleanolic acid (**RP12**).

g) was separated into two sub-fractions (fr. 1 and 2) by silica-gel CC eluted subsequently with 2 L mixture of EtOAc/Hexane/MeOH/NH_4OH (70:25:5:5) and EtOAc/Hexane/MeOH/NH_4OH (90:15:40:5). Dissolved in $CHCl_3$ fr. 2 was subjected to liquid/liquid extraction against H_2O/H^+ (pH ~ 2.8, HCl) resulted in a dark aqueous layer (II.1), a white chloroform layer (II.2) and an intermediate insoluble layer (II.3). Based on TLC analysis fr. II.2. is subjected to further purification with CC against Diaion HP-20. Elution was gradient with following solvent mixture (v/v): MeOH/H_2O (20/80), MeOH/H_2O (60:40), MeOH (100), MeOH/CH_2Cl_2 (50:50), CH_2Cl_2 (100%). Fraction II.2.100% was applied to a CC silica gel, eluted sequentially with mixture of EtOAc/CH_2Cl_2/Hexane/MeOH/NH_4OH (v/v) in respective proportions (5:5:8:2:0.5), (5:5:6:4:0.5) and (5:5:4:6:0.5) to yield 40 fractions with a volume of 20 mL each. Fr. II.2.100%/C (30–44) was applied to gradient CC against Polyamide CC 6 (~ 20 g) Elution of the column started from pure CH_2Cl_2 and a gradient of CH_2Cl_2/MeOH up to 100% MeOH yielded 46 fractions of 5 mL each, combined into four sub-fractions. After PTLC analysis of fr. II.2.100%/C-C (20–25) using EtoAc/CH_2Cl_2Hexane/MeOH/NH_4OH (3:6:10:5:0.5) pure compound II.2.100%/C-C-3 (**RP12**) 5.3 mg was obtained.

Identification of isolated compound RP12

Compound **RP12** was isolated as a whitish powder. In the ^{1}H NMR spectrum of **RP12**, seven signals are observed at d 0.93 (s, 3H, CH_3), d 0.94 (s, 3H, CH_3), d 0.83 (s, 3H, CH_3), d 1.28 (s, 3H, CH_3), d 0.99 (s, 3H, CH_3), d 1.00 (s, 3H, CH_3), d 0.94 (s, 3H, CH_3) corresponding to seven methyl groups characteristic of oleanane-type triterpenoids (Suppl. material 1: figs S1, S1b). In ^{13}C NMR spectrum, chemical shifts at d 122.2 (C-12) and d 144.7 (C-13) were observed, corresponding to the double bond. The signal at d 80.1 (C-3) is indicative of a substituted hydroxyl group. The presence of a carboxyl group is proved by signal at d 167.2 (C-9') (Suppl. material 1: figs S2, S4). After comparing the data with literature, the sapogenin of **RP12** was identified as oleanolic acid (Seebacher et al. 2003). In the ^{1}H NMR spectrum of **RP12** is also observed 3 signals corresponding to aromatic protons. In the COSY spectrum, the correlations between d 6.71 (d *J* 15.92 Hz, 1H, H-7') d 8.05 (d *J* 15.92 Hz, 1H, H-8'), as well as the correlations in the HMBC spectrum between d 145.5 (C-7') and d 7.23 (dd, *J* 1.73, 8.27 Hz, 1H, H-2'), d 126.7(C-1') and d 7.7 (s, 1H, H-6') testify to the presence of an aromatic nucleus substituted by two hydroxyl groups OH-4' and OH-5' (Suppl. material 1: fig. S3). The presence of two olefinic protons at d (d, *J* 15.92 Hz, 1H, H-8') and d 8.05 (d, *J* 15.78 Hz, 1H, H-7'), as well as their correlation in the HMBC spectrum with the carbonyl carbon atom d 167.2 (C-9'), testify to the presence of an acidic residue of 3, 4-dihydroxycinnamic acid (caffeic acid). The HMBC correlation between d 4.86 (dd, *J* 4.47, 11.92, 1H, H-3) and the carbonyl d 167.2 (C-9') confirms our assumption of substitution precisely at the C-3 position by the oleanolic sapogenin (Fig. 1). From the considerations made, the structure of compound **RP12** was determined to be 3-*O*-caffeoyloleanolic acid (Fig. 2) (Fuchino et al. 1995; Chen et al. 1999). In the work, this compound was isolated for the first time from the genus *Robinia* and from the species *R. pseudoacacia*.

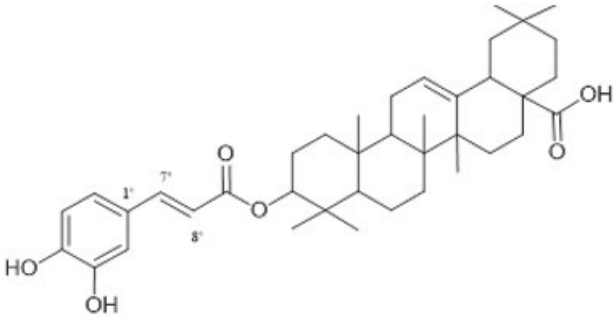

Figure 2. Structures of isolated 3-*O*-Caffeoyloleanolic acid from the stem bark of *R. pseudoacacia*.

Conclusion

In this study, we focused on the stem bark of *R. pseudoacacia* and successfully isolated and identified a rare terpenoid compound, 3-*O*-Caffeoyloleanolic acid. However, care must be taken as all parts of the plant except the flowers contain toxic compounds, especially robinin and robitin, which can have adverse effects on humans and animals. The resistance of the bark to decay has been attributed to the antifungal flavonoids dihydrorobinetin and robinetin. *R. pseudoacacia* contains a wide range of biologically active compounds, making it a valuable source of potential antimicrobial, antitumor, antifungal, antiviral, and antioxidant agents. Previous reports of *in vitro* cytotoxicity against the A549 cell line displayed 3-*O*-caffeyl oleanolic acid as an effective anti-lung cancer agent, with CC_{50} values of less than 20 μg/mL at 48 h of MTT assay, outperforming cisplatin used as positive control (Liao et al. 2014). In addition, 3-*O*-caffeyl oleanolic acid showed moderate NO inhibitory activity, with IC_{50} values of 32.6, which can be related with phenylpropanoid ester at C-3 position. The isolation of this rear triterpenoid from this new source introduces novel opportunities for exploring its pharmacological characteristics in subsequent research. This research contributes also to a deeper understanding of *R. pseudoacacia*'s chemical constituents and highlights its potential for use in herbal medicinal products for treating various diseases.

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Supplementary material 1

Supplementary images

Authors: Yancho Zarev

Data type: docx

Explanation note: **figure S1.** ^{1}H NMR spectra (C_5D_5N) of 3-*O*-Caffeoyloleanolic acid (RP12). **figure S2.** ^{13}C NMR spectra (C_5D_5N) of 3-*O*-Caffeoyloleanolic acid (RP12). **figure S3.** COSY spectrum (C_5D_5N) of 3-*O*-Caffeoyloleanolic acid (RP12). **figure S4.** COSY spectrum (C_5D_5N) of 3-*O*-Caffeoyloleanolic acid (RP12). **figure S5.** LC-HRESI-MS of 3-O-Caffeoyloleanolic acid.

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