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Raw coffee based dietary supplements contain carboxyatractyligenin derivatives inhibiting mitochondrial adenine-nucleotide-translocase

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ABSTRACT

Capsules, powders and tablets containing raw coffee extract are advertised to the consumer as antioxidant rich dietary supplements as part of a healthy diet. We isolated carboxyatractyligenin (**4**), 2-O-β-D-glucopyranosyl carboxyatractyligenin (**6**) and 3′-O-β-D-glucopyranosyl-2′-O-isovaleryl-2β-(2-desoxy-carboxyatractyligenin)-β-D-glucopyranoside (**8**) from green coffee and found strong inhibitory effects on phosphorylating respiration in isolated mitochondria similar to the effects of the known phytotoxin carboxyatractyloside. LC-MS/MS analysis of commercial green coffee based dietary supplements revealed the occurrence of carboxyatractyligenin, 3′-O-β-D-glucopyranosyl-2′-O-isovaleryl-2β-(2-desoxy-carboxyatractyligenin)-β-D-glucopyranoside, and 2-O-β-D-glucopyranosyl carboxyatractyligenin in concentrations up to 4.0, 5.7, and 41.6 μmol/g, respectively. These data might help to gain first insight into potential physiological side-effects of green coffee containing dietary supplement.
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1. Introduction

Due to appropriate advertising strategies, the consumer perceives frequent consumption of food rich in antioxidant phytochemicals as dietary means to prevent the development of life-style diseases like diabetes type 2, Alzheimer's disease or various types of cancer, all of which are considered oxidative stress associated illnesses (Kempf et al., 2010; Eskelinen et al., 2009; Larsson and Wolk, 2007). Due to its high content of chlorogenic acids (Clifford, 1999), food products have been proposed to be supplemented with raw coffee extracts (Hoelzl et al., 2010) to deliver functional foods providing "natural" antioxidants. A series of dietary supplements containing raw coffee extract are already available on the market and are advertised to the health-conscious consumer as a rich source of natural antioxidants, or as weight loss inducing agents. Recently, the detection of mycotoxin contamination in raw coffee based dietary supplements suggested to initiate toxicological investigations of such products with respect to consumer safety (Vaclavik et al., 2013).

From raw coffee we recently isolated compounds structurally related to the known adenine-nucleotide translocase (ANT-) inhibiting phytotoxins atractyloside and carboxyatractyloside (**1** and **2** in Fig. 1). While intoxications with plant material containing **1** and **2** are reported to induce fatal liver and kidney necrosis (see Obatomi and Bach, 1998 for a review; Georgiou et al., 1988), LD50 values have only been investigated in rodents and dogs (Santi and Luciani, 1978), and any information on the dose of carboxyatractyloside exhibiting acute toxic effects in human is lacking.

The compounds we isolated from raw coffee were glucosides of atractyligenin (**3**) and carboxyatractyligenin (**4**), namely 2-O-β-glucopyranosyl-atractyligenin (**5**), 2-O-β-glucopyranosyl-carboxyatractyligenin (**6**), and 3′-O-β-D-glucopyranosyl-2′-O-isovaleryl-2β-(2-desoxy-atractyligenin)-β-D-glucopyranoside (**7**). Individual incubation of these compounds with liver mitochondria from mice showed that carboxyatractyligenin glucoside **6** interfered with oxidative phosphorylation, as measured by oxygen consumption, similar to carboxyatractyloside (Lang et al., 2013) while the atractyligenin related derivatives **3**, **5** and **7** were inactive. Whether this ANT-inhibiting activity renders **6** a potentially hazardous substance is not clear yet. However, the finding that raw coffee contains compounds which show the same mode of action as a known phytotoxin prompted us to investigate raw coffee based dietary supplements for carboxyatractyligenin-derivatives.

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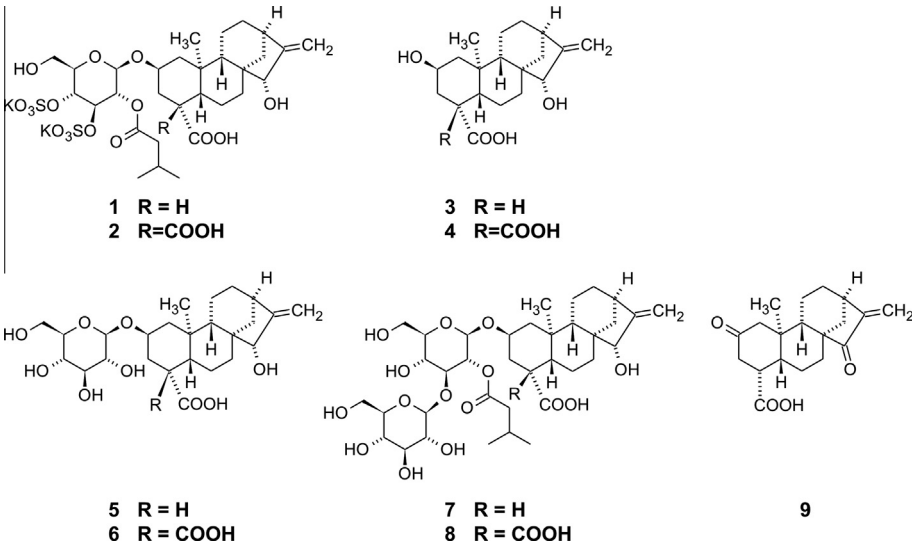

Fig. 1. Structures of the known phytotoxins atractyloside (**1**) and carboxyatractyloside (**2**), the structurally related compounds atractyligenin (**3**), carboxyatractyligenin (**4**), 2-O-β-glucopyranosyl-atractyligenin (**5**), 2-O-β-glucopyranosyl-carboxyatractyligenin (**6**), 3′-O-β-D-glucopyranosyl-2′-O-isovaleryl-2β-(2-desoxy-atractyligenin)-β-D-glucopyranoside (**7**) and 3′-O-β-D-glucopyranosyl-2′-O-isovaleryl-2β-(2-desoxy-carboxyatractyligenin)-β-D-glucopyranoside (**8**), and the internal standard 2,15-didehydro-atractyligenin (**9**).

The aims of the study were to optimize the procedures for isolation and toxicological characterization of pure carboxyatractyligenin compounds from raw coffee to serve as standard compounds. Further, raw coffee based dietary supplements were investigated for carboxyatractyligenin glycosides.

2. Materials and methods

All chemicals and enzymes used for isolation, purification or chemical synthesis were obtained from Sigma–Aldrich (Taufkirchen, Germany). Coffee samples (Arabica Brazil Santos) for isolation of the target compounds and dietary supplements were purchased via the internet (www.amazon.de).

*2.1. Isolation and purification of carboxyatractyligenin-derivatives **4**, **6** and **8** from coffee*

Elevated heat or ultrasonic treatment were avoided during the isolation procedure due to the susceptibility of carboxyatractyligenin-derivatives to decarboxylate and turn into the inactive atractyligenin derivatives. The following isolation procedure therefore is a modification of the procedure reported recently (cf. Lang et al., 2013).

Raw coffee beans (Arabica Brazil) were frozen in liquid nitrogen and powdered in a mill. The fines (100 g) were covered with aqueous methanol (70%, v/v, 300 mL) and stirred (room temperature, 48 h). Then methanol (100 mL) was added, the suspension mixed and filtered under vacuum. The solvent was reduced by rotary evaporation (25 °C bath temperature), the aqueous suspension diluted with water (~50 mL), filtered and separated by flash chromatography on RP18 (50 mm × 150 mm) with water (0.1% formic acid, eluent A) and acetonitrile (0.1% formic acid, eluent B). The injection volume was 20 mL. At a flow of 40 mL/min, solvent B was increased from 5% to 10% within 10 min, then to 40% within 30 min and finally to 100% within 5 min. Prior to the next injection, the column was re-equilibrated by rinsing with 100% B (5 min) and 5% B (10 min). The column effluent was monitored at 200 nm. Individual fractions (40 mL) were collected and each fraction screened by MS (ESI^-, loop injection, scan range m/z 115–1000) for compounds **5**–**8** (Fig. 1). Fractions 23–27 contained 2-O-β-D-glucopyranosyl-carboxyatractyligenin (**6**, m/z 525 $[M-H]^-$) and 2-O-β-D-glucopyranosyl-atractyligenin (**5**, m/z 481 $[M-H]^-$), fractions 30–32 contained 3′-O-β-D-glucopyranosyl-2′-O-isovaleryl-2β-(2-desoxy-carboxyatractyligenin)-β-D-glucopyranoside (**8**, m/z 771, $[M-H]^-$) and fractions 33–35 contained 3′-O-β-D-glucopyranosyl-2′-O-isovaleryl-2β-(2-desoxy-atractyligenin)-β-D-glucopyranoside (**7**, m/z 727, $[M-H]^-$). The MPLC-fractions were individually combined according to the results from the MS (ESI^-) screening. The solvent was reduced by rotary evaporation at 25 °C and subjected to preparative HPLC (RP18, Hyperclone, 250 × 21.4 mm, 5 µ, Phenomenex, Darmstadt, Germany) to isolate the pure compounds. Eluents were 0.1% aqueous formic acid (A) and acetonitrile (B) delivered at a flow of 20 mL/min. The effluent was monitored at 200 nm by means of a UV detector.

2.1.1. Isolation of target compounds from fraction 23–27

The eluent composition during chromatographic separation of the combined MPLC-fraction 23–27 was 15% B for 5 min followed by a linear gradient from 15–30% B within 30 min, then to 100% B within 3 min (3 min isocratic elution), re-establishment of the starting conditions within 2 min, re-equilibration (7 min). 2-O-β-D-glucopyranosyl-carboxyatractyligenin (**6**, Rt. 11.4 min) and 2-O-β-D-glucopyranosyl-atractyligenin (**5**, Rt. 13.8 min) were collected and dried by lyophilisation. The mass spectrometric (ToF, ESI^-) and nuclear resonance spectrometric (^1H, ^{13}C) data were in accordance with the data reported recently (Lang et al., 2013).

2.1.2. Isolation of target compounds from fraction 30–32

Using the same gradient given above for analysis of fractions 23–27, 3′-O-β-D-glucopyranosyl-2′-O-isovaleryl-2β-(2-desoxy-carboxyatractyligenin)-β-D-glucopyranoside (**8**, Rt. 14.2 min) was collected and dried by lyophilisation. The mass spectrometric (ToF, ESI^-) data were in accordance with the data reported recently (Lang et al., 2013). See Table 1 for NMR data of the intact molecule.

2.1.3. Isolation of target compounds from fraction 33–35

The eluent composition during chromatographic separation of the combined MPLC-fraction 33–35 was 20% B for 5 min followed by a linear gradient from 20–35% B within 30 min, then to 100% B within 3 min (3 min isocratic elution), re-establishment of the starting conditions within 2 min, re-equilibration (7 min). 3′-O-β-D-glucopyranosyl-2′-O-isovaleryl-2β-(2-desoxy-atractyligenin)-β-D-glucopyranoside (**7**, Rt. 21.8 min). The mass spectrometric (ToF, ESI^-) and nuclear resonance spectrometric (^1H, ^{13}C) data were in accordance with the data reported recently (Lang et al., 2013).

*2.2. Enzymatic liberation of carboxyatractyligenin (**4**) from 2-O-β-D-glucopyranosyl-carboxyatractyligenin (**6**) and purification*

Isolated 2-O-β-D-glucopyranosyl-carboxyatractyligenin (10 mg, ~19 µmol) was dissolved in sodium acetate buffer (5 mL, 3 g/L, pH adjusted to 4.8 with glacial acetic acid). After addition of β-glucuronidase (from *helix pomatia*, 150,000 units, 400 µL), the mixture was incubated whilst slow stirring (37 °C, 5 d). The solution was membrane-filtered (0.45 µm) and carboxyatractyligenin (**4**, Rt. 24.9 min, 3.4 mg 9 µmol, 47% yield) was isolated by preparative HPLC (RP18, Hyperclone, 250 × 21.4 mm, 5 µ, Phenomenex, Darmstadt, Germany). The fraction containing carboxyatractyligenin was collected and dried by lyophilization. Eluents were 0.1% aqueous formic acid (A) and acetonitrile (B) delivered at a flow of 20 mL/min. The effluent was monitored at 200 nm. The eluent composition during chromatographic separation was 95/5A/B (5 min isocratic after injection) to 55/45 A/B within 35 min.

Carboxyatractyligenin (**4**): UPLC-ToF-MS (ESI^-) m/z 363.1808 calculated for $C_{20}H_{27}O_6$ ($[M-H]^-$), found m/z 363.1806 (Δ – 0.2 mDa); UPLC-ToF-MS/MS (ESI^-) m/z 319.1909 calculated for $C_{19}H_{27}O_4$ ($[M-CO_2-H]^-$), found m/z 319.1909 (Δ ± 0.0 mDa). See Table 1 for NMR data.

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