Fitoterapia 117 (2017) 22–27

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Traditionally used medicinal plants against uncomplicated urinary tract infections: Hexadecyl coumaric acid ester from the rhizomes of *Agropyron repens* (L.) P. Beauv. with antiadhesive activity against uropathogenic *E. coli*

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ARTICLE INFO

Article history:
Received 21 November 2016
Received in revised form 20 December 2016
Accepted 26 December 2016
Available online 29 December 2016

Keywords:
Agropyron repens
Adhesion
Bladder cells
Coumaric acid ester
Uropathogenic *E. coli*

ABSTRACT

The rhizomes from *Agropyron repens* are traditionally used for the treatment of uncomplicated urinary tract infections. Extracts prepared with solvents of different polarity did not show any cytotoxic effects against different strains of uropathogenic *E. coli* (UPEC) and human T24 bladder cells under *in vitro* conditions. Significant antiadhesive activity against the bacterial attachment to human T24 bladder cells was found for an acetone extract (AAE) at concentrations >250 μg/mL. More hydrophilic extracts did not influence the bacterial attachment to the eukaryotic host cells. Bioassay guided fractionation of AAE led to the identification of (*E*)-hexadecyl-3-(4-hydroxyphenyl)-acrylate (hexadecyl-coumaric acid ester) **1** as the compound responsible for inhibiting the UPEC adhesion to T24 bladder cells. **1** reduced the bacterial invasion into the bladder cells as shown by a specific invasion assay. Additionally, **1** was obtained by chemical synthesis, and also the synthetic structural analogs **2** and **3** were tested for their potential antiadhesive activity, indicating that a shorter alkyl chain at the ester function as well as the lack of hydroxylation of the phenyl moiety will abolish the antiadhesive activity.

1. Introduction

Extracts from the rhizomes of *Agropyron repens* (L.) BEAUV. (couch grass, quackgrass, graminis rhizoma) are traditionally used in Europe for the treatment of uncomplicated urinary tract infections (UTI). This traditional use within the European Union is reflected by a recently published official community herbal monograph of the European Medicines Agency on *A. repens* rhizomes which supports the traditional use of aqueous extracts for adjuvant therapy in minor urinary complaints due to an increased urine flow [1]. On the other side critical review of published scientific literature does not unambiguously explain a distinct mode of action or prove clinical evidence.

Animal experiments in rats indicated diuretic activity of an aqueous extract after oral and intraperitoneal application [2]. Two uncontrolled clinical studies, with a low impact of evidence due to inadequate study design indicated positive effects of hydroalcoholic extracts from couch grass for cystitis, irritable bladder, prostatitis and urethritis [3] as well as for prostatic adenoma and cystitis [4] A recent *in vitro* investigation indicated absence of cytotoxic effects of hydroalcoholic extracts from couch grass against uropathogenic *E. coli* (UPEC), but pinpointed strong antiadhesive effects against the receptor-mediated recognition and adhesion of T24 bladder cells by UPEC [5].

From the phytochemical point of view couch grass rhizomes contain polysaccharides (mainly 3–8% of a inulin-like branched triticin) [6], quercetin and luteolin glycosides [7,8], phenolic glucosides, mainly the 5-glucosides of 5-hydroxyindole-3-acetic acid and 5-hydroxytryptophan [9,10], phenolic acids [11], about 0.02% of volatile oil [12], sugar alcohols [13], *p*-hydroxcycinnamic esters [14,15], and fatty acids.

UTIs are one of the most common infectious diseases, caused in 90% of all cases by uropathogenic *E. coli* (UPEC) [16]. Antibiotics are used as standard treatment for UTI but antibacterial resistance and high recurrence rates emphasize the importance to develop alternative preventive therapy strategies. Compounds interfering with the bacterial attachment of UPEC, *e.g.* FimH inhibitors [17], compounds interfering with the fimbriae assembly [18], probiotics against UPEC [19] or vaccination [20] are still under development. In this context the consumption of herbal medicinal products is widely used in patients with recurrent UTI [5].

Abbreviations: BCR, bacteria-cell ratio; FCPC, fast centrifugal partition chromatography; FITC, fluorescein isothiocyanat; UPEC, uropathogenic *E. coli*; UTI, uncomplicated urinary tract infections.

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http://dx.doi.org/10.1016/j.fitote.2016.12.010
0367-326X/

The following study aimed to investigate potential antiadhesive effects of couch grass in detail and pinpoint the relevant plant secondary compound, responsible for the inhibitory activity against UPEC.

2. Materials and methods

2.1. Materials and general experimentation procedures

If not stated otherwise, solvents, reagents and consumables were obtained from VWR International (Darmstadt, Germany) in analytical quality. Water was produced by a Millipore Simplicity 185 system (Schwalbach, Germany). Dried root material from *Agropyron repens*, batch 10,034,705, was obtained by Caeser & Loretz GmbH (Hilden, Germany). The material was identified by A.H. A voucher specimen of the material is retained in the archives of the Institute of Pharmaceutical Biology and Phytochemistry, Münster, Germany, under the designation IPBP 263.

2.2. Preparation of Agropyron repens extracts

Ten g of freshly pulverized plant material were suspended in 100 mL of different solvents (water, methanol, ethanol-water 1:1 (v/v), ethanol-water 7:3 (v/v), acetone) and were extracted 3× for 5 min by rotor-stator homogenizer (Ultra-Turrax®) on ice. The resulting suspension was centrifuged at 3000 ×*g* for 5 min. After removal of the organic solvents by rotary evaporation under vacuum the remaining aqueous residues were lyophilized and stored at −20 °C. Yield of the so obtained extracts (% (w/w), related to the dried plant material) and respective abbreviations used in the subsequent investigations:

Water extract (AWE) 15%, methanol extract (AME) 1.5%, ethanol-water 1:1 extract (AEE 50%) 16.1%, ethanol-water 9:1 (AEE 90%) 9%, acetone extract (AAE) 1.3%.

2.3. Liquid-liquid extraction of AAE

Ten g of AAE were dissolved in 100 mL of chloroform and extracted 3× with 50 mL water. The solvent of the organic phase was removed under vacuum to yield 3.3 g (43%, related to ACE) of the Agropyron Chloroform Extract (ACE).

2.4. Fractionation of ACE by fast centrifugal partition chromatography (FCPC)

Equipment: FCPC ascending mode (CPC-Kromaton, Kromaton Technologies, Angers, France) with Knauer HPLC pump (Knauer GmbH, Berlin, Germany). Stationary (lower) phase: methanol; mobile (upper) phase: heptan. Flow rate: 0.8 mL/min, 900 rpm, ascending mode. Sample preparation: 2 g of ACE were dissolved in 750 mL of the upper phase; the solution was filtered (polypropylene filters, 0.45 μm, VWR, Germany) and injected into FCPC. After 150 mL eluate the run was stopped and the stationary phase was pumped out. After removal of the organic solvent by rotary evaporation at a 40 °C not exceeding temperature, followed by lyophilization, six different fractions (ACE_1 to ACE_6) were obtained. The respective yields (%, w/w, related to the amount of ACE used for FCPC) were as follows: ACE_1: 12%, ACE_2: 15.5%, ACE_3: 23.3% (corresponding to 0.1%, related to the herbal material), ACE_4: 5.8%, ACE_5: 6%, ACE_6: 12.3%.

2.5. UHPLC/+ESI-qTOF-MS

ACE, dissolved in cyclohexane (5 mg/mL) was separated on a Dionex Ultimate 3000 RS Liquid Chromatography System over a Dionex Acclaim® RSLC 120, C18 column (2.1 × 100 mm, 2.2 μm) with a binary gradient (A: water with 0.1% formic acic; B: acetonitrile with 0.1% formic acid) at 0.8 mL/min. 0 to 9.5 min: linear from 5% to 100% B; 9.5 to 12.5 min: isocratic at 100% B; 12.5 to 12.6 min: linear from 100% to 5% B; 12.6 to 15.0 min: isocratic at 5% B. The injection volume was 2 μL. Eluted compounds were detected using a Dionex Ultimate DAD-3000 RS over a wavelength range of 200–400 nm and a Bruker Daltonics micrOTOF-QII time-of-flight mass spectrometer equipped with an Apollo electrospray ionization source in positive mode at 2 Hz over a mass range of *m/z* 50–1500 using the following instrument settings: nebulizer gas nitrogen, 4 bar; dry gas nitrogen, 9 L/min, 200 °C; capillary voltage, −4500 V; end plate offset, −500 V; transfer time, 100 μs; prepulse storage, 6 μs; collision energy, 8 eV. MS/MS scans were triggered by AutoMS2 settings within a range of *m/z* 200–1500, using a collision energy of 40 eV and a collision cell RF of 130 Vpp. Internal data set calibration (HPC mode) was performed for each analysis using the mass spectrum of a 10 mM solution of sodium formate in 50% isopropanol that was infused during LC re-equilibration using a divert valve equipped with a 20 μL sample loop. Data were processed with DataAnalysis 4.0 SP5 (Bruker Daltonics) using an in-house VBA script analyzing Full-MS Dissect spectra and exporting the resulting exact monoisotopic masses as well as MS/MS Peak Lists. KNApSAcK and METLIN databases were used to identify the dissected compounds of ACE based on their monoisotopic masses and characteristic fragments [21,22]

2.6. General experimental procedure for the preparation of esters of trans-cinnamic acid and p-coumaric acid

A solution (25 mL) of equimolar quantities of *trans*-cinnamic acid (Fluka, Switzerland) (0.01 mol/L), or *p*-coumaric acid (Sigma, Germany) (0.01 mol/L), cetyl alcohol (Merck, Germany) (0.01 mol/L) or 1-octanol (Merck, Germany) (0.01 mol/L) and *p*-toluenesulfonic acid (Merck, Germany) (0.001 mol/L) as a catalyst, in toluene was refluxed for 6 h and the azeotrope was collected in a Dean–Stark trap. After completion of the reaction (monitored by TLC, stationary phase: silica gel G60 F_{254}, mobile phase: acetone/cyclohexane (1:9 v/v), detection by anisaldehyde/sulfuric acid reagent), the mixture was cooled to room temperature and 2 g of solid sodium bicarbonate were added. After stirring the mixture for another 30 min at room temperature, the suspension was filtered and the resulting filtrate was concentrated under reduced pressure. The resulted crude products were purified by column chromatography (column dimensions 50 mm × 61 cm) on silica gel 60 (Merck Millipore, Germany) by using a solvent system of an acetone/cyclohexane (1:9, *v*/v) mixture.

NMR spectra were recorded on an Agilent DD2 400 MHz spectrometer (Agilent Technologies, Santa Clara, CA, USA). Samples were dissolved in chloroform-d^1 (purity >99.8%, δ = 7.26 ppm) and the respective solvent peak was set as reference.

2.7. (E)-Hexadecyl-3-(4-hydroxyphenyl)-acrylate (hexadecyl-coumaric acid ester) **1**

White solid; yield 90%; ESI-MS *m/z* found 389.3028 (calc. 389.3028), $C_{25}H_{40}O_3$ $[M + H]^+$; ^{1}H NMR (400 MHz, CDCl3): δ 7.62 (d, 1H, J = 15.9 Hz), δ 7.43 (d, 2H, J = 8.2 Hz), δ 6.84 (d, 2H, J = 8.2 Hz), δ 6.30 (d, 1H, J = 16.0 Hz), δ 5.47 (s, 1H), δ 4.19 (t, 2H, J = 6.7 Hz), δ 1.66 (m, 4H), δ 1.38 (m, 22H), δ 0.88 (t, 3H, J = 6.8 Hz). See also [23].

2.8. (E)-Octyl-3-(4-hydroxyphenyl)-acrylate (octyl-coumaric acid ester) **2**

Bright yellow semi-oil; yield 95%; ESI-MS *m/z* found 277.1986 (calc. 277.1725), $C_{17}H_{24}O_3$ $[M + H]^+$; ^{1}H NMR (400 MHz, CDCl3): δ 7.62 (d, 1H, J = 15.9 Hz), δ 7.43 (d, 2H, J = 8.3 Hz), δ 6.85 (d, 2H, J = 8.3 Hz), δ 6.30 (d, 1H, J = 15.9 Hz), δ 5.50 (br s 1H), δ 4.219 (t, 2H, J = 6.7 Hz), δ 1.70 (m, 2H), δ 1.27 (m, 10H), δ 0.87 (t, 3H, J = 6.6 Hz); see also [23].