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1 Research Article

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3 # *Antiadhesive hydroalcoholic extract from* ***Apium graveolens*** **seeds prevents bladder and**
4 **kidney infection against uropathogenic *E. coli***

5

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21 **Keywords**: *Apium graveolens*, adhesion, bladder cells, celery seeds, uropathogenic *E. coli.*

22

23 **Abbreviations:** AHL: acetylated homoserine lactone; BCR: Bacteria-cell ratio; CSE: Celery
24 seed extract; FACS: fluorescence-activated cell sorting, flow cytometry; FimH: type 1 fimbriae,
25 mannose sensitive; q.s.: *quorum sensing*; UHPLC: ultrahigh pressure liquid chromatography;
26 UPEC: uropathogenic *E. coli*; UTI: uncomplicated urinary tract infections.

27

28 **Abstract**

29 *Ethnopharmacological relevance:* The seeds from *Apium graveolens* are used traditionally in
30 Persian and European medicine for the treatment of uncomplicated urinary tract infections. No
31 data are available on the influence of Celery extract on the interplay between uropathogenic *E.*
32 *coli* and the eukaryotic host cells and on the *quorum sensing* of the bacteria.

33 *Aim of the study:* The aim of the present study was to characterize a potential antiadhesive and
34 *anti quorum sensing* effect of a phytochemically characterized Celery extract by specific *in*
35 *vitro* assays and to correlate these effects with *in vivo* data obtained from an animal infection
36 model.

37 *Materials and methods*: Hydroalcoholic extract CSE (EtOH-water, 1:1) from *A. graveolens*
38 seeds was characterized by UHPLC/+ESI-QTOF-MS and investigated on antiproliferative
39 activity against uropathogenic *E. coli* (strain NU14) and human T24 bladder cells. Antiadhesive
40 properties of CSE were investigated within two different *in vitro* adhesion assays (microplate
41 fluorescence assay and flow cytometric adhesion assay). For *in vivo* studies BALB/c mice were
42 used in an UPEC infection model. The effect of CSE on bacterial load in bladder tissue was
43 monitored within a 4- and 7 days pretreatment (200 and 500 mg/kg) of the animals.

44 *Results*: CSE was dominated by the presence of luteolin-glycosides and related flavon
45 derivatives besides furocoumarins. CSE had no cytotoxic effects against UPEC strain NU14
46 and against T24 bladder cells within the tested concentration range (0.1 to 1 mg/mL). CSE
47 exerts a dose dependent antiadhesive activity against UPEC strains NU14 and UTI89. CSE
48 inhibited in a concentration-dependent manner bacterial *quorum sensing*. 4 - and 7-day
49 pretreatment of animals with CSE (200 and 500 mg/kg/day),transurethrally infected with UPEC
50 NU14, significantly reduced the bacterial load in bladder tissue.

51 *Conclusion*: CSE is assessed as a strong antiadhesive plant extract for which the traditional use
52 in phytotherapy for UTI might be justified.

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1. Introduction

Extracts from the seed of *Apium graveolens* L. (*Fructus Apii graveolens*, Celery seeds) are used in traditional Persian medicine (Bahmani et al., 2016) but also in Europe for the treatment of uncomplicated urinary tract infections (UTI) (Blaschek (2016). From the phytochemical point of view Celery seeds contain about 2 to 3% volatile oil with R(+)-limonen (about 60%), β-selinen (up to 13%) and phtalids (20-30 %) as main components. Further on the seeds contain a complex mixture of phtalidglycosides, about 0.2% of furocoumarins and a complex mixture of flavonoids and flavonoid glycosides. The presence of lignanglucosides, phenolglycosides and a high amount of triglycerides with about 40-60% of petroselinic acid has been described (for review see Blaschek, 2016).

Preclinical *in vivo* data or clinical studies on positive effects of celery seed extracts for UTI have not been published until now, but reports on potential antimicrobial effects under *in vitro* conditions are available (Zhou et al., 2009; Shanmugapriya and Ushadevis 2014). Within the last years investigations of new drug candidates with antiadhesive properties against uropathogenic *E. coli* (UPEC) have been described, indicating that the blocking of bacterial outer membrane adhesins (especially FimH) effectively prevents infection of eukaryotic host cells by the pathogen (Beydokthi et al., 2017a; Beydokthi et al., 2017b; Rafsanjany et al., 2015a, Rafsanjany et al., 2015b, Kleeb et al., 2016).

From this point of view the present study aims to investigate potential antimicrobial and antiadhesive effects of Celery seed extract in detail under *in vitro* and *in vivo* conditions and to characterize the respective extract by LC-MS methodology concerning its phytochemical composition.

Materials and Methods

General experimentation procedures

If not stated otherwise, solvents, reagents, and consumables were obtained from VWR International (Darmstadt, Germany). All solvents and reagents were of analytical quality. Water was produced by a Millipore®Simplicity 185 system (Schwalbach, Germany). Dried seeds from *A. graveolens* was obtained from a local medicinal plant market (Teheran, Iran). The material was identified by S.B. and A.H. A voucher specimen of the material is retained in the archives

87 of Institute of Pharmaceutical Biology and Phytochemsitry, Münster, Germany under the
88 designation IPBP 407.

89 *Preparation of hydroalcoholic Celery seed extract (CSE)*

90 Ten grams of freshly powdered plant material were extracted with 100 mL of ethanol-water
91 (1:1 v/v) for 15 min (3 × 5 min) under ice-cooling by rotor-stator extractor (Ultraturrax®) at
92 maximum rotor speed. After centrifugation of the resulting suspension (5.000 × g, 15 min) the
93 clear supernatant was concentrated by rotary evaporator, follCSEd by lyophilization to yield
94 2.0 g of dry extract (herbal material : extract ratio = 5:1), which was termed in the following as
95 Celery seed extract CSE. CSE was stored at -20°C in sealed containers under vacuum.

96 *LC-MS dereplication of CES*

97 For the preparation of LC-MS samples CSE was dissolved in methanol to a concentration of
98 10 mg/mL. Separation was performed on a Dionex Ultimate 3000 RS Liquid Chromatography
99 System over a Dionex Acclaim RSLC 120, C18 column (2.1 × 100 mm, 2.2 µm) with a binary
100 gradient (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid) at 0.4 mL/min.
101 0 to 5 min: isocratic at 5% B; 5 to 37 min: linear from 5 to 100% B; 37 to 47 min: isocratic at
102 100% B; 47 to 48 min: linear from 100 to 5% B; 48 to 55 min: isocratic at 5% B. The injection
103 volume was 2 µL. Eluted compounds were detected using a Dionex Ultimate DAD-3000 RS
104 over a wavelength range of 200-800 nm and a Bruker Daltonics micrOTOF-QII time-of-flight
105 mass spectrometer equipped with an Apollo electrospray ionization source in positive mode at
106 2 Hz over a mass range of *m/z* 50-1500 using the following instrument settings: nebulizer gas
107 nitrogen, 4 bar; dry gas nitrogen, 9 L/min, 200°C; capillary voltage -4500 V; end plate offset -
108 500 V; transfer time 100 µs, prepulse storage 6 µs, collision energy 8 eV. MS/MS scans were
109 triggered by AutoMS2 settings within a range of *m/z* 200-1500, using a collision energy of
110 40 eV and and collision cell RF of 130 Vpp.. Internal dataset calibration (HPC mode) was
111 performed for each analysis using the mass spectrum of a 10 mM solution of sodium formate
112 in 50% isopropanol that was infused during LC reequilibration using a divert valve equipped
113 with a 20 µL sample loop.

114 *Uropathogenic E. coli (UPEC) strains and growth conditions*

115 Bacterial strains: UPEC strains NU14 and UTI89 (both clinical isolates obtained from cystitis
116 patient and a patient with an acute bladder infection respectively) (Chen et al., 2006), UPEC
117 pyelonephritis isolate CFT073(O6:K2:H1) (Guyer et al., 1998), *E. coli* 2980 (DSM 10791)

118 (Hacker and Ott, 1986) were used in this study. Bacteria from the second passage were used for
119 all tests and cultivated from frozen stocks.

120 Agar grown bacteria were harvested and used for inoculation of Loeb agar, supplemented with
121 0.2 % $CaCl_2$ which is supposed to increase the type 1 fimbria expression (Connel et al., 1996).
122 For liquid culture one colony of agar grown bacteria was transferred to 10 mL of LB medium
123 or pooled human urine supplemented with 5% of LB medium. The cultures were incubated in
124 50 mL Falcon tubes under shaking (200 rpm/37°C) for 17 h.

125 *Cell culture*

126 T24 cell line (ATCC HTB-4), derived from human urinary bladder carcinoma (Bubenik et al.,
127 1973) and known to be suitable for adhesion and invasion *in vitro* assays with UPEC (Miyazaki
128 et al., 2002) were kindly provided by Prof. Straube (University of Jena, Germany). Cells were
129 cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose with L-glutamine)
130 (Merck Millipore, Darmstadt, Germany), supplemented with 10 % (v/v) FCS (Merck Millipore,
131 Darmstadt, Germany) and 1 % penicillin/streptomycin (Merck Millipore, Darmstadt,
132 Germany) at 5 % CO_2 / 37 °C. Passaging took place at 80 to 90% confluence of T24 cells.

133 A498 cell line (ATCC HTB-41), derived from human kidney carcinoma (Fogh, 1978) was
134 kindly provided by Dr. Carina Hillgruber (Klinik für Hautkrankheiten, Münster, Germany).
135 Cells were cultured in Eagle's Minimum Essential Medium (Merck Millipore, Darmstadt,
136 Germany), supplemented with 10 % (v/v) heat-inactivated FCS (Merck Millipore, Darmstadt,
137 Germany), and 1 % penicillin/streptomycin (Merck Millipore, Darmstadt, Germany) at 5 %
138 CO_2 / 37 °C. Passaging took place at 80 to 90% confluence of A498 cells.

139 *Agar diffusion assay* (*E. coli* strains 2980 and NU14)

140 Agar grown bacteria were harvested, suspended in sterile PBS and adjusted to an OD_{640} nm of
141 0.2. 100 µL of the bacterial suspension were transferred to a Loeb agar plate and spread
142 homogenously. Subsequently, five holes were provided at equal intervals on the agar plate with
143 sterile Pasteur pipets. 100 µL of CSE at different concentrations (500 to 2000 µg/mL in water,
144 sterile filtered, 0.22 µm) were pipetted into the wells. Norfloxacin (50 µg/mL) (Fluka, Buchs,
145 Switzerland) served as positive control. Plates were incubated for 24 h / 37°C.

146 *Determination of cell viability (MTT assay)*

147 To examine influence of CSE on the cell viability and to exclude cytotoxic effects of CSE
148 towards T24 bladder and A498 kidney cells, the MTT assay was performed (Mosmann, 1983).
149 T24 cells were seeded into 96-well plates with 2.5×10^4 cells per well (100 µL), incubated for
150 24 h at 37°C with 5 % CO_2 and washed with 200 µL/well of PBS. Incubation of the cells with
151 100 µL of CSE at different concentrations (500 to 2000 µg/mL in water, sterile filtered, 0.22
152 µm) was performed for 24 h at 37°C / 5 % CO_2. The supernatant was removed and cells were
153 washed twice with PBS (200 µL/well). 50 µL of MTT reagent were added to each well and
154 after an incubation period of 24 hours at 37°C / 5 % CO_2, crystals of the blue formazan product
155 were dissolved in 50 µL of DMSO and the absorbance was determined in a microplate reader
156 (λ = 492/690 nm).

157 *Adhesion assay by microplate assay*

158 T24 cells (5×10^4 cells/well) were cultured in a black 96-well plate (Costar®) and incubated for
159 24 h to reach the 90 % confluency. The medium was removed and the cells were washed once
160 with DMEM. The suspension containing FITC-labeled bacteria was adjusted to an OD_{640} nm
161 of 4.0/mL and 100 µL of this suspension was mixed with 900 µL of test solution or DMEM as
162 an untreated control. Finally, 100 mL of each mixture were added to each well. After 1 h of
163 incubation the cells were washed two times with 200 µL PBS to dispose unattached bacteria.
164 The fluorescence was measured by a fluorescence reader at $\lambda_{ex/em}$ = 485/538 nm. The relative
165 bacterial adhesion was calculated by the following equation.

166 *Adhesion assay by quantitative flow cytometry* (Rafsanjany et al., 2015a; Rafsanjany et al.,
167 2015b).

168 T24 bladder cells and A498 kidney cells (2.5×10^5 cells/well) were seeded into 6-well plates
169 and incubated at 37°C / 5 % CO_2 for 24 h until 90 % confluence was reached. After incubation
170 T24 cell culture medium was removed, cells were washed once with PBS, and 2 mL DMEM
171 were added to each well 2 h prior to the assay.

172 10 mL of sterile urine, containing 5 % of UPEC LB medium and 2 mg/mL CSE were inoculated
173 with one CFU of agar grown bacteria and incubated in a rotary shaker (200 rpm/37°C). Pooled
174 urine supplied with 5% of UPEC liquid medium inoculated with one CFU of agar grown
175 bacteria served as untreated control.

176 Bacteria were centrifuged after overnight incubation (~15 h) at 7.000 × g for 10 min, washed
177 once with PBS, and the suspension was adjusted to an OD_{640nm} of 0.6 in saline solution for

178 FITC-labeling. FITC-labeling of UPEC and flow cytometric adhesion assay was performed as
179 described by Rafsanjany et al. (2013a) and Messing et al. (2014). All further steps with FITC-
180 labeled *E. coli* (OD_{640} 0.4/mL) were carried out under light protection. After fluorescence-
181 labeling the density of bacteria were adjusted to an OD_{640} of 0.4/mL in DMEM. Bacteria were
182 added to T24 cells in 6-well plates and incubated for 2 h at 37°C. Finally, bacterial adhesion
183 was quantified by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg,Germany).
184 Instrument settings were as follows: FCS (Detector): E-1 (Voltage), 3.0 (AmpGain), Lin
185 (Mode); SSC: 352, 1.00, Lin; FL1: 360, 1.00, Log.

186 *Quorum sensing inhibition activity*

187 To examine the *quorum sensing* inhibition activity of CSE a transformed *E. coli* biosensor
188 reporter strain was used as reported in a recent study (Vila-Sanjurjo (submitted). *E. coli* strain
189 Top10 was transformed by use of plasmid pSB1A3 - BBa_T9002, carrying the BBa_T9002
190 genetic device (Registry of Standard Biological Parts: http://parts.igem.org/Part:BBa_T9002)
191 ligated into vector pSB1A3, introduced by chemical transformation and stored as a 30%
192 glycerol stock at –80ºC. The sequence BBa_T9002, comprised the transcription factor (*LuxR*),
193 which is constitutively expressed but it is active only in the presence of the exogenous cell-cell
194 signaling molecule $3OC_6HSL$. At an adequate concentration, two molecules of $3OC_6HSL$ bind
195 to two molecules of LuxR and activate the expression of GFP (output), under the control of the
196 *lux* pR promoter from *Vibrio fischeri*. *Growth media and conditions*: Bacterial strains were
197 cultivated on LB7 and M9 minimal medium (Becton, Dickinson, Heidelberg, Germany). 10 mL
198 of LB broth, supplemented with 200 µg/mL ampicillin were inoculated with one colony from a
199 freshly streaked plate of Top10 containing BBa_T9002 and incubated for 18 h at 37ºC under
200 shaking at 100 rpm. Each culture was then diluted 1:1000 into 20 mL M9 minimal medium
201 supplemented with 0.2% casamino acids and 1 mM thiamine hydrochloride plus 200 µg/mL
202 ampicillin (AppliChem, Germany). The culture was maintained under the same conditions until
203 the OD_{600} reached 0.15 (~ 5 h). Then, 500 µL of overnight culture were mixed with 500 µL of
204 30% sterile glycerol and stored at -80 °C. Prior to the biosensor assay, 40 µL of the overnight
205 culture were cultivated in 20 mL M9 medium in the presence of 200 µg/mL ampicillin. Once
206 the culture OD_{600} reached OD 0.04 to 0.07 (~ 4 h), it was considered as ready for the biosensor
207 assay.

208 *E. coli* Top10 biosensor assay. The $3OC_6HSL$ was dissolved in acetonitrile to a stock
209 concentration of 100 mM and stored at –20ºC until usage. Prior to each experiment serial
210 dilutions from the stock solution were prepared in water to produce solutions with a

211 concentration ranging from 10 nM to 100 mM. 10 µL of $3OC_6HSL$ solution were mixed with
212 10 µL of CSE (1, 5, 10 and 20 mg/mL) in the wells of a flat-bottomed 96-well plate (Greiner
213 Bio-One, Germany), and each well was then filled with 180 µL aliquots of the bacterial culture
214 to test for QS inhibition activity. Several controls were also included. Blank 1 contained 180
215 µL of M9 medium and 20 µL of MilliQ water to measure the absorbance background. Blank 2
216 contained 180 µL of bacterial culture and 20 µL of MilliQ water, thus allowing measuring the
217 absorbance of background corrected for the cells. Finally, the positive control wells contained
218 10 µL of water plus 10 µL $3OC_6HSL$ solution and 180 µL of the bacterial culture to measure
219 of the fluorescence background. In order to allow for the effect of CSE themselves on OD_{600}
220 and fluorescence, 10 µL of $3OC_6HSL$ solution were mixed with 10 µL of CSE in the wells and
221 each well was then filled with 180 µL M9 medium, and it was measured in identical conditions
222 as for the rest of the assays.

223 The plates were incubated in a Safire Tecan-F129013 Microplate Reader (Tecan, Crailsheim,
224 Germany) at 37 ºC and fluorescence measurements were registered automatically using a
225 repeating procedure ($\lambda_{ex/em}$ = 480/510 nm, 40 µs, 10 flashes, gain 100, top fluorescence),
226 absorbance measurements (OD_{600}) (λ = 600 nm absorbance filter, 10 flashes) and shaking (5 s,
227 orbital shaking, high speed). The interval between the measurements was 6 min. For each
228 experiment, the fluorescence intensity (FI) and OD_{600} data were corrected by subtracting the
229 values of absorbance and fluorescence backgrounds and expressed as the average for each
230 treatment. All measurements consisted in a minimum of three biological replicates.

231

232 *In vivo infection experiments: Mice infection model*

233 The study was performed at Pasteur Institute of Iran according the international guidelines for
234 animal studies and instructions of the local ethical committee (allowance number
235 IR.PII.REC.1394.80). Female BALB/c mice, 20 to 22 g and aged 6-8 weeks were obtained from
236 the breeding station of, Pasteur Institute, Karaj, Iran, and were maintained on a 12 h light/dark
237 cycle, at constant temperature at 21°C and free access to water and standard dietary food.
238 Treatment of animals: application of test/control solutions was performed by intragastric
239 application of 500 µL of the respective test solutions (untreated control: water; treated groups:
240 CSE at different concentrations). Gavage was performed using a 20 gauge-feeding needle
241 1465LL (Acufirm Ernst Kratz, Dreieich, Germany). The test extract CSE was dissolved in
242 distilled water for preparation of the test solution.

243 Animals were randomly distributed into different treatment groups:

244 Pretreatment of animals for 4 resp. 7 days prior to infection of the animals with UPEC strain
245 NU14: CSE was used at two different doses of 500 and 750 mg/kg body weight/day from day
246 -4 and -7, prior to infection of the animals with UPEC NU14 at day 0. Mice were infected 6 h

247 The infection of the animal was performed according to the method described by Lane et al.,
248 2005). Animals were anesthetized with ketamine/xylazine (70 mg/kg + 5 mg/kg) (Alfasan,
249 Netherland) and inoculated transurethrally with 20 µL of the respective UPEC suspension,
250 containing either 2 × 10^8 CFU NU14 or 1 × 10^8 CFU CFT073 per mouse by using a sterile
251 polyethylene catheter PE-10 (inner diameter 0.28 mm; outer diameter 0.61 mm) (Warner
252 Instruments, U.S.A.) After the experiments mice were sacrificed by means of cervical
253 dislocation. The bladders were aseptically removed and homogenized in 1.0 mL of PBS in a
254 sterile 1.5 mL Micro Tube by using either a G50 Tissue Grinder (Coyote Bioscience Inc.
255 Columbia, MD, U.S.A.) at a grinding speed of 8.000 rpm for 10 sec for 3 consecutive times or
256 2 mL Dounce Tissue Grinder™ (B. Braun Biotech, International).

257 The resulting suspension was spiral-plated onto plain LB agar plates, follCSEd by incubation
258 for 24 h at 37°C. CFU per mL homogenate and per animal tissue were counted and quantified
259 for each tissue of each animal.

260 A group size of n = 6 mice was used for untreated controls (UC) and CSE treated groups.

261 *Statistical analysis*

262 One-way ANOVA, Student's t-test, and the Tukey HSD test were used to compare the
263 differences between the mean values of the groups. The results of infection experiments were
264 also analyzed by Kruskal–Wallis test with Dunn's multiple comparisons, using GraphPad Prism
265 software. $p < 0.05$ of all results was considered as significant.

266

267 **3. Results and Discussion**

268 Dried seed from *A. graveolens* were extracted with ethanol-water (1:1, v/v). Lyophilisation
269 yielded the dry extract CSE (20% w/w, related to the dried seed material). UPLC/+ESI-QTOF-
270 MS analysis with high-resolution MS was used to obtain detailed information on the
271 phytochemical composition of CSE. MS data were processed with DataAnalysis 4.1 SP5 using
272 an inhouse-VBA script to dissect compound peaks and monoisotopic masses were determined.
273 Among the dissected compounds, 10 peaks were selected for further structural investigations
274 due to their high intensity (Fig. 1). Compounds with similar and known monoisotopic mass
275 found for *A. graveolens* in KNApSAcK and REAXYS® database were correlated to the
276 respective peaks. The respective fragmentation pattern of the related compounds was compared
277 with METLIN, MassBank and MetFrag database and ultimately a list of 6 tentative compounds
278 was generated (Table 1 and Fig. 2). From this analytical profiling the presence of luteolin
279 glycosides (**1**, **2**, **3**) and related derivatives (**4**, **5**) besides the furocoumarin **8** got obvious.

280 For functional testing of CSE the influence of the extract on cell viability of UPEC (strain
281 NU14) and human T24 bladder cells was investigated. The influence of CSE on mitochondrial
282 activity of T24 cells was determined over a 24 h incubation interval by MTT assay (Mosmann,
283 1983). The respective data indicated that high concentrations (0.5 and 1 mg) of CSE caused a
284 slight reduction of T24 cell viability while lower concentrations had no significant influence on
285 mitochondrial activity (Fig. 3). Shorter incubation intervals of the bladder cells with the test
286 extract (4 h) did not result in any changes in cell vitality (data not shown). From these results
287 CSE is assessed to have no influence on the proliferation of UPEC over a 24 h incubation time
288 in the concentration range from 0.2 to 2 mg/mL (data not shown).

289 Subsequently *in vitro* antiadhesion assays were performed with CSE to investigate potential
290 blocking effects against bacterial adhesion to T24 bladder cells. The principle of this bioassay
291 is based on the fluorescence labeling of *E. coli*, strain NU14 with fluorescein isothiocyanate.
292 The FITC-labeled bacteria were coincubated together with CSE and T24 bladder cells for 2 h.
293 Non-adherent bacteria were washed off from the cell monolayer and the fluorescence intensity
294 of the cells was directly quantified within a microwell plate by fluorescence reader. Mannose
295 (2.7 mmol) served as positive control, leading to a significant reduction of the bacterial
296 adherence (Fig. 4A). CSE exert a concentration-dependent inhibition of the bacterial adhesion
297 with significant reduction between 30 und 40% at concentrations > 0.1 mg/mL (Fig. 4A).

298 The antiadhesive effect of CSE was additionally confirmed by a flow cytometric assay using
299 UPEC strains NU14 and UTI89. In principle FITC-labeled bacteria were coincubated with CSE
300 and T24 bladder cells for 2 h, non-adherent bacteria are washed off from the cell monolayer
301 which was subsequently trypsinzed and the fluorescence intensity of the individual cells was
302 determined by flow cytometry (Rafsanjany et al., 2015a; Rafsanjany et al., 2015b). As shown
303 in Fig. 4B CSE at 0.5 mg/mL exerts a significant reduction in bacterial adhesion of both NU14
304 and UTI89 strains with NU14 being more sensitive compared to UTI89.

305 As CSE strongly influences UPEC adhesion it was worth to investigate also the influence of
306 the extract on bacterial cell-cell communication by *quorum sensing*, which is mediated by small
307 molecular signals that are termed autoinducers (Waters and Bassler, 2005). *Quorum sensing*
308 regulates the gene expression in response to the changes of cell-population density and controls
309 many physiological activities, including virulence factor production, motility and biofilm
310 formation (Miller and Bassler, 2001). To investigate a potential influence of CSE on *quorum*
311 *sensing* an *E. coli* Top 10 reporter biosensor strain was used. This strain constitutively expresses
312 the LuxR receptor which is only active in the presence of the exogenous cell-cell signaling
313 molecule $3OC_6HSL$. At an adequate concentration, two molecules of $3OC_6HSL$ will bind to
314 two molecules of LuxR and activate the expression of GFP (output), under the control of the
315 *lux* pR promoter from *Vibrio fischeri*. As shown in Fig. 5 a concentration-dependent inhibition
316 of *quorum sensing* was observed for CSE-treated *E. coli*, which was evidenced as the reduction
317 on bacterial fluorescence intensity. At the same time, CSE had no effect on bacterial
318 proliferation. Therefore these results demonstrate that CSE has a strong inhibition activity of
319 *quorum sensing* activity regulated by AHL.

320 From these data it can be concluded that hydroalcoholic Celery seed extract has an antiadhesive
321 and anti *quorum sensing* potential against UPEC under *in vitro* conditions.

322 To correlate these *in vitro* data with a potential *in vivo* activity an UPEC infection model in
323 mice was used. In principle, female BALB/c mice were orally pretreated for 4 and 7 days with
324 CSE (200 and 500 mg/kg/day). 6 to 8 hours after the last application of CSE animals were
325 infected transurethrally with 2×10^8 CFU of UPEC strain NU14, known to interact with bladder
326 tissue. 24 h post infection animals were sacrificed, the bladder tissue was removed,
327 homogenized and the bacterial load in the tissue was quantified after cultivation of serial
328 dilutions on LB agar.

329 As displayed in Fig. 6 the 4-day pretreatment evoked a strong reduction in bacterial load in 330 bladder tissue from about 10^7 to about 10^5 colonies per bladder both treated groups. 331 Prolongation of the pretreatment interval from 4 to 7 days increased CSE efficiency 332 approximately by 40% related to the 4-days treatment with the same test concentration (200 333 mg/mL). Applying a 7-day pretreatment the number of colonies in bladder tissue were reduced 334 almost 3 logarithmic steps (10^3 CFU) in comparison to the untreated control (10^6 CFU). It was 335 not possible to use a standard antibiotic treatment in this protocol as positive control as a 4 day 336 pretreatment of the animals with norfloxacin (100 mg/kg) did not have any influence on the 337 bacterial load (probably due to its fast elimination rate) and could therefore not be used as 338 positive control (data not shown). Instead the suitability of the test system was validated by use 339 of a 4 day posttreatment with norfloxacin (100 mg/kg) which significantly reduced bacterial 340 load (data not shown).

341 From these data it can be concluded that pretreatment of the mice with CSE prior to infection 342 can significantly reduce infection severity of UPEC in mice significantly and could be used for 343 prevention of potential infection to reduce recurrence rate. This finding is assessed to be in good 344 congruence with the observed antiadhesive effects from the *in vitro* experiments and indicates 345 that the ascending infection of UPEC along the urogenital epithelia is strongly reduced by CSE, 346 probably due to the antiadhesive effects of the extract.

347

348 **Conclusion**

349 From these points of view the antiadhesive potential of extracts obtained from *A. graveolens* 350 rationalizes and validates the traditional use of this herbal material for UTI. Further *in vivo* 351 studies and clinical investigations have to be performed for establishing high quality and 352 registrated phytopharmaceuticals based on this herbal material.

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Legends to Figures

Figure 1: UPLC/+ESI-QTOF-MS chromatogram of CSE. Dissect compounds overlaid with the base peak chromatogram (black line) represent the elution profiles of tentatively identified compounds 1 to 10.

Figure 2: Structural features of tentatively identified major compounds from CSE

Figure 3: Influence of CSE at concentrations of 0.1 to 1 mg/mL on cell viability (MTT assay) of T24 bladder cells after 24 h incubation time with the test extract. UC: untreated control; PCX: positive control (10% FCS supplemented media). Values represent the mean ± SD from 3 independent experiments with 6 technical replicates. * : $p < 0.05$.

Figure 4: **A** Influence of different concentrations of CSE (0.1 to 1 mg/mL) on the relative adhesion of FITC-labeled *E. coli*, strain NU14 to T24 bladder cells after 60 min of coincubation as quantified by microplate fluorescence assay. Data indicate the adhesion related to the untreated control (UC = 100%). ** $p < 0.01$. *** $p < 0.001$. PC: positive control, D-mannose 2.77 mmol. Values represent the mean ± SD of 3 independent experiments with 6 technical replicates.

B: Comparison of the influence of CSE (0.5 mg/mL) on the relative adhesion of FITC-labeled UPEC (strains NU14 and UTI89) to T24 bladder cells after 1 h coincubation as quantified by flow cytometric assay. Values represent the mean ± SD of 3 independent experiments with 3 technical replicates.** $p < 0.01$. *** $p < 0.001$. PC: untreated control.

Figure 5: Effect of CSE on the relative *quorum sensing* response of *E. coli* in relation to the untreated control (UC). Data represent the mean and ± SD of three independent experiments with three biological replicates each; *** $p < 0.01$, *** $p < 0.001$.

Figure 6: Influence of a 4 and 7 days pretreatment of female Balb/c mice with OCSE (200 and 500 mg/kg body weight) on the bacterial load in bladder after transurethral infection of the animals at day 0 with 2×10^8 cells of UPEC strain NU 14; animals were sacrified 24 h after infection, complete bladder had been removed, homogenized and the bacterial load was determined by quantification of the colony forming units (CFU) after plating on LB agar. Each spot represents the CFU/bladder from one animal; bars represent the mean value. * $p < 0.05$. ** $p < 0.01$.

Table 1: Peak assignment, analytical data and tentatively identified compounds from CSE.

401 **Table 1:** Peak assignment, analytical data and tentatively identified compounds from CSE

Cmpd.	t_R [min]	*m/z* found $[M+H/Na]^+$	Ion formula	Fragments [*m/z*]	λ max [nm]	Tentative identification	Sum formula	Exact masse [Da]	Reference
1	5.01	581.1527 $[M+H]^+$	$C_{26}H_{29}O_{15}$	287	208, 270, 384	Luteolin-7-apiosyl-(1>2)-D-glucoside	$C_{26}H_{28}O_{15}$	580.1428	KNApSAcK C00004281
2	5.08	449.1085 $[M+H]^+$	$C_{21}H_{21}O_{11}$	391, 287	208, 270, 384	Luteolin-7-O-D-glucoside Quercetin-3-O-L-rhamnoside	$C_{21}H_{20}O_{11}$ $C_{21}H_{20}O_{11}$	448.1006 448.1006	MassBank ID: TY000145; Zhou et al., 2009 KNApSAcK C00005374, MassBank ID: PR101049
3	5.31	565.1571 $[M+H]^+$	$C_{26}H_{29}O_{14}$	395, 271	212, 268, 340	Apiin	$C_{26}H_{28}O_{14}$	564.1479	KNApSAcK C000041019 Zhou et al., 2009
4	5.39	595.1673 $[M+H]^+$	$C_{27}H_{31}O_{15}$	365, 301	212, 268, 340	Graveobioside B Capreoside	$C_{27}H_{30}O_{15}$ $C_{27}H_{30}O_{15}$	594.1585 594.1585	KNApSAcK C00004342 MassBank ID: TY000189
5	5.52	463.1247 $[M+H]^+$	$C_{22}H_{23}O_6$	301	220, 276, 324	Chrysaoeriol-7-O-glucoside	$C_{22}H_{22}O_6$	462.1162	KNApSAcK C00004338 Zhou et al., 2009
6	5.75	429.1381 $[M+H]^+$	$C_{26}H_{21}O_6$	393	n.d.	--	$C_{26}H_{20}O_6$	428.1260	--
7	8.49	209.1187 $[M+H]^+$	$C_{12}H_{17}O_3$	194,168	n.d.	--	$C_{12}H_{16}O_3$	208.1099	--
8	8.59	407.2276 $[M+H]^+$	$C_{20}H_{23}O_9$	215, 193	224, 280	2,3-Dihydro-9-O-β-D-glucosyloxy-2-isopropenoyl-7H-	$C_{20}H_{22}O_9$	406.1264	KNApSAcK C00019835 Ahluwalia et al., 1988

						furo[3,2-g][1]benzopyran-7-one			
9	9.22	411.2533 [M+Na]$^+$	$C_{24}H_{36}NaO_4$	217, 195, 177	n.d.	--	$C_{24}H_{36}O_4$	388.2614	--
10	10.08	357.2015 [M+Na]$^+$	$C_{20}H_{30}NaO_4$	257, 235, 217	n.d.	--	$C_{20}H_{30}O_4$	334.2144	--

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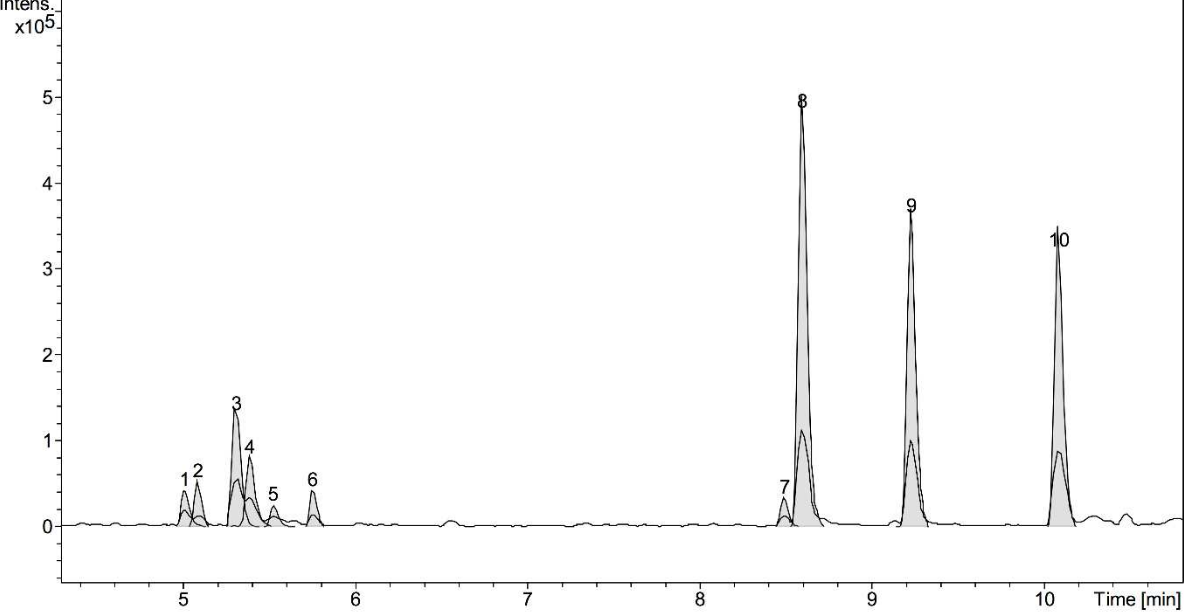
405 Figure 1



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408 Figure 2

Cmpd.	Structure	Cmpd.	Structure
1		**4**	OR
2	OR	**5**	
3		**8**	

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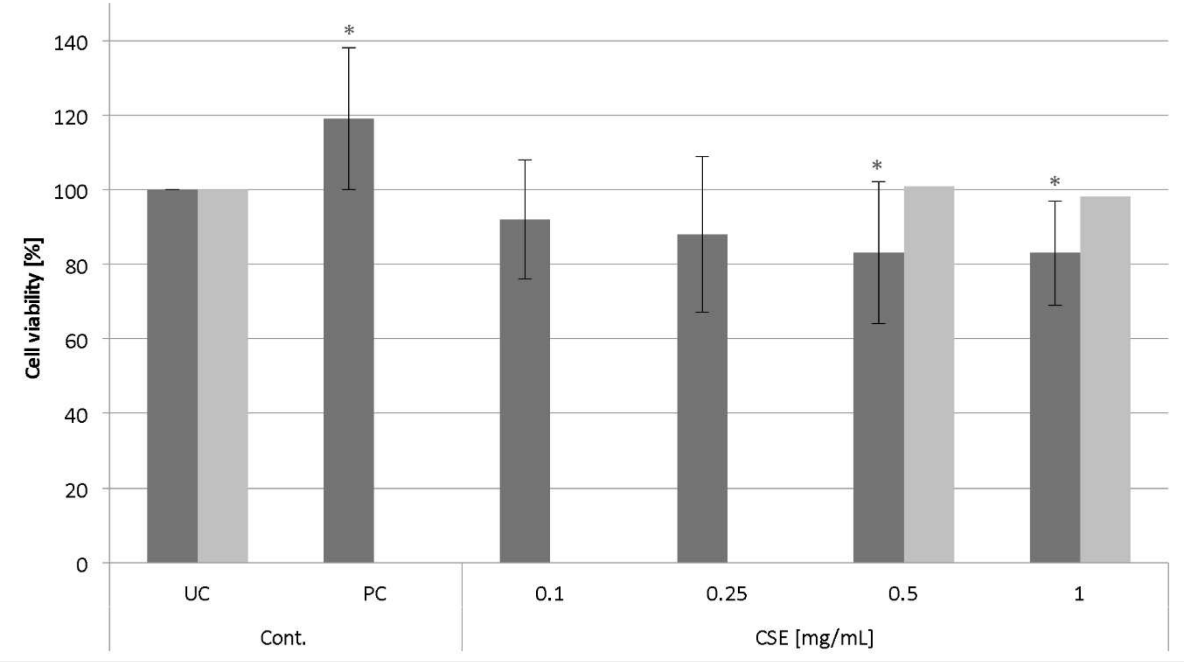
411 Figure 3



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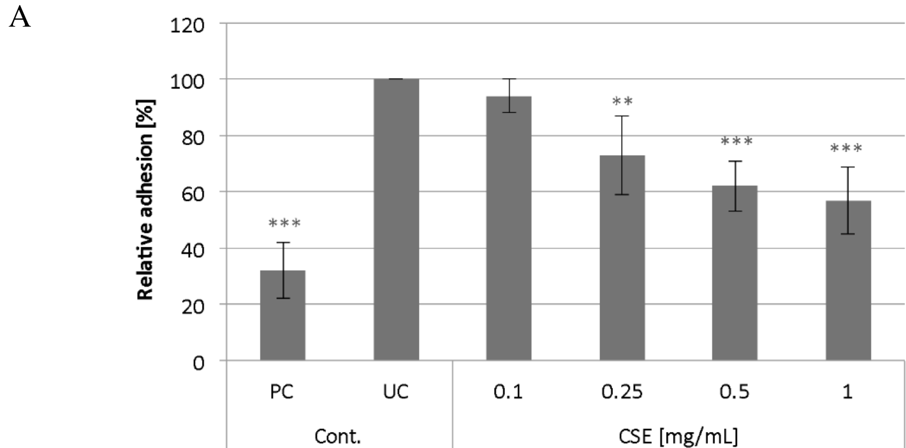
414 Figure 4

A



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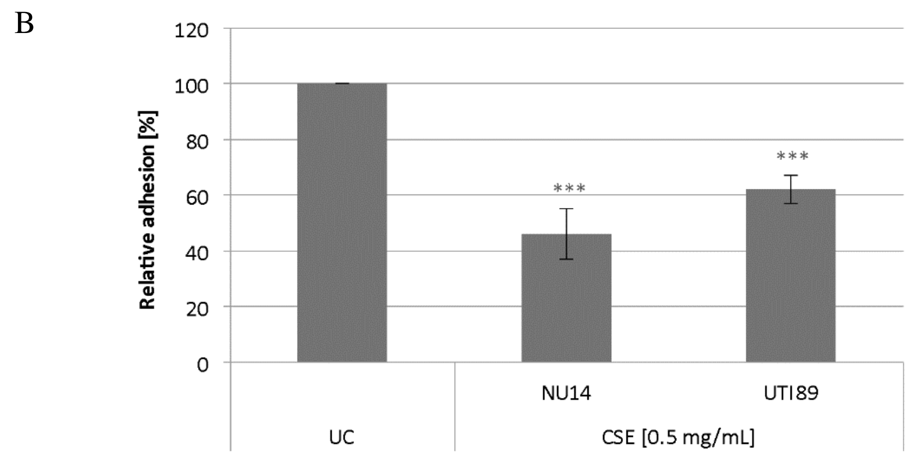

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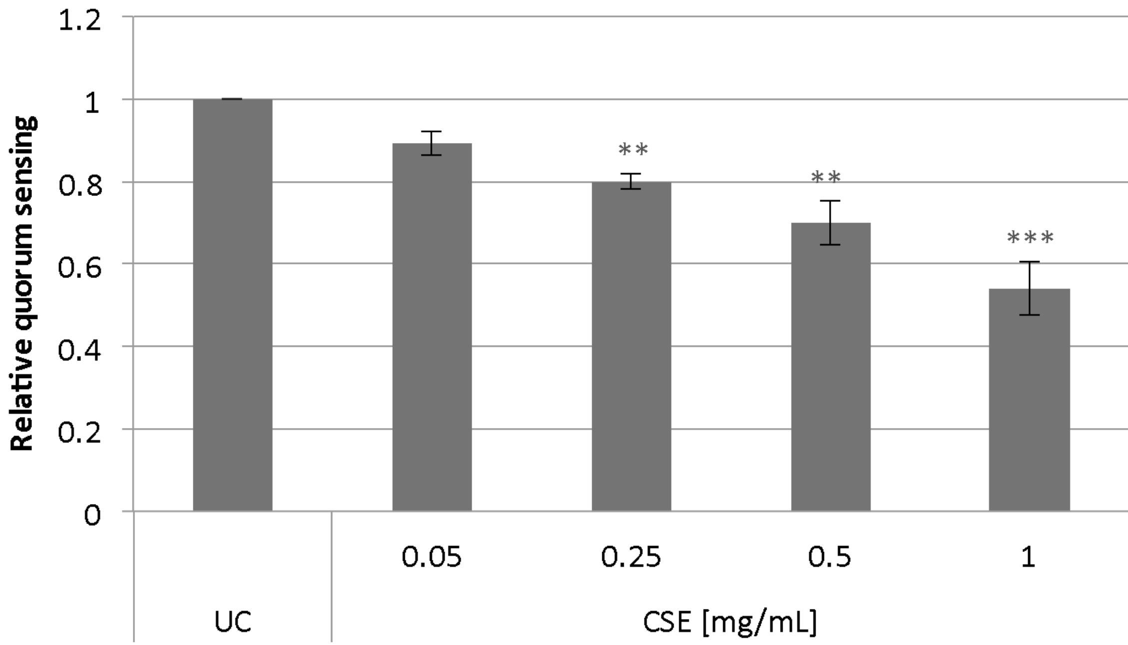
417 Figure 5



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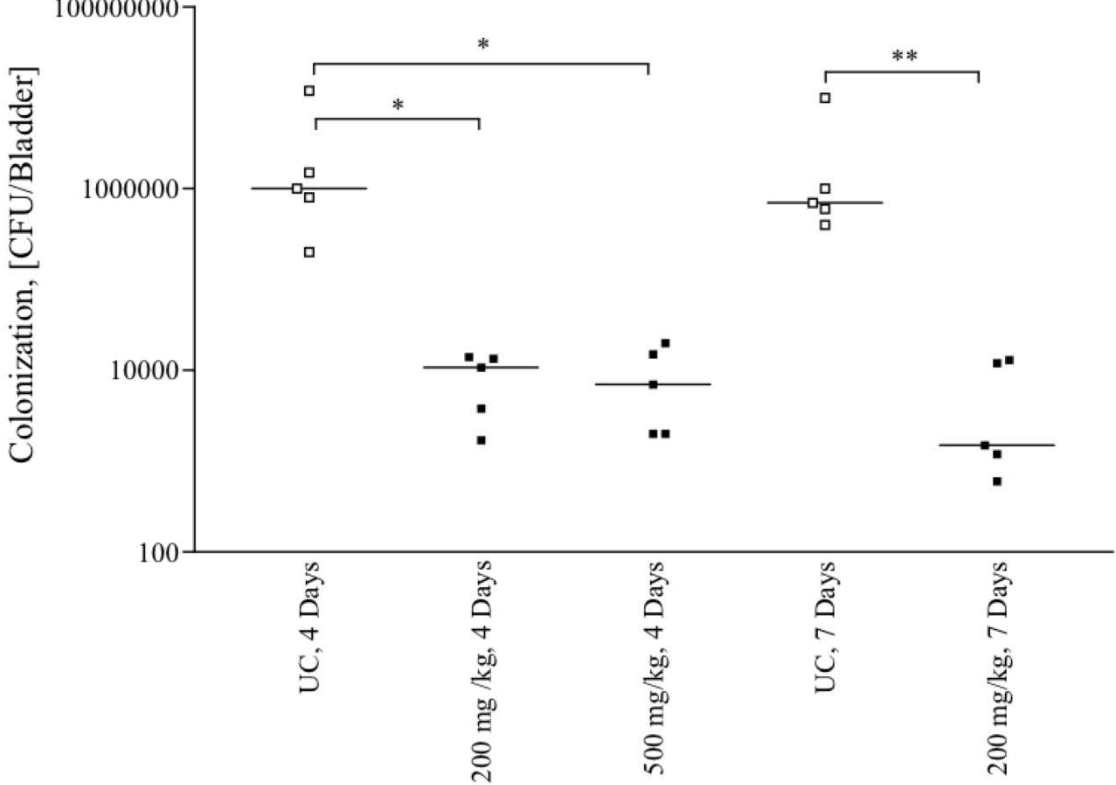
420 Figure 6



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