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Research Paper

A novel compound from celery seed with a bactericidal effect against *Helicobacter pylori*

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Abstract

Objectives The aim was to purify and characterise an antimicrobial component from celery (*Apium graveolens*) seeds, which have been used for centuries as a herbal medicine with reported antibacterial effects.

Methods A crude alcoholic extract of celery seeds was fractionated by organic solvent extractions, column chromatography and HPLC. Fractions were assayed for antimicrobial activity against the gastric pathogen *Helicobacter pylori* and other bacteria. The purified antibacterial component was characterised via MS and NMR. Preliminary investigation of its mechanism of action included morphological studies, incorporation of macromolecular precursors, membrane integrity and two-dimensional protein electrophoresis.

Key findings The purified component, termed 'compound with anti-*Helicobacter* activity' (CAH), had potent bactericidal effects against *H. pylori*; the minimum inhibitory concentration and minimum bactericidal concentration were 3.15 μg/ml and 6.25–12.5 μg/ml, respectively. CAH (M_r = 384.23; empirical formula $C_{24}H_{32}O_4$) had specific inhibitory effects on *H. pylori* and was not active against *Campylobacter jejuni* or *Escherichia coli.* MS and NMR data were consistent with a dimeric phthalide structure. The results appeared to rule out mechanisms that operated solely by loss of membrane integrity or inhibition of protein or nucleic acid synthesis.

Conclusions CAH may be suitable for further investigation as a potent agent for treating *H. pylori* infections.

Keywords *Apium graveolens*; bactericidal activity; celery seed; *Helicobacter pylori*; phthalide

Introduction

The discovery of *Helicobacter pylori* (formerly *Campylobacter pylori*) and its aetiologic role in the development of peptic ulcers, together with its role as a major contributory factor to gastric cancer, has led to development of chemotherapeutic agents to eliminate infection caused by this pathogen.[1–5] *H. pylori* is also associated with the development of acute and chronic gastritis, gastric adenocarcinoma and gastric lymphoma (MALT), and has been classified as a class I carcinogen in humans.[6] Infection with *H. pylori* can generally be treated by a combination of a proton pump inhibitor with multiple antibiotics, but these are costly drugs.[7] Also, while the infection can be eradicated in up to 90% of patients, side effects, poor compliance and the resistance of the bacterium to the antibiotics are common causes of treatment failure.[8]

Seeds of the celery plant (*Apium graveolens* (Apiaceae)) have been used for thousands of years in Ayurvedic medicine. In pharmacognosy, celery seed has been reported to be useful for treatment of urinary calculi, gut diseases, relief of flatulence and griping pains, reduction of visceral spasm and stimulation of the smooth muscle of the womb. All of these effects have been related to the phthalide constituents of celery seed.[9–11] Celery seed also promotes hypoglycaemia and may therefore be useful in treating diabetes mellitus.[12] The principal active constituents of celery seed are contained within its 1.5–3% volatile oil component. The major components of these essential oils are monoterpene hydrocarbons (46%) and phthalides (42.3%).[13,14] Friedman *et al.*[15] showed that celery seed extracts had bactericidal activity against *Campylobacter jejuni, Escherichia coli, Listeria*

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monocytogenes and *Salmonella enterica.* Constituents of a methanolic extract of celery seed have been reported to have antifungal, mosquitocidal and nematicidal activity and ethanolic extracts of celery seeds have also exhibited anti-inflammatory and antiulcer activity in rodent models.[16–21] An ethanol/water extract of celery leaves and a component from this, known as apiin, have recently been found to inhibit inducible nitric oxide (iNOS, NOS-II) and nitric oxide (NO) production *in vitro* and croton oil ear inflammation in mice.[22]

In an initial search for a new agent to combat *H. pylori* infection, Rainsford and Liu[23] observed that crude ethanolic extracts of celery seed exhibited anti-*H. pylori* properties. In this study, those antibacterial effects have been characterised in isolated components of the ethanolic extract of celery seed and a component has been isolated which has been found to be active against *H. pylori.*

Materials and Methods

Celery seed materials

The crude ethanolic extract of celery seed (A-CSE) was obtained from Beagle International Ltd (Brisbane, Queensland, Australia). Two other preparations from the same source of celery seed were obtained from Professor M. W. Whitehouse (Griffith University and University of Queensland, Australia), comprising the CO_2 critical fluid (C-CSE) and methyl ethyl ketone (ME-CSE) extracts. All the celery seed extracts were dark green, highly viscous liquids and were routinely dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions (100 mg/ml).

Bacterial strains and culture conditions

H. pylori strain 26695 was obtained from Professor David Kelly (University of Sheffield).[24] The identity of the strain was confirmed by Gram stain, urease reaction and the unique 16S rRNA gene sequence.[1,25] The bacteria were stored at –80°C in 1-ml amounts of Brucella broth containing 15% (v/v) glycerol.[26] *H. pylori* was cultured at 37°C under microaerobic conditions (10% CO_2, 3% O_2 and 87% N_2) on chocolate agar plates or in liquid Brucella broth (BBL, Franklin Lakes, NJ, USA) supplemented with foetal bovine serum (5% v/v; Invitrogen, Paisley, UK) immediately before use.[27]

Antimicrobial activity test

Microplate antimicrobial activity assays were performed using 24-well plates. Each assay contained 0.1 ml sample together with 1.0 ml early log phase *H. pylori* culture in Brucella broth plus foetal bovine serum (prepared as above). Plates were incubated at 37°C under microaerobic conditions and bacterial growth was monitored at 550 nm using a microplate Multilabel Counter (Wallac Victor^2 – 1420, Finland). Samples (1 mg) were dissolved in 10 μl solvent (DMSO or isopropyl alcohol) to obtain a concentration of 100 mg/ml, and then diluted 100-fold into Brucella broth plus foetal bovine serum to give 1 mg/ml, from which 0.1 ml was added to each assay as detailed above. Minimum inhibitory concentrations (MICs) were determined by using serial dilution assays of the samples. Control experiments where the sample (but not the solvent) was omitted indicated that the solvents (DMSO and isopropyl alcohol) did not have significant antimicrobial activity at the concentrations employed.

To distinguish between bacteriostatic and bactericidal activity, *H. pylori* were cultured in liquid media in 24-well plates with serial dilutions of relevant samples as described above, together with negative (solvent only) control. After incubation for five days, the cells were harvested from each culture by centrifugation (16 000*g*, 5 min, room temperature). The cell pellets were then resuspended in the original volume of Brucella broth plus foetal bovine serum without antimicrobial agent and cultured under the same conditions for a further three days. Bactericidal activity was inferred if the cells did not re-grow when transferred to the medium without antimicrobial agent.

Organic extraction and separation of celery seed extract

A-CSE (100 g) was extracted sequentially with petroleum ether (Sigma, Poole, Dorset, UK) (2.0 L) and then water (500 ml) to yield a petroleum ether phase and an aqueous phase. Assay of the two phases revealed that the petroleum ether phase contained the anti-*Helicobacter* activity. This bioactive phase (i.e. having inhibitory activity) was fractionated by silica gel 60 200 g (VWR, Lutterworth, Leicestershire, UK) column chromatography in 5 cm diameter glass columns by sequential elution with hexane–ethyl acetate (7 : 3, v/v), ethyl acetate (AR) and methanol (AR).[28] Analytical thin-layer chromatography (TLC) was used to assess the purity of fractions and was performed on precoated fluorescent silica gel plates (GF254, Merck, Darmstadt, Germany), using hexane–ethyl acetate (7 : 3, v/v) as the mobile phase. Spots were located by their absorption under UV light (254 and 366 nm wavelength). Sixteen fractions were obtained and the peak of anti-*H. pylori* activity was identified by performing growth inhibition assays on the fractions. Further fractionation of the active material was performed by using silica gel column chromatography as above, except that the column was eluted with hexane–diethyl ether (70 : 30, v/v). Fractions with spots of the same retention factor (R_f) values on TLC were combined to give 10 pooled fractions. The anti-*Helicobacter* assay on these pooled fractions revealed that one of the fractions, referred to as SF12-I, was the most bioactive fraction. SF12-I was further purified by preparative RP-HPLC (Phenomenex, C18, 5 μm, 250 × 10 mm) using acetonitrile/water (70 : 30) as the mobile phase at a flow rate of 6.0 ml/min. For HPLC separations, a Waters 510 pump system (Waters, Milford, MA, USA) was used and the UV absorbance was recorded online with a Waters 486 photodiode-array detector and Sp4290 integrator with detection at 212 nm. Three fractions were obtained and the peak of anti-*H. pylori* activity was identified by performing growth inhibition assays on the fractions as above. The most potent fraction was termed CAH.

NMR and mass spectrometry

Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ at room temperature using a Bruker Ac – 250 NMR

spectrometer (^{1}H, 250 MHz; ^{13}C, 62.9 MHz) (Silberstreifen, Germany). 2-D NMR spectra were recorded in a Bruker DRX-500 (^{1}H, 500 MHz; ^{13}C, 125.8 MHz).

Mass spectra of the CAH fraction were obtained on several mass spectrometers including a Kratos Concept ISQ for electron ionisation spectrum and accurate mass measurement, an API Q-star mass spectrometer (Applied Biosystems, Foster City, CA, USA) for MALDI mass spectrum and an LCQ ion trap (Thermo Scientific, Waltham, MA, USA) using APCI for tandem MS studies. HPLC-UV data were obtained on a Waters Alliance 2690 HPLC with a Waters 996 diode array detector.

2-D Electrophoresis and protein identification

Bacteria obtained from liquid cultures (5 ml) with CAH at half of the MIC under microaerophilic conditions for three days were harvested by centrifugation (16 000*g*, 5 min), washed with sterile water and then recentrifuged. The cell pellets were resuspended in Sample Rehydration buffer (Invitrogen, Paisley, UK) containing 8 M urea, 0.5% CHAPS and 200 mM dithiothreitol (DDT) to obtain the protein extracts. After sonication for 30 min (Branson 1210 sonicator, 47 kHz, 80 W, UK), samples of approximately 300 μg total protein per sample were obtained. Total protein concentration was determined using the Bradford assay.[29] Isoelectric focusing was performed using 7-cm immobilised pH gradient (IPG) strips, pH 3-11L (Invitrogen) according to recommendations of the manufacturer. After loading the protein sample (10 μg) isoelectric focusing was performed at 200 V for 20 min, 450 V for 15 min, 700 V for 15 min and finally 2000 V for 3.5 h. The strips were then incubated in LDS sample buffer with reducing reagent (Invitrogen) for 15 min and subsequently in LDS sample buffer with iodoacetamide for 15 min. The second step was performed on 1-mm polyacrylamide mini-gels (gradient 4–12%, Invitrogen) by using MES running buffer at 200 V for 45 min. Silver stain (Invitrogen) was used to visualise the protein spots. Gel images were analysed by using 2-D Gel Analysis Software Version (Phoretix 2-D Expression v2004). Gels from CAH-treated samples were compared with control gels from samples treated with the solvent without CAH. The experiments were performed in triplicate.

Silver-stained spots were excised from 2-D electrophoresis gels and transferred into a Proteome System (Shimadzu-Biotech, Milton Keynes, UK) and processed according to the manufacturer's instructions. In-gel digestion of the excised protein spots was performed using trypsin (1 μg/ml) and the sample was then mixed (1 : 1) with a saturated α-cyano-4-hydroxycinnamic acid solution in 50% acetonitrile–0.3% trifluoroacetic acid. A 2 μl sample was applied to the sample template of a Voyager Elite matrix-assisted laser desorption ionization (MALDI) mass spectrometer (Perseptive). The resulting peptide fingerprint data were analysed by using Mascot software (http://www.matrixscience.com/) and the search employed the protein database of the National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov), which contains the complete genome of *H. pylori* 26695. The accuracy of MH^+ mass determination was $\leq 0.01\%$ and possible modification of cysteine residues by acrylamide and methionine oxidation were taken into consideration.

Effect of CAH on *H. pylori* membrane potential

Samples (1 ml) from a three-day culture of *H. pylori* were centrifuged at 16 000*g* for 5 min at 20°C. The supernatant was discarded and the cell pellets were washed by resuspension in phosphate-buffered saline (PBS; Invitrogen) and centrifugation under the same conditions. Ethylene glycol-bis(2-aminoethylether)-*N,N,N,N′*-tetra-acetic acid (EGTA) (200 μl, 0.1 mM) was added to resuspend each pellet and these were incubated for 5 min to permeabilise the outer membrane. The cells were centrifuged (as above) and the pellets resuspended in 200 μl rhodamine 123 (5 μg/ml, Sigma, Poole, Dorset, UK) and incubated in the dark for 30 min at 20°C. The cells were then centrifuged (as above) and the supernatant discarded. The cell pellet was resuspended in 200 μl CAH (25 μg/ml). Three controls were performed in parallel using cells prepared in an identical way and then, in place of CAH, treated with an equal volume of PBS; carbonyl cyanide-3-chlorophenylhydrazone (CCCP; 15 μM) in PBS; or PBS plus the same concentration of isopropyl alcohol used in the reaction with CAH.[30–33] After 4 h, the samples were analysed by flow cytometry (Becton Dickinson FACScan), with side scatter thresholds set for the analysis of the bacteria, as follows. Fluorescence emissions were acquired using a 4-decade log amplifier calculated on an arbitrary linear scale (1–10 000) in the fluorescence 1 channel (630 nm). The excitation and emission wavelengths were set at 600 and 665 nm, respectively. After selecting a well-defined region of interest (ROI) on forward vs side scatter, the fluorescence intensity of the bacteria falling within this ROI (the same fluorescent intensity) was measured as described.[34] The extracellular probe was not fluorescent and therefore did not disturb the reading. Bacteria were distinguished from debris and background noise on the basis of their forward- and side-scatter characteristics. The yellow fluorescence of 10 000 bacteria was analysed by LYSIS software (Becton Dickinson). The mean fluorescent intensity represents that in the metabolically active cells in which the membrane potential does not change.[35]

Effect of CAH on gross protein and nucleic acid synthesis

^{3}H-labelled precursors (Amersham Biosciences, Amersham, UK) were used to investigate the effect of celery seed extracts on synthesis of proteins, RNA and DNA in *H. pylori* 26695, as described by Onishi *et al.*[36] Bacteria were grown to early log phase in liquid culture as described above. Samples of culture were transferred to sterile tubes containing various concentrations of CAH, together with radioactive precursors as follows: [^{3}H]leucine at 2.5 μCi/ml to determine protein synthesis; [^{3}H]uracil at 0.3 μCi/ml to measure RNA synthesis; and [^{3}H]thymidine at 0.75 μCi/ml plus uridine (50 μg/ml) to determine DNA synthesis.[36] After 24, 48 or 72 h (as stated for each experiment), the reactions were stopped by adding trichloroacetic acid (TCA; final concentration of 10%, w/v). The precipitates were collected with a cell harvester and washed five times with 10% (w/v) TCA. The filters carrying the washed precipitates were placed into scintillation tubes with 2 ml

scintillation fluid solvent, comprising 7 g PPO (2,5-diphenyloxazole), 0.6 g dimethyl-POPOP (1,4-bis[2]4-methyl-5-phenyloxazolyl benzene), and 100 ml Bio-Solv BBS-3.[37] Radioactivity was determined in a Packard 3320 liquid scintillation counter (Minnesota, USA).

Electron microscopy

A sample of *H. pylori* strain 26695 was cultured in Brucella broth plus foetal bovine serum with 5 μg/ml CAH under microaerobic conditions for three days as above. For the negative control, the cells were cultured without CAH under the same conditions as above. Both samples were centrifuged (10 000*g*; 5 min; 4°C) and the supernatant removed. The pellets were fixed in 2% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M PBS, followed by 2% (w/v) osmium tetroxide in water, then dehydrated by a graded series of ethanol treatment and air dried. These samples were examined by scanning electron microscopy at 20 kV (Phillips 500 SEM, University of Sheffield). The mean cell diameters were calculated from measurements of 50 cells. A drop of bacterial sample was applied to each grid and negatively stained with 1% (w/v) phosphotungstic acid (pH 7.2) for 20 s before transmission electron microscopy (TEM) (FEI Tecnai 20 TEM, University of Sheffield).[38]

Statistics

The Student's *t*-test and analysis of variance test were used to compare means, with significance assessed at the 95% confidence level. Statistical significance of morphological differences between treated and untreated *H. pylori* cells observed using TEM was assessed by using the Chi-squared test and analysis of variance.

Results

Antibacterial activity of celery seed extracts and purification of the active component

The three various celery seed extracts (A-CSE, C-CSE and ME-CSE) were initially tested for their inhibitory effects against *H. pylori* strain 26695. All three fractions showed activity against *H. pylori* 26695 with the MIC values being 300 (A-CSE), 25 (C-CSE) and 50 μg/ml (ME-CSE), respectively (Figure 1). Since the optical density at 550 nm of culture treated with C-CSE and ME-CSE decreased, it was likely that these extracts induced lysis of *H. pylori* cells.

A-CSE (100 g) was dissolved in water and fractionated by sequential petroleum ether and diethyl ether extractions using the scheme described in the Materials and Methods and shown in Figure 2. Among the fractions, the most active against *H. pylori* (MIC = 25 μg/ml) was the petroleum ether fraction; the values for MIC being 50 and >500 μg/ml for the diethyl ether and final aqueous phases, respectively (Figure 3). After evaporation of the solvent under vacuum, the petroleum ether phase which showed greatest antibacterial activity had a pale yellow oily appearance with a distinct celery odour. After evaporation of the solvent, the diethyl ether fraction had the appearance of a dark a green viscous liquid with a slight celery odour.

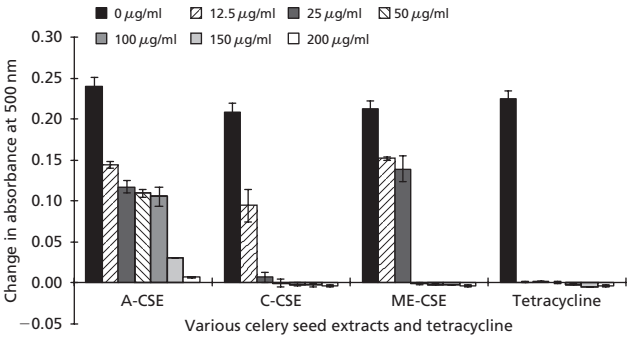


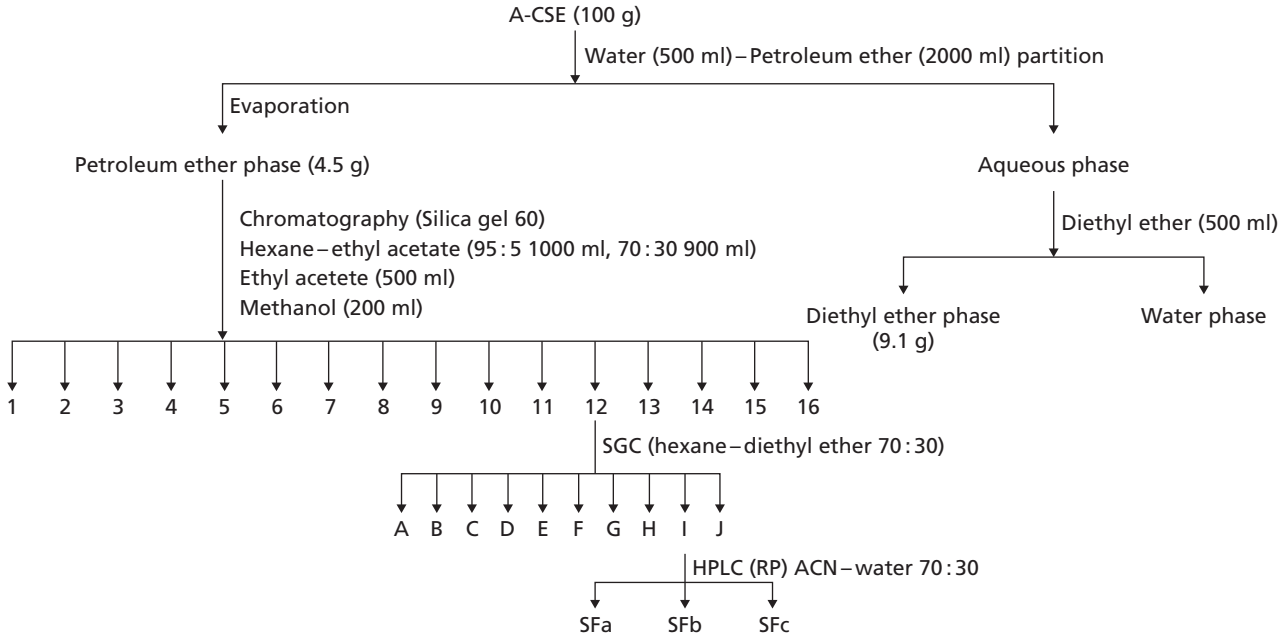
Figure 1 The effects of various celery seed extracts on growth of *Helicobacter pylori* 26695. The CO_2 critical fluid extract of celery seed (C-CSE) at concentrations greater than 25 μg/ml all significantly decreased the growth of the bacteria compared with the solvent control ($P < 0.01$) following a seven-day incubation. The error bars show the standard deviations of the experiments, which were performed five times. A-CSE, crude ethanolic extract of celery seed; ME-CSE, methyl ethyl ketone extract of celery seed.

The most bioactive phase, the petroleum ether phase, was then subjected to further fractionation by column chromatography on a silica gel 60 column, eluted with hexane/ethyl acetate (95 : 5, 1000 ml), hexane–ethyl acetate (70 : 30, 900 ml), pure ethyl acetate (500 ml) and finally pure methanol (200 ml) as mobile phases (Figure 2). Spots with the same R_f values on TLC analysis were combined to yield 16 major subfractions (SF1–16; Figure 2). Each subfraction was tested for antibacterial activity against *H. pylori* strain 26695 and the MIC values were determined.

The most pronounced inhibitory activity was found in subfraction SF12 (MIC = 12.5 μg/ml) and there was a minor peak of antimicrobial activity in SF6 (MIC = 25 μg/ml). The remaining subfractions had MIC values >50 μg/ml. The appearance of SF6 was that of a light yellow oil with a distinct celery odour, compared with that of SF12, which was a green oil with a slight celery odour.

SF12 was subjected to column chromatography on a silica gel 60 column (4.5 cm) using hexane–diethyl ether (70 : 30, 800 ml) as the mobile phase. Fractions with the same TLC patterns were combined to yield 10 major subfractions (SF12-A–J; Figure 2). Each of these subfractions was tested for activity against *H. pylori*; the most potent antimicrobial activity resided in the SF12-I fraction (MIC = 6.25 μg/ml).

The nonvolatile material in SF12-I was dissolved in isopropyl alcohol to give a concentration at 100 mg/ml and then further purified by using preparative RP-HPLC, eluted with acetronitrile–water (70 : 30) as the mobile phase. The eluted material was collected as three fractions: SFa was a pool of at least three unresolved peaks, whereas fractions SFb and SFc were each well-resolved peaks (Figure 4). Each of the three RP-HPLC fractions was evaluated for antibiotic activity against *H. pylori* 26695. The MIC values of SFa, SFb and SFc were 50, 12.5 and 3.15 μg/ml, respectively (Figure 5). The fraction with the most pronounced antimicrobial activity was SFc and was designated '*c*ompound with *a*nti-*Helicobacter*' activity (CAH). This had the appearance of colourless oil and appeared as a single component on TLC. The MIC values of these most active fractions compared



MIC: SF6: 25 μg/ml; SF12: 12.5 μg/ml; SF12-I: 6.25 μg/ml; SFc: 3.15 μg/ml

Figure 2 Scheme for fractionation of celery seed extract. A-CSE, crude ethanolic extract of celery seed; SGC, silica gel chromatography; HPLC (RP), reverse phase high-performance liquid chromatograph; ACN, acetonitrile; MIC, minimum inhibitory concentration.

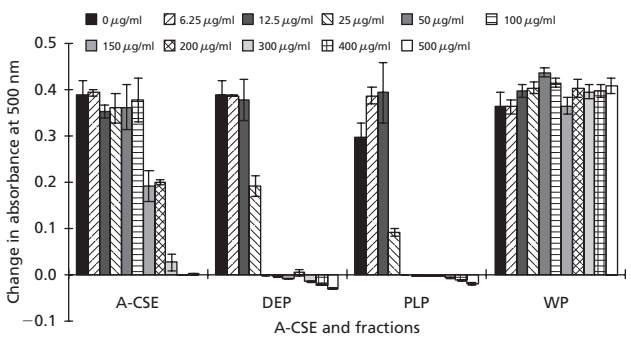


Figure 3 Treatment of *Helicobacter pylori* 26695 with celery seed extract petroleum ether phase or diethyl ether phase. Treatment resulted in a significant inhibition of growth ($P \leq 0.01$) compared to untreated controls during incubation for seven days. The water phase (WP) had no significant effect on the growth of *H. pylori* up to 500 μl/ml. The error bars show the standard deviations of experiments, which were performed five times. A-CSE, crude ethanolic extract of celery seed; PEP, petroleum ether phase; DEP, diethyl ether phase.

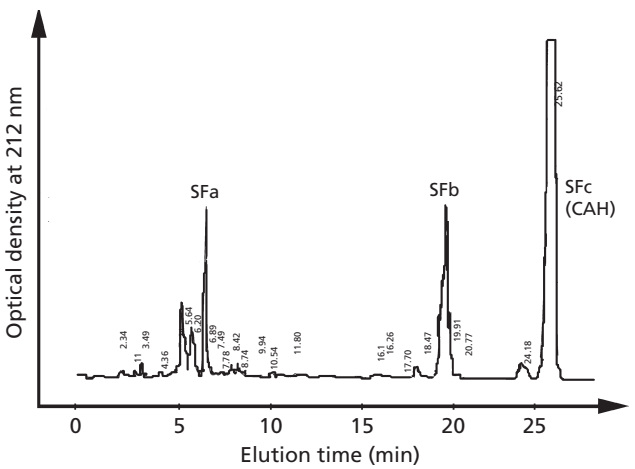


Figure 4 Absorbance trace from the RP-HPLC separation of sample SF12-I from celery seed extract. The three major fractions were collected and designated SFc (or CAH; the principally active component), SFa and SFb, respectively. System conditions: ODS semi-preparative column, mobile phase: acetonitrile/water, flow rate: 6.0 ml/min; wavelength: 212 nm.

favourably with that of the antibiotic tetracycline, which was found to have inhibitory effects at 3–5 μg/ml in parallel experiments (data not shown).

Chemical characterisation of the potent anti-*H. pylori* major fraction, CAH

The MALDI mass spectrum of CAH showed ions at *m/z* 385.2367, 407.2225 and 423.1965 (Figure 6). These ions represent $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$, respectively, for a compound of molecular weight 384. Electron ionisation mass spectrometry showed a weak M^+ ion at *m/z* 384 (1%) and prominent fragment ions at *m/z* 193 (100%), 192 (80%) and 107 (55%) (Figure 7). The molecular ion was measured as 384.22943 by peak matching and the empirical formula calculated as $C_{24}H_{32}O_4$ (within 1.6 ppm of the theoretical value). Tandem MS studies on an ion trap mass spectrometer showed the prominent product ion from the $[M+H]^+$ ion at

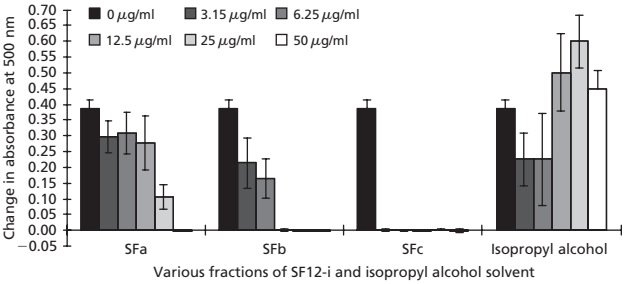


Figure 5 Inhibitory effects of subfractions of SF12-I from celery seed extract on the growth of *Helicobacter pylori* strain 26695. Incubation was for five days. CAH (the purified component, the compound with anti-*H. pylori* activity, SFc) at concentrations of 3.15 μg/ml or greater significantly decreased the growth of the bacteria compared with the corresponding solvent controls ($P < 0.01$ for all values). The error bars are the standard deviations from experiments that were performed five times.

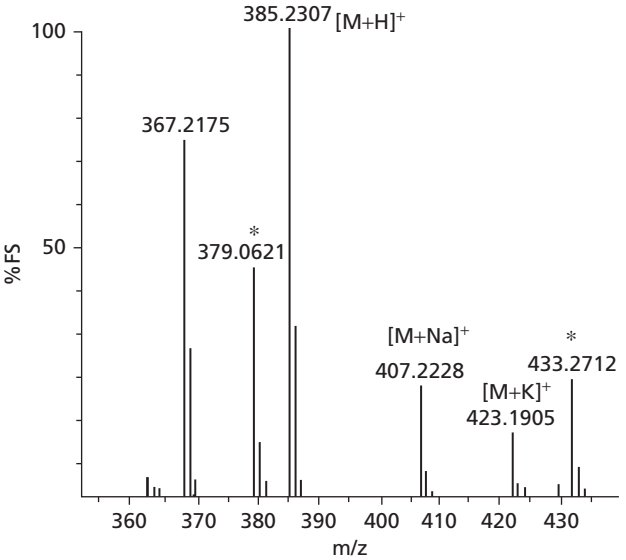


Figure 6 MALDI mass spectrum of the purified component from celery seed extract, termed 'compound with anti-*Helicobacter* activity'. Peaks arising from the MALDI matrix are indicated with asterisks.

385 to be a water loss to *m/z* 367, and the major products of this ion were at *m/z* 349, 321, 193, 175 and 147.

^{1}H NMR spectra collected in $CDCl_3$ showed the following signals: δ7.27 (5H, m, Ar), 5.10–5.15 (1H, m), 4.87–4.90 (1H, m), 2.93–2.98 (2H, m), 2.43–2.50 (1H, m), 2.07–2.11 (2H, m), 1.74–1.90 (6H, m), 1.25–1.65 (3H, m) and 0.85–0.95 (10H, m). The ^{13}C and ^{1}H–^{13}C correlation spectra indicated that there were 24 carbon signals, including two carbonyls (C=O), one of sp^2 CH, one of sp^2 C, seven of sp^3 carbons as CH and CH_3 groups and 12 sp^3 carbons as C and CH_2 groups. Also, two of the CH groups had quite a low chemical shift that could indicate nearby oxygen and there were two terminal $-CH_3$ groups.

These MS and NMR data were entirely consistent with CAH being an asymmetric phthalide dimer. Sedanenolide (**1**; Figure 8), $C_{12}H_{16}O_2$, (molecular weight 192) is a significant phthalide monomer in celery seed oil and a dimer of this would

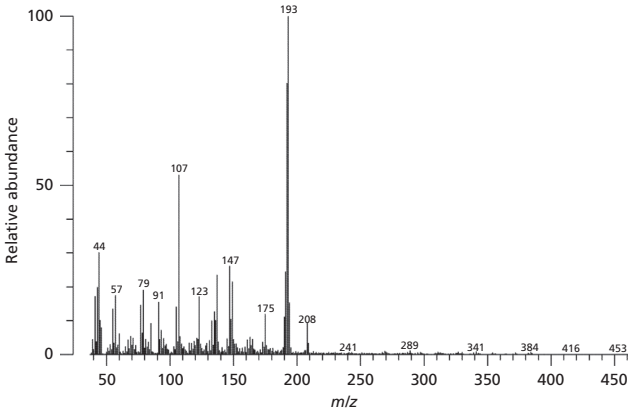


Figure 7 Electron ionisation mass spectrum of the purified component from celery seed extract, termed 'compound with anti-*Helicobacter* activity'

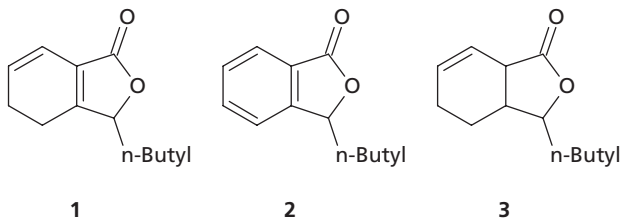


Figure 8 Structures of sedanenolide (**1**), n-butylphthalide (**2**) and sedanolide (**3**)

have the required monomeric formula.[13] n-Butylphthalide (**2**, molecular weight 190; Figure 8) and sedanolide (**3**, molecular weight 194; Figure 8) were also present as monomers in celery seed oil, and dimerisation of these would also produce a structure conforming to the determined mass. However, the intense product ion at *m/z* 193 by different MS techniques argued strongly for two equal mass monomers, but (based on NMR data) not of identical substructures.

A comprehensive literature search showed the only reports of phthalide dimers of this formula related to partial hydrogenation products prepared in the characterisation of the more unsaturated natural phthalide dimers, angeolide and *Z*-6.6′,7.3a′-diligustilide found in other members of the Apiaceae family.[39,40] The genus *Angelica* (Apiaceae) is a particularly rich known source of phthalides and phthalide dimers, with other examples of the latter including riligustilide, tokinolides A and B, and gelispirolide.[41–43] There are many theoretically possible phthalide dimers with the formula $C_{24}H_{32}O_4$, as dimerisation of phthalides can occur across any pair of double bonds, and also in the case of sedanenolide via Diels-Alder addition.

Full structural elucidation of CAH will require considerably more spectroscopic data.

Bactericidal effect of CAH on *H. pylori*

To determine whether CAH had bactericidal as well as bacteriostatic activity, bacteria were initially cultivated in the presence of the compound and subsequently recultured in its absence.[44] The minimum bactericidal concentration (MBC)

is defined as the lowest concentration at which a compound gives complete killing. The results indicated that CAH was bactericidal having an MBC of 6.25–12.5 μg/ml, thus showing that it was approximately twofold higher than the MIC. Also, at concentrations ≤ 500 μg/ml CAH did not inhibit the growth of *Campylobacter jejuni* under the same microaerobic conditions, nor did it inhibit growth of *Escherichia coli* under standard aerobic culture conditions (data not shown).

A significant morphological change in *H. pylori* induced by CAH treatment

Figure 9 shows scanning electron micrographs (SEM) of *H. pylori* strain 26695 that were cultured in the presence of CAH at more than the MIC (5 μg/ml) for 72 h. There were significant numbers of cells in the coccoid forms with all samples since the cultures were in the stationary phase. In the treated sample there were 19.7% in the rod form (226/1141) and 80.3% in the coccoid form (917/1141). In the solvent-treated (control) sample, 29.8% of the bacterial cells were in the rod form (206/692) and 71.2% in the coccoid form (486/692). This observed increase in the proportion of coccoid cells was statistically significant ($P < 0.05$; $X^2_{(0.05)}$ test; 1 d.f.). Inoue *et al.*[45] reported a similar morphological change in *H. pylori* ATCC43504 in the presence of antibactericidal agents. The average sizes (1.5 ± 0.3 μm $\times$ 0.15 μm; $n = 50$) of the cells treated with CAH were smaller than the untreated samples (3.0 ± 0.6 μm $\times$ 0.2 μm). These changes were significant, as assessed via the chi-squared test ($P < 0.05$; $X^2_{(0.05);}$ 1 d.f.). Following TEM examination, there was a marked disappearance of regions of less electron dense material in the cells treated with CAH (data not shown).

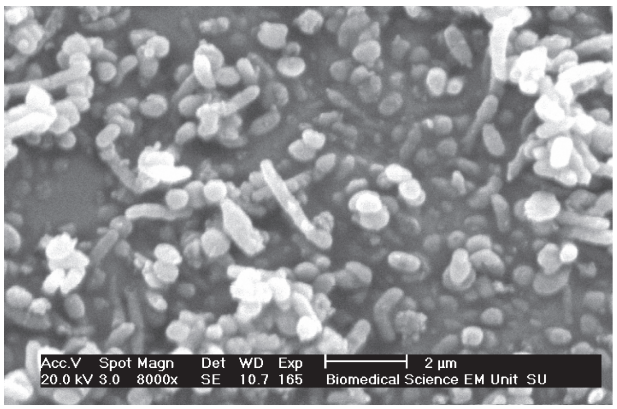
Effect of CAH on protein expression profiles

The protein-expression profiles of *H. pylori* strain 26695 treated with CAH and untreated were analysed by using 2-D electrophoresis and they were performed in triplicate. Representative gels are shown in Figure 10. 2-D electrophoresis profiles of *H. pylori* grown under normal and CAH-treated conditions exhibited distinct spots between masses 14 and 100 kDa in the pI 3 to 9 range. There were 338 spots found in the untreated gel and there were 189 of the spots which matched those in the CAH-treated gel. Of these, five spots were chosen that clearly decreased in intensity or disappeared when CAH was added (Figure 10). These proteins were characterised and presumptively identified using MS correlated with bioinformatics. The identity of these proteins is shown in Table 1.

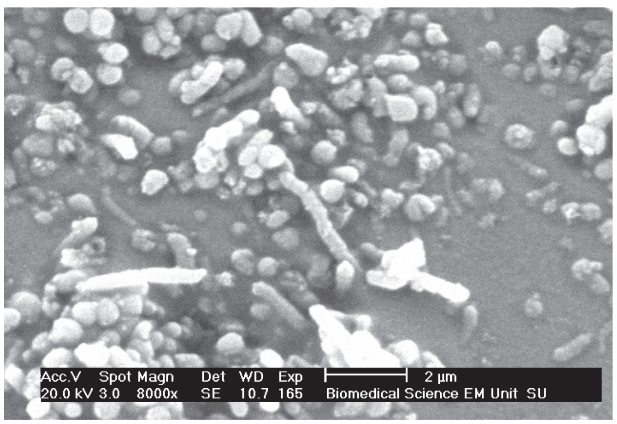
CAH significantly inhibited protein and nucleic acid synthesis in *H. pylori*

To identify whether CAH inhibited overall synthesis of protein, DNA or RNA, specific radiolabelled-precursors for these macromolecules were used to measure their rates of synthesis. At various time intervals after treating with CAH (3.15 μg/ml), incorporation of the radiolabel was quantified by scintillation counting of TCA-insoluble material (Figure 11). None of the treated samples showed substantial change in cell numbers

(a) Untreated control



(b) Treated with CAH



(c) Treated with 0.05% (v/v) isopropyl alcohol (solvent)

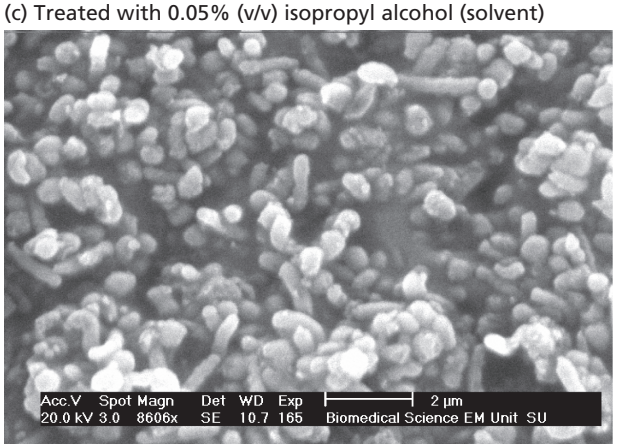


Figure 9 Scanning electron micrographs of *Helicobacter pylori* 26695. Incubation, under microaerobic conditions, was for 72 h, (a) without treatment, (b) treated with 5 mg/ml CAH (the purified component from celery seed extract, termed the compound with anti-*H. pylori* activity), added as a solution in isopropyl alcohol, and (c) treated with the same amount of isopropyl alcohol as was present as solvent in (b).

as judged by the optical density at 595 nm, whereas the solvent controls continued to grow in the absence of CAH. After three days exposure to CAH, the results indicated partial inhibition of protein, DNA and RNA synthesis in *H. pylori*, compared with controls without CAH (Figure 11).

CAH did not compromise *H. pylori* membrane potential

The lipophilic dye rhodamine 123 (Rh123) was employed to determine the proportion of viable cells, since it accumulates within bacteria in an energy-dependent manner. This enables the identification of cells with active membrane potentials and is reversible by treatment with compounds (e.g. carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)) which uncouple the membrane potential.[33] Rh123 staining was used to discriminate between viable and dead cells in cultures following treatment with CAH for 4 h (Table 2). Flow cytometric analysis revealed that the fluorescence intensity of *H. pylori* cells treated with CAH was slightly greater (133.12 ± 5.7) (mean ± SD, cell numbers), as was the *H. pylori* cells treated with solvent (133.6 ± 9.9), compared with that of the untreated cells (120.2 ± 4.7) at the same conditions (fluorescence 1 channel, 630 nm), although these effects were not statistically significant (χ^2-test, $P > 0.05$). For confirmation, cells were treated with the membrane uncoupler, CCCP. Upon treatment with CCCP, the average fluorescence intensity was significantly decreased by 90.86 ± 4.0% (χ^2-test, $P < 0.01$), compared with that in untreated cells.

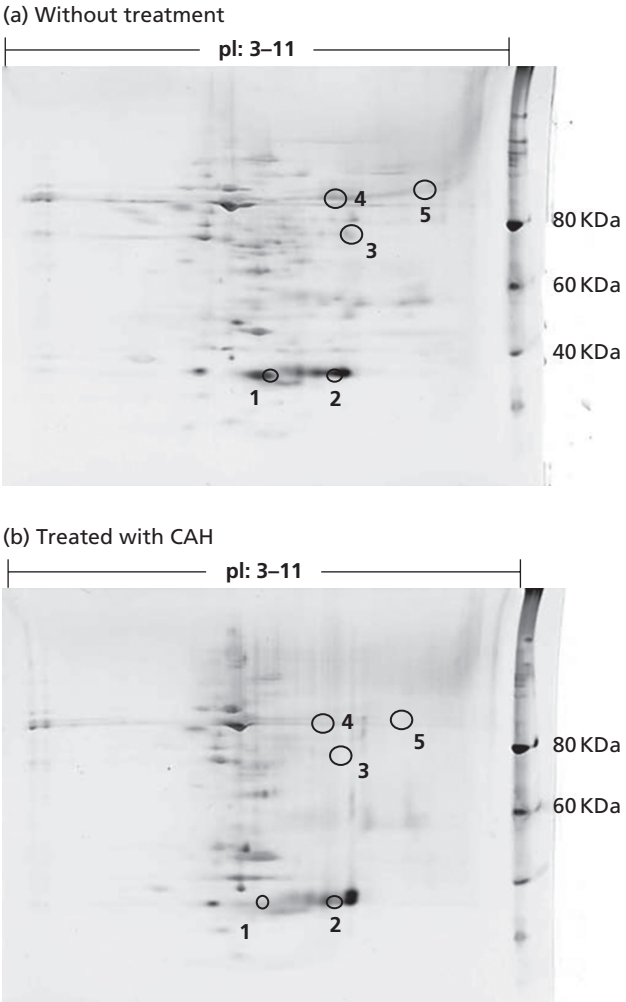


Figure 10 Two-dimensional gels showing the effects of CAH, from celery seed extract, on protein expression profiles of *Helicobacter pylori* 26695. Incubation, under microaerobic conditions, was for 72 h, (a) without and (b) with half of the minimum inhibitory concentration of CAH (the purified component from celery seed extract, termed the compound with anti-*H. pylori* activity). The five major protein spots whose intensity decreased on treatment with CAH are indicated. Their tentative identification is shown in Table 1.

Discussion

The results of this study have shown that CAH is a novel component from celery seed with effective and specific inhibition against *H. pylori.* Since the primary mechanism of action appeared to differ from the most common paradigms, it was conceivable that CAH acted via a novel mechanism. As a natural component of celery seeds, it may be safer for use than other antimicrobials and may avoid the alterations in gastrointestinal flora caused by using antibiotics.

The exact stereochemical structure of CAH remains to be determined, but the structural data presented here were consistent with a dimeric phthalide structure. Antibiotic phthalides from fungal sources and some synthetic analogues have been shown to be very effective and highly specific against *H. pylori*, and the presence of a spiroketal ring was shown to greatly enhance this activity.[46,47] While several known phthalide dimers from the family Apiaceae do contain a spiroketal ring, this occurs through a dimerisation process involving cyclisation of a butylidene phthalide side-chain, which is not present in monomeric sedanenolide, sedanolide or n-butylphthalide, which all have a saturated n-butyl side-chain.

Current commercially available antibiotics for effective *H. pylori* treatment are confined to amoxicillin, clarithromycin, metronidazole and tetracycline.[48] Our results indicated the bioactivity of CAH against *H. pylori* was comparable with tetracycline *in vitro*. Also, this bioactivity of CAH was

Table 1 The tentative identification of the five major protein spots as seen in Figure 10

Spot no.	Hp no.	Gene	Protein identification	Matching peptides	T*pI*	T*Mr* (Da)
1	HP-0389	sodB	Superoxide dismutase	12	6.0	24518
2	HP-1240	Maf	maf-like protein	6	6.8	21159
3	HP-0239	hemA	Glutamyl-tRNA reductase	5	8.4	51903
4	HP-0705	uvrA	uvrABC system protein A	29	7.3	104062
5	HP-1072	copA	Copper-transporting ATPase	9	8.3	81853

Spot no. refers to the numbers assigned in Figure 10. Hp no. refers to the 26695 genome annotation (http://www.tigr.org). Matching peptides refer to the number of matching tryptic digest fragments. T*pI* and T*Mr* are the theoretical isoelectric point and molecular mass, respectively.

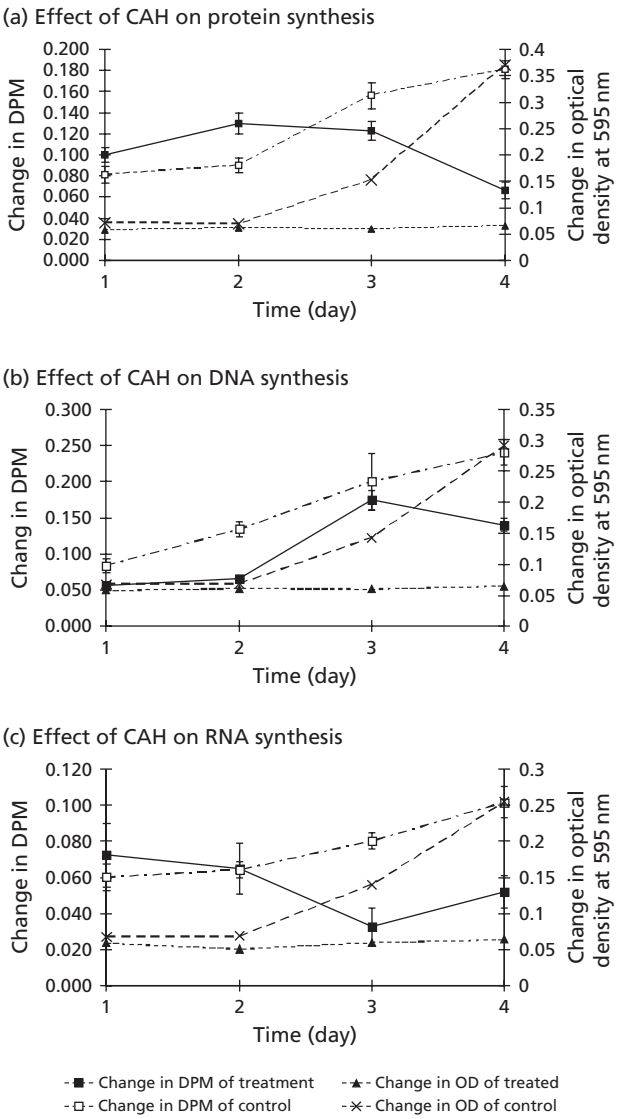


Figure 11 Effects of CAH, from celery seed extract, on protein and nucleic acids synthesis. *Helicobacter pylori* was treated with the minimum inhibitory concentration of CAH added as a solvent in isopropyl alcohol. Controls contained the same amount of solvent without CAH. Changes in culture of the optical density (OD) at 595 nm were measured at the same time as the amount of radioactive label (DPM), associated with the macromolecular (trichloroacetic acid-precipitated) fraction. Values of DPM are expressed as means ± SD of three independent experiments.

specific for *H. pylori*, since no effect on the growth of *C. jejuni* or *E. coli* was observed.

Changes in bacterial cell morphology resulting from starvation are well known. In the case of *H. pylori*, the cells change from the helical form to the coccoid form and this conversion is a passive process that results from cell death.[35] Inoue *et al.*[45] reported that the same morphological change happened in the presence of bactericidal agents against *H. pylori* ATCC43504. From our study, the effects of CAH on the morphology of *H. pylori* were modest and comprised the shapes and sizes of rods and coccoids; coincident with the bactericidal effects, the bacteria changed from the rod to coccoid shapes and shrank in size for survival.

Table 2 Flow cytometric analysis of *Helicobacter pylori* following rhodamine 123 staining

Treatments	Fluorescence intensity of *H. pylori* (mean ± SD, $P < 0.01$)
Negative control	120.2 ± 4.7
– with CAH	133.12 ± 5.7
– with solvent	133.6 ± 9.9
– with CCCP	90.86 ± 4.0

Fluorescence of *H. pylori* stained cells in the defined region of interest (ROI) at a fluorescence intensity (channel) at 630 nm. Values are expressed as means ± SD of four independent experiments. Rhodamine 123 was used as a fluorescent probe for membrane potential. The uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used as a positive control. CAH, the purified component, termed compound with anti-*H. pylori* activity, from celery seed extract.

The results showed partial inhibition of protein and nucleic acid synthesis after inhibition of cell growth by CAH. This was consistent with a reduction in the rate of bulk macromolecular synthesis as a secondary effect after inhibition of cell growth and metabolism owing to action of CAH at its currently unknown primary target.

To establish the effects of CAH on the protein expression in *H. pylori* we used 2-dimension protein expression profiles combined with gel digestion and mass spectrometry of the five selected spots. Of these, glutamyl-tRNA reductase and superoxide dismutase (SOD) were identified and were shown to have reduced expression in response to treatment with CAH. Their low level of expression may have been linked to antimicrobial activity. SOD has been shown to be a virulence factor for this pathogenic microaerophile.[49] SOD is part of the mechanism that protects *H. pylori* against toxic stress and low SOD upon treatment with CAH is consistent with a breakdown of the cells' stress defense mechanism.[50]

Glutamyl-tRNA reductase has been demonstrated to be involved in the NADPH-dependent reduction of the initial step of tetrapyrrole biosynthesis in *E. coli*.[51] Glutamyl-tRNA reductase (HemA protein) reduced glutamate to form glutamate-1-semialdehyde, which was then converted to 5-aminolevulinic acid (ALA) and could be necessary for growth of nutritionally versatile organisms including *H. pylori.* Previously it had been identified as a target for development of another novel antimicrobial agent.[52] The combination of reduced expression of some or all of these five identified proteins may have accounted for the antimicrobial effects of CAH.

Although several meta-analyses of *H. pylori* eradication regimens employing antibiotics and proton pump inhibitors have been published recently, eradication rates still vary in different regions and countries.[53] These existing therapies fail by leading to increased resistance of *H. pylori* and are often associated with significant side effects, poor compliance and high costs.[54] For vaccination treatment or prevention strategies, recent research has been disappointing and at present there is no vaccine to prevent infection.[5] Therefore, novel approaches to treatment against *H. pylori*

are currently being sought. There have been a number of reports that natural plants and foods such as garlic, honey, and capsaicin can inhibit *H. pylori in vitro* and each report has suggested that such ingredients could be used for treatment of the infection.[55] From our study, the active component from celery seed, CAH, may be a satisfactory solution for *H. pylori* eradication.

Antibiotic resistance is one of the most common reasons for treatment failure and a growing problem worldwide.[56] Mégraud[57] concluded that the frequency of primary resistance of *H. pylori* ranged from 1.7 to 25% for clarithromycin, from 9 to 62.7% for metronidazole, up to 0.5% for tetracycline and up to 0.9% for amoxicillin. Despite improved efforts for the rational use of existing agents, there remains a strong need for novel antibiotics.[58] Thus CAH from celery seed may become the starting material for the synthesis and development of new anti-*Helicobacter* drugs.

The increasing cost for these regimens is one of the problems of current anti-*H. pylori* therapy. For the cost of prescriptions in England in 1991–1993, spending on histamine antagonists remained static at about £180 million, while spending on omeprazole and other proton pump inhibitors had increased from £20 million to £94 million in two years.[59] Due to their low cost and absence of side effects, plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years.[60] The World Health Organisation estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components. The concept of using celery seed as medicine is not new. It has been used as a herb medicine for thousands of years for a variety of reasons.[61] During ancient times, India's Ayurvedic physicians (vaidvas) prescribed celery seed as a diuretic and as a treatment for colds, flu, water retention, indigestion, various types of arthritis, and ailments of the liver and spleen.[12,61] Through CAH and possible future derivatives thereof, the celery seed may yield a valuable agent for treating gastric infections due to *H. pylori*.

Conclusions

A component of celery seed, which may be a dimeric phthalide, has been purified and shown to have potent antimicrobial activity against *H. pylori*. This compound, CAH, has potency comparable with tetracycline and may represent a new class of antimicrobial agents for possible future development for treatment of *H. pylori* infections.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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