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An extract of *Apium graveolens* var. dulce leaves: structure of the major constituent, apiin, and its anti-inflammatory properties

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Abstract

Flavonoids, natural compounds widely distributed in the plant kingdom, are reported to affect the inflammatory process and to possess anti-inflammatory as well as immunomodulatory activity in-vitro and in-vivo. Since nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is one of the inflammatory mediators, the effects of the ethanol/water (1:1) extract of the leaves of *Apium graveolens* var. dulce (celery) on iNOS expression and NO production in the J774.A1 macrophage cell line stimulated for 24 h with *Escherichia coli* lipopolysaccharide (LPS) were evaluated. The extract of *A. graveolens* var. dulce contained apiin as the major constituent (1.12%, w/w, of the extract). The extract and apiin showed significant inhibitory activity on nitrite (NO) production in-vitro (IC50 0.073 and 0.08 mg mL^{-1} for the extract and apiin, respectively) and iNOS expression (IC50 0.095 and 0.049 mg mL^{-1} for the extract and apiin, respectively) in LPS-activated J774.A1 cells. The croton-oil ear test on mice showed that the extract exerted anti-inflammatory activity in-vivo (ID50 730 μg cm^{-2}), with a potency seven-times lower than that of indometacin (ID50 93 μg cm^{-2}), the non-steroidal anti-inflammatory drug used as reference. Our results clearly indicated the inhibitory activity of the extract and apiin in-vitro on iNOS expression and nitrite production when added before LPS stimulation in the medium of J774.A1 cells. The anti-inflammatory properties of the extract demonstrated in-vivo might have been due to reduction of iNOS enzyme expression.

Introduction

Apium graveolens L. var. dulce (celery) is an edible plant of the Umbelliferae family that grows in the Mediterranean area (Rao et al 2000). The plant is cultivated for its seeds and essential oil, which is used as a flavouring due its characteristic smell, and is often used as a spice. The composition of the volatile oil has been largely studied (Rao et al 2000; Deng et al 2003). Its leaves are rich in bioactive compounds which have medicinal uses and it is commonly used as a food in the Mediterranean diet. The leaves are an important source of natural dietary flavonoids (Crozier et al 1997; Miean & Mohamed 2001) and contain large amounts of antioxidant molecules (α-tocopherol) (Ching & Mohamed 2001), which can protect against several chronic diseases. The juice was shown to be protective ex-vivo against lipid peroxidation of microsome membrane hepatocytes induced by CCl_4 (Gazzani et al 1998; Woods et al 2001). Compounds extracted from the seeds (sedanolide, senkyunolide-N and -J, L-tryptophan, chromenone and indole derivatives) showed antioxidant, cyclooxygenase and topoisomerase inhibitory activity (Momin et al 2002). Butylphthalide, sedanolide, sedanenolide from the essential oil have been reported for the treatment of inflammatory conditions (Daunter et al 1995). Dried powders from the leaves are useful for topical antibacterial and deodorant preparations, detergents, soaps, dentifrices and shampoos (Lion Corp. 1984; Sakuma 2002), which prevent lipid peroxide formation, inflammation and ageing (Totsuka & Bandai 2001). Studies involving crude ethanol extract of the whole plant showed antinociceptive and anti-inflammatory activity, the latter tested in rats by the carrageenan-induced paw oedema (Al-Hindawi 1989) and cotton pellet granuloma test (Atta & Alkofahi 1998).

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The anti-inflammatory activity of the phytochemicals present in food merits further study to help those suffering chronic inflammation to ameliorate their inflammatory conditions by offering a proper selection of food. Thus, in this study we have prepared the ethanol/water (1:1) extract of the leaves, containing polyphenols and particularly flavone glycosides. The extract was characterized for its total content of polyphenols by the Folin–Ciocalteu colorimetric method and for its ratio of apiin by HPLC. To improve our understanding of the anti-inflammatory properties of *A. graveolens*, we have investigated the effects of the extract and apiin, its major constituent, on nitrite production and inducible nitric oxide synthase (iNOS) expression in the J774.A1 macrophage cell line stimulated for 24 h with *Escherichia coli* lipopolysaccharide (LPS). The croton-oil ear test in mice was used to evaluate in-vivo anti-inflammatory activity.

Materials and Methods

Materials

E. coli LPS was obtained from Fluka (Milan, Italy). 3-(4,5-Dimethyl-thiazolyl-2yl) 2,5 diphenyl tetrazolium bromide (MTT), phosphate buffer solution (PBS), bovine serum albumin (BSA), 6-mercaptopurine (6-MP) and 2-propanol were obtained from Sigma Chemicals Co. (Milan, Italy). Kodak X-Omat film, ECL detection system, Hybond polyvinyldene difluoride membrane were from Millipore (Bedford, MA). Materials for Western blot analysis of iNOS (Picerno et al 2005) were obtained from Transduction Laboratories (Lexington, KY) and the peroxidase secondary antibody was purchased from Jackson (West Grove, PA). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, HEPES, glutamine, fetal calf serum (FCS) and horse serum were from Hy Clone (Euroclone-Cellbio, Pero, Milan, Italy). For the croton oil ear test, croton oil and indometacin were from Sigma (St Louis, MO). Ketamine hydrochloride was from Virbac S.r.l. (Milano, Italy).

Apparatus

UV spectra were recorded on a UV-2101PC, UV/vis scanning spectrophotometer (Shimadzu Italia srl, Milan, Italy). A Bruker DRX-600 NMR spectrometer, operating at 599.2 MHz for ^{1}H and at 150.9 MHz for ^{13}C was used for NMR measurements; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD; coupling constants, *J*, are in hertz. 1D- and 2D-NMR experiments were obtained using the conventional pulse sequences as described in the literature. Electrospray ionization mass spectrometry (ESIMS) was performed using a Finnigan LC-Q Deca instrument (Thermoquest San Jose, CA) equipped with Xcalibur software. Samples were dissolved in MeOH and infused in the ESI source by using a syringe pump (capillary temperature, 220°C; flow rate, 3 μL min^{-1}). High resolution mass spectra (HREIMS) were recorded using a Q-Star Pulsar (Applied Biosystems) triple-quadrupole orthogonal time-of-flight (TOF) instrument, equipped with electrospray ionization source. Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden). HPLC separations were performed on a Waters 590 series pumping system (Waters Corporation, Milford, USA) equipped with a W R401 refractive index detector and a U6K injector. HPLC quantitative analysis were carried out on an Agilent 1100 series system equipped with a model G-1312 pump, and a DAD G-1315 A detector.

Plant material

Leaves of *A. graveolens* L. var. dulce were collected at Trezzo sull'Adda, Italy in May 2005 and identified by Dr A. Facchini, Respharma. A voucher sample (RES 001.05) was deposited at the Herbarium of Respharma s.r.l., Trezzo sull'Adda, Italy.

Fractionation procedure

Dried and powdered leaves (700 g) were extracted in a Soxhlet with EtOH/H_2O 1:1 to give 4.7 g of residue. This was partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH soluble portion (3.1 g). A sample (2 g) of the *n*-BuOH extract was chromatographed over a Sephadex LH 20 column (1 m × 3 cm i.d.) using MeOH as eluent (flow rate 0.5 mL min^{-1}). Fractions (8 mL each) were collected and checked by TLC (Si-gel, *n*-BuOH–AcOH–H_2O (60:15:25), $CHCl_3$–MeOH–H_2O (7:3:0.3)). Fractions with similar R_f values were joined giving three major fractions. Fraction II containing flavone glycosides was purified further by RP-HPLC on a C_{18} μ-Bondapack column (30 cm × 7.8 mm, flow rate 2.0 mL min^{-1}) using MeOH–H_2O (45:55) as the eluent to yield pure **1** (19.1 mg, Rt = 20.5 min).

*Apigenin-7-O-β-D-apiofuranosyl(1->2)]-β-D-glucopyranoside (apiin, **1**)* White amorphous powder. $[\alpha]^D_{25} = -42.2$ (c 0.05, MeOH); HREIMS *m/z* 564.5560 for $C_{26}H_{828}O_{14}$; calculated, 564.2254; ESI-MS *m/z* 565 $[M+H]^+$, *m/z* 563 $[M-H]^-$, 431 $[(M-H)-132]^-$, 269 $[(M-H)-(132+162)]^-$; ^{1}H and ^{13}C NMR data see Table 1.

Quantitative HPLC analysis of the extract

Quantitative HPLC was conducted using a 150 × 3.9 mm i.d. C-18 μ-Bondapack column. The solvents were TFA 0.1% in H_2O (solvent A) and MeOH (solvent B). The elution gradient used was as follows: 0–>10 min, 30% B; 10–>20 min, 30–>45% B; 20–>35 min, 45% B (isocratic); 35–>60 min, 45–>100% B. Analyses were carried out in triplicate, at a flow rate of 1 mL min^{-1} with DAD detector set at λ330. Reference standard solutions of **1** were prepared at three concentration levels in the range of 17.5–70 μg mL^{-1}. The standard curve was analysed using the linear least-squares regression equation derived from the peak area (regression equation y = 758.79x – 1026.2, r = 0.999, where y is the peak area and x the concentration). The peak associated with compound **1** was identified by retention time, UV and MS spectra compared with standard and confirmed by co-injection. The extract was redissolved in MeOH and analysed under the same chromatographic conditions. The results showed that **1** represented 1.12% w/w of the extract.

Table 1 ^{13}C NMR and ^{1}H NMR spectral data[a] of compound **1** in CD_3OD

Position	δ_C	δ_H[a]	HMBC correlations
2	166.3		106.8 (C-10), 166.3 (C-2)
3	103.7	6.68 s	
4	184.2		
5	162.9		
6	101.0	6.50 d (2.0)	164.3 (C-7), 106.8 (C-10), 95.7 (C-8)
7	164.3		
8	95.7	6.82 d (2.0)	
9	158.9		
10	106.8		
1′	122.5		
2′	129.9	7.92 d (8.8)	166.3 (C-2), 163.8 (C-4′), 129.9 (C-2′)
3′	117.5	6.95 d (8.8)	122.5 (C-1′)
4′	163.8		
5′	117.5	7.92 d (8.8)	
6′	129.9	6.95 d (8.8)	
Glc-1″	100.1	5.17 d (7.5)	164.3 (C-7)
Glc-2″	78.6	3.68 dd (8.5, 7.5)	110.8 (Ap-1‴)
Glc-3″	78.9	3.66 t (8.5)	
Glc-4″	71.1	3.43 t (8.5)	
Glc-5″	78.2	3.56 m	
Glc-6″	62.2	3.74 dd (12.0, 4.5); 3.95 dd (12.0, 3.0)	
Ap-1‴	110.8	5.48 d (2.0)	78.6 (Glc-2″)
Ap-2‴	77.9	3.99 d (2.0)	
Ap-3‴	80.0		
Ap-4‴	75.2	4.07 d (10.0); 3.84 d (10.0)	
Ap-5‴	65.4		

Glc, glucopyranosyl; Ap, apiofuranosyl. [a]J values in Hz in parentheses and assignments were from 2D COSY, HSQC, HMBC experiments.

Quantitative determination of total phenols

The *A. graveolens* extract, dissolved in MeOH, was analysed for its total phenolic content according to the Folin–Ciocalteu colorimetric method (Aquino et al 2002). Total phenols, expressed as apigenin equivalents, were $171 \pm 0.6\ \mu g$ (mg extract)$^{-1}$.

Cells

J774.A1, murine macrophage cell line, was grown in adhesion on Petri dishes and maintained at 37°C as described by Picerno et al (2005). HEK-293 (human epithelial kidney cells) were maintained in adhesion on Petri dishes with DMEM supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin.

Cytotoxic activity

Potential cytotoxic activity of the extract (0.01–0.1 mg mL^{-1}) and compound **1** (0.005–0.05 mg mL^{-1}) in PBS solutions was evaluated in cell cultures (J774.A1 and HEK-293 cell lines) by MTT assay as previously described (Mosmann 1983; Picerno et al 2005). The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with tested compounds and 6-MP was calculated as: % dead cells = 100–(OD treated/OD control) × 100. Data on cell viability were expressed as percentages of viability vs negative controls (PBS-treated cells).

Analysis of nitrite

Monolayers of J774.A1 cells were routinely harvested by gentle scraping with a Teflon cell scraper, diluted in fresh medium and cultured to confluency at 37°C. Before each experiment cells were harvested, plated to a seeding density of 1.5×10^6 in P60 well plates. After 2 h of cell adhesion, the extract (0.01–0.1 mg mL^{-1}) or **1** (0.005–0.05 mg mL^{-1}) in PBS solution was added to the culture medium 1 h before and simultaneously to LPS (6×10^3 U mL^{-1}/24 h). Nitrite accumulation, indicator of nitric oxide (NO) release, was measured in the culture medium by the Griess reaction (Green et al 1982) 24 h after LPS challenge, according to Picerno et al (2005). The amount of nitrite in the samples was calculated using a sodium nitrite standard curve freshly prepared in culture medium. Results were expressed as percentages of inhibition calculated vs NO production of cells treated with LPS alone.

Western blot analysis for iNOS

After 24 h of incubation with LPS, medium was removed, cells were lysed and Western blot analysis was performed according to Picerno et al (2005).

Topical anti-inflammatory activity

Topical anti-inflammatory activity was evaluated as inhibition of the croton-oil-induced ear oedema in mice (Tubaro et al 1985). All experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609 ECC). Male CD-1 mice (28–32 g; Harlan-Italy, Udine, Italy) were anaesthetized with ketamine hydrochloride (145 mg kg^{-1}, i.p.). Cutaneous inflammation was induced on the inner surface of the right ear (surface: approximately 1 cm^{-2}) of anaesthetized mice by application of 80 μg croton oil dissolved in 42% aqueous ethanol (v/v), used as vehicle for extract and its control. Control mice received only the irritant solution, whereas the other mice received the irritant and the test substance. At the maximum oedematous response, 6 h later, mice were killed and a plug (6-mm diameter) was removed from the treated (right) and the untreated (left) ears. The oedematous response was measured as the weight difference between the two plugs. Anti-inflammatory activity was expressed as percent reduction of the oedema in treated mice compared with control mice. The non-steroidal anti-inflammatory drug (NSAID) indometacin was used as the reference drug.

Statistical analysis

Data regarding NO production and iNOS expression were expressed as percentages of inhibition calculated vs cell treated with LPS alone. Data on cell viability were expressed as percentages of viability vs negative controls (PBS-treated cells). All data were calculated from mean ± s.e.m. values of three independent determinations. Statistical analysis was performed by analysis of variance test and multiple comparisons by Bonferroni test (Picerno et al 2005). All experiments in-vitro were made three times, each time with three independent observations. The IC50 value (dose giving 50% inhibition of NO production or iNOS expression) was calculated by graphic interpolation of the concentration–effect curves.

Oedema was expressed as mean ± s.d. of the mean. Oedema values were analysed by one-way analysis of variance followed by Dunnett's test for multiple comparison of unpaired data. A probability level lower than 0.05 was considered significant. The ID50 value (dose giving 50% oedema inhibition) was calculated by graphic interpolation of the dose–effect curve.

Results

The dried leaves of *A. graveolens* var. dulce were extracted with ethanol/water 1:1. The total phenolic content, determined by the Folin–Ciocalteu method and expressed as apigenin equivalents, was $171 \pm 0.6\,\mu g$ (mg extract)$^{-1}$. To characterize the extract, it was partitioned between water and *n*-BuOH and subjected to a purification by gel filtration on a Sephadex LH-20 column and RP-HPLC. This gave **1** as the major constituent.

NMR and MS spectra in comparison with literature data (Agrawal 1989) indicated that **1** had apigenin as aglycon and two sugar residues. The ESI-MS spectra in negative mode of compound **1** exhibited a quasi-molecular ion peak at *m/z* 563 $[M - H]^-$, and high-resolution measurements (HREIMS *m/z* 564.5560) indicated the molecular formula $C_{26}H_{28}O_{14}$, in accordance with ^{13}C NMR data. Major fragments at *m/z* 431 and 269 were assigned to the loss of a pentose unit (132 amu), and the successive loss of a hexose unit (162 amu). The nature of the terminal sugar unit as β-D-apiofuranosyl was deduced by the following evidence: the ^1H NMR spectrum indicated an anomeric signal at δ 5.48 (H-1‴, d, $J = 2.0$ Hz); in the 1D TOCSY experiment, selective excitation of the signal at δ 5.48 led to the enhancement only of H-2‴ (δ 3.99, d, $J = 2.0$ Hz); and the multiplicity of H-2‴ may be derived only from the presence of a quaternary carbon at C-3, characteristic of an apiofuranosyl structure. The ^{13}C NMR spectrum gave 11 carbon signals for the sugar moiety, of which three methylenes were ascribable to C-4‴ (δ 75.2) and C-5‴ (δ 65.4) of an apiofuranosyl unit and to C-6″ (δ 62.2) of a glucopyranosyl unit, respectively. Analysis of the correlated ^{13}C NMR signals in the HSQC spectrum and of the resonances of the quaternary carbon signal (δ 80.0) matched well with a terminal β-D-apiofuranosyl linked to an inner β-D-glucopyranosyl. C-2″ (δ 78.6) of the glucopyranosyl unit was shifted downfield (β-effect) by approximately 3.5 ppm with respect to a terminal glucopyranosyl (Picerno et al 2003), demonstrating the (1->2) linkage between the apiosyl and glucosyl units.

Finally, the interglycosidic linkage of apiofuranosyl was confirmed unambiguously to be at C-2″ based on the HMBC cross-peak, due to $^3J_{C\text{-}H}$ long range coupling, between the anomeric proton signal (δ 5.8) of the apiofuranosyl and C-2″ (δ 78.6) of the glucopyranosyl unit. Correlations due to long-range HMBC couplings were also observed between H-1″ (δ 5.17) of the glucosyl and C-7 (δ164.3) of the aglycon, confirming that the disaccharide chain was bonded by a *O*-glycosidic linkage to C-7. Therefore the structure of **1** was apigenin-7-*O*-β-D-apiofuranosyl(1->2)]-β-D-glucopyranoside (apiin, Figure 1). It was isolated from celery by Galensa & Herrmann (1979), but its complete spectral data and biological activity have been reported here for the first time.

Compound **1** was used as biological marker of the extract for quantitative HPLC analysis by a direct calibration method. We found that the whole extract was composed of a high ratio (1.12% w/w) of apiin (**1**).

It is well known that in inflammatory disease NO production is elevated by the constitutive and the inducible nitric oxide synthase, and NO produced by iNOS is an important inflammatory mediator. Therefore, we investigated the in-vitro activity of the extract and compound **1** on inducible nitric oxide synthase expression (iNOS) and on NO production by LPS-stimulated J744.A1 macrophages. Cytotoxicity was evaluated using cell cultures (J774.A1 (murine monocyte/macrophage) and HEK-293 (human epithelial kidney)).

NO release in the cellular medium of J774.A1 macrophages incubated with compound **1** (0.005–0.05 mg mL^{-1}) or the extract (0.01–0.1 mg mL^{-1}) 1 h before LPS stimulation was evaluated 24 h after LPS (6×10^3 U mL^{-1}) challenge. Results were expressed as % of inhibition calculated vs controls (Aquino et al 2002). As shown in Figures 2 and 3, extract (0.01, 0.05 and 0.1 mg mL^{-1}) and apiin (**1**; 0.01 and 0.05 mg mL^{-1}) added 1 h before and simultaneously with LPS inhibited NO release significantly and in a concentration related manner; so that the IC50 value was calculated as 0.073 mg mL^{-1} of extract and 0.08 mg mL^{-1} of apiin. To establish whether the inhibitory effect of compound **1** and of the extract on NO release was related to the modulation of iNOS induction, iNOS expression was evaluated by Western blot analysis on cell lysates obtained by J774.A1 incubated with **1** (0.005–0.05 mg mL^{-1}) or the extract (0.01–0.1 mg mL^{-1}), 1 h before and simultaneously with LPS. Compound **1** (IC50

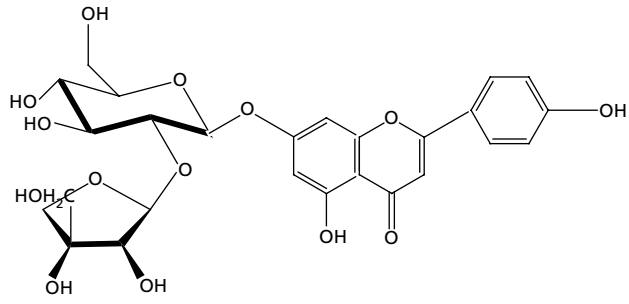

Figure 1 Apiin (**1**) isolated from *A. graveolens* leaves.

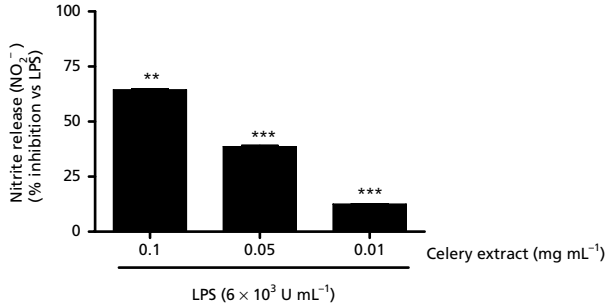

Figure 2 Concentration-related effect of the celery extract (0.01–0.1 mg mL^{-1}) in-vitro on nitrite (NO_2^-) release by LPS-stimulated J774.A1 macrophages. Values, mean ± s.e.m., are expressed as % inhibition of at least three independent experiments with three replicates each. Comparisons were performed using a one-way analysis of variance test. ***$P < 0.001$, **$P < 0.01$ calculated vs LPS alone.

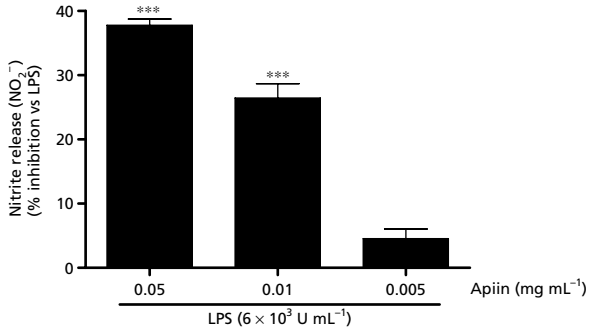

Figure 3 Concentration-related effect of apiin (**1**) (0.005–0.05 mg mL^{-1}) in-vitro on nitrite (NO_2^-) release by LPS-stimulated J774.A1 macrophages. Values, mean ± s.e.m., are expressed as % inhibition of at least three independent experiments with three replicates each. Comparisons were performed using a one-way analysis of variance test. ***$P < 0.001$ calculated vs LPS alone. No significant inhibition was observed at 0.005 mg mL^{-1}.

0.049 mg mL^{-1}) and the extract (IC50 0.095 mg mL^{-1}) showed a significant and concentration-dependent inhibition of iNOS expression ($P < 0.1$, compound **1** 0.05 and 0.01 mg mL^{-1}; $P < 0.01$, extract 0.1 and 0.05 mg mL^{-1}) (Figures 4 and 5).

To verify the effects on cell viability, the extract (0.01–0.1 mg mL^{-1}) and compound **1** (apiin) (0.005–0.05 mg mL^{-1}) were tested on two different cell lines, J774.A1 (murine macrophage cells) and HEK-293 (human epithelial kidney cells) using the MTT test. Our results indicated that they did not affect cell viability (data not shown).

As to the in-vivo topical anti-inflammatory activity of the extract, tested using the croton oil ear test in mice, the anti-oedematous effect of the extract, at doses of 100, 300 or 900 μg cm^{-2}, is reported in Table 2. The dose–activity relationship of the extract was investigated in comparison with indometacin (ID50, dose inducing 50% oedema inhibition = 93 μg cm^{-2}). The extract provoked a significant and dose-dependent oedema inhibition with potency seven-times lower than that of indometacin (ID50 730 μg cm^{-2}).

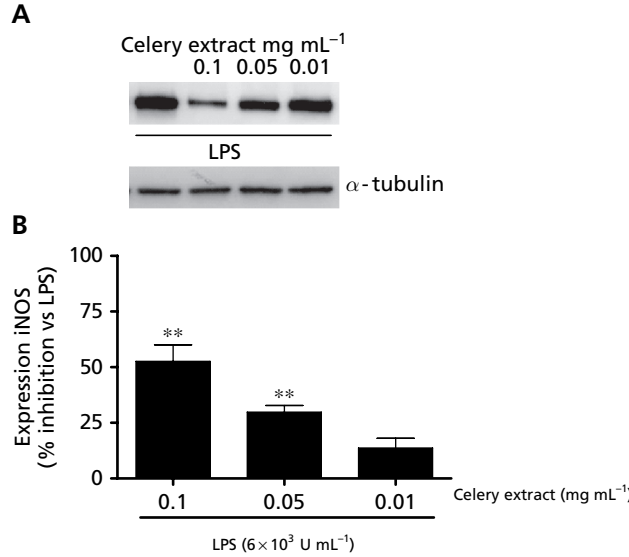

Figure 4 A. Representative Western blot of iNOS expression. B. Densitometric analysis of concentration-dependent effect of the celery extract (0.01–0.1 mg mL^{-1}) on LPS-induced iNOS expression in J774.A1 macrophages. The extract was added 1 h before and simultaneously with LPS challenge. Values, mean ± s.e.m., are expressed as % inhibition of at least three to six independent experiments with three replicates each. Comparisons were made using a one-way analysis of variance test. **$P < 0.01$ calculated vs cells containing LPS alone. No significant inhibition was observed at 0.01 mg mL^{-1}.

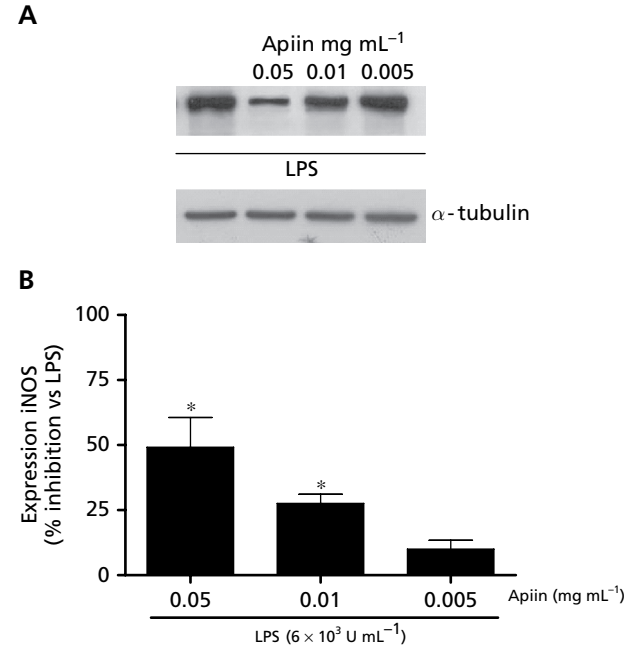

Figure 5 A. Representative Western blot of iNOS expression. B. Densitometric analysis of concentration-dependent effect of apiin (**1**) (0.005–0.05 mg mL^{-1}) on LPS-induced iNOS expression in J774.A1 macrophages. Apiin was added 1 h before and simultaneously with LPS challenge. Values, mean ± s.e.m., are expressed as % inhibition of at least three to six independent experiments with three replicates each. Comparisons were made using a one-way analysis of variance test. *$P < 0.1$ calculated vs LPS alone. No significant inhibition was observed at 0.005 mg mL^{-1}.

Table 2 Anti-inflammatory activity of *A. graveolens* extract after 6-h induction of croton oil mouse ear oedema

Substance	Dose ($\mu g\ cm^{-2}$)	No. of mice	Oedema (mg)	Oedema reduction (%)
Controls	–	10	6.7 ± 0.2	–
Extract	100	10	5.6 ± 0.3*	16
	300	10	4.4 ± 0.2*	34
	900	10	2.6 ± 0.4*	61
Indometacin	100	10	2.8 ± 0.3*	59

Oedema values are expressed as mean ± s.d. *$P < 0.05$ at the analysis of variance, as compared with controls.

Discussion

Flavones are a class of natural products with a large number of derivatives in plants and they are common components in the human diet. They are reported to possess a wide range of biochemical and pharmacological effects including antioxidant, anti-inflammatory, anticancer, antimicrobic and immunomodulatory activity (Gryglewski et al 1987; Middleton & Kandaswami 1992; Cooks & Samman 1996).

As it has been reported, their mechanisms of action could be explained with the inhibition of different enzymes such as prostaglandin synthase, lipoxygenase and cyclooxygenase, involved in the inflammatory process and tumorigenesis, and with the induction of detoxifying enzymes such as glutathione S-transferase (Cooks & Samman 1996; Comalada et al 2006; Horinaka et al 2006; Vargo et al 2006).

Despite the large use of *A. graveolens* var. dulce in traditional remedies, there are not enough data in the literature on its leaf components as anti-inflammatory agents and on their mechanism of action. In-vitro cyclooxygenase and topoisomerase inhibitory activity has been reported for compounds of different chemical structures (sedanolide, senkyunolide-N and -J, L-tryptophan, chromenone and indole derivatives), isolated from the seeds of *A. graveolens* (Momin & Nair 2002). Crude ethanol extracts of *A. graveolens* showed in-vivo anti-inflammatory activity in rats on carrageenan-induced paw oedema (Atta & Alkofahi 1998) and on cotton pellet granuloma (Al-Hindawi et al 1989). No significant anti-exudative effect was observed in xylene-induced ear oedema in mice with the same extract (Al-Hindawi et al 1989). Previously water extract of celery, a rich source of apigenin, was shown to enhance prostaglandin E_2 (PGE_2) production in the absence of LPS, and ethyl acetate extract of celery enhanced PGE_2 production in the presence of LPS in RAW264.7. Wu & Huang (2001) reported that the flavone aglycone, apigenin, inhibited the production of PGE_2. It is also well known that in inflammatory disease NO production is elevated by the constitutive and the inducible nitric oxide synthase, and NO produced by iNOS is another important inflammatory mediator. The results of this study indicated that polar extract of *A. graveolens* contained apiin as the major constituent and a high content of polyphenols that may all have contributed to the biological activity of celery. Moreover, apiin and celery extract were able to inhibit significantly and in a concentration-related manner in-vitro NO release and iNOS expression when added 1 h before LPS stimulation, with IC50 values of $0.049\ mg\ mL^{-1}$ ($86.8\ \mu M$) and $0.08\ mg\ mL^{-1}$ ($141.8\ \mu M$), respectively. Different results were obtained by Kim et al (1999) using different experimental conditions. They did not observe inhibition of NO release and iNOS expression when flavone glycosides, such as apiin, were added simultaneously (from 1 to $100\ \mu M$) with LPS in the macrophage cell line RAW264.7, whereas they reported the inhibition of NO production for flavones, such as apigenin (IC50 $23\ \mu M$), under the same experimental conditions.

The activity of the celery extract was confirmed by our results obtained in-vivo that firstly indicated the topical anti-inflammatory capability of the extract of celery leaves to ameliorate inflammation or other conditions in which enhanced expression of iNOS could be observed. These results were in agreement with recent researches on flavonoids as anti-inflammatory and antioxidative agents (Chu et al 2002; Ninfali & Bacchiocca 2003), as well as with studies on their mechanisms of action (Cooks & Samman 1996; Comalada et al 2006; Horinaka et al 2006; Vargo et al 2006).

References

Agrawal, P. K. (1989) (ed.) *Carbon-13 NMR of flavonoids*. Elsevier, London

Al-Hindawi, M. K., Al-Deen, I. H., Nabi, M. H., Huang Ismail, M. A. (1989) Anti-inflammatory activity of some Iraqi plants using intact rats. *J. Ethnopharmacol*. **26**: 163–168

Aquino, R., Morelli, S., Tomaino, A., Pellegrino, M., Saija, A., Grumetto, L., Puglia, C., Ventura, D., Bonina, F. (2002) Antioxidant and photoprotective activity of a crude extract of *Culcitium reflexum* H.B.K. leaves and their major flavonoids. *J. Ethnopharmacol*. **79**: 183–191

Atta, A. H., Alkofahi, A. (1998) Anti-nociceptive and anti-inflammatory effects of some Jordanian medicinal plant extracts. *J. Ethnopharmacol*. **60**: 117–124

Ching, L. S., Mohamed, S. (2001) Alpha-tocopherol content in 62 edible tropical plants. *J. Agric. Food Chem*. **49**: 3101–3105

Chu, Y.-F., Sun, J., Wu, X., Liu, R. H. (2002) Antioxidant and antiproliferative activities of common vegetables. *J. Agric. Food Chem*. **50**: 6910–6916

Comalada, M., Ballester, I., Bailon, E., Sierra, S., Xaus, J., Galvez, J., de Medina, F. S., Zarzuelo, A. (2006) Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure activity relationship. *Biochem. Pharmacol*. **72**: 1010–1021

Cooks, N. C., Samman, S. (1996) Flavonoids – Chemistry, metabolism, cardioprotective effects and dietary sources. *J. Nutr. Biochem*. **7**: 66

Crozier, A., Michael, E. J. L., McDonald, M. S., Black, C. (1997) Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce, and celery. *J. Agric. Food Chem*. **45**: 590–595

Daunter, B. (1995) Butylphthalide, sedanolide, sedanenolide, and related compounds as therapeutic agents for treatment of inflammation. *PCT Int. Appl*. WO 9500157

Deng, C., Song, G., Zheng, X., Hu, Y., Zhang, X. (2003) Analysis of the volatile constituents of *Apium graveolens* L. and *Oenanthe* L. by gas chromatography – mass spectrometry, using headspace solid-phase microextraction. *Chromatographia* **57**: 805–809

Galensa, R., Herrmann, K. (1979) Flavone glycosides of the leaves and tubers of celery (*Apium graveolens* L.). 9. Flavonols and flavones of vegetables. *Zeitschrift fuer Lebensmittel-Untersuchung und -Forschung* **169**: 170–172

Gazzani, G., Papetti, A., Daglia, M., Bertè, F., Gregotti, C. (1998) Protective activity of water soluble components of some common diet vegetables on rat liver microsome and the effect of thermal treatment. *J. Agric. Food Chem*. **46**: 4123–4127

Green, L. C., Wagner, D. A., Glogowsky, J., Skipper, P. L., Wishnok, J. J., Tannebaum, S. R. (1982) Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. *Anal. Biochem*. **126**: 131–138

Gryglewski, R. J., Korbut, R., Robak, J., Sueis, J. (1987) On the mechanism of antithrombotic action of flavonoid. *Biochem. Pharmacol*. **36**: 317

Kim, H. K., Cheon, B. S., Kim, Y. H., Kim, S. Y., Kim, H. P. (1999) Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochem Pharmacol*. **58**: 759–765

Horinaka, M., Yoshida, T., Shiraishi, T., Nakata, S., Wakada, M., Sakai, T. (2006) The dietary flavonoid apigenin sensitizes malignant tumor cells to tumor necrosis factor-related apoptosis-inducing ligand. *Mol. Cancer Ther*. **5**: 945–951

Lion Corp. Japan (1984) Dentifrices containing plant extracts, carvone and/or menthol. *Jpn. Kokai Tokkyo Koho*, JKXXAF JP 59013712

Middleton, E. J. R., Kandaswami, C. (1992) Effect of flavonoids on immune and inflammatory cell function. *Biochem. Pharmacol*. **43**: 1167–1179

Miean, K. H., Mohamed, S. (2001) Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. *J. Agric. Food Chem*. **49**: 3106–3112

Momin, R. A., Nair, M. G. (2002) Antioxidant, cyclooxygenase and topoisomerase inhibitory compounds from *Apium graveolens* Linn. seeds. *Phytomedicine* **9**: 312–318

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**: 55–63

Ninfali, P., Bacchiocca, M. (2003) Polyphenols and antioxidant capacity of vegetables under fresh and frozen conditions. *J. Agric. Food Chem*. **51**: 2222–2226

Picerno, P., Mencherini, T., Lauro, M. R., Barbato, F., Aquino, R. (2003) Phenolic constituents and antioxidant properties of *Xanthosoma violaceum* leaves. *J. Agric. Food Chem*. **51**: 6423–6428

Picerno, P., Autore, G., Marzocco, S., Meloni, M., Sanogo, R., Aquino, R. (2005) Anti-inflammatory activity of Verminoside from *Kigelia africana* and evaluation of cutaneous irritation in cell cultures and reconstituted human epidermis. *J. Nat. Prod*. **68**: 1610–1614

Rao, L. J. M., Nagalakshmi, S., Naik, J. P., Shankaracharya, N. B. (2000) Studies on chemical and technological aspects of celery (*Apium graveolens*. Linn) seed. *J. Food Sci. Technol*. **37**: 631–635

Sakuma, K. (2002) Mass production of dried powders of plant leaves and their applications. *Jpn. Kokai Tokkyo Koho*, JKXXAF JP 2002205952

Totsuka, Y., Bandai, Y. (2001) Antiaging cosmetics containing plant extracts and metal ions. *Jpn. Kokai Tokkyo Koho*, JKXXAF JP 2001139484

Tubaro, A., Dri, P., Delbello, G., Zilli, C., Della Loggia, R. (1985) The croton oil test revised. *Agents Actions* **17**: 347–349

Vargo, M. A., Voss, O. H., Poustka, F., Cardounel, A. J., Grotewold, E., Doseff, A. I. (2006) Apigenin-induced-apoptosis is mediated by the activation of PKCdelta and caspases in leukemia cells. *Biochem. Pharmacol*. **72**: 681–692

Woods, J. A., Jewell, C., O'Brien, N. M. (2001) Sedanolide, a natural phthalide from celery seed oil: effect on hydrogen peroxide and tert-butyl hydroperoxide-induced toxicity in HepG2 and CaCo-2 human cell lines. *In Vitro Mol. Toxicol*. **14**: 233–240

Wu, M.-C., Huang, C.-J. (2001) Inhibition of prostaglandin E2 production of a macrophage cell line by some phytochemicals. *Food Sci. Agric. Chem*. **3**: 59–71