JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Kaiware Daikon (*Raphanus sativus* L.) Extract: A Naturally Multipotent Chemopreventive Agent[#]

JESSICA BARILLARI,[†] RENATO IORI,*[,†] ALESSIO PAPI,[‡] MARINA ORLANDI,[‡] GIOVANNA BARTOLINI,*[,‡] SIMONE GABBANINI,[||] GIAN FRANCO PEDULLI,[§] AND LUCA VALGIMIGLI*[,§]

Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Centro di Ricerca per le Colture Industriali (CRA-CIN), via di Corticella 133, 4129 Bologna, Italy; Dipartimento di Biologia Evoluzionistica e Sperimentale (BES), Università di Bologna, via Selmi 3, 40126 Bologna, Italy; R&D Department, BeC srl, via C. Monteverdi 49, 47100 Forlì; and Dipartimento di Chimica Organica "A. Mangini", Università di Bologna, via San Giacomo 11, 40126 Bologna, Italy

Brassica vegetables are attracting major attention as healthy foods because of their content of glucosinolates (GLs) that release the corresponding isothiocyanates (ITCs) upon myrosinase hydrolysis. A number of studies have so far documented the chemopreventive properties of some ITCs. On the other hand, single nutrients detached from the food itself risk being somewhat "reductive", since plants contain several classes of compounds endowed with a polyhedral mechanism of action. Our recent finding that 4-methylthio-3-butenyl isothiocyanate (GRH-ITC) and 4-methylsulfinyl-3-butenyl isothiocyanate (GRE-ITC), released by the GLs purified from Japanese (Kaiware) Daikon (*Raphanus sativus* L.) seeds and sprouts, had selective cytotoxic/apoptotic activity on three human colon carcinoma cell lines prompted further research on the potential chemopreventive role of a standardized Kaiware Daikon extract (KDE), containing 10.5% w/w GRH and 3.8% w/w GRE, compared to its isolated components. KDE administered in combination with myrosinase at doses corresponding to 50 μM GRH-ITC plus 15 μM GRE-ITC (50 μM KDE-ITC) to three human cancer cell lines (LoVo, HCT-116 and HT-29) significantly reduced cell growth by 94−96% of control in six days ($p < 0.05$), outperforming pure GRH-ITC or GRE-ITC at the same dose. On the other hand, the same treatment had no significant toxicity on normal human T-lymphocytes. A 50 μM concentration of KDE-ITC had relevant apoptosis induction in all tested cancer cell lines, as confirmed by annexin V assay (e.g., 33% induction in LoVo compared to control, $p < 0.05$), Bax protein induction (e.g., +20% in HT-29, $p < 0.05$), and Bcl2 downregulation (e.g.−20% in HT-29, $p < 0.05$), and induced caspase-1 and PARP-1 activation in all cancer cells as shown by Western blot analysis. Unlike pure GRH or GRH-ITC, KDE also had significant chain-breaking antioxidant activity, retarding the AAPH-initiated autoxidation of methyl linoleate in SDS micelles at concentrations as low as 4.4 ppm (−50% in oxygen consumption rate), as monitored by Clark-type microelectrode oxygen-uptake kinetics, and induced very fast quenching of DPPH· radical in methanol with $t_{1/2}$ (s) = (1.47 ± 0.25) × 10^{-2}/[KDE; (g/L)], measured by stopped-flow UV−vis kinetics at 298 K. The potential chemopreventive role of KDE is discussed.

KEYWORDS: ***Raphanus sativus*; daikon; antioxidant; apoptosis; cancer**

INTRODUCTION

There is growing attention regarding the chemopreventive role of vegetable-rich dietary approaches. Several vegetables have become the topic of specific interest because of their content in health-promoting chemicals, such as carotenoids, polyphenols, and vitamins (particularly C, E, and A). In some cases single nutrients have been investigated as chemopreventive agents with the aim of "boosting" the "healthy" properties of their natural sources. This approach has often generated disappointing or contradicting results, showing, interestingly, that health cannot be boosted: it is instead the result of a delicate equilibrium.

For instance, despite the well-known health-promoting role of carrots, tomatoes, and other carotenoid-rich vegetables, it has been found that administration of β-carotene (pro-vitamin A) increased oxidative stress in rats and produced damage to DNA

* Corresponding authors. (R.I.) E-mail: r.iori@isci.it, fax: +39 051 374857; (G.B.) e-mail: giovanna.bartolini@unibo,it, fax: +39 051 2094286; (L.V.) e-mail: luca.valgimigli@unibo.it, fax: +39 051 2095688.

[#] We dedicate this paper to our friend and co-author, Prof. Giovanna Bartolini, who died July 20, 2008.

[†] CRA-CIN.

[‡] Dipartimento BES—Università di Bologna.

[||] R&D Department, BeC srl—Forlì.

[§] Dipartimento "A. Mangini"—Università di Bologna.

10.1021/jf8011213 CCC: $40.75
Published on Web 07/30/2008

(*1*). Similarly, diet supplementation with β-carotene in humans produced an increase in the incidence of lung cancer in smokers and asbestos workers and authoritative clinical trials over the years have failed to prove the long-term beneficial impact of β-carotene supplementation (*2*).

Studies focusing on single nutrients detached from food itself risk being somewhat "reductive", since plants contain several classes of compounds endowed with a polyhedral mechanism of action, which often act synergistically on the same target. The intake of healthy foods, in "whole" or "extract" forms, in place of diet-supplementation with high-dose single constituents therefore appears to be an interesting option.

Among healthy foods, cruciferous vegetables (Brassicaeae) have recently gained major attention because of their content in glucosinolates (GL), which release isothiocyanates (ITC) upon myrosinase hydrolysis (*3*) A number of studies have already documented the chemopreventive properties of some ITCs (*4*), but some potential threads linked to the administration of pure GLs (as opposed to the corresponding vegetable source) have also been reported (*5, 6*). Our ongoing research in the field has recently highlighted the selective cytotoxic/apoptotic activity on three human colon carcinoma cell lines of 4-methylthio-3-butenyl isothiocyanate (GRH-ITC; raphasatin) and 4-methylsulfinyl-3-butenyl isothiocyanate (GRE-ITC; sulforaphene) released from the corresponding GL glucoraphasatin (GRH) and glucoraphenin (GRE) contained in Japanese (Kaiware) Daikon (*Raphanus sativus* L.) seeds and sprouts (*7*). We have also shown by several experimental approaches that GRH and GRH-ITC have interesting direct antioxidant/radical-scavenging properties (*7, 8*).

Previous studies by our group described the preparation of an extract of Daikon sprouts (Kaiware Daikon Extract, KDE) containing 10.5% w/w of GRH and 3.8% w/w of GRE, that possessed interesting choleretic properties in vivo, and had antioxidant properties comparable to or higher than other vegetable choleretic extracts (e.g., laurel and artichoke), as judged by common tests like TEAC (trolox equivalent antioxidant capacity), or rac (relative antioxidant capacity) (*9*). With the aim of assessing whether this extract had different biological properties and antioxidant behavior with respect to the pure GLs it contains, and of investigating its potential as a chemopreventive agent, we undertook a detailed study on the cytotoxic activity of KDE in human colon carcinoma cell lines LoVo, HCT-116, HT-29, and on normal human T lymphocytes, under experimental conditions closely similar to those previously employed for GRH-ITC and GRE-ITC (*7*), to allow a direct comparison of the experimental results. We also measured the kinetics of quenching by KDE of the DPPH• radical (2,2-diphenyl-1-picrylhydrazyl), a persistent mimic of peroxyl radicals, and we performed controlled autoxidation studies of methyl linoleate in SDS micelles to investigate the chain-breaking antioxidant activity of KDE.

MATERIALS AND METHODS

Chemicals. All solvents and chemicals, except when noted, were of the highest grade commercially available (Fluka-Aldrich-Sigma) and were used without purification. DPPH• (2,2-diphenyl-1-picrylhydrazyl radical) was purchased from Aldrich and stored at +5 °C; fresh solutions in methanol were prepared immediately prior to use. (*R*,*R*,*R*)-α-Tocopherol (Aldrich) was purified by column chromatography on silica gel as previously described (*10*). Methyl linoleate (Sigma) was fractioned in 50 μL aliquots, sealed under nitrogen in glass ampules immediately upon receipt, and then stored at −20 °C. AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride; CAS No. 2997-92-4] was available from previous studies and stored at −20 °C.

Preparation of Kaiware Daikon Extract (KDE). KDE was prepared according to a standardized procedure previously described (*9*). *R. sativus* major seeds, cultivar OP 38 (Brassicaceae), were supplied by Suba & Unico (Longiano, FC, Italy). Six-day-old sprouts were grown at room temperature by using a Freshlife model 2000 germinator (Tribest Corp.). The crude extract was prepared as follows. First, freeze-dried sprouts were treated by boiling 70% aqueous ethanol (1:20 w/v) to produce a quick deactivation of endogenous myrosinase. The solid residue was removed by centrifugation and re-extracted using the same weight/volume ratio. The two solutions were collected, and the ethanol was then removed by concentration using a rotary evaporator at a temperature of 45 °C. The concentrated extract was maintained in an ice bath overnight. Finally, the precipitated proteins were removed by centrifugation and the extract was freeze-dried.

The content of GRH and GRE was assessed by the EU official method (ISO 9167-1) (*11*), based on the HPLC analysis of desulfo GLs obtained through the removal of the sulfate group of GLs via sulfatase-catalyzed hydrolysis. The amount of these two GLs was 10.5% w/w and 3.8% w/w, respectively, determined using sinigrin as the internal standard and their relative response factor.

Preparation of Myrosinase, GRH, GRE and Derived ITCs. GRH and GRE were purified from *Raphanus sativus* L. sprouts and seeds, respectively (*8*), in two sequential steps, by anion exchange and size exclusion chromatography, according to the previously reported method (*12*). Individual GLs were characterized by ^{1}H and ^{13}C NMR spectrometry, and the purity was assayed by HPLC analysis of the desulfo derivative according to the ISO 9167-1 method (*11*), proving to be about 99% based on peak area value and more than 95% on weight basis due to their high hygroscopic properties. The enzyme myrosinase was isolated from seeds of *Sinapis alba* L. according to a reported method (*13*) with some modifications. The stock solution used in the present study had a specific activity of ∼60 units/mg of soluble protein and was kept at 4 °C after dilution in sterile distilled H_2O at ∼30 U/mL. One myrosinase unit was defined as the amount of enzyme able to hydrolyze 1 μmole/min of sinigrin at pH 6.5 and 37 °C. Prior to use, GLs or KDE were dissolved in H_2O at a concentration of 10 mM and kept at −20 °C. The relative ITCs (GRH-ITC, GRE-ITC, and KDE-ITC) were produced via myrosinase-catalyzed hydrolysis that was performed by an in situ method (*14*). In every treatment, 5 μL of myrosinase was added to 0.5 mL of the medium (buffered at pH 7.4) containing increasing concentrations of GLs or KDE, to which cells were added.

Preparation of Reference Compound GRH-ITC. GRH-ITC was produced via myrosinase-catalyzed hydrolysis of pure GRH, performed in 0.1 M phosphate buffer pH 6.5 at 37 °C. The total conversion of GL into ITC was confirmed by HPLC analysis of the desulfo derivative (*11*), which allowed us to monitor the reduction until complete disappearance of GRH in the reaction mixture. GRH-ITC was purified by EXtrelut NT20 (Merck) column extraction. The mixture (20 mL) was applied to the dry column filled with granular EXtrelut NT20, and elution was carried out using dichloromethane that extracted GRH-ITC from the aqueous phase into the organic phase. The solutions containing GRH-ITC were concentrated to a few milliliters in a rotary evaporator at 45 °C, and finally the solvent was completely removed under a stream of nitrogen. The GRH-ITC structure was ascertained by GC-MS spectrometry using a Hewlett-Packard GCD G1800A, equipped with a 30 m × 0.25 mm capillary column HP-5 (*15*): (EI+, 70 EV) *m*/*z*: [163 (<0.1%), 161 (∼3%), 159 (30%); $M^{+\bullet}$], 87 (100%), 72 (20%), 45 (42%).

Autoxidation Studies. Autoxidation experiments in the presence and in the absence of antioxidants were carried out by monitoring the oxygen concentration with a miniaturized Clark-type electrode (Instech, Plymouth Meeting, PA) provided with an automatic data recorder (World Precision Instruments, Sarasota, FL). Experimental procedures were similar to those previously reported in other heterogeneous media (*16*). The measurement chamber (internal volume of 0.6 mL) was kept at a constant temperature by circulating water and was protected from room light to avoid initiator photodecomposition. The oxidizable substrate was methyl linoleate (1% v/v ≈ 3×10^{-5} M) dispersed in buffered (pH 7.4) micellar solution of sodium dodecyl sulfate (50 mM) in water. After thermal equilibration of the mixture into the measure-

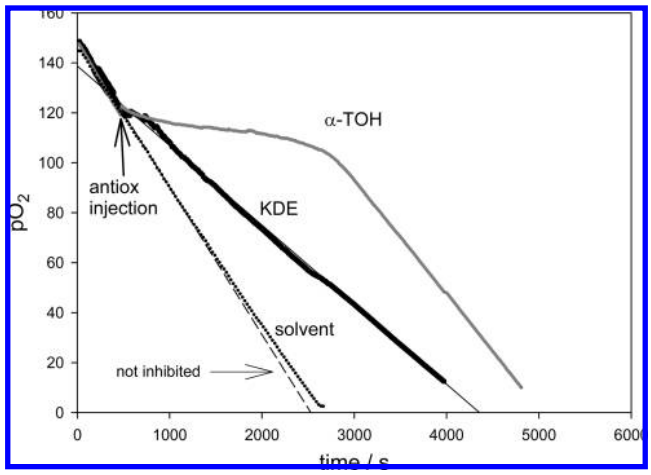

Figure 1. Oxygen uptake traces during AAPH (8.3×10^{-3}M) induced autoxidation of methyl linolate (3×10^{-5} M) in SDS micelles at 37 °C (pH 7.4) in the absence of an inhibitor (slashed), after injection of methanol (dotted), in the presence of α-tocopherol 2.6×10^{-6} M (gray line), and of KDE 4.4 ppm (black line). The arrow shows antioxidant (or solvent) injection.

ment chamber at 37 °C, the appropriate amount of AAPH (final concentration of 8.3×10^{-3} M) was injected into the cell at the beginning of data collection. After a few minutes, 10−20 μL of a concentrated methanol (or methanol/water) solution of the antioxidant (or extract) was added to obtain the desired final concentration. Reference experiments were performed using α-tocopherol or its synthetic analogous 2,2,5,7,8-pentamethyl-6-chromanol as standard reference antioxidant at final concentrations of $1-5 \times 10^{-6}$ M.

Kinetic Measurements with DPPH· Radical. The kinetics of the reaction with DPPH· radicals was investigated for KDE (20−100 mg/L) dissolved in methanol/water (90:10) using α-tocopherol ($5-100 \times 10^{-5}$ M) as a reference antioxidant. The solution of KDE was prepared to achieve a concentration of GRH ranging from 9×10^{-5} M to 5×10^{-4} M, considering an average content of GRH as 0.22 mol/kg of KDE. A diluted stock solution of DPPH· radical ($1-5 \times 10^{-5}$M) in methanol was rapidly mixed (in 1:1 ratio) with a solution of KDE containing at least a 10-fold excess of GRH or with a methanol solution of α-tocopherol, at 298 ± 2 K using a stopped-flow apparatus (HI-TECH SFA-20 Rapid Kinetic Accessory) with the mixing chamber sitting in the optical path of a Jasco V-550 spectrophotometer. The reaction was monitored by following the time-course of DPPH· absorbance at $\lambda_{max} = 517$ nm ($\epsilon = 12400$ M^{-1} cm^{-1}). Good pseudo-first-order decays were obtained, that afforded pseudo-first-order rate constants k_1 by analyzing the upper 1/3 of the decay according to eq 1.

$$\ln[\mathrm{DPPH}]_t - \ln[\mathrm{DPPH}]_0 = A - k_1 t \qquad (R^2 > 0.98) \qquad (1)$$

These values were compared to the measured rates of decay of DPPH· in the presence of α-tocopherol considering that second-order rate constants k_2 bear the relationship described in eq 2 with pseudo-1-order constants k_1.

$$k_2 = k_1[\mathrm{antiox}] \qquad (2)$$

Cell Cultures. The human cell lines were maintained in RPMI (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Cambrex, East Rutherford, NJ), 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin and grown at 37 °C in a humidified air with 5% CO_2.

The human tumor cell lines HT-29, LoVo, and HCT-116 were obtained from Interlab Cell Line Collections (Genoa, Italy). We generated the ITCs in situ by adding myrosinase to the respective GL precursor, as described above (*14*). For all cell treatments, KDE was added in the form of a water stock solution, which contained 10 mM GRH and 3 mM GRE.

MTT Assay. The effect of ITCs on cell viability was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, based on the reduction of the number of metabolically active cells, and the results were expressed as a percentage of the controls. Briefly, 3×10^3 cells/well were seeded into 96-well plates and treated with several concentrations of KDE-ITC, GRH-ITC, and GRE-ITC, for 3 and 6 days, after which 0.5 mg/mL of MTT (Sigma, St. Louis, MO) were added to each well and incubated for 4 h at 37 °C. Following the incubation, a solution containing 10% sodium dodecyl sulfate and 0.01 M HCl was added. After at least 18 h at 37 °C, the absorbance of each well was measured in a microplate reader (Bio-Rad, Hercules, CA) at 570 nm. The results were expressed as a percentage of the controls (*17*).

Sulforhodamine Assay. In order to confirm the data obtained by the MTT assay, we evaluated the amount of cells in samples by the sulforhodamine (SRB) assay. The SRB assay is based on the ability of SRB dye to bind protein basic amino acid residues. The amount of dye incorporated by the cells indicates the number of cells. Cells were plated in 96-well plates (3×10^3/well) and treated in the same way as the MTT assay. At the end of treatment, cell culture medium was eliminated and only RPMI was added (50 μL/well). The cells were fixed using 25 μL/well of 50% aqueous trichloroacetic acid for 1 h at 4 °C and rinsed with water several times, and 50 μL/well sulforhodamine B solution was added (0.4%) for 30 min. After being rinsed with 1% acetic acid and solubilized in 10 mM Tris for 5 min, the absorbance of each well was measured in a microplate reader (Bio-Rad) at 570 nm. The results were expressed as a percentage of the controls (*18*).

Cytotoxicity Test on T-Cells. T lymphocytes from blood samples of several donors (men and women) were isolated by density gradient centrifugation and erythrocyte resetting. T cells (8×10^4 cells/mL) were cultured in RPMI 1640 containing 10% FBS and 20 μg/mL phytohemagglutinin P (Difco Laboratories, Detroit, MI) (*19*). Viability was determined using MTT assay after treatment with KDE-ITC, GRH-ITC, and/or GRE-ITC.

Annexin V Apoptosis Detection Assay. Experiments were performed on cells seeded in six-well plates with slides, at a density of 8×10^4 cells/well, treated for one day with KDE-ITC and GRH-ITC plus GRE-ITC. Samples were prepared using 0.5 μg of annexin V FITC (Bender MedSystems, Austria) for 15 min in the dark. During this incubation, annexin V FITC binds only cells with PS flipped (apoptotic cells). After incubation, the cells were fixed with paraformaldehyde at 4 °C for 30 min and marked with 10 μL (1 μg/mL) of propidium iodide per 100 μL assay buffer. After being washed with PBS (phosphate-buffered saline) and water, samples were observed under a fluorescence microscope using a dual filter set for FITC and rhodamine (*20*).

Western Blot Analysis. To determine Bax, Bcl2, caspase-9, and poly(ADP-ribose) polymerase (PARP-1) levels, cells were plated and treated with KDE-ITC at different concentrations. The cells were detached and collected by centrifugation at 300*g* for 10 min, and pellets were resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5% Triton X-100, 5 μM Na_3VO_4) and sonicated on ice, in the presence of protease inhibitors. Protein concentration was determined by the method of Lowry (*21*).

Cell lysates (50 μg of protein per lane) were size-fractionated in 10% SDS-polyacrylamide gel prior to transfer to Hybond TM-C Extra membranes (GE Healthcare, Buckinghamshire, U.K.) by standard protocols. Membranes were blocked for 2 h with 5% milk in transfer buffer saline (Tris-HCl, 2.42%, NaCl, 8%, Tween 20, 0,1%, pH 7.4) (TBS) at RT. The membranes were incubated overnight at 4 °C with antibodies diluted 1:300: anti-Bax (Calbiochem, Darmstadt, Germany), anti-Bcl2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anticaspase-9 (Sigma, St. Louis, MO), obtained in rabbit and with antibody anti-PARP-1, obtained in mouse (Santa Cruz Biotechnology Inc.), and dissolved in TBS-milk 5%. The membranes were washed twice with TBS-milk 5% and were incubated for 1 h with the respective peroxidase-conjugated antibodies. Both the antirabbit and the antimouse peroxidase conjugated antibodies were diluted 1:1000 with TBS-milk 5%. The proteins were detected by luminol (GE Healthcare). Bands were quantified by using densitometric image analysis software (Image Master VDS, Pharmacia Biotech, Uppsala, Sweden). The amount of protein in each lane was the same, as confirmed by Actin (Sigma, St. Louis, MO).

Statistical Analysis. Data were expressed as mean ± SE. Differences were analyzed by Student's *t* test and considered statistically significant at $p < 0.05$ between the control and experimental samples.

RESULTS

Autoxidation Studies. The kinetics of oxygen consumption in a closed system were investigated during the AAPH-initiated controlled autoxidation of methyl linoleate in water/SDS micelles, by a Clark-Type microelectrode at 37.0 ± 0.1 °C. Under the experimental conditions employed, the recorded trace of oxygen consumption was linear and reflected the usual reaction scheme represented by eqs 3−7 where LH is the oxidizable substrate (e.g., methyl linoleate) and AH is a generic chain-breaking antioxidant (e.g., α-tocopherol) (*22*).

Initiation:

$$\mathrm{AAPH} \xrightarrow{\mathrm{O_2}} \mathrm{ROO^{\bullet}} \qquad (\mathrm{rate} = R_i) \tag{3}$$

$$\mathrm{ROO^{\bullet}} + \mathrm{LH} \rightarrow \mathrm{ROOH} + \mathrm{L^{\bullet}} \tag{4}$$

Propagation:

$$\mathrm{L^{\bullet}} + \mathrm{O_2} \xrightarrow{\mathrm{diff}} \mathrm{LOO^{\bullet}} \tag{5}$$

$$\mathrm{LOO^{\bullet}} + \mathrm{LH} \xrightarrow{k_p} \mathrm{LOOH} + \mathrm{L^{\bullet}} \tag{6}$$

Termination:

$$\mathrm{LOO^{\bullet}} + \mathrm{LOO^{\bullet}} \xrightarrow{k_t} \text{molecular products} \tag{7}$$

Injection of α-tocopherol, or its synthetic analogous 2,2,5,7,8-pentamethyl-6-chromanol, in the reaction mixture at concentrations as low as 1×10^{-6} M produced a neat inhibition of oxygen consumption for a period τ during which reactions 6 and 7 were outperformed by reactions 8 and 9, until all the antioxidant AH was consumed.

$$\mathrm{LOO^{\bullet}} + \mathrm{AH} \xrightarrow{k_{inhib}} \mathrm{LOOH} + \mathrm{A^{\bullet}} \tag{8}$$

$$\mathrm{LOO^{\bullet}} + \mathrm{A^{\bullet}} \xrightarrow{\mathrm{diff}} \text{molecular products} \tag{9}$$

As a control experiment injection of solvent (methanol) did not affect the rate of oxygen uptake (**Figure 1**)

On the other hand, injection of a methanol/water solution of KDE into the oxidizing mixture also inhibited the autoxidation process, but not completely, thereby yielding a "retarded" rate of oxygen consumption, which depended on the amount of KDE, hence on the relative rates of the inhibition and propagation processes.

For instance, the rate of oxygen consumption was reduced by 50%, with respect to an uninhibited autoxidation, by injection of KDE at a final concentration of 4.4×10^{-4} g/100 mL (4.4 ppm), as shown in **Figure 1**, in comparison with the injection of a ten times lower amount (by weight) of α-tocopherol under identical experimental settings.

Reaction with DPPH·Radicals. Rapid mixing of KDE (dissolved in methanol/water 98:2) with a methanol solution of DPPH· at 298 K produced very fast decay of the DPPH· radical, as judged from the change in absorbance at the characteristic $\lambda_{max} = 517$ nm. The kinetics of the reaction were therefore followed spectrophotometrically using a stopped-flow apparatus, as recently described for GRH and GRH-ITC purified from Daikon sprouts (*7*). The concentrations of the two reactants was initially adjusted on the basis of the GRH content in the extract (10.5% w/w). However, a much faster than expected DPPH· decay was observed, forcing us to dilute the mixing solutions. As shown in **Figure 2**, good decay traces were obtained by 1:1 mixing of 10−20 mg/L solutions of KDE with 2.0×10^{-5} M DPPH·. Pseudo-first-order kinetic treatment of the upper 1/3 decay curve afforded reasonably linear plots (see the insert in **Figure 2**). Processing of the pseudo-first-order rate constants with eq 2 made it possible to calculate the lifetime of DPPH· as a function of KDE concentration as reported in eq 10.

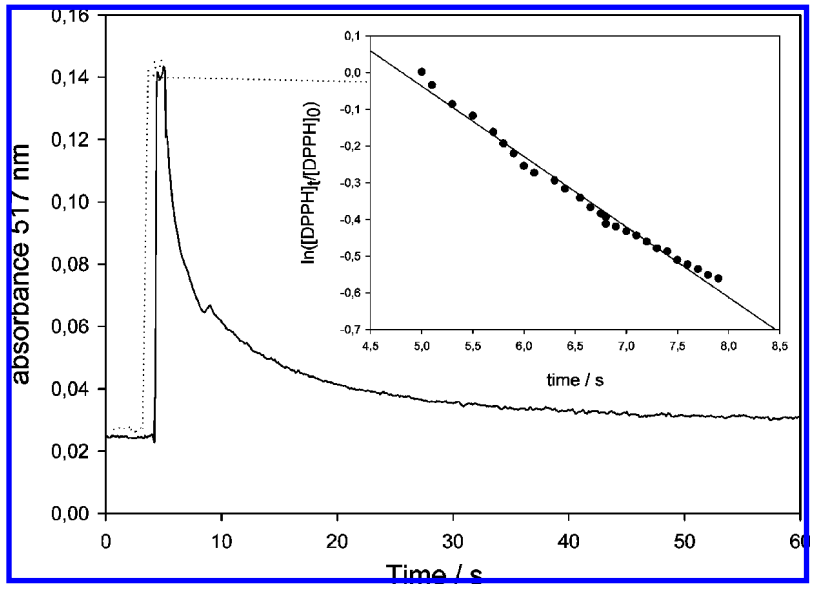

Figure 2. Kinetics of decay of DPPH· signal at 517 nm in methanol at 298 ± 2 K after rapid 1:1 mixing of a 2.0×10^{-5} M solution of DPPH· with the solvent (spontaneous decay, dotted line) or with a 10 mg/L solution of KDE in 98:2 methanol/water (full line). Insert: first order plot.

$$t_{1/2}\text{: (s)} = (1.47 \pm 0.25) \times 10^{-2}/[\text{KDE; (g/L)}] \quad (10)$$

For comparison, the lifetime obtained in **Figure 2** would have been produced (under identical conditions) by injecting a solution of α-tocopherol ~ 1×10^{-3} M (0.4 g/L) to quench the DPPH· radical.

Growth Inhibition. The effects of KDE-ITC on cell growth in three colon cancer cell lines (LoVo, HCT-116, and HT-29) were measured by MTT and sulforhodamine assay. KDE-ITC reduced cell proliferation in a dose-dependent manner, as shown in **Figure 3**. We compared the antiproliferative effect of KDE-ITC with GRH-ITC, GRE-ITC, and GRH-ITC plus GRE-ITC (at the same molar ratio as contained in KDE). Interestingly, while our results show that ITCs derived from Daikon are potent inhibitors of colon cancer cell growth, neither KDE nor GRH nor GRE alone, in the absence of myrosinase, showed growth inhibitory activity. Also, treatment with myrosinase alone had no effect on cell growth (data not shown). All the results obtained upon cell treatment with GL + myrosinase must therefore be attributed to the effect of the corresponding ITC. It is evident from **Figure 3** that KDE-ITC was markedly more effective in inhibiting cell growth in all cell lines with respect to any of its purified ITCs alone at the same dose. Interestingly, KDE was also slightly more effective that the mixture of GRE and GRH used at the same doses found in the extract.

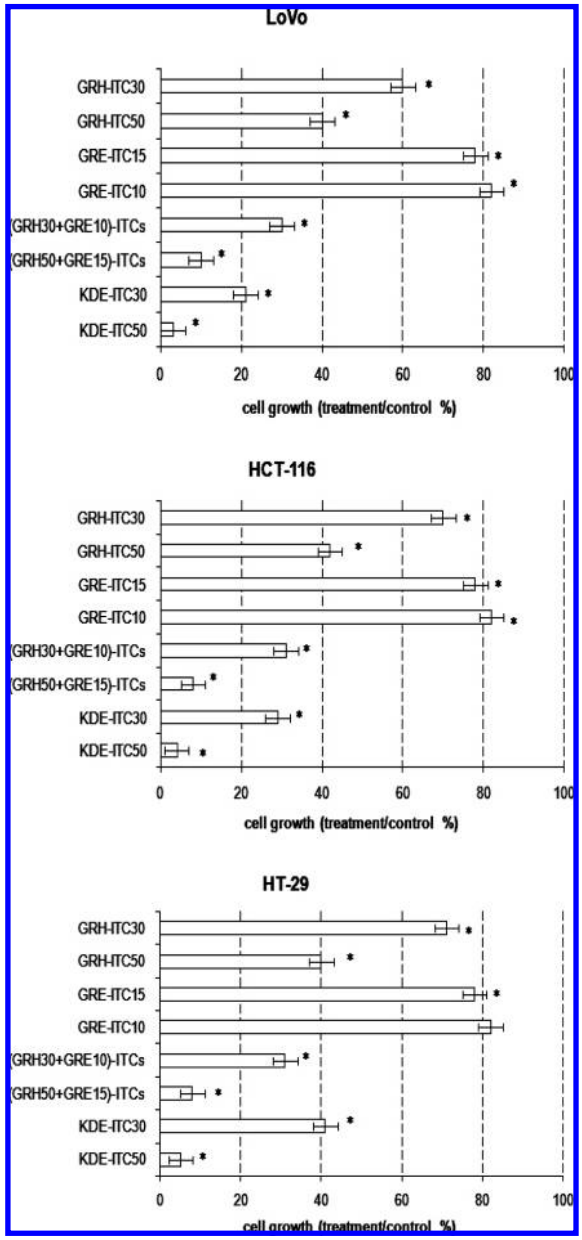

Figure 3. Effect after six days of different doses (μM) of KDE-ITC, GRH-ITC, GRE-ITC, and GRH + GRE ITCs on LoVo, HCT-116, and HT-29 cell growth as assessed by MTT assay. KDE-ITC dose refers to the final concentration of GRH-ITC (GRE-ITC dose was ~1/3 of GRH-ITC). Each bar represents the mean (±SE) of six replicate cultures from three independent experiments. *, $p < 0.05$.

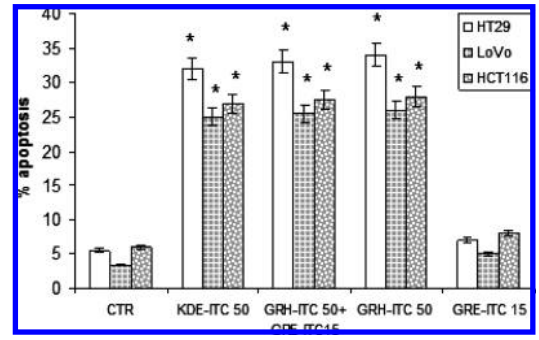

Figure 4. Apoptosis induction by (μM) KDE-ITC, GRH-ITC, GRE-ITC, and GRH + GRE ITCs in HT-29, LoVo, and HCT-116 cells after 24 h exposure as assessed by annexin V apoptosis assay. KDE-ITC dose refers to the final concentration of GRH-ITC (GRE-ITC dose was ~1/3 of GRH-ITC). Apoptosis was evaluated by counting FITC-labeled cells in five random fields at least and expressed as a percentage of total cells. CTR: untreated cells. Each bar represents the mean (±SE) of four experiments. *, $p < 0.05$.

Apoptotic Activity. In order to evaluate whether the inhibition of cell proliferation caused by ITC was accompanied by apoptosis, annexinV/propidium iodide staining analysis was performed. FITC-labeled annexin V binds the newly exposed phophatidylserine (PS) at the outer membrane leaflet. As demonstrated by annexinV analysis, KDE-ITC displayed apoptotic activity in the three lines tested; furthermore, apoptotic activity induced by KDE-ITC was as high as that induced by GRH-ITC: in HT-29 cells, treatment with 50 μM KDE-ITC or GRH-ITC increased the percentage of apoptosis to 33% and to 34%, respectively (**Figure 4**.) Addition of GRE-ITC only contributes minimally to apoptosis in all tested cell lines. In fact, when the compound was used alone, it did not produce significant variations with respect to controls.

Bcl-2 and Bax Expression. We evaluated the effect of KDE-ITC on Bcl-2 and Bax protein expression (**Figure 5**.) As demonstrated by Western blot analysis, KDE-ITC caused a strong downregulation of Bcl-2 protein and upregulation of Bax in all colon cancer lines tested. Indeed, proteins of the Bcl-2 family comprise both pro-apoptotic (e.g., Bax) and antiapoptotic (e.g., Bcl-2) members and are the main regulators of the intrinsic mitochondrial pathway to apoptosis. The ratio of pro- to antiapoptotic proteins is believed to play an important role in determining cell death (*23*).

Hence, both variations observed here for Bax and Bcl-2 proteins in the three investigated cell lines, treated with KDE-ITC, clearly confirm induction of apoptosis.

Caspase-9 and PARP-1 Expression. Activation of caspase-9 and PARP-1 is a very important step in the apoptotic process. As shown in **Figure 6A**, in HT-29 cell line we found that 50 μM KDE-ITC and 50 μM GRH-ITC plus 15 μM GRE-ITC doses induced a strong cleavage of the 47-kDa pro-enzyme caspase-9 into its active 35-kDa form, which was observed starting from 3 days treatment onward. We also observed (**Figure 6B**) the PARP-1 cleavage, seen as a typical 89-kDa band, which was almost complete after 3 days of treatment.

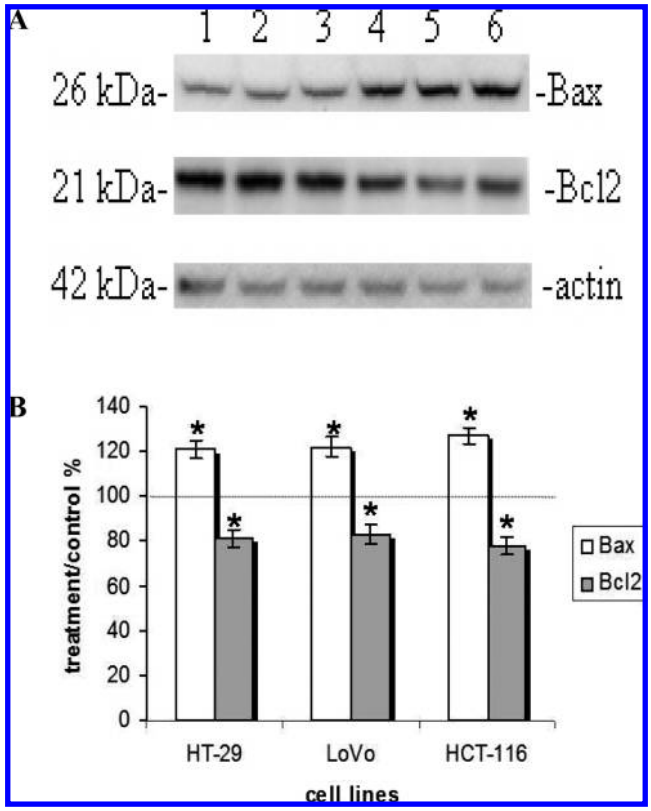

Figure 5. (A) Bax and Bcl2 protein levels in HT-29 (1), LoVo (2), and HCT-116 (3) cell lines before and after 48 h of treatment with 50 μM KDE-ITC (4:HT-29, 5: LoVo; 6: HCT-116), as assessed by Western blot. KDE-ITC dose refers to the final concentration of GRH-ITC (GRE-ITC dose was ~1/3 of GRH-ITC). (B) Densitometric data from Western blot expressed as percentage of treated samples with respect to control (CTR). Each bar represents the mean (±SE) of three independent experiments. The dotted line indicates the control value (100%). Asterisks indicate significant difference with respect to CTR: *, $p < 0.05$.

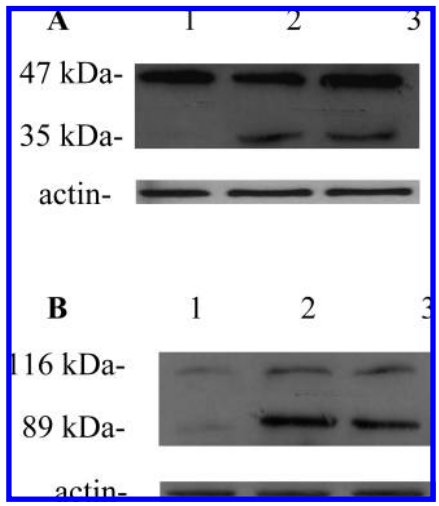

Figure 6. Western blot analysis for caspase-9 (**A**) and PARP-1 (**B**) protein in HT-29 cells, after 3 days exposure to 50 μM KDE-ITC (2), 50 μM GRH-ITC plus 15 μM GRE-ITC (3). 1: untreated cells. Representative blots from one of three experiments. KDE-ITC dose refers to the final concentration of GRH-ITC (GRE-ITC dose was ~1/3 of GRH-ITC).

These results were confirmed also in LoVo and HCT-116 colon cancer cell lines (data not shown).

Lymphocyte Cell Viability. To assess the cytotoxic effect of ITCs contained in Daikon sprouts on healthy human lymphocytes, normal T-lymphocytes were exposed for three and six days to KDE-ITC 30 μM, GRH-ITC 30 μM, GRE-ITC 30 μM or 10 μM, and GRH-ITC plus GRE-ITC at the same molar ratio as found in KDE (3:1). The same treatment scheme was then reproduced using the 50 μM dose. Data are collected in **Figure 7** and statistically significant ($p < 0.05$) variations from control are marked by an asterisk. It is shown that, after six days of treatment, only 50 μM GRE-ITC and 50 μM GRH-ITC + 15 μM GRE-ITC caused a statistically significant reduction of lymphocyte proliferation with respect to the untreated control, while at a 30 μM dose GRE-ITC was the only treatment that significantly reduced lymphocyte viability after 6 days. All the remaining treatments only produced nonsignificant inhibitions with respect to the control. Interestingly, therefore, while GRE-ITC at 50 μM caused a 50% growth inhibition (after 6 days) and the combination of GRH-ITC and GRE-ITC a smaller but significant toxicity (−19% cell viability after 6 days), treatment with 50 μM KDE-ITC, i.e. at the same dose as its purified ITCs, produced no significant toxicity after 3 or 6 days of treatment.

DISCUSSION

Michael Heinrich's concept of the drug as a "magic bullet" ideally having to target a single receptor or enzyme, i.e. a single biological partner at molecular level, has guided medicinal chemists since the 1980s, through about two decades of illuminated drug discovery. Although such selectivity has never actually been achieved, aiming at that utopia has led toward the development of "cleaner and cleaner" drugs with specific medical indications and limited side effects. This approach has however failed to find answers to complex illnesses such as cancer. Medicinal scientists are now interestingly looking in the opposite direction: the most promising approach to treat complex disorders like cancer is apparently "playing dirty", i.e. looking for "magic shotguns", molecules or mixtures of molecules able to simultaneously target multiple points and have multiple biological targets, so as to prevent compensatory mechanisms by the cells (*24*).

In this vision, a vegetable extract made up of hundreds of molecules, including, as in the present case, several biologically active compounds, is potentially an interesting tool for achieving this goal. The results presented here, particularly when combined with previous investigations on KDE (*9*) or single GLs and ITCs purified from Daikon (*7, 8, 25*), certainly compose a complex picture on the potential biological effects of KDE.

Our current results show that KDE-ITCs produces a significant inhibition of cell proliferation on human colon carcinoma cell lines (HT-29, LoVo, and HCT-116). This effect is sustained by a pro-apoptotic action, confirmed by different approaches. Activation of caspase 9 and PARP-1 cleavage suggests that apoptosis might be initiated through an internal mitochondrial way, which is supported also by reduction of Bcl-2 that would normally block apoptosis by inhibiting the release of mitochondrial proteins and phosphatidyl serine (PS) exposure. Indeed, the exposure of PS residues at the outer plasma membrane serves as a trigger for phagocytosis, and it has been shown to occur early during the *effector phase* of apoptosis, preceding the loss of plasma membrane integrity and DNA fragmentation. Treatment with 50 μM KDE-ITC, similarly to treatment with the pure ITCs contained in the extract (GRH-ITC and GRE-ITC), induces PS exposure, as demonstrated by the annexin V test.

Interestingly, although KDE-ITC induces parallel mechanisms of apoptosis as compared with its "bioactive" components at the same dose as contained in the extract, its antiproliferative effect on the three tested cancer cell lines was noticeably higher than that of GRE-ITC both at 30 and 50 μM doses. When the two main ITCs contained in the extract (as GRH and GRE precursors) were used in combination at the same molar ratio

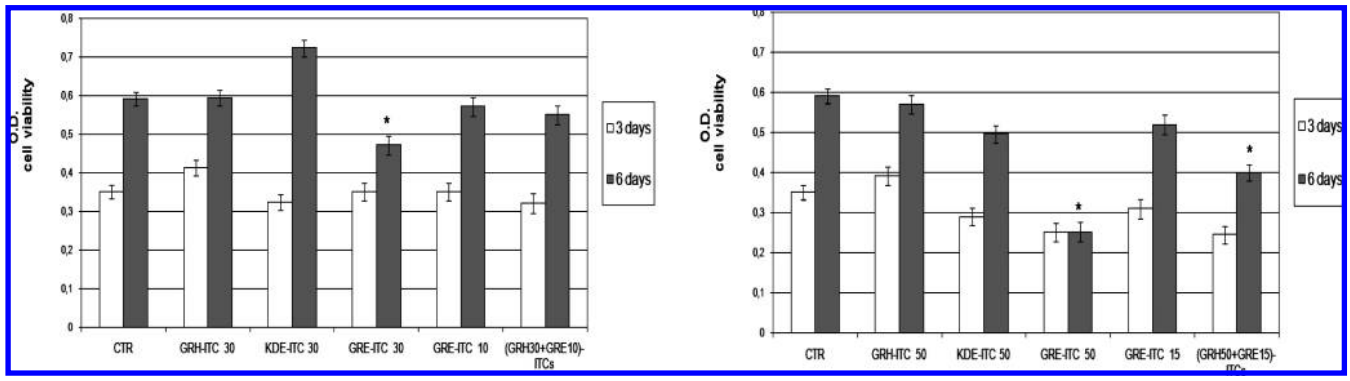

Figure 7. Effect after three and six days of treatment with KDE-ITC 30 μM (left) and 50 μM (right) on lymphocyte cell viability as assessed by MTT assay. KDE-ITC dose refers to the final concentration of GRH-ITC (GRE-ITC dose was ~1/3 of GRH-ITC). The data represent the viability of cells, expressed as optic density (O.D.). CTR: control. *, $p < 0.05$.

found in KDE, KDE itself still showed a slightly higher activity, possibly indicating that some additional bioactive component is contributing to the effect (vide infra).

Despite toxicity in cancer cell lines, the toxicity on normal human T-lymphocytes was significant only after 6 days of treatment at the highest test dose of 50 μM GRE-ITC and was negligible at 30 μM. Also from **Figure 7** it is apparent that KDE-ITC at 30 μM or 50 μM shows lower toxicity than GRH-ITC and GRE-ITC or a combination of the two.

The antioxidant behavior of KDE is certainly the most obvious property that differentiates the extract from its purified GLs or ITCs. Unlike GRH and GRH-ITC (*7*), KDE possesses relevant chain-breaking antioxidant behavior. Indeed it was able to significantly retard the forcedly initiated autoxidation of methyl linoleate in water/SDS micelles. It should be noted that the results reported in **Figure 1** (50% reduction of the rate of autoxidation) were obtained with a "dose" of KDE corresponding to the administration of about 300 mg of extract to an individual weighing 70 kg, i.e. at a dose totally compatible with its use as a chemopreventive diet supplement. Looking at **Figure 1** also clearly indicates that the ability of KDE to stop methyl linoelate autoxidation is lower than other natural antioxidants such as vitamin E (α-tocopherol), which was able to produce complete inhibition (as compared to retarding) of the autoxidation at a ten times lower dose (by weight). A more quantitative kinetic treatment and a more detailed discussion of the results obtained from autoxidation studies are not possible at this stage, partly because the actual rate of oxygen consumption in heterogeneous media would depend on the actual distribution of the antioxidant and oxidizable substrate in the micelles as well as on the rate of exchange (diffusion) of the species involved in the process (antioxidant and peroxyl radicals) between micelles and water (*26, 27*) but mostly because it would require knowledge of what are the actual antioxidant species in KDE. By comparing these results to those previously reported on GRH, GRE, and the corresponding ITCs (*7*), it is obvious that the chain-breaking antioxidant activity of KDE is not due to these components but can instead be attributed to other antioxidants possibly contained in the extract, e.g. ascorbate, phenols, or thiols. Previous studies (*8, 9*) and our ongoing analytical investigation (vide infra) suggest that the content of ascorbate is negligible in our standardized KDE and is not likely to significantly contribute to the observed antioxidant behavior. On the other hand, KDE was reported to have a total phenolic content of 62%, expressed as gallic acid equivalents, determined by the Folin−Ciocolteu reagent and spectrophotometric analysis (*9*). This widely employed method, however, is known to be subject to interference by many reducing substrates (*28*), clearly including GRH itself. The reported value therefore certainly overestimates the actual content of phenolic components. For this reason we undertook, for several components of KDE, a detailed analytical characterization, which is currently in progress and will be the subject of a specific publication. Preliminary results (*29*) suggest that the antioxidant composition might significantly differ from the pattern reported by Takaya et al. for a methanol extract of Daikon sprouts (*30*). The very high reactivity in reducing DPPH• radicals also applies for the considerable radical-scavenging potential of KDE. For the sake of comparison, not only did KDE quench DPPH• radicals impressively more effectively than we would have expected on the basis of its content in GRH, but the lifetime of DPPH• in our experiments was as short as we would have obtained with an approximately 40-fold amount by weight of pure α-tocopherol (*7*). This clearly suggests that, beside GRH, some other antioxidant components are significantly contributing to the antioxidant and radical-scavenging ability of KDE.

Although detailed knowledge on the structure of the actual antioxidant species would be needed for deeper discussion, from current data it would appear that, in KDE, the antioxidant/radical-scavenging activity is not related to the apoptotic activity, since this latter activity is mostly due to its content of ITCs, which, in turn, give limited contribution to the overall radical-scavenging ability of the extract and no contribution to its chain-breaking antioxidant activity (*7*).

In conclusion, the results reported herein show that KDE has a similar spectrum of chemopreventive activity compared with the use of its purified GLs/ITCs. The pattern of apoptotic activity was largely superimposable on that obtained with GRH-ITC plus GRE-ITC at the doses corresponding to their content of the extract, and the cytotoxic activity on three human colon carcinoma cell lines was actually increased in KDE-ITC, with an interesting decrease in the cytotoxic activity on healthy (normal) T-lymphocytes. On the other hand, the radical-scavenging activity of KDE largely outperforms that of its GLs and ITCs and, unlike them, it possesses considerable chain-breaking antioxidant activity. When seen together with the results of previous investigations on the same extract and on its components, these data profile a very interesting and broad spectrum of biochemical properties that make KDE a promising, multipotent chemopreventive agent.

ACKNOWLEDGMENT

We thank Dr. M. Brigotti for T-cells and Drs. R. Amorati and E. Plescia for assistance with autoxidation studies.

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Received for review April 10, 2008. Revised manuscript received May 28, 2008. Accepted June 19, 2008. Financial support from the University of Bologna (Italy) and MIUR (Rome, Italy), as well as from the Research Centre for Industrial Crops (Bologna, Italy) and Agricultural Research Council (Rome, Italy), is gratefully acknowledged.

JF8011213