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Vasomodulating Potential of Mediterranean Wild Plant Extracts

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The incidence of cardiovascular disease and endothelial dysfunction is low in the Mediterranean area, where the major proportion of daily calories comes from plant food, high in antioxidant polyphenols. It has been shown that a reduced production or enhanced inactivation of endothelium-derived nitric oxide (NO) is involved in the onset of endothelial dysfunction. We investigated the effects of Mediterranean wild plant, that is, wild artichoke and thyme, phenolic-rich extracts on NO release by porcine aortic endothelial cells (PAECs; by using indirect methods) and by cerebral cell membrane homogenates (by using direct NO detection). NO release by PAECs was significantly potentiated by 234% and 135% by wild artichoke and thyme extracts (10^{-6} mol/L), respectively. Direct detection of NO release by brain membranes also showed significantly increased NO production after wild artichoke addition (+35.4%). Further, the release of another vasorelaxant factor by PAECs, that is, prostacyclin, was significantly increased by wild artichoke and thyme (10^{-6} mol/L) (+269% and +190%, respectively). Investigation of the mechanism(s) of action of wild artichoke and thyme suggests maintenance of an intracellular reduced environment, as previously shown for ascorbate. Even though these data require in vivo confirmation, they suggest that regular intake of bioactive compounds from Mediterranean wild plants contributes to maintenance of proper vasomotion and to the low incidence of atherosclerosis and endothelial dysfunction recorded in the Mediterranean area.

KEYWORDS: Nitric oxide; Mediterranean diet; antioxidants; polyphenols; endothelial dysfunction

The lower incidence of coronary heart disease (CHD) in the Mediterranean basin stimulated interest in the diet adopted by populations in this area. In particular, the cardioprotective effects of this diet have been attributed to the high proportion of plant foods, either in the form of complex carbohydrates or as fresh vegetables, spices, legumes, and fruits; consumption of olive oil as the principal source of fat; and moderate consumption of wine with meals (*1*). The products of plants' secondary metabolism, namely, flavonoids and polyphenols, are being actively investigated, as research is disclosing several "pharmacological" activities of such compounds (*2*). For example, the antioxidant properties of polyphenols might be partly responsible for the lower incidence of atherosclerosis, while modulation of some enzymatic activities relevant to cardiovascular disorders might confer further cardioprotective potential to a plant-food-rich diet (*3*).

Endothelial dysfunction is a major complication of atherosclerosis (*4*), and evidence is accumulating of an involvement of oxidative stress in its onset and maintenance (*5*, *6*). Reduced production/availability of the vasorelaxant factor nitric oxide (NO) plays a major role in the oxidative-stress-related development of endothelial dysfunction (*5*, *7*). Accordingly, administration of antioxidants, for example, vitamin C and flavonoids from tea and wine, has been shown to ameliorate endothelial function and vasomotion (*7*, *8*) and increasing evidence over the past decade shows that several dietary factors may partly modulate nitric oxide synthase (NOS) activity (*9*).

The project "Local Food Nutraceuticals" (QLK-2001-00173) was undertaken to investigate the effects of extracts obtained from selected, phenol-rich wild plants traditionally eaten in the Mediterranean area on the production of NO and prostacyclin by cultured aortic endothelial cells. In particular, we report results obtained with *Cynara cardunculus* (wild artichoke, LYNC) and *Thymus pulegioides* (wild thyme, LTHY) extracts.

MATERIALS AND METHODS

Plant samples were collected in Southern Italy (Castelmezzano) and were dried, extracted with 90% hot ethanol under reflux, and provided by the group of Dr. Michael Heinrich, School of Pharmacy, University of London. The extraction and characterization procedure will be fully reported elsewhere by that group. The total polyphenolic content of the extracts was determined by the Folin−Ciocalteau method, using gallic acid as the reference compound (*10*).

The extracts were analyzed by HPLC coupled with ESI and DAD-UV detectors, using a Waters Xterra MS-C18 column and a H_2O−AcN/MeOH gradient elution. Major peaks were identified by comparison with authentic standards and a database.

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Primary Cell Culture of Porcine Aortic Endothelial Cells (PAECs). PAECs were harvested from pig aorta using standard techniques (*11*) and were kindly provided by Drs. Shane R. Thomas and John F. Keaney, Jr., Boston University.

The cells were grown in gelatin-precoated Petri dishes, with M199 medium supplemented with 15% fetal bovine serum and penicillin−streptomycin. Heparin (50 μg/mL) was added to the medium in order to prevent potential proliferation of fibroblasts. In all experiments, PAECs of passage five to seven were used, as they maintain unaltered eNOS activity in response to calcium ionophore (Dr. Shane R. Thomas, personal communication).

Total protein was estimated by using the Bradford method, with albumin as the standard (*12*).

Assay of Nitric Oxide Synthase Activity. Confluent cells grown in 12 well plates were incubated for 16 h in M199 medium containing 20% fetal bovine serum and the indicated compounds. At the end of the incubation period, the medium was replaced with 500 μL of 22 mmol/L HEPES buffer, pH 7.4, containing 1 mmol/L $MgCl_2$, 1.5 mmol/L $CaCl_2$, 10 μM indomethacin, and 200 μmol/L 3-isobutyl-1-methylxanthine. The cells were equilibrated for 30 min at 37 °C. Then, 200 pmol/L of L-[^{14}C]arginine was added to each well, and 10 min later the cells were stimulated with the calcium ionophore A23187 (2 μmol/L). After 10 min of incubation at 37 °C, the reaction was stopped by the addition of ice-cold phosphate-buffered saline (PBS), containing 5 mmol/L l-arginine and 4 mmol/L EDTA.

The cells were lysed by the addition of NaOH 0.1 N, neutralized with HCl 0.1 N, and l-[^{14}C]citrulline was separated by ion exchange chromatography with AG 50W-X8 resin, sodium form.

After the addition of scintillation cocktail (Beta Blend; ICN, Costa Mesa, CA), radioactivity was determined by scintillation counting.

In some experiments, the eNOS inhibitor *N*ω-nitro-L-arginine (L-NNA) was added to the medium.

Assays of Endothelium-Derived NO and of Prostacyclin. Confluent PAECs in 12 well plates were equilibrated for 30 min in HEPES buffer. After incubation, the cells were stimulated with 2 μmol/L A23187.

We assayed endothelium-derived NO as the accumulation of cGMP, with a radioimmunoassay (Amersham). Further, the production of prostacyclin was evaluated as accumulation of its stable metabolite 6-keto-$PGF_{1\alpha}$, with a commercially available enzyme immunoassay kit (Cayman).

Direct Measurement of NO Release. Due to the low levels of directly detectable NO production by PAECs (as evaluated in preliminary experiments), an additional experimental model where NO can be released in relatively abundant quantities was set up. We directly measured NO production by brain cell membranes with the use of an NO-specific amperometric electrode (ISO-NO MarkII, World Precision Instruments), placed inside a Faraday's cage. The sensor was calibrated with *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), according to the manufacturer's instructions and as described by Zhang et al. (*13*). Each experiment was repeated three times. A rat brain was homogenized in PBS containing EGTA (100 μmol/L), dithiothreitol (DTT, 100 μmol/L), and a cocktail of protease inhibitors. Following centrifugation to remove cellular debris (120*g* for 10 min), cell membranes were separated by ultracentrifugation (100 000*g* for 60 min). The protein content of the pellet was assessed according to Lowry et al. (*14*). The assays were performed at 37 °C with constant, gentle mixing. Cell membrane protein (100 mg) was added to a mixture of calmodulin (1 μmol/L), flavin adenine dinucleotide and flavin mononucleotide (1 μmol/L), tetrahydrobiopterin (50 μmol/L), DTT (1 mmol/L), NADPH (1 mmol/L), and $CaCl_2$ (2 mmol/L). The reaction was started by the addition of A23187 (2 μmol/L).

Western Blot Analysis. Confluent cells were treated as for the eNOS activity experiments. At the end of the incubation period, cells were removed in the presence of protease inhibitors. Aliquots of the samples were run on a 10% polyacrylamide gel, under denaturating conditions. Proteins were blotted onto a nitrocellulose membrane and were incubated with a 3% solution of defatted dried milk in TBS-T containing 0.1% Tween 20, to block nonspecific binding sites. Then, a mouse monoclonal antibody (anti-nitric oxide synthase; Calbiochem, San Diego, CA) diluted in TBS-T was added (1:1000), and the membranes were incubated overnight.

The membranes were then incubated for 1 h, at room temperature, in TBS-T containing the second antibody, a horseradish peroxidase labeled anti-mouse IgG (1:5000; Biorad, Hercules, CA). Extensive washing in TBS-T followed each step. Antigen detection was achieved by incubation with ECL (Amersham Biosciences, Little Chalfont, U.K.) detection solution and exposure to film (Kodak; Rochester, NY).

Extraction of Total RNA from PAECs. Total RNA was isolated from PAECs after the treatments with the compounds, as described above. The cell monolayers were lysed in denaturing buffer containing guanidium thiocyanate. Ethanol precipitation was then performed on the lysate, and the resultant pellet was resuspended in denaturing buffer. A chloroform extraction was performed and was followed by 2-propanol precipitation. The resultant pellet was washed in 75% ethanol, briefly air-dried, and resuspended in diethylpyrocarbonate (DEPC)-treated water. The integrity and purity of the total RNA were assessed by gel electrophoresis, and its yield was determined by spectrophotometry.

Preparation of cDNA and Polymerase Chain Reaction (PCR). One microgram of total cellular RNA from PAECs was reverse-transcribed into first strand cDNA.

Reverse transcription was performed in cDNA synthesis buffer added with 25 μmol/L Oligo-dT, 0.5 mmol/L dNTP, 0.5% DEPC water, 5 mmol/L DTT, 2 U/μL RNasi OUT, 0.025 U/μL Thermoscript (Invitrogen, Carlsbad, CA), and 2 μg mRNA.

Reverse transcription was performed for 50 min at 50 °C, 5 min at 85 °C, and 10 min at 15 °C.

PCR was performed in a total volume of 50 μL of PCR buffer added with 0.2 μmol/L dNTP, 2.5 U/μL TAQ polymerase (Amersham Life Science, Cleveland, OH), 1.5 mmol/L $MgCl_2$, 0.5 μmol/L primer (eNOS and β-actin), 0.4 μg cDNA, and DEPC water.

PCR was performed with a Perkin-Elmer Gene Amp PCR system 2400, after an initial DNA-denaturation (5 min at 95 °C) step, as follows: 30 cycles of denaturation for 30 s at 94 °C; annealing and extension for 6 min at 68 °C.

We used the following primers: eNOS sense gene-specific primer 5′-GAACACGAGACGCTGGTGCTGGTGG-3′ and eNOS anti-sense gene-specific primer 5′-GGAGCCGAGCCCGAACACACAGAAC-3′ (Genenco, Cornaredo, Italy); β-actin sense gene-specific primer 5′-TGGAGAAGAGCTACGAGCT-3′ and β-actin anti-sense gene-specific primer 5′-ACATCTGCTGGAAGGTGGAC-3′ (Invitrogen, Paisley, U.K.). The gene-specific primers were designed based on a highly conserved region of the human eNOS c-DNA. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide, and the relative intensities of the bands were quantified using a scanning densitometer (software: Quantity One, Biorad, Hercules, CA).

Cytotoxicity Assay. The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was assessed by a slight modification of the method of Denizot et al. (*15*). MTT was stored as a 5 mg/mL stock solution in PBS pH 7.5. Before use, it was filtered through a 0.22 μm filter and diluted to 0.5 mg/mL. Confluent PAECs in 12 well plates were supplemented with the extracts, and at the end of the incubation period the medium was replaced with 500 μL of MTT solution. After 3 h of incubation at 37 °C, the solution was removed and a mixture of 2-propanol and DMSO (90:10; v/v) was added to each well. After 20 min of incubation at 37 °C, the absorbance was read at 550 nm.

Statistical Analysis. All data are given as means ×b1 standard deviation (SD) of three to five independent experiments. Concentrations are expressed as gallic acid equivalents (GAE). The statistical significance was determined by analysis of variance with Bonferroni's correction for multiple comparisons.

RESULTS

HPLC−MS analysis of wild artichoke and wild thyme extracts (**Figure 1**) confirmed their high proportion in phenolic compounds, as previously shown by other investigators (*16*, *17*).

Supplementation of PAECs with wild artichoke or thyme extracts (10^{-6} mol/L GAE) significantly increased eNOS activity by 234% and 135%, respectively, as assessed by increased

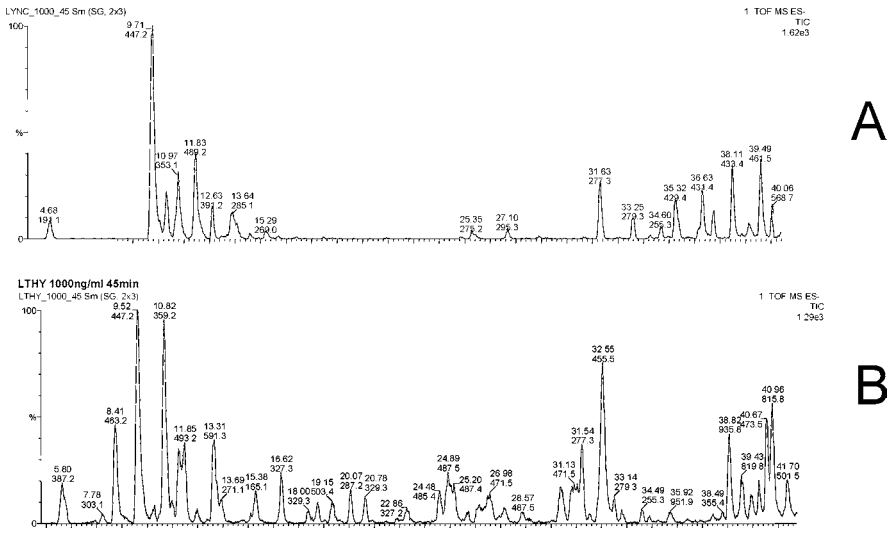

Figure 1. HPLC–ESI/DAD analysis of wild artichoke (**A**) and wild thyme (**B**) extracts. Phenolic compounds are luteolin-7-glycoside (9.71 min), apigenin (15.29 min), rosmaric acid (10.82 min), asiatic acid (24.89 min), ursolic acid (32.55 min), and flavonoid glucoside yet to be identified (8.41 and 9.52 min).

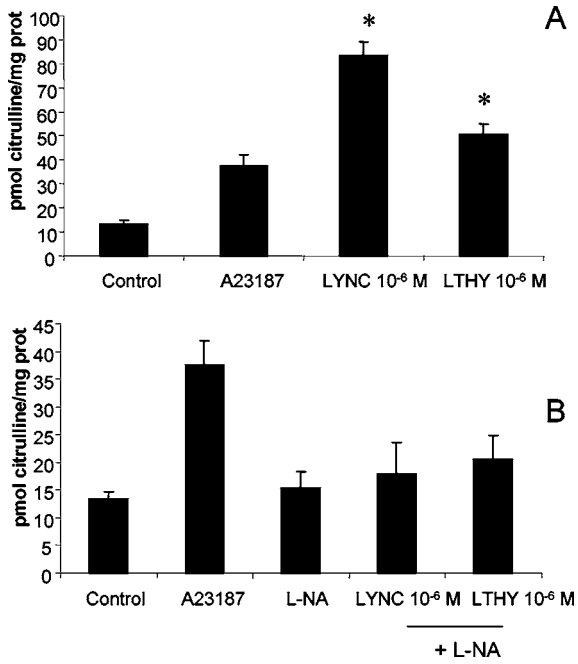

Figure 2. Effect of wild plant extracts on eNOS activity in porcine aortic endothelial cells. Confluent PAECs were incubated with *C. cardunculus* (LYNC) or *T. pulegioides* (LTHY) extracts for 16 h. The medium was replaced with HEPES buffer, and eNOS activity was triggered by the addition of the calcium ionophore A23187 (2 μmol/L) and determined by ion-exchange chromatography as the conversion of L-[^{14}C]arginine to L-[^{14}C]-citrulline. eNOS activity was determined either in the absence (**A**) or in the presence (**B**) of the eNOS inhibitor L-NNA (300 μmol/L).

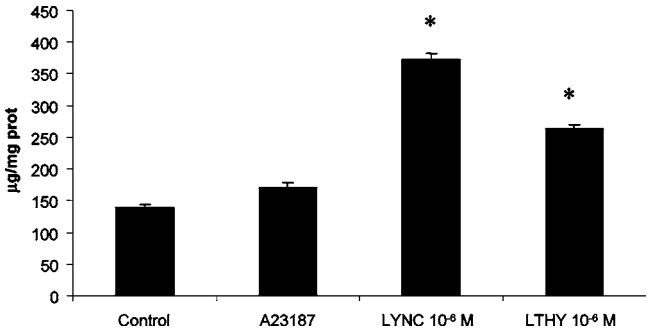

Figure 3. Effect of wild plant extracts on prostacyclin production by porcine aortic endothelial cells. Confluent PAECs were incubated with *C. cardunculus* (LYNC) or *T. pulegioides* (LTHY) extracts for 16 h. The medium was replaced with HEPES buffer, and cycloxygenase activity was triggered with the calcium ionophore A23187 (2 μmol/L). Prostacyclin production was assessed as the formation of its stable metabolite 6-keto-$PFG_{1\alpha}$, which was evaluated by immunoassay.

conversion of L-arginine to L-citrulline (**Figure 2**, panel A). This effect was inhibited by co-incubation with L-NNA (300 μM), further suggesting that the tonic effects of plant extracts are due to enhanced enzymatic activity (**Figure 2B**).

The production of another vasomodulating agent, that is, prostacyclin, was also significantly increased by wild artichoke and thyme supplementation (+269% and +190%, respectively) (**Figure 3**).

Enhanced NO production by wild artichoke addition was further verified (in a rat brain homogenate) by a direct method, that is, by the use of a sensitive NO sensor. Co-incubation with wild artichoke extract (10^{-5} M) increased NO production by 35.4%, that is, from 56.2 ± 3.6 nmol/L to 75.6 ± 4.1 nmol/L peak values over a 5 min time period (**Figure 4**). This effect was also inhibited by L-NNA (300 μmol/L) (data not shown).

When the effects of extract supplementation on NO major effector, that is, cGMP, were assessed, no significant change was noted, even though a trend toward increased cGMP production was recorded (**Figure 5**).

To investigate the effects of wild artichoke and thymus on eNOS expression, both Western blot analysis of the enzyme and RT-PCR of its DNA were performed. As reported in **Figures 6** and **7**, a comparable eNOS expression in all samples, that is, controls or supplemented, was found under all experimental conditions.

The possibility that wild artichoke and thyme extracts were toxic to PAECs, at least at the concentrations we used, was ruled out by cytotoxicity assays (**Table 1**).

DISCUSSION

This study demonstrates that phenolic-rich extracts of wild plants collected in the Mediterranean area, that is, wild artichoke and wild thyme, are able to increase NO production by stimulated endothelial cells and by a brain homogenate. This finding adds to the body of basic research supporting the epidemiological evidence of a lower CHD incidence in the

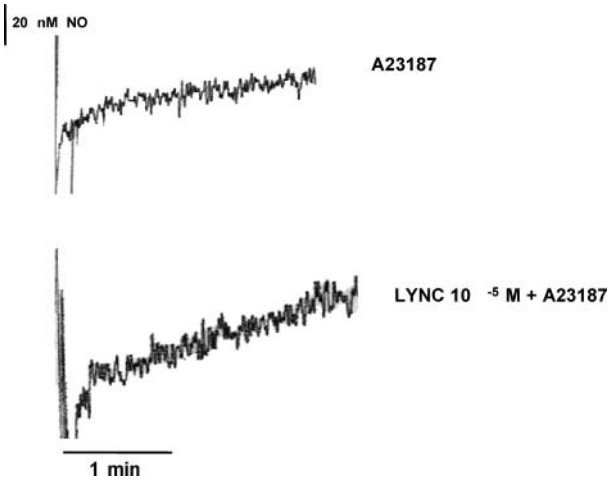

Figure 4. Real time representation of the effect of a *C. cardunculus* extract (LYNC) on NO production by brain cell membranes. NO production was stimulated by the addition of the calcium ionophore A23187 (2 μmol/L) and was continually recorded with a NO-specific amperometric electrode, calibrated with SNAP (*13, 46*). Experiments were repeated four times.

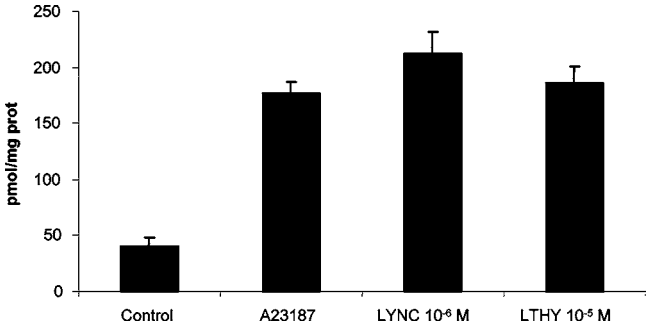

Figure 5. Effect of wild plant extracts on cGMP production in porcine aortic endothelial cells. Confluent PAECs were incubated with *C. cardunculus* (LYNC) or *T. pulegioides* (LTHY) extracts for 16 h. The medium was replaced with HEPES buffer, and PAECs were stimulated by the addition of the calcium ionophore A23187 (2 μmol/L). cGMP accumulation was determined by immunoassay.

Mediterranean area; further, it suggests that maintenance of correct vasomotion by bioactive components of the Mediterranean diet might contribute to its cardioprotective properties. Indeed, most studies have thus far focused on the role of macronutrients, for example, low intake of saturated fat and high intake of fiber and complex carbohydrates, in modulating the plasma lipid profile of people on the Mediterranean diet. However, accumulating evidence suggests that the incidence of "classic" risk factors for CHD is not much different between populations of the Mediterranean region and those of other areas such as northern Europe and the United States (*18*, *19*). As a consequence, the hypothesis was formulated that other, as yet not fully identified, risk factors play a role in the onset and development of CHD (*20*). Among such factors, enhanced oxidative stress leading to modification of low-density lipoprotein (LDL) or to impaired vascular reactivity has been implicated in cardiovascular morbidity and mortality and is nowadays being recognized as one of the risk factors for CHD (*21*, *22*). Even though antioxidant therapy, namely, with vitamin E, does not appear to decrease atherosclerosis and cardiovascular mortality (*23*) and yielded equivocal results when applied to the treatment of endothelial dysfunction (*24*), clinical data are supportive of a salubrious effect of vitamin C administration (*8*). Thiol agents

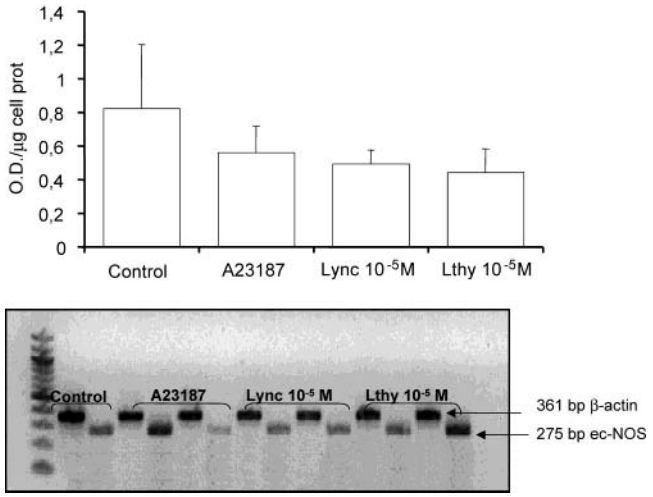

Figure 6. Effect of wild plant extracts on eNOS levels in porcine aortic endothelial cells. Confluent PAECs were incubated with 10^{-5} mol/L *C. cardunculus* (LYNC) or *T. pulegioides* (LTHY) extracts for 16 h, and eNOS activity was triggered by the addition of the calcium ionophore A23187 (2 μmol/L). Cells were then lysed, proteins were resolved by SDS-PAGE, and Western blots were performed as described under Materials and Methods with anti-eNOS antibody (Calbiochem).

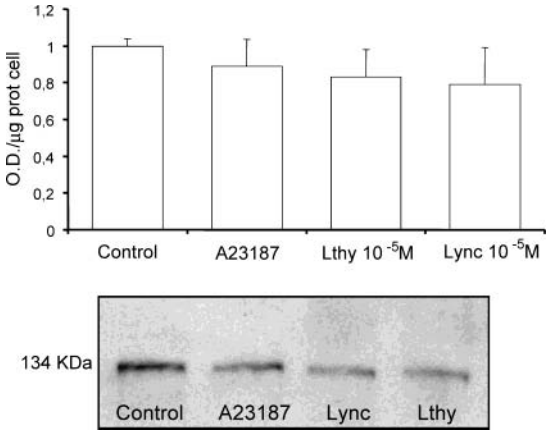

Figure 7. Effect of wild plant extracts on eNOS RNA expression in porcine aortic endothelial cells. Confluent PAECs were incubated for 16 h with or without *C. cardunculus* (LYNC) or *T. pulegioides* (LTHY) extracts (10^{-5} mol/L). eNOS activity was triggered by the addition of the calcium ionophore A23187 (2 μmol/L), and total RNA was harvested and reverse-transcribed into first strand cDNA. PCR was then performed, and its products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. The relative intensities of the bands were quantified using a scanning densitometer.

Table 1. Cytotoxicity Assay of *C. cardunculus* (LYNC) and *T. pulegioides* (LTHY) Extracts[a,b]

sample	10^{-5} M	10^{-6} M
LYNC	95 ± 1.3	100 ± 2.4
LTHY	98 ± 0.1	99 ± 1.6

[a] Extracts were added to PAECs at the indicated concentrations, and cytotoxicity was assessed by the MTT test (*15*). [b] Data represent the percent survival and are means (SD) of quadruplicate experiments.

such as lipoic acid and L-2-oxothiazolidine-4-carboxylic acid (OTC) have also been shown, in vitro (*25*, *26*) and in vivo (*27*), to increase endothelium-derived NO production. Likely, water-soluble or amphiphilic antioxidants (such as the ones included in the ethanolic extracts we tested) acting at the lipid−water

interface might more effectively scavenge free radicals or modulate enzymatic processes relevant to cardiac and vascular functions.

The free radical scavenging properties of artichoke (*Cynara scolimus*) and of wild artichoke (*C. cardunculus*) have been previously described (*16*, *28*−*34*), and this plant is traditionally used for its diuretic, choleretic, and hepatoprotective effects. Thyme is also rich in phenolic compounds (*17*) endowed with antioxidant and additional biological activities, demonstrated in vitro (*17*, *35*−*39*). The data reported here show that wild artichoke and thyme extracts can enhance the production of NO by vascular cells; hence, our investigation discloses additional properties that might, if confirmed in vivo, add to the salubrious properties of such plants. A tonic effect was also noted with respect to the production of prostacyclin, whose pathways of production are independent from those of NO. Thus, enhanced production of prostacyclin might confer additional vasoprotective benefits to wild artichoke and thyme.

The mechanism(s) of action responsible for the enhancement of NO production are as yet to be fully elucidated. However, our data exclude any effect on eNOS levels (**Figures 6** and **7**) and suggest that maintenance of an intracellular reduced environment and the consequent preservation of cofactors such as BH_4 prevent eNOS uncoupling (*5*) and facilitate its activity, as already been shown for ascorbic acid (*11*, *25*, *40*, *41*) and suggested for thiol agents (*26*). Finally, facilitated hydroxylation of eNOS, which is the first step in NO production by this enzyme (*42*), might also explain the potentiating activities of wild plant extracts: hence, wild artichoke and thyme phenolic constituents such as lutein (**Figure 1**), in addition to exerting strong antioxidant activities due to the presence of hydroxyl groups in the ortho-position (catechols), might facilitate eNOS activation.

Current techniques to evaluate NO production usually rely on indirect methods, such as conversion of L-arginine to L-citrulline. In our investigation, NO production was also assessed directly, that is, by means of a NO sensor on a brain homogenate (chosen as a model in which larger quantities of NO are produced, as compared with endothelial cells), further corroborating data obtained with the indirect method. As far as the production of NO major effector, that is, cGMP, is concerned, a trend toward increased production after supplementation with the extracts was noted. However, this effect did not reach statistical significance, in part questioning the true vasorelaxant effects of supplementation. Future in vivo experiments will eventually clarify this aspect.

Whether this vasomodulating potential can be observed in vivo is a matter of future investigations. The current scarceness of knowledge on the absorption and metabolism of oligonutrients (*43*), especially of wild artichoke and thymus bioactive components, does not allow for speculation regarding their potential vasomodulating activities in vivo. However, administration of catecholic antioxidants, for example, with tea (*44*) and cocoa (*45*), has been shown to ameliorate endothelial function.

In conclusion, our data suggest that phenolic-rich extracts of some wild plants traditionally consumed in the Mediterranean area and endowed with antioxidant activity are able to increase the production of NO by endothelial cells and that they might provide useful grounds for the formulation of appropriate, cardioprotective diets.

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