JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2005, 56, Suppl 1, 37–49
www.jpp.krakow.pl

Review article

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MEDITERRANEAN FOOD AND HEALTH: BUILDING HUMAN EVIDENCE

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Adherence to a Mediterranean style diet affords protection from degenerative diseases such as cardiovascular disorders and cancer. Identification of the active constituents of the Mediterranean diet is crucial to the formulation of appropriate dietary guidelines. Also, research on the pharmacological properties of the "minor components" of this diet, eg vitamins and polyphenols, is very active and might lead to the formulation of functional foods and nutraceuticals. Even though in vitro data are plentiful, human studies are difficult to perform due to ethical and practical reasons. Yet, intervention trials represent the best approach to validate claims of healthful activities. This article reviews human evidence of the biological properties of olive oil and tomato constituents and illustrates a research approach by which the bioactive elements of a wild plant (*Cynara cardunculus*) are first studied in vitro to build biochemical evidence, then in vivo to obtain proof of their vasomodularoty activity.

Key words: *Mediterranean diet; antioxidants; tomato; olive oil; polyphenols; endothelial dysfunction*

The traditional dietary habits of the Mediterranean area have been consistently associated with lower incidence of cardiovascular disease (CHD) and cancer (1-3). Though the healthful properties of the Mediterranean diet as a whole have gained recognition, basic researchers are nowadays concentrating their efforts on individual food items, e.g. cereals, fruits, vegetable, olive oil, and their components, e.g. fibers, vitamins, polyphenols (4). By singling out the contribution of micronutrients to the protective activities, one can better focus dietary guidelines and, possibly, formulate appropriate functional foods or nutraceuticals (5).

As an example, olive oil and tomato consumption have been linked to CHD- and chemoprotection, but biochemical studies of their components are still accumulating. In this respect, we pioneered research on the biological properties of olive oil polyphenols, whose wide range of pharmacological activities might provide a partial explanation for the high longevity and low incidence of degenerative diseases observed in the Mediterranean basin, where olive oil is the predominant source of fat (6). Moreover, in recent research we investigated the effects of tomato supplementation, by providing healthy volunteers with purified tomato extracts in an attempt to identify the contribution of individual tomato components, e.g. carotenoids such as lycopene (7,8).

Another approach is based on in vitro observations of potentially healthful properties of plant extracts or their isolated components. There is indeed extensive literature that reports on the antioxidant and enzyme-modulating activities of numerous herbs and their extracts (9). However, the transposition of these basic observations into claims of human effects is, at present, unsubstantiated (10). Human studies are difficult to perform, because of ethical and practical reasons. Hence, even though in vitro studies abound, human (and even animal) intervention studies are scant. Thus, it is mandatory to confirm in vitro observations in in vitro studies before definitive claims can be made. This is why we recently followed an in vitro-to-in vivo approach to test the vasomodulatory properties of plant food extracts, selected within a EU-funded project on Local Food Nutraceuticals (www.biozentrum.uni-frankfurt.de/Pharmakologie/EU-Web/index.html).

This manuscript reports some examples of such approaches, aimed at building human evidence to sustain claims that the Mediterranean diet and its components do indeed exert healthful activities.

OLIVE OIL

Olive oil is the principal (often exclusive) and most typical source of visible fat of the Mediterranean diet. The healthful properties of olive oil have been often attributed to its high monounsaturated fatty acid (MFA) content, namely in the form of oleic acid (18:1n-9). However, there is currently no consensus on the effects of MFA on circulating lipids and lipoproteins: the effects of high monounsaturated fatty acid intakes on serum cholesterol might be indirect and due to the associated replacement of saturated fatty acids (11). Yet, some studies (12) attributed a direct, although modest, cholesterol-lowering effect to MFA alone, when they equicalorically replace carbohydrates. It should also be underlined that oleic acid is one of the predominant fatty acid in worldwide largely-consumed animal foods, such as poultry and pork; thus, the percentage of oleic acid in the Mediterranean diet is only slightly higher than that of other kinds of Western diets, *e.g.* the North American one (13). Finally, several seed oils obtained through genetic selection, such as sunflower, soybean, and rapeseed oils are nowadays rich in monounsaturates, albeit devoid of phenolics (14), and are

commercially available. It is therefore unlikely that oleic acid is exclusively accountable for the healthful properties of olive oil. In turn, even though the salubrious effects of a high proportion of oleic acid intake - including reduced endothelial activation (15,16) and lower susceptibility of LDL to oxidation (17,18) should not be overlooked, what really sets extra virgin olive oil apart from other vegetable oils is its content in phenolic compounds.

Olives are rich in phenolic components (19,20). Approximately 10 years ago, we started our investigations on the antioxidant properties of olive oil phenolics (21) and, subsequently, our and others' groups (6,22) followed up on this. In synthesis, hydroxytyrosol and oleuropein have been shown to be potent scavengers of superoxide anion and other reactive species (peroxynitrite, hypochlorous acid) possibly implicated in the onset of CHD and mutagenesis. Moreover, hydroxytyrosol and oleuropein are also capable to modulate enzymatic processes (22), some of which might be relevant to CHD. As an example, hydroxytyrosol has been shown to inhibit platelet aggregation, suggestive of an antithrombotic potential.

Even though human absorption and metabolism of olive oil phenolics have been well documented (23-29), in vivo studies of antioxidant potential and biological activity are still scarce and results are not univocal (30). However, research in this field is rapidly progressing and data proving in vivo activities of olive oil polyphenols do accumulate (31-33).

To further investigate the in vivo activities of olive oil phenolics, we undertook a series of human studies, the most recent of which (the VOLOS), was carried out in mildly dyslipidemic patients (34). In the VOLOS (virgin olive oil study) we evaluated the antioxidant capacity and the serum TXB_2 levels of 22 mildly dyslipidemic patients who were given 40 ml/day of either extra virgin phenolic rich oil or refined, phenol poor olive oil, with a cross over design. Each treatment was carried out for 7 weeks with 4 weeks of wash out in between. Seven weeks of EVOO supplementation resulted in a marked (-20%) decrease in serum TXB_2 production, both in the first and in the second arm of the study. This effect was reverted upon subsequent ROO administration. This is showed in *Figure 1*: the reduction in serum TXB_2 concentrations (μg/ml) in subjects administered EVOO is evident at 28 days. On the other hand, subjects administered ROO do not exhibit any reduction of serum TXB_2 concentrations. The reduction in TXB_2 serum production confirms the antithrombotic potential suggested for extra virgin olive oil polyphenols (see above).

Plasma antioxidant capacity (measured as μM of Cu^{++} reduced) was also evaluated. *Figure 2* shows how 7 weeks of EVOO consumption is associated with an improvement in antioxidant capacity, while no effect was noted with ROO administration.

One of the interesting aspects of the results obtained in the VOLOS is that they were obtained by providing doses of phenolic compounds comparable to those currently consumed by many population groups in the Mediterranean area.

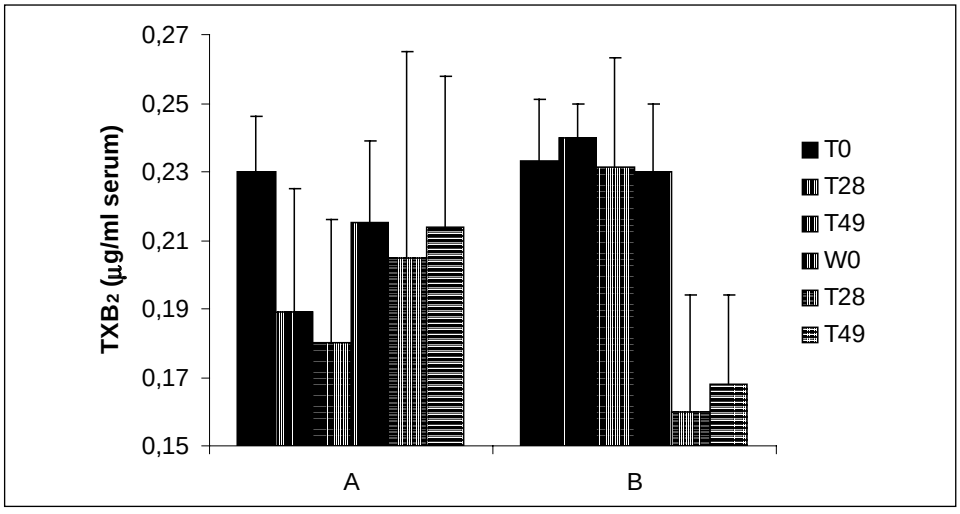

Figure 1 Thromboxane B_2 (TXB_2) serum levels (µg/ml) at baseline, 28 days, and 49 days after administration of 40 ml/day of extra virgin olive oil (EVOO) or refined olive oil (ROO) consumption by 22 mildly dyslipidemic patients. All subjects were randomly divided into two groups (A and B). The first one (A) was randomly assigned to the administration of EVOO (the second group was assigned ROO) in the first 49 days and after the washout period all subjects were switched treatment for an additional 7 weeks.
Data are means ± S.D., n = 22. From Visioli et al. (34) with permission of the publisher.

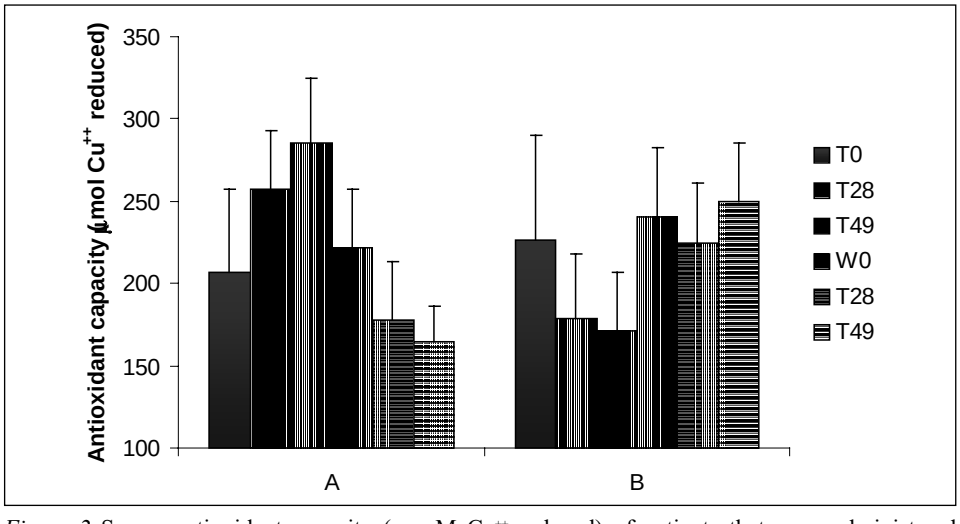

Figure 2 Serum antioxidant capacity (as µM Cu^{++} reduced) of patients that were administered EVOO or ROO (40 ml/day) for 7 weeks. After that period, all subjects were switched treatments for an additional 7 weeks.
All subjects were divided into two groups (A and B). Each group consumed the two kinds of oil in a crossover fashion.
Data are means ± S.D., n = 22. From Visioli et al. (34) with permission of the publisher.

Lipidic parameters, namely cholesterolemia ad triglyceridemia, were also evaluated in the VOLOS, but no significant improvement was noted, as in other previous studies (32).

TOMATO

Tomato was imported in the Mediterranean area from South America at the beginning of the XVIII century and is now an important component of the Mediterranean diet. Among the minor components of tomato, carotenoids such as beta-carotene, lycopene, lutein, and zeaxanthin have been extensively investigated because of their relative abundance in human plasma (35) and their antioxidant properties (36,37). Accordingly, both basic research and epidemiological studies concur to suggest the cardioprotective and chemopreventive activities of carotenoids, in particular of beta-carotene and lycopene. However, it should be noted that the results of the ATBC and CARET trials (38,39) conducted among smokers (who are exposed to enhanced oxidative stress) supplemented with beta-carotene demonstrated excess risk for cancer and cardiovascular endpoints (40). In addition, other clinical trials did not demonstrate any effect of beta-carotene supplementation on cancer (41,42). Finally, a recent meta-analysis even suggested harmful effects of carotenoids supplementation, which has been associated with increased total mortality (43). The major conundrum in carotenoid research now is: Why is it that consumption of tomato and tomato products is associated with lower cancer and cardiovascular risks whereas supplementation with beta-carotene is yet to be proven beneficial? Suggestions include the co-carcinogenic and pro-oxidant potential of beta-carotene under certain conditions (44,45), the presence of concomitant liver disease in ATBC and CARET patients (46), and the confounding interference by smoking habits and alcohol consumption on overall outcomes (47). As a final point, the healthful effects of tomato consumption might not be limited to its carotenoid content, as suggested by a recent study we performed (7).

In an attempt to discern the contribution of tomato components to human health, we undertook some intervention studies with carefully standardized diets, added with well-defined amounts of carotenoids. The intervention study of Riso et al. (8) was performed to verify if regular consumption of tomato products could protect lymphocytes and plasma from oxidative stress. As show in *Table 1*, after the first week of controlled diet (t=0), carotenoids concentrations decrease significantly in plasma ($P< 0.05$); after the three-week tomato diet there was a significant decrease in plasma lutein, zeaxanthin, and β-cryptoxanthin concentrations ($P<0.01$). This is probably due to the effects of the standard diet, low in carotenoids. Analysis of lymphocyte carotenoids concentrations showed a slight decrease (between 10% and 30%), but that was significant only for zeaxanthin ($P<0.01$) and β-cryptoxanthin ($P<0.05$). Lycopene concentrations increased significantly after tomato intervention both in plasma (+53%) and in lymphocytes (+72%). This result confirms that of

Table 1 Plasma and lymphocyte carotenoids concentrations before and after tomato intervention

Carotenoids		
Variable	T=0	T=21
	Plasma (mol/l)	
Lutein	0.56 ± 0.06	0.50 ± 0.05[a]
Zeaxanthin	0.04 ± 0.01	0.03 ± 0.01[a]
β-Cryptoxanthin	0.32 ± 0.23	0.23 ± 005[a]
α-Carotene	0.07 ± 0.01	0.07 ± 0.06[a]
β-Carotene	0.44 ± 0.06	0.38 ± 0.06
Lycopene	0.34 ± 0.03	0.52 ± 0.03[b]
	Lymphocytes (nmol/mg prot)	
Lutein	0.028 ± 0.011	0.023 ± 0.016
Zeaxanthin	0.004 ± 0.003	0.003 ± 0.001[a]
β-Cryptoxanthin	0.018 ± 0.015	0.012 ± 0.009[a]
α-Carotene	traces	traces
β-Carotene	0.011 ± 0.008	0.009 ± 0.007
Lycopene	0.010 ± 0.004	0.017 ± 0.008[a]

Twelve healthy, young women were instructed to follow a "basal diet" low in carotenoids and free from tomato products for one week (t= -7). The basal diet was followed during the experimental period, which lasted for three weeks, but this time subjects were instructed to consume well-characterized tomato products daily. Fasting blood samples were drawn at t= -7 and 21. Carotenoids plasma and lymphocytes concentrations were performed by HPLC. Data are mean ± s.d. [a]P<0.01, [b]P<0.001. From Riso et al. (8). Reprinted by permission from Eur J Clin Nutr: 58: 1350-1358, copyright (2004) Macmillan Publishers Ltd.

another study by Porrini et al. (48) and data in literature. In fact, many data have been published on carotenoids uptake by lymphocytes (48-50) and buccal mucosal cells (51,52). Conversely, limited literature exists on the intracellular concentrations of lycopene, although this is very important for understanding the role of this antioxidant on tissue activity.

For this reason, we focused our research on lycopene and its biological effects (53). Our purpose was to verify that the daily intake of a beverage called Lyc-o-mato®, containing a natural tomato extract (Lyc-o-mato® oleoresin 6%), was able to modified plasma carotenoids concentration and that this intake could protect DNA in lymphocytes from oxidative stress. In *Table 2* we report the carotenoids and vitamin E plasma concentrations (μmol/l) before and after interventions. Lycopene, phytoene, phytofluene and beta-carotene concentrations increased significantly by

about 68%, 92%, 61%, and 28%, respectively, after 26 days of Lyc-o-mato® drinking, but not after placebo intake. Conversely, the consumption of Lyc-o-mato® did not affect the plasma concentrations of lutein, zeaxanthin, β-cryptoxanthin and β-carotene, which were not present in the drink; plasma concentrations of α-tocopherol also did not change throughout the intervention, possibly due to the relatively low amount of this vitamin present in the Lyc-o-mato® drink.

The second aim of this study was to verify if the increased of carotenoids concentration was able to increase cellular defences against the oxidative stress. *Figure 3* shows the percentage of DNA in the tail of lymphocytes subjected to the comet assay decreased by about 42% ($P<0.0001$), as calculated by considering the variation between the percentage of DNA in the tail registered before and after Lyc-o-mato® intake with respect to the value recorded before Lyc-o-mato®. This is in agreement with studies that showed observed significant decreases of leukocyte 8-OHdG/dG (-21%) in patients with prostate adenocarcinoma who consumed tomato sauce for 3 weeks (30 mg lycopene daily) before prostatectomy (54). In addition, a decrease in 8-OHdG/dG was observed in the prostate tissue. Also, Rao et al. found that one week of supplementation with lycopene or tomato products let to a fall in leukocyte 8-OHdG/dG level (55).

Table 2. Plasma and lymphocyte carotenoids concentrations before and after Lyc-o-mato® intervention.

	Before placebo		After placebo		Before Lyc-o-mato®		After Lyc-o-mato®	
Variable	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Lycopene	0.34	0.12	0.32	0.12	0.31	0.17	0.52[a]	0.17
Phytofluene	0.23	0.10	0.22	0.10	0.23	0.12	0.37[a]	0.17
Phytoene	0.13	0.08	0.14	0.08	0.12	0.09	0.23[a]	0.16
β-Carotene	0.60	0.14[b]	0.56	0.39	0.54	0.35	0.69[a]	0.43
α-Carotene	0.11	0.09	0.12	0.18	0.11	0.11	0.12	0.11
Lutein	0.53	0.26	0.50	0.23	0.50	0.28	0.49	0.23
Zeaxanthin	0.04	0.04	0.05	0.04	0.04	0.04	0.04	0.04
β-Criptoxanthin	0.30	0.20	0.32	0.20	0.32	0.29	0.29	0.20
α-Tocopherol	48.6	23.7	47.8	24.2	47.3	20.7	48.3	22.5

Twenty-six healthy men and women were recruited and divided into two groups: group 1 was assigned to the sequence placebo/wash-out/Lyc-o-mato®; group 2 followed the sequence Lyc-o-mato®/wash-out/placebo. Each period lasted for 26 days. Blood samples were collected at the beginning and the end of each treatment period, before and after receiving placebo or Lyc-o-mato®. Carotenoids and vitamin E plasma concentration was performed by HPLC. ([a] significantly different from each other point of the same group, $P<0.0001$; [b] significantly different from other points, $P<0.05$). From Porrini et al. (53) with permission.

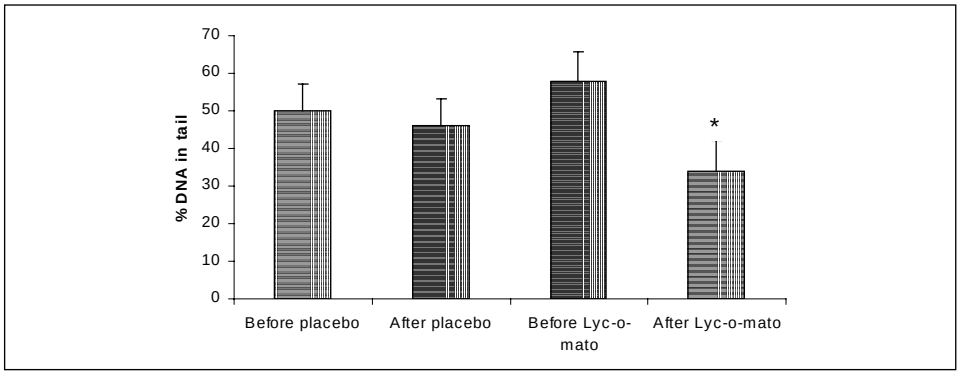

Figure 3 Lymphocytes DNA damage (as percentage of DNA in the tail), recorded before and after each experimental period and evaluated after the oxidative treatment of the cells (*significantly different from each of the other points, P<0.0001). Twenty-six healthy man and women were recruited and divided into two groups: group 1 was assigned to the sequence placebo/wash-out/Lyc-o-mato®; group 2 followed the sequence Lyc-o-mato®/wash-out/placebo. Each period lasted for 26 days. Blood samples were collected at the beginning and the end of each treatment period, before and after receiving placebo or Lyc-o-mato®. The resistance of lymphocyte DNA to oxidative stress was evaluated by means of the Comet assay. From Porrini et al. (53) with permission.

One of the most interesting aspects of the potentially cardioprotective and chemopreventive properties of carotenoids is that lycopene is more bioavailable from tomato products, e.g. paste, puree, and sauce, than from raw tomatoes (56). Also, addition of olive oil and cooking further promotes absorption (57,58). In addition to raw tomatoes, which are important constituents of salads, consumption of processed, cooked tomato products might provide additional benefits deriving from lycopene.

WILD PLANTS AND ENDOTHELIAL FUNCTION

Endothelial dysfunction is a major complication of atherosclerosis (59,60) and accumulating evidence suggests that oxidative stress plays a major role in its onset and maintenance (61,62). Reduced production/availability of the vasorelaxant factor nitric oxide (NO) plays a major role in the oxidative stress-related development of endothelial dysfunction (61,63). Indeed, administration of some antioxidants, e.g. vitamin C and flavonoids from tea and wine, has been shown to ameliorate endothelial function and vasomotion (63,64) and increasing evidence over the past decade shows that several dietary factors may partly modulate nitric oxide synthase (NOS) activity (65). The project "Local Food Nutraceuticals" 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.

NO is an uncharged gaseous radical with a half-life between 3 and 6 seconds (66). It plays a quintessential role in regulation of systemic vascular tone (62) and

remodeling of the vascular wall (63). The vasodilatory action of NO stems from its rapid diffusion and direct activation of soluble guanylyl cyclase forming cGMP which lowers intracellular calcium in the VSM, leading to relaxation of the muscle. Endothelial NO is formed by endothelial nitric oxide synthase (eNOS) via a five electron reduction of the terminal guanidino group of L-arginine yielding L-citrulline as a secondary product (62). Thus, one of the most popular methods to indirectly detect NO production relies on the measurement of the conversion of L-arginine to L-citrulline, by using radioactive substrates.

As reported in *Fig 4*, supplementation of porcine aortic endothelial cells (PAEC) with *Cynara cardunculus* or *Thymus pulegioides* extracts increases NO production. Moreover, enhanced secretion into the medium of prostacyclin, another important vasorelaxant factor, further confirms the vasomodulatory potential of these wild plants. Recently, we further tested a *Cynara cardunculus* extract on isolated aortic rings and after supplementation to aged rats. The results (Rossoni et al, in preparation) confirm that the vasorelaxant properties of *Cynara cardunculus* are maintained in vivo, suggesting that part of the lower incidence of endothelial dysfunction and the higher vascular health observed in the Mediterranean area are to be attributed to the consumption of wild plants (67).

CONCLUSIONS

Adherence to a Mediterranean-like diet affords protection from CHD and cancer (68). As the major proportion of caloric intake in that area derives from plant foods (1), pharmacologists are concentrating their efforts on the identification of novel biological activities of plant minor constituents, which are

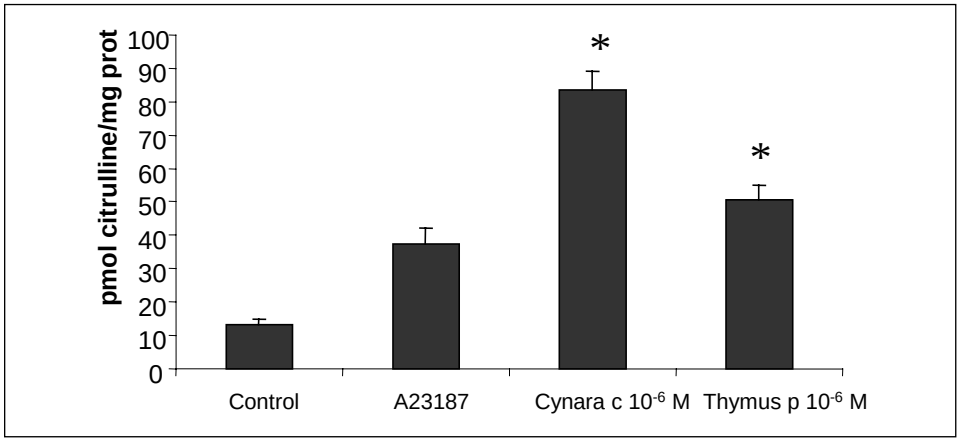

Figure 4 Effect of wild plant extracts on eNOS activity in porcine aortic endothelial cells. Confluent PAECs were incubated with *Cynara cardunculus* or *Thymus pulegioides* extracts for 16 h. The medium was replaced with HEPES buffer and eNOS activity was triggered by the addition of the calcium ionophore A23187 (2 μmol/L) and determined by ion-exchange chromatography as the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline. From Grande et al. (72) with permission.

many (69). In vitro studies abund, but human trials (which would provide the most useful information) are still insufficient and do not always support the notion that supplementation with plant foods or their extracts does affect surrogate markers of CHD, also due to the current scarcity of appropriate biomarkers (70,71). However, future availability of appropriate techniques to evaluate the in vivo activities of food items or of their isolated components will likely resolve this issue. For the time being, the advice to incorporate proper quantities of plant foods in the diet has strong epidemiological grounds, which for the most part derive from studies of the Mediterranean diet.

Acknowledgments: Work in our laboratory has been supported by EU grants FAIR CT 97 3039 and QLK-2001-00173, and by Carapelli s.p.a (Tavarnelle Val di Pesa, Italy). Lycored Natural Products Industries Ltd (Beer-Sheva, Israel) supplied the Lyc-o-mato®.

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Received: January 31, 2005
Accepted: February 15, 2005

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