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## ANTIOXIDANT PROPERTIES OF MEDITERRANEAN FOOD PLANT EXTRACTS: GEOGRAPHICAL DIFFERENCES

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Locally grown, wild food plants seasonally contribute a considerable portion of the daily diet in certain Mediterranean areas and it has been suggested that the beneficial effects of the Mediterranean diet on human health partly originate from the antioxidant effect of flavonoid-rich food plants. The nutrient content of most wild plants is higher than that of cultivated ones and may vary depending on the prevailing environmental conditions. Accordingly, three local Mediterranean plant foods (i.e. *Cichorium intybus*, *Sonchus oleraceus*, *Papaver rhoeas*) were collected in Greece (Crete), southern Italy, and southern Spain in order to assess possible differences in their *in vitro* antioxidant potential. The biological assays revealed diverse intra-plant specific antioxidant effects for the tested extracts ranging from no activity to almost complete protection. Furthermore, substantial differences in the polyphenol content were found for the nutritionally used part of the same plant originating from different locations. However, no clear correlations between the polyphenol content and the extracts' antioxidant activities were found. Taken together, the data suggest that certain local Mediterranean plant foods possess promising antioxidant activity and that the observed biological effects are possibly influenced by the geographically-dependent environmental conditions prevailing during plant growth.

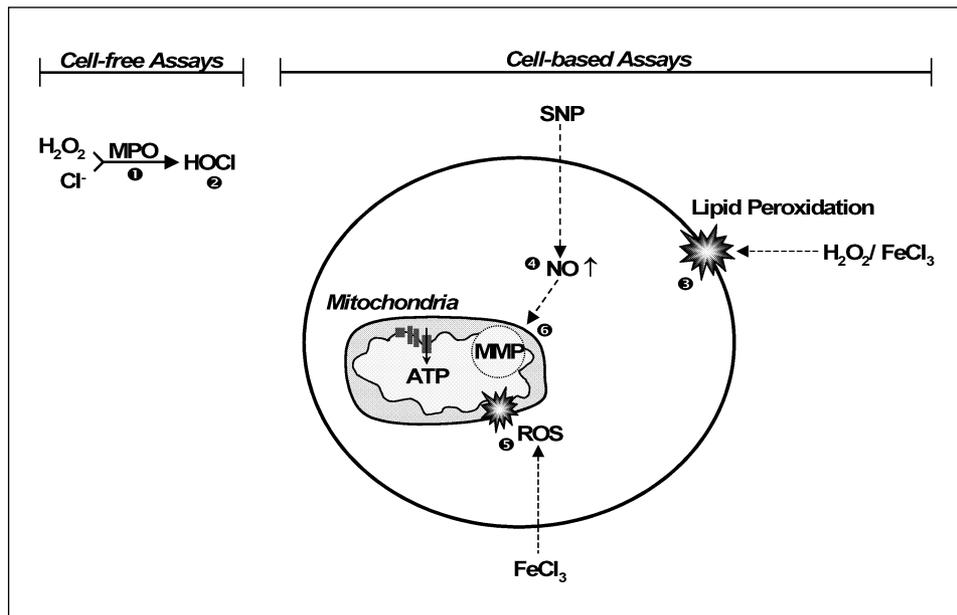
*Key words: Mediterranean diet, antioxidants, oxidative stress, polyphenols*

### INTRODUCTION

The interest in the Mediterranean diet originated from findings about low cardiovascular diseases (CVD) mortality in southern Europe, particularly for coronary heart disease (CHD) (1). Furthermore, recent reviews of

epidemiological data collected in the past decades also suggest a lower incidence of certain other diseases, including for example cancer, in Mediterranean populations (2). Latter observations have been linked to health-beneficial effects of the Mediterranean diet mediated by specific food components, in particular polyphenols (PPs) and unsaturated fatty acids (UFAs) (3,4).

An imbalance in the production and removal of reactive oxygen and nitrogen species (ROS and RNS) leads to the occurrence of deleterious oxidative stress, a hallmark of most cardiovascular and neurodegenerative diseases as well as cancer (5-7). PPs attenuate oxidative stress by acting as effective ROS- and RNS-scavengers as shown *in vitro* (8,9) and, though to a lesser degree, *in vivo* (10-12). The amount of PPs present in plants, however, heavily depends on environmental factors determined by growing season (13) and location (14). Within the EU-funded project "Local Food-Nutraceuticals", three wild or semi-cultivated plants (*Table 1*) were collected in southern Italy, southern Spain and Greece (Crete), respectively, to investigate potential effects of different geographical locations on the plants' *in vitro* antioxidant activities. All plant samples were collected in spring 2002 or 2003. The extracts prepared from the sample plants were assessed for their antioxidant effects in a multi-method approach in order to select those with high bioactivity (*Fig. 1*).



*Fig 1.* Overview on the cell-free and cell-based (whole brain homogenate or dissociated brain cells) assays utilized for antioxidant profiling (1, myeloperoxidase-catalysed guaiacol oxidation; 2, HOCl-induced oxyhemoglobin bleaching; 3, lipid peroxidation (MDA); 4, intracellular nitric oxide levels; 5, mitochondria-associated ROS; 6, mitochondrial membrane potential(MMP)).

## MATERIALS AND METHODS

### *Materials used*

Myeloperoxidase, 4,5-Diaminofluorescein diacetate, and Rhodamine 123 were purchased from Calbiochem (Darmstadt, Germany). Sephadex G-25 was obtained from Amersham Bioscience (Uppsala, Sweden), the dialysis membrane (20x32 mm) from MAGV (Rabenau-Londorf, Germany), DHR 123 from Molecular Probes (Eugene, Oregon, USA), and freeze-dried hemoglobin from ICN (Eschwege, Germany). All other reagents were of the highest purity available and were purchased from Sigma Chemical (Munich, Germany) or Merck (Darmstadt, Germany).

### *Plant extraction procedure*

Briefly, 50 g of air-dried material were extracted by reflux with ethanol (90%) for 30 min, and then pressed, the resultant liquids pooled and cooled at room temperature prior filtration. The extracts were then concentrated with a rotary evaporator (40°C), freeze-dried and stored at -50°C. The detailed extraction procedure will be published elsewhere.

### *Inhibition of MPO-catalysed guaiacol oxidation*

The inhibitory effect of plant extracts at a concentration of 0.2 mg/mL on MPO-catalysed guaiacol oxidation has been measured according to (15).

### *Oxyhemoglobin bleaching assay*

The potential of plant extracts at a concentration of 0.2 mg/mL to prevent HOCl-induced oxyhemoglobin bleaching has been measured according to (15).

### *Preparation of dissociated neurons*

Female NMRI mice were housed in groups of 6-8 animals with *ad libitum* access to food and water. All experiments were in accordance with the national guidelines for animal welfare. Mice were sacrificed by decapitation, and brains were quickly dissected on ice using a modified method according to (16). After removing the cerebellum, the tissue was minced in medium I with a scalpel and further dissociated by trituration through a nylon mesh with a Pasteur pipette. The resulting suspension was filtered by gravity through a fresh nylon mesh with a smaller pore diameter, and the dissociated cell aggregates were washed twice with medium II by centrifugation. 50  $\mu$ L of the suspension were used for protein determination. After centrifugation, cells were re-suspended in HBSS and incubated with extracts at a concentration of 0.2 mg/mL for 30 min. After incubation, cells were washed and 500  $\mu$ L/well were distributed on a 48-well plate for the measurement of mitochondrial membrane potential (MMP), NO scavenging and mitochondria-associated ROS.

### *Lipid peroxidation*

Lipid peroxidation has been measured in brain homogenates of mice as malondialdehyde (MDA) in the absence and presence of plant extracts (0.2 mg/mL) using the commercial Lipid Peroxidation Assay Kit (Calbiochem, Darmstadt, Germany).

### *Intracellular nitric oxide (NO) scavenging*

Dissociated neurons from young mice were plated in 48-well plates. The fluorescence dye 4,5-diaminofluorescein diacetate was used in a concentration of 10  $\mu$ M to monitor NO levels (17) before and after addition of the NO-donor SNP to cells loaded with the extracts (0.2 mg/mL).

Following the incubation with the extract, cells were washed 3 times with Hanks' balanced salt solution and the fluorescence was determined with a fluorescence reader (Victor® multilabel counter, Perkin Elmer, Rodgau-Jügesheim, Germany) at 490/535 nm.

### Scavenging of mitochondria-associated ROS

The fluorescence dye dihydrorhodamine (DHR) was used to quantify mitochondria-associated ROS production. The capacity of extracts to lower basal and FeCl<sub>3</sub>-induced production of ROS in dissociated brain cells isolated from young mice was tested at an extract concentration of 0.2 mg/mL.

### Mitochondrial membrane potential (MMP)

The MMP of dissociated mouse neurons was measured using the fluorescence dye Rhodamine 123 at a concentration of 0.4 μM for 15 min (18). The transmembrane distribution of the dye depends on the MMP. Cells were incubated with SNP in the presence or absence of extracts. Fluorescence was determined with a fluorescence reader (Victor® multilabel counter) at 490/535 nm.

## RESULTS

*Cichorium intybus* (Fig. 2). The PP content for extracts prepared from the three *Cichorium intybus* samples differed by more than 100% (Table 1). The extract originating from the Greek plant, having the lowest PP content, still efficiently scavenged mitochondria-associated ROS (>50%). This effect is in accordance with the observed maintenance of the MMP, a parameter sensitive for ROS-induced modification. However, the same extract demonstrated only low to medium activity in terms of NO and HOCl scavenging as well as in the prevention of lipid peroxidation. Similarly, the potential to inhibit myeloperoxidase-catalysed guaiacol oxidation was low. In contrast, the extract prepared from the Spanish *Cichorium intybus* sample showed high activity in the prevention of lipid peroxidation and removal of NO. The effects on mitochondria-associated ROS scavenging and protection of the MMP were less pronounced. Whereas the same extract inhibited MPO-catalysed guaiacol oxidation by more

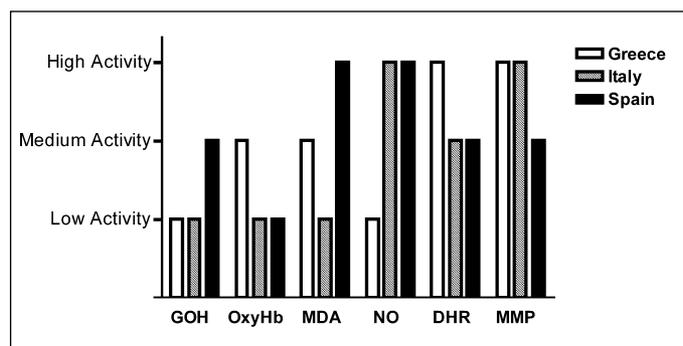


Fig. 2. Antioxidant activity of extracts prepared from *Cichorium intybus* L. collected in Italy, Spain, and Greece (for definition of activity range see Table. 2)

Table 1. Botanical information of local Mediterranean plant foods used in this study and polyphenol concentration of ethanolic plant extracts

PLANT NAME	PLANT FAMILY	ORIGIN	PARTS USED	POLYPHENOLS (mg/g)
<i>Cichorium intybus</i> L.	Asteraceae	Greece	Leaves	48
		Italy	Leaves	65
		Spain	Leaves	107
<i>Sonchus oleraceus</i> L.	Asteraceae	Spain	Aerial parts	75
		Greece	Aerial parts	122
		Italy	Aerial parts	157
<i>Papaver rhoeas</i> L.	Papaveraceae	Italy ( <i>Lucania</i> )	Aerial parts	34
		Greece	Aerial parts	120
		Italy ( <i>Calabria</i> )	Aerial parts	286

than 50%, its HOCl-scavenging potential was low. The Italian plant produced an extract with high NO scavenging and MMP protection potential. All other parameters, however, were of medium to low activity.

*Sonchus oleraceus* (Fig. 3). The PP content for extracts prepared from the three *Sonchus oleraceus* samples also differed by more than 100% (Table 1). The extract prepared from the Greek plant sample had the lowest PP content and showed a low activity in all screening assays except the maintenance of the MMP (medium activity). Similarly, the Spanish *Sonchus oleraceus* extract revealed only medium or high activity in two (i.e. scavenging of HOCl and mitochondria-associated ROS) of the six assays, whereas the extract derived from the Italian plant, possessing the highest PP content, only failed to significantly affect the two parameters linked to the HOCl metabolism. All other activities were of medium or high activity for this latter plant extract.

*Papaver rhoeas* (Fig. 4). This plant has been collected in two regions in Italy as well as in southern Spain. The two Italian extracts, though possessing a more

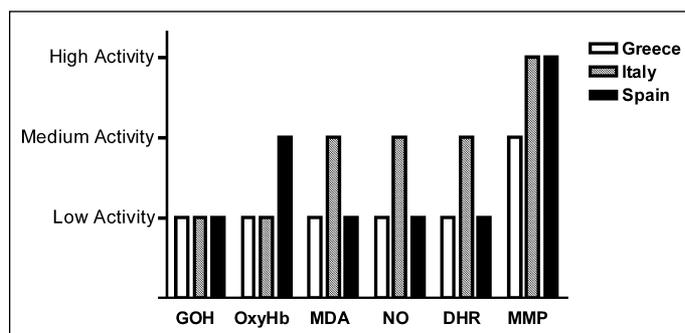


Fig. 3. Antioxidant activity of extracts prepared from *Sonchus oleraceus* L. collected in Italy, Spain, and Greece (for definition of activity range see Table. 2)

Table 2. Definition of activity range (% of control) for the performed antioxidant assays.

ASSAY	ACTIVITY		
	LOW	MEDIUM	HIGH
Inhibition of MPO-catalysed guaiacol (GOH) oxidation	< 50%	50-75%	> 75%
Prevention of HOCl-induced oxyhemoglobin (OxyHb) bleaching	< 25%	25-50%	> 50%
Prevention of lipid peroxidation (MDA)	< 50%	50-75%	> 75%
Scavenging of nitric oxide (NO)	< 25%	25-50%	> 50%
Scavenging of mitochondria-associated ROS (DHR)	< 25%	25-50%	> 50%
Protection of mitochondrial membrane potential (MMP)	< 50%	50-75%	> 75%

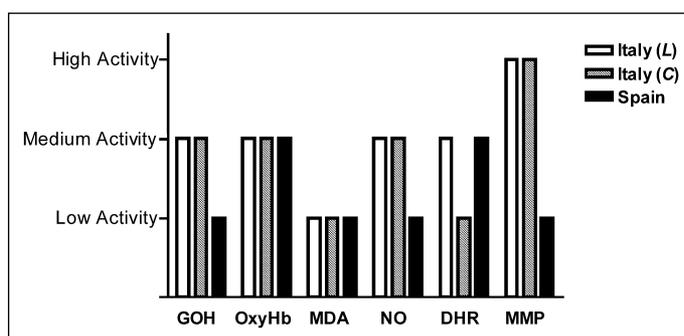


Fig. 4. Antioxidant activity of extracts prepared from *Papaver rhoeas* L. collected in Italy (Lucania (L) and Calabria (C) region) and Greece (for definition of activity range see Table. 2)

than 8-fold difference in their PP content, showed a very similar activity profile with medium or high activity in all tests with exception in the scavenging of mitochondria-associated ROS (extract prepared from *Papaver rhoeas* collected in Calabria, Italy). In contrast, the Spanish *Papaver rhoeas* sample failed to efficiently inhibit MPO, to scavenge NO, to prevent lipid peroxidation, and to maintain the MMP. The antioxidant activity assessed by the two other assays was also only in the medium range.

## DISCUSSION

Dietary habits play an important role in the promotion of and vice versa protection from common morbidities, such as cancer, diabetes, and CVD. The Mediterranean diet is characterised by an abundant intake of plant foods, such as vegetables, fruits, nuts, and cereals (19). Though recent studies revealed distinct differences in the dietary composition between certain Mediterranean countries (20,21), it is common for rural Mediterranean populations, for example in Greece, to seasonally enrich their daily diet with the intake of wild or semi-cultivated greens (22). However, the biological effects of these plants, which often contain high amounts of antioxidants such as quercetin, have only been investigated in a

limited number of studies (22,23) without taking into consideration a possible geographical impact on the plant nutritional quality. Abiotic stress, e.g. drought or coldness, significantly enhanced the levels of PPs in *Crataegus* leaves subsequently leading to an increase antioxidant capacity of the leave extract (24). The removal of potentially deleterious ROS and RNS through antioxidants has been suggested to be an important mediator for the protection of human health (25). In the present paper, antioxidant data on three wild or semi-cultivated plants each obtained in different Mediterranean regions are reported. As stated before, numerous PPs are known to possess excellent antioxidant effects, especially *in vitro* and the amount of PPs present in a plant preparation has been suggested to correlate with the antioxidant activity. Chinnici et al. and Leontowicz et al., for example, found good correlations between total PPs and the total antioxidant activity of  $r=0.944$  and  $r=0.902$ , respectively (26,27). In our study, however, no such correlation was found, suggesting that other extract constituents, for example fatty acids or vitamins, contributed to the observed biological effect of the sample extracts. This observation is in accordance with a recent report on the lack of correlation between the antioxidant activity of Mediterranean plant extracts and their PP content (28).

Significant intra-plant specific differences in the antioxidant profile have been detected for the three subsets of *Cichorium intybus*, *Sonchus oleraceus*, and *Papaver rhoeas*, indicating a strong impact of the geographical background on the plant antioxidant composition. This effect is most prominent for *Sonchus oleraceus* where the extract prepared from the Greek sample showed only below average antioxidant effects in contrast to the Italian extract with medium or high activity in all but two assays. The high activity of the Italian *Sonchus oleraceus* extract is in contrast to a recent study showing low antioxidant activity (<35%) for this plant in three *in vitro* tests (23), thus rather confirming the screening data for the extract prepared from the Greek *Sonchus oleraceus* plant. In the same study, an approximately 60% inhibition of xanthine oxidase, an enzyme involved in the promotion of oxidative stress, has been shown for an extract prepared from *Cichorium intybus* leaves collected in Italy. As stated previously, MPO catalyses the formation of HOCl, a potentially detrimental metabolite when produced in excess. The inhibition of enzymes participating in the production of ROS has been suggested as therapeutically approach to prevent oxidative stress (29). In our study, the Spanish *Cichorium intybus* extract inhibited the pro-oxidant enzyme MPO also by more than 50%, whereas the other two extracts prepared from this plant showed only low inhibitory activity. The addition of  $Fe^{2+}$  or  $Fe^{3+}$  is a common approach to experimentally induce oxidative stress *in vitro* and has also been used in two of our assays (induction of lipid peroxidation and intracellular ROS formation). A recent study by El et al. (30) found for *Cichorium intybus*, *Sonchus oleraceus*, and *Papaver rhoeas* high  $Fe^{2+}$ -chelating activity, possibly explaining some of our observed antioxidant effect in mouse brain tissue. Moreover, these plants also demonstrated promising radical scavenging towards

DPPH. A spatial impact on the total phenolic content has also been reported for *Poacynum henersonii* collected at three sites in China (14). Moreover, the same authors found a significant seasonal impact (April < July > October) on the total phenolic content. All plant samples investigated in the present study have been collected in spring, although not always exactly on the same day. Hence, a certain impact of the collection date on the plant sample composition and thus the obtained biological effects cannot be completely ruled out. Also, storage and transport conditions might have induced changes in the PP concentration of the plant extracts. However, long-term (> 40 weeks) storage (refrigerator or controlled atmosphere) of apples has been reported not to influence polyphenol concentration or antioxidant activity (31).

The consortium "Local Food-Nutraceuticals" applied a large-scale screening approach of local food plants (total of 127 plants) with presumed health-beneficial effects in order to ensure an optimal capture of the true bioactivity of target plants or plant families. Taken together, our multi-method antioxidant activity assessment of a *Cichorium intybus*, *Sonchus oleraceus* and *Papaver rhoeas* clearly highlights a geographical impact on the antioxidant profile of extracts prepared from these Mediterranean plants collected at different locations, thus supporting the consortium's scientific approach. However, more detailed analytical information on the constituents mediating the observed biological effects are needed prior the promotion or development of effective and safe foods or food supplements, e.g. nutraceuticals, for human consumption.

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