SII-5

PHENOLIC ANTIOXIDANT CONTENT OF OLIVE OILS AND THEIR POTENTIAL IN THE PREVENTION OF CANCER

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Abstract

The traditional (European) Mediterranean diet is characterized by an abundance of plant foods such as bread, pasta, vegetables, salad, legumes, fruit, nuts; olive oil as the principal source of fat; low to moderate amounts of fish, poultry, dairy products and eggs; only small amounts of red meat; low to moderate amounts of wine, normally consumed with meals. This diet is low in saturated fatty acids, rich in carbohydrate and fibre, and has a high content of monounsaturated fatty acids (MUFA). These are primarily derived from olive oil. Despite a wealth of general knowledge concerning the major classes of compounds present in olives and olive oil, detailed knowledge of the phenolic antioxidant content has been lacking. Therefore the aim of the study was to evaluate the phenolic antioxidant content in a range of olive and seed oils. While seed oils were devoid, on average, the olive oils contained 196 ± 19 mg/kg total phenolics as judged by HPLC analysis, but the value for extravirgin (232 ± 15 mg/kg) was significantly higher than that of refined virgin olive oil (62 ± 12 mg/kg; $P < 0.0001$). Appreciable quantities of simple phenols (hydroxytyrosol and tyrosol) were detected in olive oils, with significant differences between extravirgin (41.87 ± 6.17) and refined virgin olive oils (4.72 ± 2.15; $P < 0.01$). The major linked phenols were secoiridoids and lignans. Although extravirgin contained higher concentrations of secoiridoids (27.72 ± 6.84) than refined olive oils (9.30 ± 3.81) this difference was not significant. On the other hand the concentration of lignans was significantly higher ($P < 0.001$) in extravirgin (41.53 ± 3.93) compared to refined virgin olive oils (7.29 ± 2.56). All classes of phenolics were shown to be potent antioxidants. In future epidemiolgic studies, both the nature and source of olive oil consumed should be differentiated in ascertaining cancer risk.

Key words: antioxidants, HPLC, Mediterranean diet, phenolic compounds, reactive oxygen species

THE MEDITERRANEAN DIET

Background

The term the Mediterranean diet was first popularized by Keys and Keys (1975) in their book How to Eat Well and Stay Well: the Mediterranean way[1]. This preceded the publication[2,3] of studies which showed that Mediterranean countries have diets associated with low incidences of coronary heart disease (CHD). Later studies have shown that the Mediterranean countries also enjoy a low incidence of cancers of the colon and breast and there is now little doubt that the Mediterranean countries enjoy a low risk of many of the diet-related diseases of affluence.

At a meeting convened by the European Commission at the Italian National Research Council in Rome 11 April 1997, European nutrition, cardiology, lipidology and public health specialists gathered to reach a health consensus on olive oil and the Mediterranean diet.

They agreed that there is strong evidence that a Mediterranean-style diet, in which olive oil is the principal source of fat, contributes to the prevention of cardio-vascular risk factors such as dyslipidaemia, hypertension, diabetes and obesity, and therefore, in the primary and secondary prevention of CHD. In addition, there is evidence suggesting that the Mediterranean diet plays a preventive role against some cancers.

In this consensus statement, the major evidence for the health benefits of the Mediterranean diet was detailed, the mechanisms by which its components are believed to contribute to the benefits were stated, and the role of the Mediterranean diet in the prevention of diseases was pointed out.

A working definition of the traditional Mediterranean diet was described[4] as follows:

The traditional (European) Mediterranean diet is characterized by an abundance of plant foods such as bread, pasta, vegetables, salad, legumes, fruit, nuts; olive oil as the principal source of fat; low to moderate amounts of fish, poultry, dairy products and eggs; only small amounts of red meat; low to moderate amounts of wine, normally consumed with meals. This diet is low in saturated fatty acids, rich in carbohydrate and fibre, and has a high content of monounsaturated fatty acids (MUFA). These are primarily derived from olive oil'

General introduction

As mentioned earlier the observation of a lower incidence of CHD and of certain types of cancers in the Mediterranean area led to the hypothesis that a diet rich in grain, legumes, fresh fruits and vegetables, wine in moderate amounts, and olive oil had beneficial effects on human health. For example in Europe mortality from breast and colorectal cancer[5-9] is considerably lower in countries of high olive oil consumption (e.g. Greece, Italy, Portugal, Spain) than in those with low consumption (e.g. Scotland, England, Denmark).

Olive oil, in particular, is the principal source of fat of the Mediterranean diet. The production of olive oil for 1997-1998 in the European Community was estimated to be 1,941,000 tons, which represents 85% of world production. Although available data is sparse the mean daily intake of olive oil in countries of the Mediterranean basin varies between a few kg/capita/year in France to approximately 15 kg/capita/year in Greece.[10] In areas outside of the Mediterranean basin this intake is considerably lower than in France even. Recently due to the increasing popularity of the Mediterranean diet, its consumption has spread to non-producer countries such as Australia, the United States, Canada, and Japan. In terms of fat content an abundance of oleic acid, a monounsaturated fatty acid, is the feature that sets olive oil apart from other vegetable oils. In particular, oleic acid (18:1n-9) ranges from 56 to 84% of total fatty acids, while linoleic acid (1 8:2n-6), the major essential fatty acid and the most abundant polyunsaturate in our diet is present in concentrations between 3 and 21 %. Depending on its chemical and organoleptic properties, olive oil is classified into different grades that also serves as a guideline for the consumers preference.[11] In many producing countries however extra virgin olive oil, i.e. the one with the highest quality accounts for just 10% of total production. In addition to triacylglycerols and free long chain fatty acids, olive oil contains a variety of non-saponifiable compounds that contribute up to 1-2% of the oil and are important for its stability and unique flavour and taste. In contrast, other edible seed oils lose most of their minor compounds during the refining stages.

Despite a wealth of general knowledge concerning the major classes of compounds present in olives and olive oil, detailed knowledge of the phenolic antioxidant content has been lacking, as evinced by a quote[12] from the Editor of the European Journal of Cancer Prevention, Professsors Michael J. Hill and Atillio Giacosa:-

There is urgent need for research into the whole subject of the Mediterranean diet and its protective role. In particular we need to know much more about the role of olive oil-the one important aspect of the Mediterranean diet to have made no impact on the food patterns of northern Europeans'

To counteract this lack of knowledge we have embarked upon a concentrated program to investigate in detail the phenolic antioxidant content of olives and olive oil, to assess their contribution to the health promoting properties of the Mediterranean diet. Data from other pertinent contributions in the field are also included to give an overview of the current knowledge in this very important field of research.

Olives

The olive is the fruit of the olive tree (*Olea europaea L.*) and belongs to the family Oleaceae and its cultivation dates back to Biblical times when it was an important part of primary civilisations. The

cultivation of the olive tree and the production of olive oil from the mature drupe (fruit) remains an essential part of farming practices in the Mediterranean basin today. In the olive groves (Fig. 1) of Europe the olive tree flowers in the spring and produces a small ovoidal bud which develops into the mature drupe between October and January depending on the area of production. During the growth and maturation periods, a number of changes in the fruit occur. When the fruits are approximately 6 months old the major phenolic components are glucosides (Fig. 2) termed ligstroside and oleuropein glucoside (termed secoiridoid glucosides) but these are not detected in the olive oil harvested at maturity. The glucoside precursors are present in the pericarp, but as the olive reaches maturity they are deglycosylated by glucosidase enzymes releasing the free secoiridoids. These along with a number of other transformation products are able to cross the water/oil barrier and therefore become important antioxidants in the harvested oil.

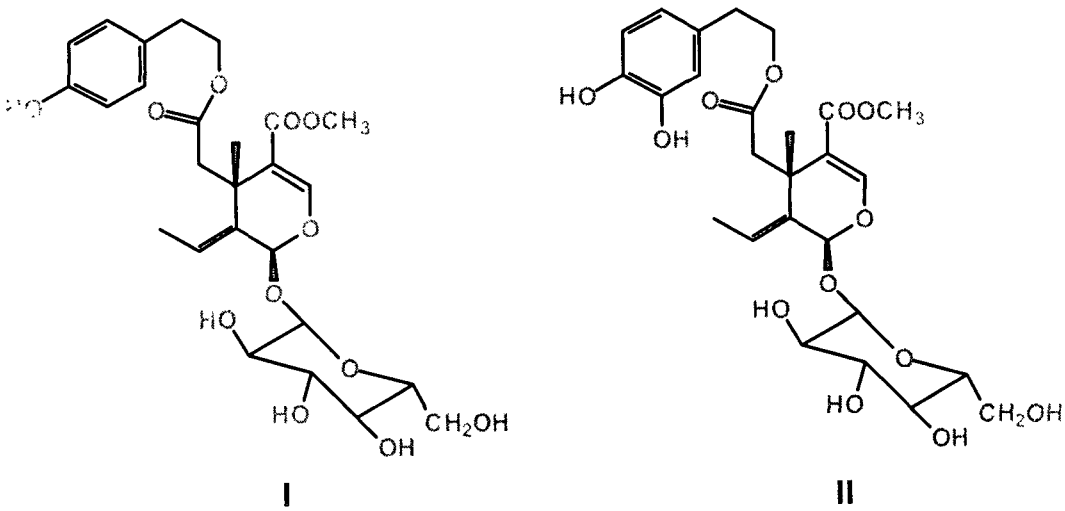

Figure 2. The major glucosides present in immature olives.
Ligstroside

Production of olive oil

The production of olive oil is steeped in tradition, although over the course of time some changes towards automation and ease of handling have inevitably occurred. The initial step in the production of olive oil is washing (Fig 3a) the olives to remove dirt, stones and other debris which may adhere to the fruit. The olives are then crushed in a hammer mill and the pomace is finely homogenised (malaxation) prior to pressing (Fig. 3b). When traditional methods are used, the pomace is fed directly to the hydraulic press plates, each of which is covered by a filtering diaphragm before the plate pile is

loaded into the press for extraction. The oil is produced under pressures of up to 5,700 p.s.i. and is separated from vegetation water by a centrifugal clarifier and a brilliant clear oil results after filtration through a filter press. When modern methods are employed a continuous horizontal centrifuge rotating at 1200-1500 rev/min is used to separate the oil from the pomace and vegetation water (Fig. 3c). Following further washing with an approximately equal proportion of clean water the oil is again centrifuged prior to harvesting, yielding (Fig. 3d) extra virgin olive oil (VOQ). High quality oils are bottled directly, but low quality oils (high acidity) are further processed yielding refined virgin oil (RVO). Oil extracted from the residual pomace or husk with organic solvents such as hexane yields a low quality refined husk oil (RHO). The quality of both RVO and RHO is usually improved by blending with VOQ.

RESULTS

Phenolic content of olive oils

Although there are have been many publications related to the identification of individual compounds in olive oil, comprehensive analyses on the solvent extractable phenolic fraction are limited. Major contributions in this area come from the data of Montedoro *et al.*,[13-14] Angerosa *et al.*[15] and more recently Owen *et al.*.[16-19] Olive oil was extracted as shown in Fig. 4, and the structures of the

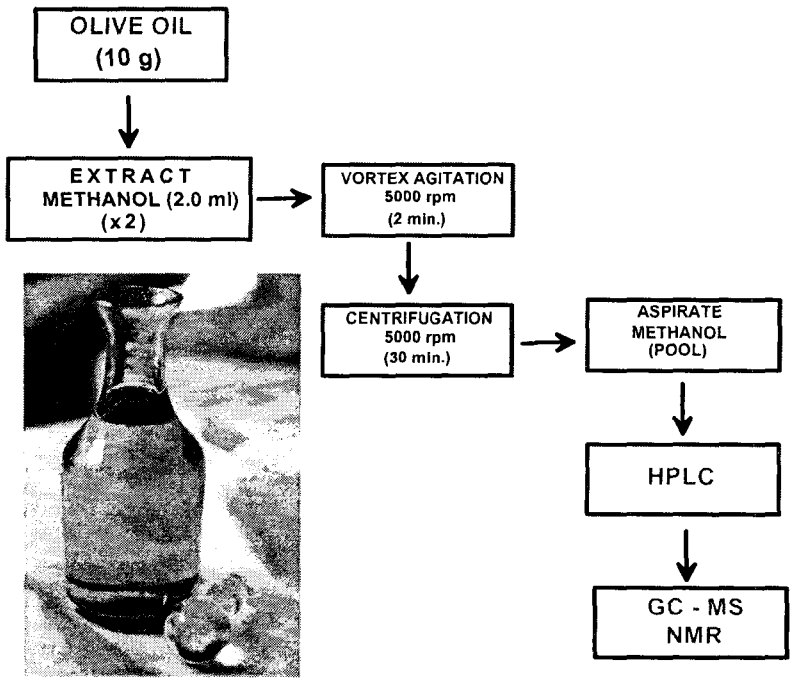

Figure 4. Protocol for the extraction and analysis of phenolic antioxidants in olive oil.

major individual phenolic compounds isolated from olive oil by semi-preparative HPLC were confirmed by NMR[13], GC-MS[14] and by ESI-MS, GC-MS and NMR.[16-19] These comprise the secoiridoids (SID), simple phenols and their precursors (Fig. 5). However the presence of lignans (Fig. 6) in olive oil was only discovered recently.[16] A typical HPLC chromatogram of a methanolic extract of extra virgin olive oil (Fig. 7) displays 7 major identifiable peaks of which 1-4 and 6-7 correspond to

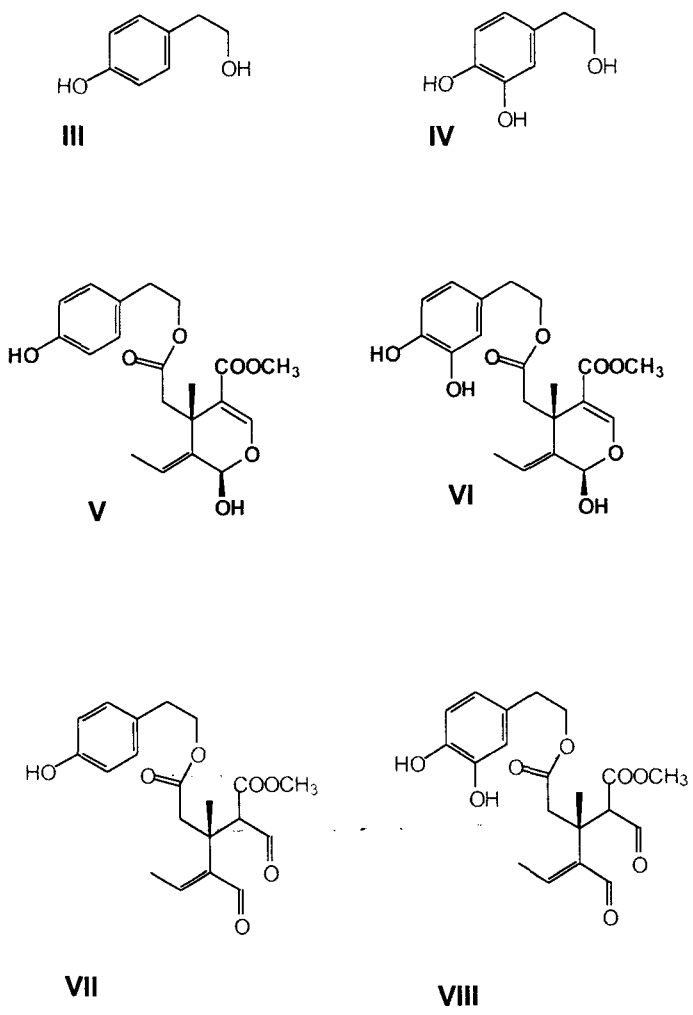

Figure 5. Strucures of the secoiridoids detected in olive oil.

Tyrosol
Hydroxytyrosol
Aglycone of ligstroside
Oleuropein
Dialdehydic form of ligstroside lacking a carboxymethyl group
Dialdehydic form of oleuropein lacking a carboxymethyl group

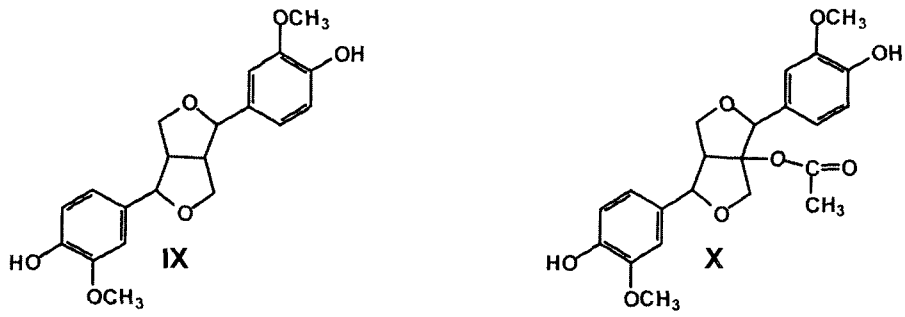

Figure 6. Structures of the lignans detected in olive oil

(+)-Pinoresinol

(+)-1-Acetoxypinoresinol

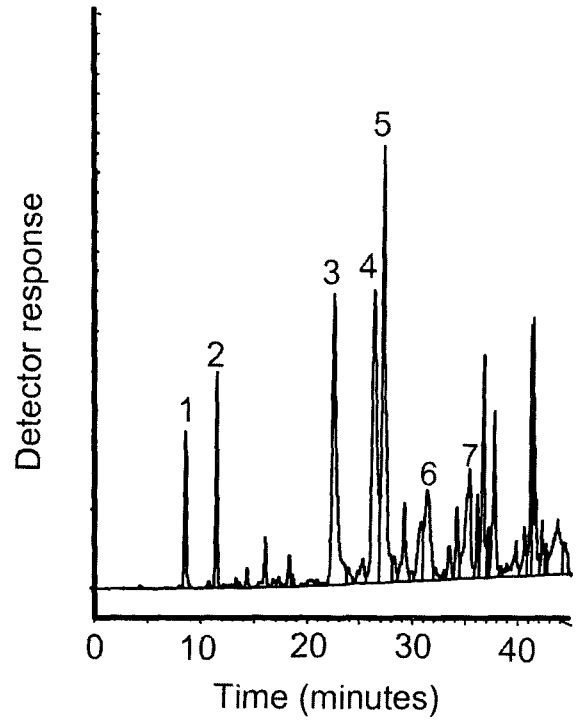

Figure 7. HPLC chromatogram of a methanolic extract of an extra virgin olive oil.

Hydroxytyrosol

Tyrosol

Dialdehydic aglycone of oleuropein glucoside lacking a carboxymethyl group

Dialdehydic aglycone of ligstroside lacking a carboxymethyl group

(+)-1-Acetoxypinoresinol and (+)-pinoresinol

Aglycone of oleuropein glucoside

Aglycone of ligstroside

hydroxytyrosol (**IV**), tyrosol (**III**), the dialdehydic form of oleuropein [SID-1] lacking a carboxymethyl group (**VI**) the dialdehydic form of ligstroside [SID-2] lacking a carboxymethyl group (**V**), the aglycone [SID-3] of oleuropein (**VIII**) and the aglycone [SID-4] of ligstroside (**VII**). Peak 5 in the chromatogram represents the lignans (+)-pinoresinol (**IX**) and (+)-1-acetoxypinoresinol (**X**) and which co-elute in this system.

Montedoro *et al.*[13-14] reported total phenolic content of olive oils over 500 mg/kg which is contrary to the data of Owen *et al.*[16-19] who showed on average, that olive oils (Table 1) contained 196 ± 19 mg/kg total phenolics as judged by HPLC analysis. However the value for VOQ (232 ± 15 mg/kg) was significantly higher than that of RVO (62 ± 12 mg/kg; $P < 0.0001$). A comprehensive evaluation of the individual phenolics in olive oils was conducted by Owen *et al.*[16] who showed that the difference in total phenolics between VOQ and RVO was also reflected in the concentration (Table 2) of the major individual components. Appreciable quantities of hydroxytyrosol and tyrosol were detected (Table 2) in olive oils as judged by HPLC with an average of 11.66 ± 2.60 [SEM] and 22.13 ± 3.82 mg/kg respectively. Again, there was a significant difference in the concentration of these phenolics in VOQ (hydroxytyrosol, 14.42 ± 3.01; tyrosol, 27.45 ± 4.05 mg/kg) and RVO (hydroxytyrosol, 1.74 ± 0,84; tyrosol, 2.98 ± 1.33 mg/kg: $P < 0.05$ and $P < 0.01$ respectively).

Table 1. The concentration of total and individual phenolic compounds in olive oils.

Phenolic compound	Olive oil			
Mg/kg	ALL (n = 23)	VOQ (n = 18)	RVO (n = 5)	*P* value*
Total	196 ± 19	232 ± 15	62 ± 12	< 0.0001
Hydroxytyrosol	11.66 ± 2.60	14.42 ± 3.01	1.74 ± 0.84	< 0.05
Tyrosol	22.13 ± 3.82	27.45 ± 4.05	2.98 ± 1.33	< 0.01
Total simple phenols (TSP)	33.79 ± 4.48	41.87 ± 6.17	4.72 ± 2.15	< 0.01
Secoiridoid-1	7.97 ± 2.57	9.62 ± 3.18	2.00 ± 0.87	ns
Secoiridod-2	15.75 ± 3.54	18.09 ± 4.31	7.30 ± 3.01	ns
Total secoiridoids (SID)	23.71 ± 5.61	27.72 ± 6.84	9.30 ± 3.81	ns
Lignans	34.09 ± 4.42	41.53 ± 3.93	7.29 ± 2.56	< 0.001
TSP + SID + lignans	91.59 ± 10.57	111.12 ± 9.99	21.31 ± 8.03	< 0.001

Data expressed in mg/kg ± SEM,7
VOQ:- extra virgin olive oil
RVO:- refined virgin oil
*VOQ versus RVO
ns:- not significant

The concentration of SID (Table 1) in olive oils was variable with mean values of 7.97 ± 2.57 mg/kg (SID-1) and 15.75 ± 3.54 mg/kg (SID-2) and were higher in VOQ (SID-1, 9.62 ± 3.18; SID-2, 18.09 ± 4.31) compared to RVO (SID-1, 2.00 ± 0.87; SID-2, 7.30 ± 3.01) but these differences were not significant. On the other hand despite appreciable inter-oil variation (Fig. 8) the concentration (Table 1) of lignans in VOQ (41.53 ± 3.93 mg/kg) was significantly higher ($P < 0.001$) than in RVO (7.29 ± 2.56 mg/kg).

The aglycones of oleuropein glucoside and ligstroside are also evident in considerable quantities in the HPLC (Fig. 7) and GC-MS chromatograms but the non-homogeneity of the peaks in many of the oils prevents definitive quantitation. Studies are in progress in our laboratory to improve their separation by HPLC.

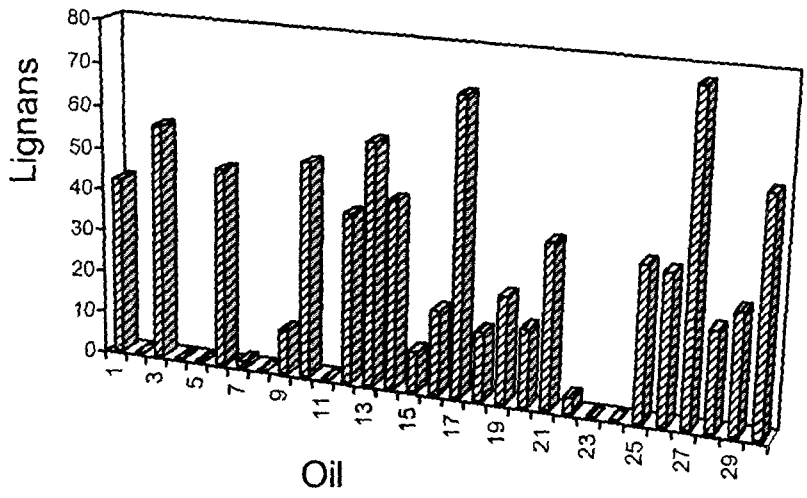

Figure 8. Content of lignans in various types of oils.

Olive column – extra virgin olive oils; green column – refined virgin oils; yellow column – seed oils

Antioxidant potential of olive oil phenolic compounds

The antioxidant potential of phenolic compounds in olive oil has also been the subject of cosiderable interest. This not only has relevance to a chemoprotective effect in humans but is also a major factor in the high stability (shelf life) of olive oils. The relatively high content (over 70%) of the monounsaturated fatty acid, oleic acid is also of importance here because it is far less susceptible to oxidation than the polyunsaturated fatty acid linoleic acid which predominates in e.g. sunflower oil.[18]

The antioxidant effect of natural phenols on olive oils was studied by Papadoupoulos and Boskou.[20] They added extracts of a VOQ containing 200 p.p.m. polyphenols to a refined, bleached and deodorised oil (depleted of phenolic antioxidants) and demonstrated significant inhibition of autooxidation (peroxide value) over time compared to samples without addition. A comparison of individual compounds which have been reported in the phenolic fraction of olive oil showed that this effect was more pronounced in the presence of hydroxytyrosol [protection factor at 20 days (PF_{20}) = 15.2] compared to caffeic acid (5.7) and protocatechuic acid (2.7) while other simple phenols were only marginally effective.

Salami *et al.*[21] investigated the formation of isoprostanes during *in vitro* oxidation of LDL by copper sulphate. They demonstrated that inclusion of hydroxytyrosol in the incubation mixture significantly inhibited the production of isoprostanes and other markers of lipid peroxidation.

Manna *et al.*[22] investigated the effects of reactive oxygen species on the intestinal epithelium using the Caco-2 human cell line as a model. Oxidative stress was induced in the apical department of these cells by the addition of 10 mM hydrogen peroxide or by the addition of 10 milli-units of xanthine oxidase in the presence of xanthine (250 μM). This caused a significant decrease in viability of the cells, whereas a significant increase in intracellular concentrations of malondialdehyde and paracellular inulin transport ensued, indicating the occurrence of lipid peroxidation and monolayer permeability changes respectively. The hydrogen peroxide and xanthine oxidase induced oxidative stress was inhibited completely however in the presence of 100-500 μM concentrations of hydroxytyrosol. Surprisingly tyrosol was ineffective up to a concentration of 500 μM. The authors therefore propose that the antioxidant effects of hydroxytyrosol in this model is dependent on a dihydroxy substituted phenol ring.

Using the hypoxanthine/xanthine oxidase model[23-24] for the generation of reactive oxygen species, Owen *et al.*[16] studied the antioxidative potential of methanolic extracts from a range of VOQ (n = 18) and RVO (n = 5) in comparison to SO (n = 7). All oil extracts were shown to exhibit antioxidant properties to a greater or lesser extent. On average, scavenging of the hydroxyl radical ($HO^{\bullet}$) was significantly higher by extracts of olive oil than those of seed oils. In fact, extracts of the seed oils exhibited minimum antioxidant activity and the potency of the VOQ extracts was significantly greater than that of SO ($P < 0.0001$) and RVO ($P < 0.05$).

In addition to their direct antioxidant capacity, extracts of olive oil were also potent inhibitors of xanthine oxidase activity as judged by HPLC analysis against a standard curve of uric acid. On average, while SO had little effect (inhibition, 6%), xanthine oxidase activity was inhibited to an extent of 48% by extracts of RVO and 73% by extracts of VOQ ($P < 0.05$ and $P < 0.0001$ in comparison to SO respectively).

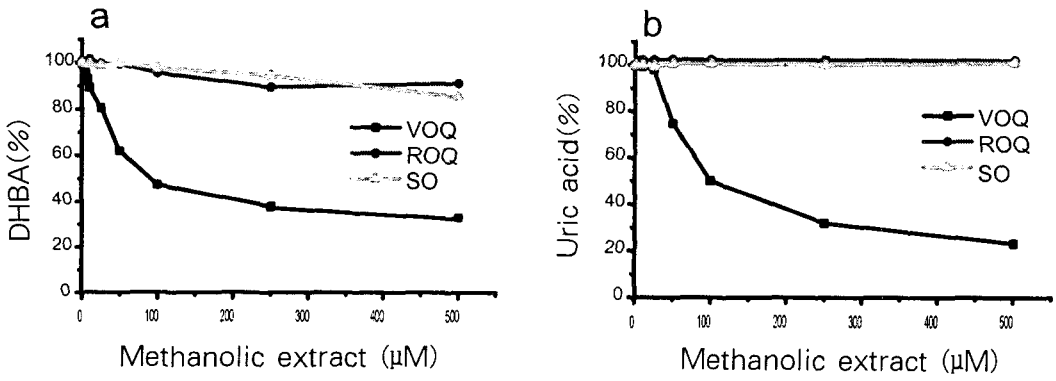

Figure 9a. Attenuation of of reactive oxygen species detection by extracts of various oils in the hypoxanthine/xanthine oxidase assay.
b. Inhibition of xanthine oxidase activity by extracts of oils.
VOQ – extra virgin olive oil; RVO – refined virgin oil; SO – seed oils, DHBA – dihydroxy benzoic acids

A comparison was also made between the antioxidant capacity of a concentration range of methanol extracts of each of the three oil types. The data shows, that while extracts of a seed oil (sunflower) and a RVO had minimal effects on the hydroxylation of salicylic acid by $HO^{\bullet}$ and on xanthine oxidase activity, a VOQ extract had significant dose dependent effects on both the hydroxylation of salicylic acid by $HO^{\bullet}$ (Figure 10a) and on xanthine oxidase activity (Figure 10b).

Also the phenolic substances isolated and purified from olive oil were potent antioxidants in comparison to the classical *in vivo* and *in vitro* free radical scavengers vitamin E (Trolox) and dimethylsulphoxide respectively. Of the three classes of phenolic substances detected in significant quantities in olive oil tyrosol (simple phenol), SID-1 (secoiridoid) and (+)-1-acetoxypinoresinol (lignan) gave stronger responses than the classical antioxidant Trolox (Fig. 11).

Owen *et al.*[23-24] have shown that the faecal matrix is capable of generating reactive oxygen species in abundance and furthermore established the potential of phenolic compounds isolated from olive oil to scavenge reactive oxygen species generated in the stool.[18] The data showed that all three classes of phenolic antioxidants significantly attenuated the signals obtained in their absence. The IC_{50} values obtained were of the same order as in the standard assay.

DISCUSSION

The data addressed in this overview reveals that olives and olive oil, especially VOQ contain significantly higher concentrations of phenolic antioxidants than seed oils and their precursors.

Therefore in an area such as the Mediterranean basin where olive oil is the cooking and garnishing fat of choice, and olives are eaten on a regular basis, intake of phenolic antioxidants in the diet is likely to be considerably higher than in other areas of Europe. Probably this is a major factor which determines the far lower incidence of cancer and heart disease in the region. But what is the mechanism?

Habitual high intakes of olives and olive oil (especially VOQ) will provide a continuous supply of antioxidants which may mediate their effects by reducing oxidative stress via inhibition of lipid peroxidation, a factor which is currently linked to a host of diseases such as cancer, heart disease and aging. The compounds described here are fat soluble and therefore a considerable proportion is likely to be absorbed and thereby should have chemopreventive effects against among others, breast cancer. The unabsorbed remainder will reach the large bowel where they can exert their chemopreventive effects against colorectal cancer.

The identification of lignans as major antioxidant components of the phenolic fraction in extra virgin olive oil especially[17] has considerable impact. The data derived from fermentation studies and mass spectrometry techniques shows conclusively that the lignan precursors in VOQ are metabolised by the bacterial flora of the gastrointestinal tract to the mammaliam lignans enterodiol and enterolactone. These enterolignans have been studied in far greater depth than other classes of phenolic antioxidants. Animal, cellular and metabolic studies have shown they possess important biological effects, which may contribute to their potential as chemopreventive agents. Lignans have been shown to inhibit skin, breast, colon and lung cancer cell growth.[25-26] In animal models consumption of flaxseed, a concentrated source of lignans, has been shown to inhibit the development of early biomarkers of breast cancer risk [27,28] and breast cancer itself.[29] Proposed mechanisms by which lignans may inhibit carcinogenesis include antiviral[30] and antioxidant[16] activities. In addition, the similarities in structure among lignans, oestradiol and the synthetic anti-oestrogen tamoxifen, suggest that lignans may also exert their anti-carcinogenic influence in part as a result of anti-oestrogenic effects.[31] In fact, lignans have been shown to inhibit placental[32] and adipocyte[32] oestrogen synthesis, to inhibit oestradiol-induced proliferation of MCF-7 human breast carcinoma cells[33] and to stimulate sex hormone-binding globulin synthesis with a subsequent decrease in free oestradiol.[34]

These observations have ramifications for the chemopreventive effect of the Mediterranean diet of which olive oil is an essential component. The differences underscored in this review, not only between VOQ and RVO, but also the variation in antioxidant content of VOQ indicates that, in future epidemiologic and case control studies, both the nature and source of olive oil consumed should be differentiated in ascertaining cancer risk. The necessity for this is justified by the data showing that olive oil phenolics scavenge reactive oxygen species generated by human biological material (faecal

matrix) which otherwise could lead to the formation of highly pro-mutagenic exocyclic DNA adducts in the colonic epithelium and other tissues.[35] Finally the study of the inter-relation between reactive oxygen species and dietary antioxidants in olive oil is an area of real promise for elucidating mechanisms of carcinogenesis and possible future chemopreventive strategies. Therefore the methodology described is to be applied in the very near future not only to the Heidelberg arm of the EPIC study, but also to a range of clinical studies in collaboration with local, national and international partners.

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References

1. Keys A, and Keys M. : How to Eat Well and Stay Well: the Mediterranean way, Doubleday and Co, New York, NY, USA M (1975)
2. Keys A, Aravanis C, Van Buchem H, *et al.* The diet and all-causes death rate in the Seven Countries Study. *Lancet* **2,** 58-61 (1981)
3. Keys A, Menotti A, Karvonen MJ *et al.* The diet and 15-year death rate in The Seven Countries Study. *Am. J. Epidemiol.* **124,** 903-15 (1986)
4. Assmann G, de Backer G, Bagnara S *et al.* International consensus statement on olive oil and the Mediterranean diet: implications for health in Europe. Eur. J. Cancer Prev. **6,** 418-21 (1997)
5. Martin-Moreno JM, Willett WC, Gorgojo L, *et al.* Dietary fat, olive oil intake and breast cancer risk. *Int. J. Cancer* **58,** 774-80 (1994)
6. Trichopoulou A, Katsouyanni K, Stuver S, *et al.* Consumption of olive oil and specific food groups in relation to breast cancer risk in Greece. *J. Nat. Cancer Inst* **87,** 110-6 (1995)
7. La Vecchia C, Negri E, Franceschi S, Decarli A, Giacosa A, Lipworth L. Olive oil, other dietary fats, and the risk of breast cancer (Italy). *Cancer Causes and Control* **6,** 545-50 (1998)
8. Braga C, La Vecchia C, Francheschi S, *et al.* Olive oil, other seasoning fats, and the risk of colorectal carcinoma. *Cancer* **82,** 448-53 (1998)
9. Levi F, Lucchini F, La Vecchia C. Worldwide patterns of cancer mortality. *Eur. J. Cancer Prev.* **3,** 109-143 (1994)
10. Visioli E, Galli C. Natural antioxidants and prevention of coronary heart disease: the potential role of olive oil and its minor constituents. *Nutr. Metab. Cardiovasc. Dis.* **5,** 306-14 (1995)
11. EC Council regulations No. 356192 (1992)

12. Hill MJ, Giacosa A. The Mediterranean diet. Eur. J. Cancer Prev. **1,** 339-340 (1992)
13. Montedoro G, Servilli M, Baldioli M, Miniati E. Simple and hydrolysable phenolic compounds in virgin olive oil. 2. Initial characterisation of the hydrolysable fraction. *J. Agric. Food. Chem.* **40,** 1577-80 (1992)
14. Montedoro G, Servilli M, Baldioli M, Selvaggini R, Miniati E, Macchioni A. Simple and hydrolysable phenolic compounds in virgin olive oil. 3. Spectroscopic characterisation of the secoiridoid derivatives. *J. Agric. Food Chem.* **41,** 2228-34 (1993)
15. Angerosa F, d'Alessandro N, Konstantinou P, Di Giacinto L. GC-MS evaluation of phenolic compounds in virgin olive oil. *J. Agric. Food Chem.* **43,** 1802-7 (1995)
16. Owen RW, Mier W, Giacosa A, Hull WE, Spiegelhalder B, Bartsch. H. Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. *Food Chem. Tox.* **38,** 647-59 (2000)
17. Owen RW, Mier W, Giacosa A, Hull WE, Spiegelhalder B, Bartsch H. Phenolic and lipid components of olive oils: Identification of lignans as major components of the phenolic fraction of olive oil. *Clin. Chem.* **46,** 976-88 (2000)
18. Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *Eur. J. Cancer* **36,** 1235-47 (2000)
19. Owen RW, Giacosa A, Hull WE, Haubner R, Würtele G, Spiegelhalder B. Bartsch, H. Olive oil consumption and health: the possible role of antioxidants. *Lancet Oncology* **1,** 107-12 (2000)
20. Papadopoulos G, Boskau D. Antioxidant effect of natural phenols on olive oil. *J. Am. Oil. Chem.* **68,** 669-71 (1991)
21. Salami M, Galli C, De Angelis L, Visioli F. Formation of F_2-isoprostanes in oxidised low density lipoprotein: inhibitory effect of hydroxytyrosol. *Pharmacological Res.* **31,** 275-9 (1995)
22. Manna C, Galletti P, Cucciolla V, Moltedo O, Leone A, Zappia V. The protective effect of the olive oil polyphenol (3,4-dihydroxyphenyl)-ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells. *J. Nutr.* **127,** 286-92 (1996)
23. Owen RW, Wimonwatwatee T, Spiegelhalder B, Bartsch H. A high performance liquid chromatography method for quantification of hydroxyl radical formation by determination of dihydroxy benzoic acids. *Eur. J. Cancer Prev.* **5,** 233-40 (1996)
24. Owen RW, Spiegelhalder B, Bartsch H. Generation of reactive oxygen species by the faecal matrix. *Gut* **46,** 225-32 (2000)
25. Kardono LB, Tsauri S, Pezzuto JM, Kinghorn AD. Cytotoxic constituents of the bark of Plumeria rubra collected in Indonesia. *J. Nat. Prod.* **53,** 1447-55 (1990)

26. Hirano T, Fukuoka F, Oka K, *et al.* Antiproliferative activity of mammalian lignan derivatives against the human breast carcinoma cell line, ZR-75-1. *Cancer Invest.* **8,** 592-602 (1990)
27. Serraino M, Thompson LU. The effect of flaxseed supplementation on early risk markers for mammary carcinogenesis. *Cancer Lett.* **60,** 135-42 (1991)
28. Serraino M, Thompson LU. The effect of flaxseed consumption on the initiation and promotional stages of mammary carcinogenesis. *Nutr. Cancer* **17,** 153-9 (1992)
29. Thompson LU, Seidl MM, Rickard SE, Orcheson LJ, Fong HH. Antitumorigenic effect of a mammalian lignan precursor from flaxseed. *Nutr. Cancer* **26,** 159-65 (1996)
30. Schroder HC, Merz H, Steffen R, *et al.* Differential *in vitro* anti-HIV activity of natural lignans. *Zeitschrift Naturforschung* **45,** 1215-21 (1990)
31. Adlercreutz H, Bannwart C, Wähälä K, *et al.* Inhibition of human aromatase by mammalian lignans and isoflavoid phytooestrogens. *J. Steroid Biochem. Mol. Biol.* **44,** 147-53 (1993)
32. Wang C, Mäkelä T, Hase T, Adlercreutz H, Kurzer MS. Lignans and flavonoids inhibit aromatase enzyme in human preadipocytes. *J. Steroid Biochem. Mol. Biol.* **50,** 205-12 (1994)
33. Mousavi Y, Adlercreutz H. Enterolactone and estradiol inhibit each others proliferative effect on MCF-7 breast cancer cells in culture. *J Steroid Biochem. Mol. Biol.* **41,** 615-9 (1992)
34. Adlercreutz H, Mousavi Y, Clark J, *et al.* Dietary phytoestrogens and cancer: *in vitro* and *in vivo* studies. *J. Steroid Biochem. Mol. Biol.* **41,** 331-7 (1992)
35. Bartsch H, Nair J, Owen RW. Dietary PUFAs and cancer of the breast and colorectum: compelling evidence for their role as risk modifiers. *Carcinogenesis* **20,** 2209-18 (1999)