PERGAMON

European Journal of Cancer 36 (2000) 1235–1247

European Journal of Cancer

www.ejconline.com

The antioxidant/anticancer potential of phenolic compounds isolated from olive oil

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Received 24 March 2000; accepted 11 April 2000

Abstract

In our ongoing studies on the chemoprevention of cancer we have a particular interest in the health benefits of the Mediterranean diet, of which olive oil is a major component. Recent studies have shown that extravirgin olive oil contains an abundance of phenolic antioxidants including simple phenols (hydroxytyrosol, tyrosol), aldehydic secoiridoids, flavonoids and lignans (acetoxypinoresinol, pinoresinol). All of these phenolic substances are potent inhibitors of reactive oxygen species attack on, e.g. salicylic acid, 2-deoxyguanosine. Currently there is growing evidence that reactive oxygen species are involved in the aetiology of fat-related neoplasms such as cancer of the breast and colorectum. A plausible mechanism is a high intake of ω-6 polyunsaturated fatty acids which are especially prone to lipid peroxidation initiated and propagated by reactive oxygen species, leading to the formation (via α,β-unsaturated aldehydes such as *trans*-4-hydroxy-2-nonenal) of highly pro-mutagenic exocyclic DNA adducts. Previous studies have shown that the colonic mucosa of cancer patients and those suffering from predisposing inflammatory conditions such as ulcerative colitis and Crohn's disease generates appreciably higher quantities of reactive oxygen species compared with normal tissue. We have extended these studies by developing accurate high performance liquid chromatography (HPLC) methods for the quantitation of reactive oxygen species generated by the faecal matrix. The data shows that the faecal matrix supports the generation of reactive oxygen species in abundance. As yet, there is a dearth of evidence linking this capacity to actual components of the diet which may influence the colorectal milieu. However, using the newly developed methodology we can demonstrate that the antioxidant phenolic compounds present in olive oil are potent inhibitors of free radical generation by the faecal matrix. This indicates that the study of the inter-relation between reactive oxygen species and dietary antioxidants is an area of great promise for elucidating mechanisms of colorectal carcinogenesis and possible future chemopreventive strategies.

Keywords: Antioxidants; Breast cancer; Colorectal cancer; HPLC; Mass spectrometry; Nuclear magnetic resonance spectroscopy; Olive oils; Phenolic compounds; Reactive oxygen species; Seed oils

1. Introduction

The Mediterranean diet with its high content of fruits, vegetables, fibre, fish and olive oil represents a healthy and disease-preventive diet [1]. Its protective effect appears quite broad in that it not only has a chemoprotective effect against cancer (especially of the colorectum and breast) but also significantly reduces mortality from heart disease [2,3]. A major component is represented by olive oil and global production (approximately 1.3 billion tonnes) is shown in Fig. 1 of which the majority is produced in Europe (Fig. 2).

Recent data suggests that the components of dietary olive oil may have a greater role in disease prevention than previously thought [4–8]. For example, the content of oleic acid a mono-unsaturated long-chain fatty acid ($C_{18\text{-}1}$) is high in olive oil compared with the seed oils, e.g. sunflower oil (Fig. 3) which are rich in polyunsaturated linoleic acid ($C_{18\text{-}2}$) a fatty acid that is especially prone to oxidation by a number of processes.

Olive oil can be consumed in the natural unrefined state known as extra virgin oil quality (VOQ), or as a

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0959-8049/00/$ - see front matter
PII: S0959-8049(00)00103-9

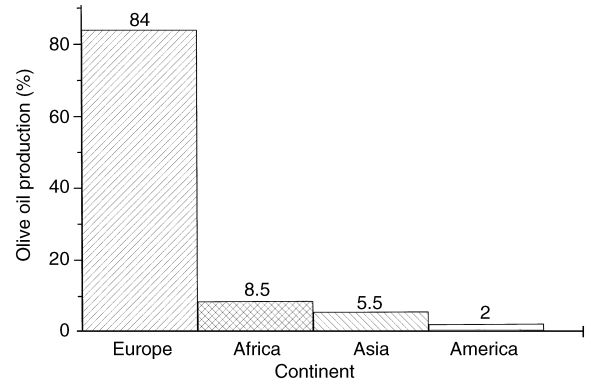

Fig. 1. Global production of olive oil.

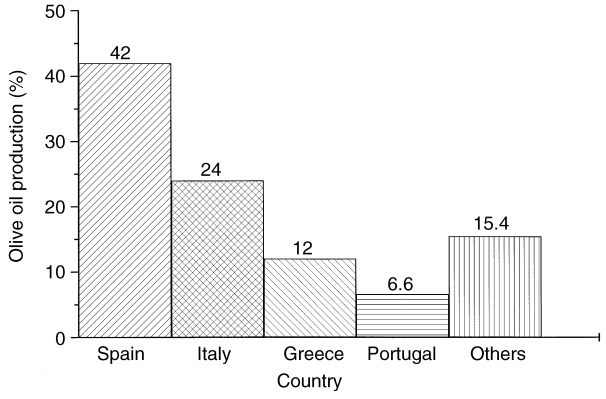

Fig. 2. European production of olive oil.

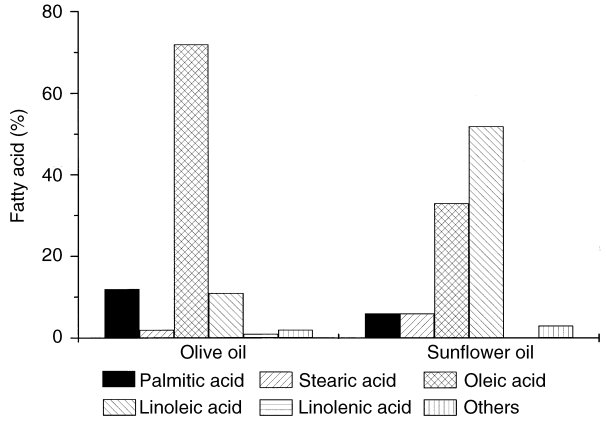

Fig. 3. Comparison of the composition of long chain fatty acids in olive and sunflower oils.

refined product. The refined product is made either from virgin oil and called refined virgin oil (RVO) or from solvent-extracted oil called refined husk oil (RHO) (Fig. 4) [9]. Virgin and refined oils differ little in fatty acid composition; oleic acid is the main component, with minor but nutritionally relevant contributions from palmitic acid and the essential linoleic acid. The proportion of linoleic acid in oils from the more southerly, warmer regions of the Mediterranean tends to be higher

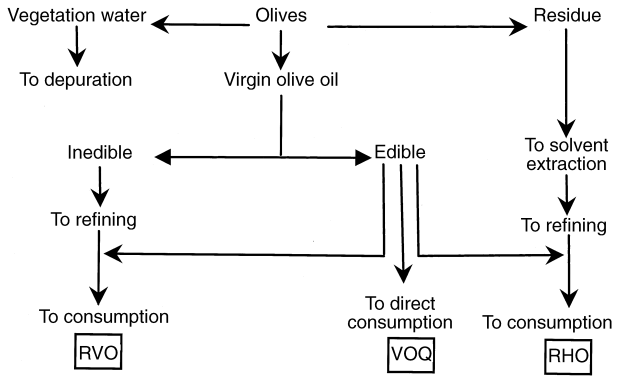

Fig. 4. Scheme for the production of various classes of olive oil.

than in other areas. VOQ, RVO and RHO differ slightly in composition if the minor components which account for 1% of VOQ to 3% of RHO, are considered [10].

These minor components, several hundred chemical substances, can be grouped in classes as follows:

Hydrocarbons (saturated, unsaturated, linear, branched).
Esters (fatty acid derivatives of short-chain alcohols, long-chain alcohols, sterols, triterpenic alcohols, monoterpenic alcohols, phenols).
Aldehydes (medium- and long-chain, monoterpenic).
Alcohols (medium- and long-chain, monoterpenic, triterpenic).
Phenols (tocopherols, epoxyphenols).
Acids (in addition to free fatty acids, triterpenic acids, phenoxy acids).
Chlorophyll.

The higher absolute amount of minor components in RHO compared with VOQ and RVO, is chiefly due to fatty acid derivatives of long-chain alcohols (waxes) and to triterpenic alcohols. Many of these compounds contribute to the classical flavour of the oil: some are detrimental to the taste, imparting bitterness or a burning sensation [11]. The high stability (shelf-life) of olive oil is partly due to its high oleic acid content, which is less prone to oxidation than linoleic and linolenic acids. In addition, a strong positive contribution comes from the simple and complex phenolic compounds (tocopherols) including Vitamin E [12].

Because refining causes a partial loss of the preservative action of antioxidants, RVO and RHO are mixed with a portion of VOQ, and this blending process restores some of the flavour and storage characteristics.

As part of our investigations into the chemopreventive effects of the Mediterranean diet, we regarded it important to obtain an unequivocal profile of the major phenolic components in olive oil. To this end, we have studied a range of olive oils in comparison with seed oils to evaluate not only their composition but also the

antioxidant potential of the major phenolic components in recently developed systems [13–16].

2. Materials and methods

2.1. Oils

Thirty oils comprising VOQ ($n=18$), RVO ($n=7$) and seed oil (SO) ($n=5$) currently on the Italian market were studied. Of these an oil (olio di oliva extravergine 'ligustro' prodotto e confezionato dalla carapelli firenze s.p.a. via b. cellini 75 tavarnelle val di pesa) shown to contain considerable quantities of linked phenols [16] was chosen for the isolation of sufficient quantities of these substances for detailed analytical and spectroscopic analyses.

2.2. Standard compounds

(*p*-Hydroxyphenyl)ethanol (tyrosol), catechol, syringic acid, *p*-hydroxybenzoic acid, vanillic acid, benzoic acid, 3,4-dihydroxy benzoic acid, homovanillic alcohol, *o*-coumaric acid, *p*-coumaric acid, gallic acid, Trolox and $Fe_2Cl_3.6H_2O$ were obtained from Sigma-Aldrich, (Steinheim, Germany). Ferulic acid, cinnamic acid, caffeic acid and ethylene diamine tetraacetic acid (EDTA) were obtained from Fluka Chemie (Buchs, Switzerland). Acetic acid, dimethylsulphoxide, methanol, hypoxanthine (HX), xanthine oxidase (XO) and salicylic acid (SA) were obtained from E. Merck (Darmstadt, Germany). Oleuropein glucoside was obtained from Extrasynthese (Z.I. Lyon Nord, Genay, France). (Dihydroxyphenyl)-ethanol (hydroxytyrosol) was synthesised from oleuropein glucoside as described by Owen and colleagues [20]. The structures of the standard compounds are given in Fig. 5.

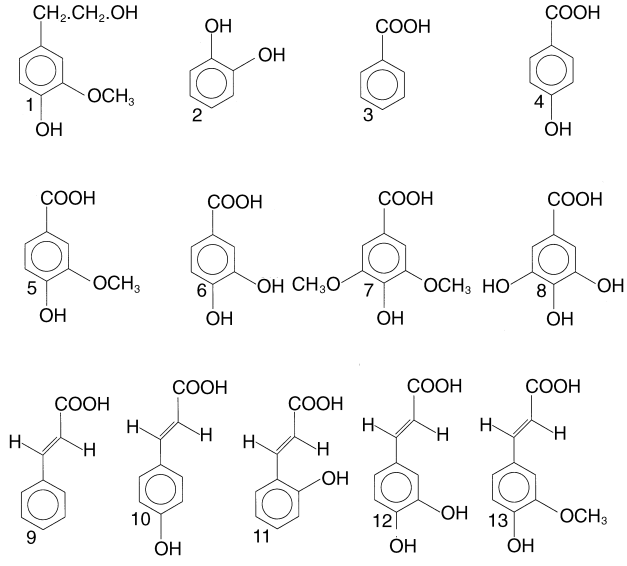

Fig. 5. Structures of the standard phenolic compounds. 1. Homovanillic alcohol; 2. Catechol; 3. Benzoic acid; 4. *p*-Hydroxybenzoic acid; 5. Vanillic acid; 6. 3,4-Dihydroxybenzoic acid; 7. Syringic acid; 8. Gallic acid; 9. Cinnamic acid; 10. *p*-Coumaric acid; 11. *o*-Coumaric acid; 12. Caffeic acid; 13. Ferulic acid.

2.3. Extraction protocol

The method used to extract the phenolic compounds from the oils is depicted in Fig. 6.

2.4. High performance liquid chromatography (HPLC)

Analytical HPLC was conducted on a Hewlett-Packard (HP) 1090 liquid chromatograph fitted with a C-18 (Latex, Eppelheim, Germany), reverse-phase 25 cm, (5 μm) column (internal diameter, 4.0 mm); semi-preparative HPLC was conducted on a similar column of 10 mm internal diameter. Detection of phenolic compounds was by means of an ultra violet (UV) detector set at an absorbance (A) of 278 nm at room temperature.

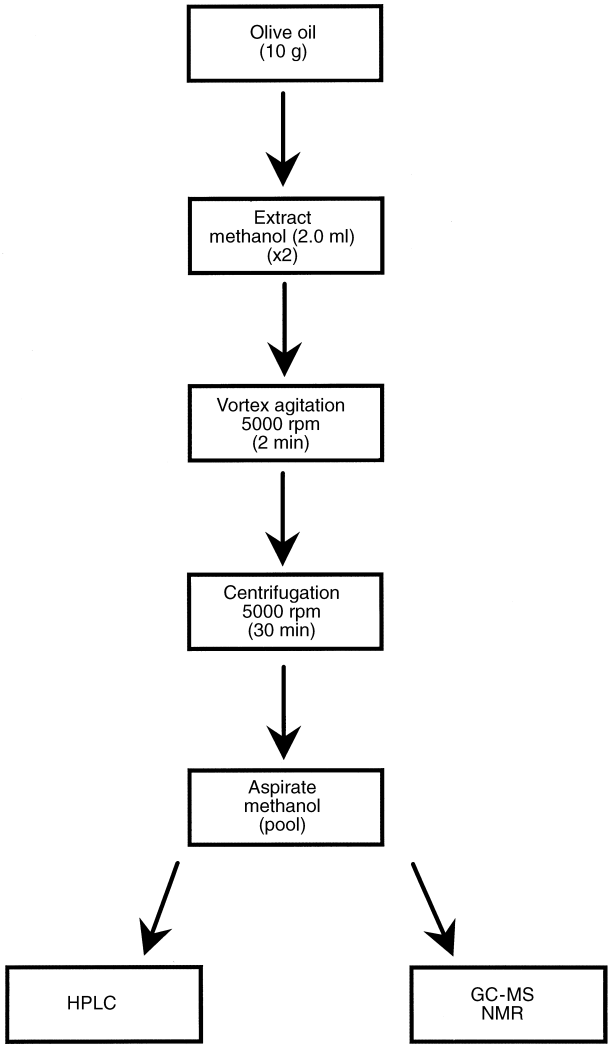

Fig. 6. Protocol for the extraction of phenolic compounds from olive oil.

For the evaluation of total phenols in the oil extracts, the mobile phase was methanol (100%) run isocratically for 20 min and, for the separation of individual compounds, the mobile phase consisted of 2% acetic acid in water (A) and methanol (B) utilising the following gradient over a total run time of 45 min: 95% A for 2 min, 75% A in 8 min, 60% A in 10 min, 50% A in 10 min and 0 A until completion of the run [15–19].

Extracts were dissolved in methanol prior to injection (20 μl) into the HPLC. The amount of hydroxytyrosol and tyrosol present in the extracts was calculated from their respective standard curves constructed from chromatograms run at A_{278} nm in the range 0–4 mM. The amounts of secoiridoids present in the extracts were determined against the standard curve of hydroxytyrosol. The flow rate of the mobile phase was 1 ml/min. Instrument control and data handling was by means of a HP Chemstation operating in the Microsoft® Windows™ software environment.

2.5. Gas-chromatography mass spectrometry (GC-MS)

Analyses were performed using a HP 5970 mass quadrupole spectrometer coupled to a HP 5890 gas chromatograph. Adequate separation of the analytes was achieved using a OV-101 capillary column, 30 m×0.25 mm I.D., 0.25 μm film thickness. Helium was used as carrier gas with a linear velocity of 38 ml/s. Sample aliquots of 1 μl were injected. The oven temperature programme was as follows: initial temperature 100°C, 100–270°C at 4°C/min, 20 min at 270°C. GC injector temperature was 250°C; the transfer line temperature was held at 320°C.

The conditions used in the electron impact (EI) mode were as follows: ion source temperature, 250°C: electron energy, 70 eV: filament current, 500 μA and electron multiplier voltage, 1200 V. Similar conditions were also utilised in the single ion monitoring mode. Prior to GC-MS, dried methanolic extracts were derivatized by addition of 100 μl of BSTFA (bis(trimethylsilyl)trifluoroacetamide) at room temperature for 15 min.

2.6. Electrospray ionisation mass spectrometry (ESI-MS)

ESI mass spectra were recorded on a Finnigan MAT TSQ 7000 mass spectrometer in methanol in both the positive- and negative-ion modes.

2.7. Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra were recorded in $CDCl_3$ or CD_3OD solvent on Bruker AC-250 and AM-500 spectrometers (Bruker Analytik, Rheinstetten, Germany) at ^1H frequencies of 250.133 MHz and 500.135 MHz, respectively, or ^{13}C frequencies of 62.896 MHz and 125.759 MHz, using conventional 1D Fourier transform techniques (^1D ^1H, ^{13}C with broadband ^1H decoupling, ^{13}C DEPT) for all compounds [20].

2.8. Measurement of antioxidant potential

2.8.1. Standard assay system

The method (Fig. 7) employed to assay the antioxidant activity of authentic phenolic standards, phenolics isolated and purified from olive oil, and, oil extracts, was based on the methods of Owen and colleagues [13–15]. Authentic standards, and individual components isolated and purified from olive oil, were tested in the range 0–4 mM against the classical *in vivo* and *in vitro* antioxidant controls, vitamin E (in the form of water soluble Trolox) and dimethylsulphoxide, respectively. The relevant concentration range in methanol, was added to 15.0 ml plastic centrifuge tubes in duplicate and the solvent was removed under a stream of nitrogen. The dried residue was resuspended in phosphate buffer (1.0 ml), and 5.0 μl of a 1:5 dilution of xanthine oxidase in NH_4SO_4 (3.20 mol/l) was added to initiate the reaction. The tubes were incubated for 3 h until reaction completion at 37°C. After incubation, 20 μl of the reaction mixture was analysed by HPLC using the mobile phase and conditions described under HPLC. The extent of diphenol (2,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid) produced by hydroxyl radical ($HO^{\bullet}$) attack on salicylic acid was determined from standard curves of the respective diphenols.

In the basic screen of the antioxidant capacity of neat oil, methanolic extracts (500 μl) were added to a 15 ml centrifuge tube and treated as described above. When concentration ranges of oil extracts were studied, 0–500 μl of methanolic extracts were added to 15 ml centrifuge tubes in duplicate and treated likewise. The concentration of individual phenolic components and the hydroxylation of hypoxanthine were monitored at *A* 278 nm, while the hydroxylation of salicylic acid was monitored at *A* 325 nm. The phenolic components of olive oil dissolved in the phosphate buffer, and endproducts of the enzyme or free radical reactions were quantitated against relevant standard curves measured at the same wavelength.

2.8.2. Faecal assay system

The methods were based on those of Owen and colleagues [13–16]. Briefly, hypoxanthine was omitted from the phosphate buffer and the generation of reactive oxygen species was initiated by the addition of freeze-dried faecal matrix (100 mg) to 10.0 ml buffer in 50 ml plastic centrifuge tubes. Incubation was conducted on a shaking water bath at 37°C for 18 h after which the solutions were centrifuged at 13 000 rpm for 10 min. Twenty microlitres of the clarified solutions was injected into the HPLC under the conditions described above for the detection of diphenols produced as a result of $HO^{\bullet}$ attack on salicylic acid.

2.8.3. Statistics

The differences between the oils in the composition of the phenolic compounds were analysed by ANOVA, while the interactions between antioxidants within the olive oils, and, inhibition of reactive oxygen species attack on salicylic acid were evaluated by multiple linear regression. Inhibitory concentrations [IC_{50}] were determined using the Table Curve programme (Jandel Scientific).

3. Results

3.1. Phenolic content of oils

The structures of the major individual phenolic compounds isolated from the large scale extract of olive oil by semi-preparative HPLC were confirmed by ESI-MS, GC-MS and NMR [20]: the structures of the secoiridoids, simple phenols and their precursors are depicted in Fig. 8 while the lignans are shown in Fig. 9. A HPLC

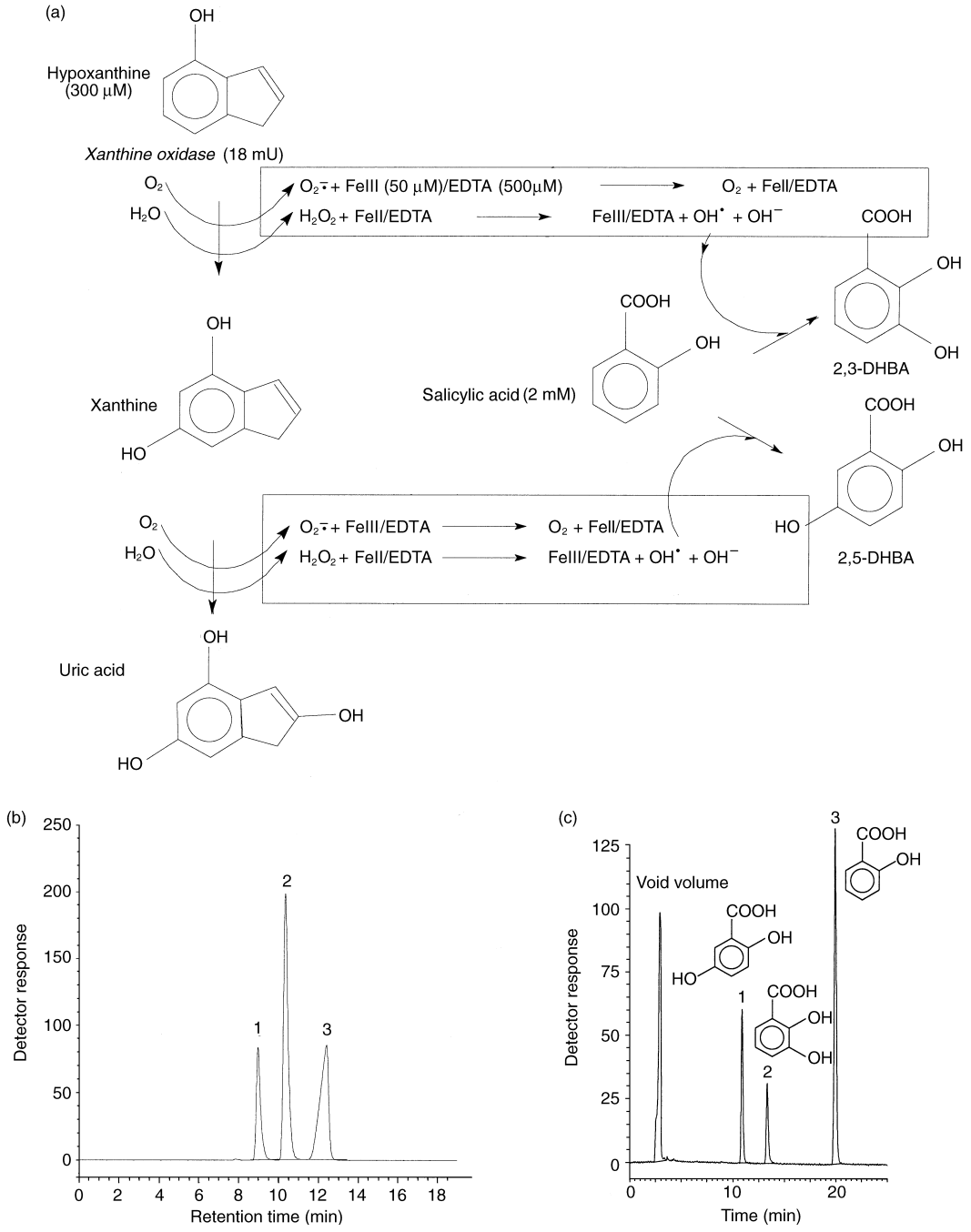

Fig. 7. Scheme for the generation of reactive oxygen species in the hypoxanthine/xanthine oxidase system. (a) Reaction. (b) HPLC chromatogram of hypoxanthine (1) and its hydroxylated products xanthine (2) and uric acid (3). (c) HPLC chromatogram of salicylic acid (3) and its hydroxylated metabolites 2,5-DHBA (1) and 2,3-DHBA (2).

chromatogram of this extract (Fig. 10) displayed seven major identifiable peaks of which one to four and six to seven corresponded to hydroxytyrosol (VIII), tyrosol (VII), the dialdehydic form of oleuropein glucoside lacking a carboxymethyl group (VI), the dialdehydic form of ligstroside lacking a carboxymethyl group (V), the aglycone of oleuropein glucoside (IV), the aglycone of ligstroside (III). Peak five in the chromatogram represented the lignans (+)-1-acetoxypinoresinol (IX) and (+)-pinoresinol (X) which co-elute in this system. Seed oils were devoid of these phenolic substances.

Other simple phenols which have been reported for extracts of olive oil (10 g) could not be detected routinely by HPLC. This was also the case when 100 g samples were extracted. However, GC-MS analysis revealed that vanillic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid and *o*-coumaric acid were present at very low concentrations. Because of this, their definitive quantitation was not attempted. This is in agreement with the data of Angerosa and colleagues [21] but is contrary to that of Montedoro and colleagues [17].

On average, olive oils (Table 1) contained 196±19 mg/kg total phenolics as judged by HPLC analysis, but the value for VOQ (232±15 mg/kg) was significantly higher than that of RVO (62±12 mg/kg; $P < 0.0001$).

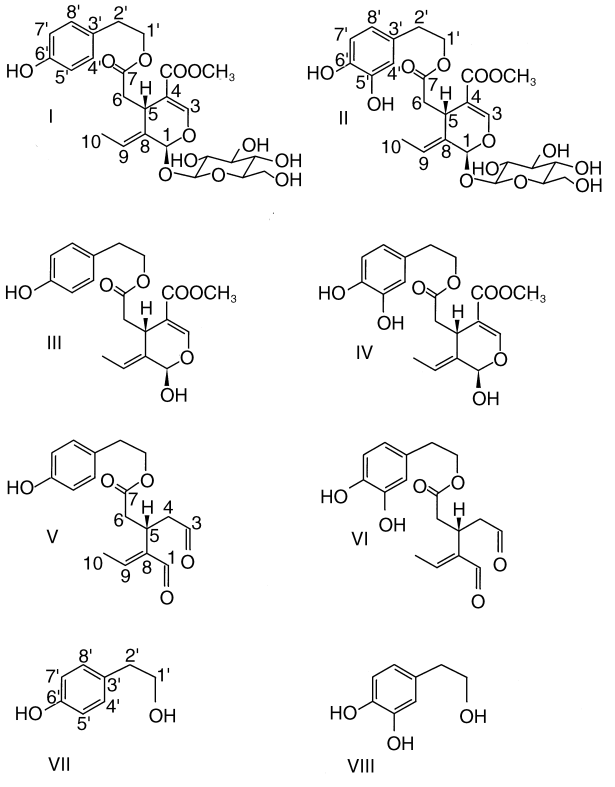

Fig. 8. Structures of the phenolic compounds and their precursors detected in olive oil. I. Ligstroside; II. Oleuropein glucoside; III. Aglycone of ligstroside; IV. Aglycone of oleuropein glucoside; V. Dialdehydic form of ligstroside aglycone lacking a carboxymethyl group; VI. Dialdehydic form of oleuropein glucoside aglycone lacking a carboxymethyl group; VII. Tyrosol; VIII. Hydroxytyrosol.

The difference in total phenolics (Fig. 11) between VOQ and RVO was also evident in the concentration (Table 1) of the major individual components. Appreciable quantities of hydroxytyrosol and tyrosol were detected (Table 1) in olive oils as judged by HPLC analysis against their respective standard curves with an average of 11.66±2.60 standard error of the mean (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).

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 with RVO (SID-1, 2.00±0.87; SID-2, 7.30±3.01) but these differences were not significant. In contrast, despite appreciable inter-oil variation (Fig. 12) 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 were also evident in considerable quantities in the HPLC (Fig. 10) and GC-MS chromatograms but the non-homogeneity of the peaks in many of the oils prevented definitive quantitation.

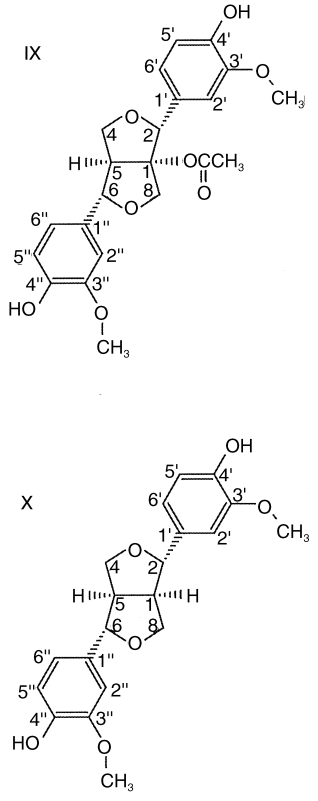

Fig. 9. Structures of the lignans detected in olive oil. IX. (+)-1-Acetoxypinoresinol; X. (+)-Pinoresinol.

3.2. Antioxidant capacity of authentic and olive oil phenolic compounds

3.2.1. Standard assay system

All of the authentic phenolic substances tested, were potent antioxidants in comparison to the classical *in vivo* and *in vitro* free radical scavengers vitamin E (Trolox) and dimethylsulphoxide respectively. The IC_{50} values are given in Table 2. All exhibited significantly stronger antioxidant properties than Trolox and in some cases dimethylsulphoxide. 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. 13).

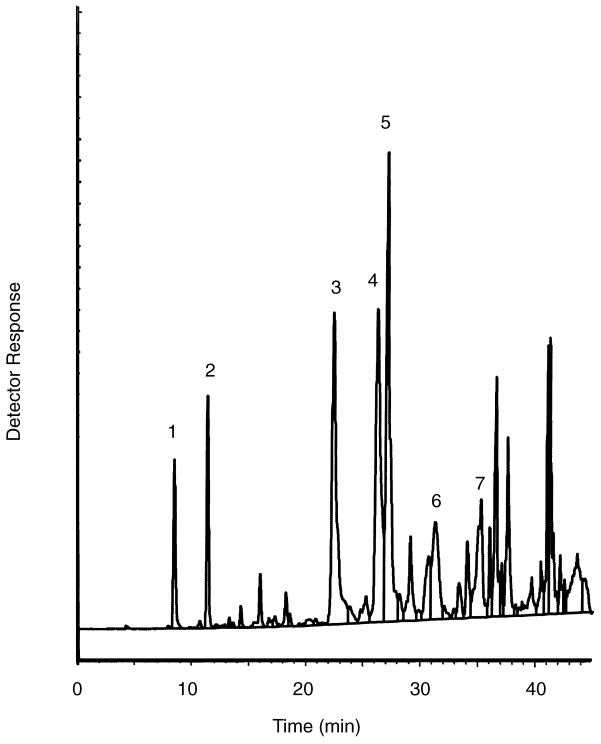

Fig. 10. HPLC chromatogram of a methanolic extract of an extra-virgin olive oil. 1. Hydroxytyrosol; 2. Tyrosol; 3. Dialdehydic aglycone of oleuropein glucoside lacking a carboxymethyl group; 4. Dialdehydic aglycone of ligstroside lacking a carboxymethyl group; 5. (+)-1-Acetoxypinoresinol and (+)-pinoresinol; 6. Aglycone of oleuropein glucoside; 7. Aglycone of ligstroside.

All oil extracts were shown to exhibit antioxidant properties to a greater or lesser extent. On average, scavenging of $HO^{\bullet}$ was significantly higher by extracts of olive oil than those of seed oils (data not shown). 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 ($P<0.05$). Furthermore, the antioxidant capacity of the oil extracts was significantly correlated ($r=-0.88$; $P<0.0001$) with the total phenolic content of the oils (Fig. 14a) and many of the individual components (Table 3) of the olive oils showed a significant correlation with antioxidant activity, the stronger being the lignans ($r=-0.81$; $P<0.0001$: Fig. 14b) followed in decreasing order by SID-2, hydroxytyrosol, tyrosol ($P<0.001$) and SID-1 ($P<0.05$).

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% (Fig. 15) by extracts of VOQ ($P<0.05$ and $P<0.0001$ in comparison to SO, respectively). Inhibition of xanthine oxidase activity was strongly associated (Fig. 14c) with total phenolic content ($r=-0.86$; $P<0.0001$), and, again many of the individual components of the olive oils showed significant correlations with decreased xanthine oxidase activity (Table 3), the stronger being SID-2, and, the lignans

Table 1
The concentration of total and individual phenolic compounds in olive oils[a]

Phenolic compound mg/kg	Olive oil			
	ALL ($n=23$)	VOQ ($n=18$)	RVO ($n=5$)	P value[b]
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
Secoiridoid-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.
[a] VOQ, extravirgin oil; RVO, refined virgin oil; ns, not significant.
[b] VOQ versus RVO.

($P < 0.0001$) followed, in decreasing order by hydroxytyrosol ($P < 0.001$), SID-1 and tyrosol ($P < 0.01$).

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 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 $HO^{\bullet}$ (Fig. 16a) and on xanthine oxidase activity (Fig. 16b).

3.2.2. Faecal assay system

In a recently developed system [13–16] it was shown that the faecal matrix is capable of generating reactive oxygen species in abundance (Fig. 17) and therefore it was of interest to establish the potential of phenolic compounds isolated from olive oil to scavenge reactive

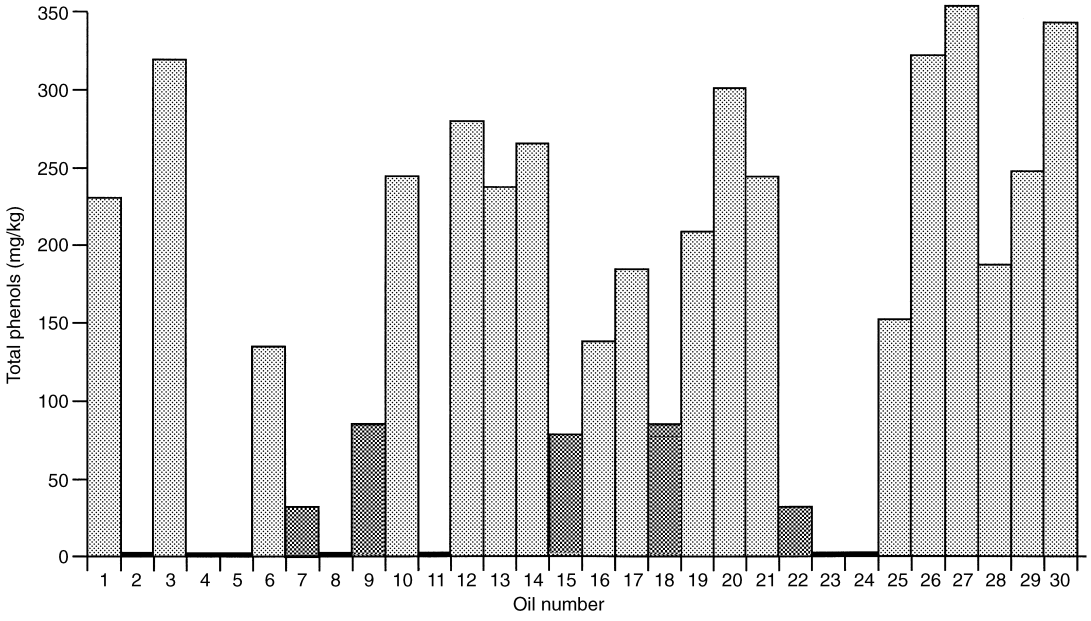

Fig. 11. Content of total phenolic compounds in the oils. ▒ extravirgin olive oils; ▓ refined virgin oils; ■ seed oils.

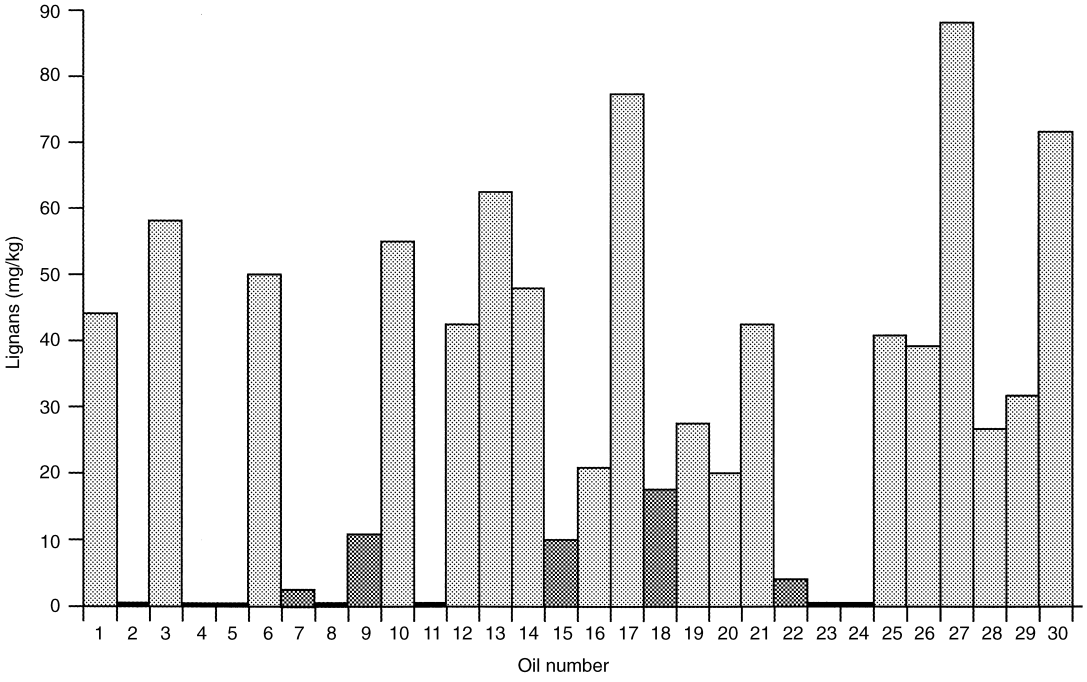

Fig. 12. Content of lignans in the oils. ▒ extravirgin olive oils; ▓ refined virgin oils; ■ seed oils.

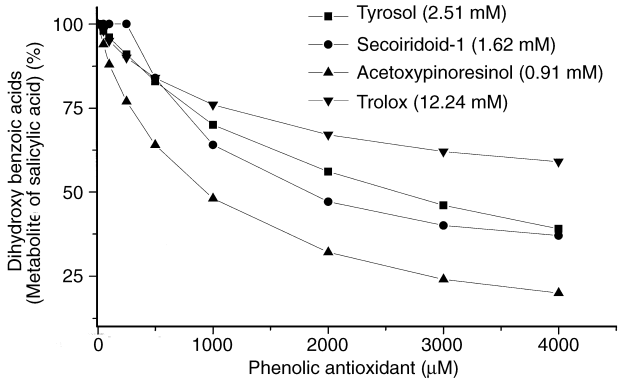

Fig. 13. Inhibitory concentration IC_{50} of a representation of phenolic compounds on hydroxyl radical attack on salicylic acid.

Table 2
Inhibitory concentration (IC_{50}) of individual phenolic compounds on reactive oxygen species attack on salicylic acid in the hypoxanthine/xanthine oxidase assay

Phenolic compound	IC_{50} (mM)
Hydroxytyrosol	1.34
3,4-Dihydroxybenzoic acid	3.03
Tyrosol	2.51
p-Hydroxybenzoic acid	1.69
Homovanillic alcohol	2.17
Vanillic acid	2.70
Caffeic acid	6.05
Syringic acid	3.19
p-Coumaric acid	2.33
Ferulic acid	1.56
o-Coumaric acid	2.32
Cinnamic acid	2.33
Benzoic acid	2.75
Catechol	2.50
(+)-1-Acetoxypinoresinol	0.91
Secoiridoid-1	1.62
Oleuropein glucoside	7.80
Trolox	12.24
Dimethylsulphoxide	2.30

oxygen species in this system. The data shows (e.g. for tyrosol; Fig. 18) 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.

4. Discussion

The content of phenolic compounds in a range of olive and seasoning oils has been assessed and compared. Seed oils were devoid of the typical phenolic compounds detected in olive oils.

The results of this study show for the first time that the lignans (+)-1-acetoxypinoresinol (IX) and (+)-pinoresinol (X) (Fig. 9) are major components of the phenolic fraction of olive oils. (+)-pinoresinol is a common component of the lignan fraction of several plants such as the Forsythia species [22] and *Sesamum indicum* seeds [23] while (+)-1-acetoxypinoresinol and its respective glucosides have been detected [24,25] in the bark of the olive tree (*Olea europaea L.*).

The amounts of total phenols (196 mg/kg) detected in the olive oil extracts were on average lower than those previously reported, and, this was also the case when VOQ oils only were compared. Montedoro and colleagues [17], differentiated their data on total phenol content in VOQ into tertiles, with ranges of 50–200, 200–500 and 500–1000 mg/kg. The data reported here, falls on average into the first tertile, but, when the olive oils were divided into VOQ (232±15, range: 135–324 mg/kg) and RVO (62±12 range: 32–87 mg/kg) the value for VOQ fell into the second tertile. However, in no case was the value for VOQ higher than 324 mg/kg. The reason for some of the differences in the quantity of total phenols in VOQ observed, are probably due to variation in the harvest areas and methods of oil production. Another plausible reason is the difference in the analytical protocol. In this study, total phenols were quantified by HPLC analysis

Table 3
The antioxidant status of individual phenolic and combined phenolic fractions within methanol extracts of olive oil

Phenolic compound	Correlation	
	ROS[a]	XO[b]
Total phenols	$r=-0.88$; $P<0.0001$	$r=-0.86$; $P<0.0001$
Hydroxytyrosol	$r=-0.57$; $P<0.001$	$r=-0.59$; $P<0.001$
Tyrosol	$r=-0.64$; $P<0.001$	$r=-0.50$; $P<0.01$
Total simple phenols (TSP)	$r=-0.66$; $P<0.0001$	$r=-0.56$; $P<0.01$
Secoiridoid-1	$r=-0.44$; $P<0.05$	$r=-0.56$; $P<0.01$
Secoiridoid-2	$r=-0.57$; $P<0.001$	$r=-0.70$; $P<0.0001$
Total secoiridoids (SID)	$r=-0.77$; $P<0.0001$	$r=-0.76$; $P<0.0001$
Lignans	$r=-0.81$; $P<0.0001$	$r=-0.70$; $P<0.0001$
TSP + SID + lignans	$r=-0.86$; $P<0.0001$	$r=-0.80$; $P<0.0001$

[a] Inhibition of reactive oxygen species (ROS) attack on salicylic acid.
[b] Inhibition of xanthine oxidase (XO).

at 278 nm, while, the method used by Montedoro and colleagues [17] was colorimetric evaluation using the Folin-Ciocalteu reagent.

The mean levels of hydroxytyrosol and tyrosol detected in the olive oils, was, however, approximately 200 times higher than the highest value reported for these phenolics by Montedoro and colleagues [17]. It should be noted that due to their method of reporting, an exact mean value for the simple phenolics present in olive oils is difficult to calculate. Furthermore, in the current study the levels of hydroxytyrosol and tyrosol totalled as simple phenolics were approximately 10 times higher in VOQ compared with RVO.

The concentration of secoiridoids detected in the oils is also difficult to compare on any basis with previous publications. Montedoro and colleagues [17], reported

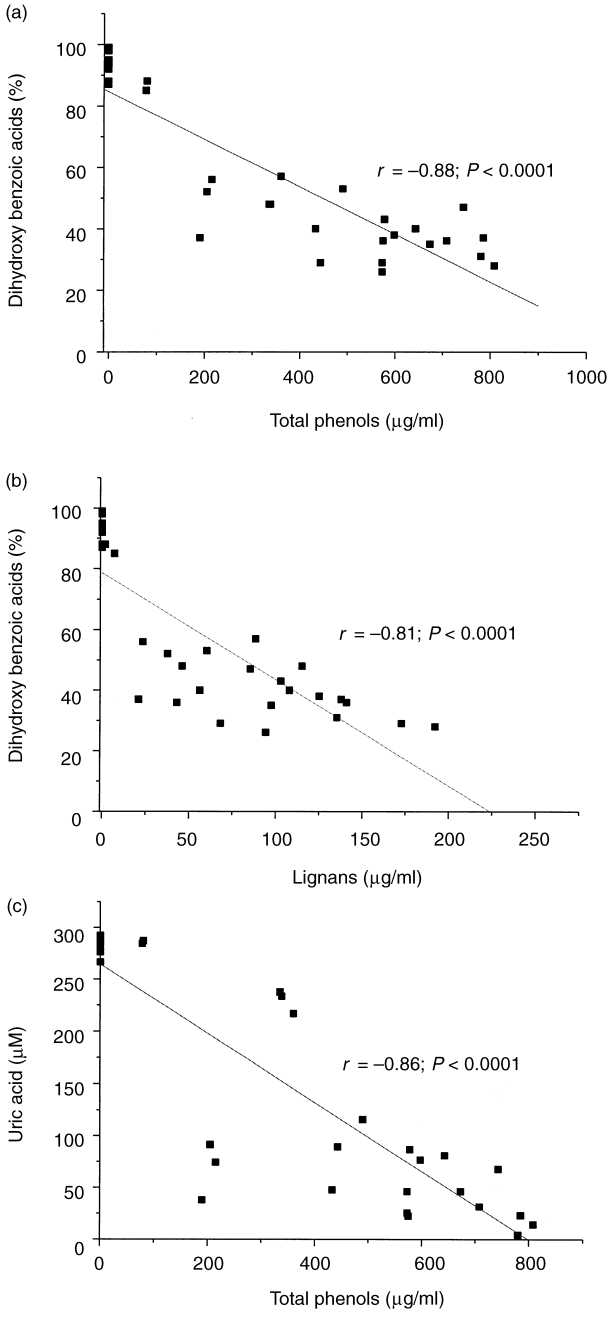

Fig. 14. (a) Effect of total phenolic compounds within olive oil on the detection of reactive oxygen species in the hypoxanthine/xanthine oxidase assay. (b) Effect of lignans within olive oil on the detection of reactive oxygen species in the hypoxanthine/xanthine oxidase assay. (c) Effect of total phenolic compounds within olive oil on xanthine oxidase activity.

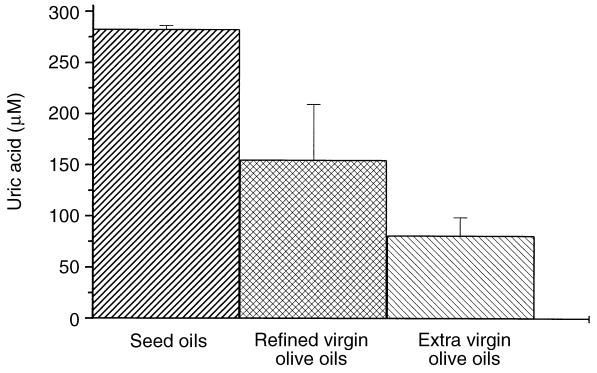

Fig. 15. Effect of oil extracts on xanthine oxidase activity as determined by the production of uric acid in hypoxanthine/xanthine oxidase assay.

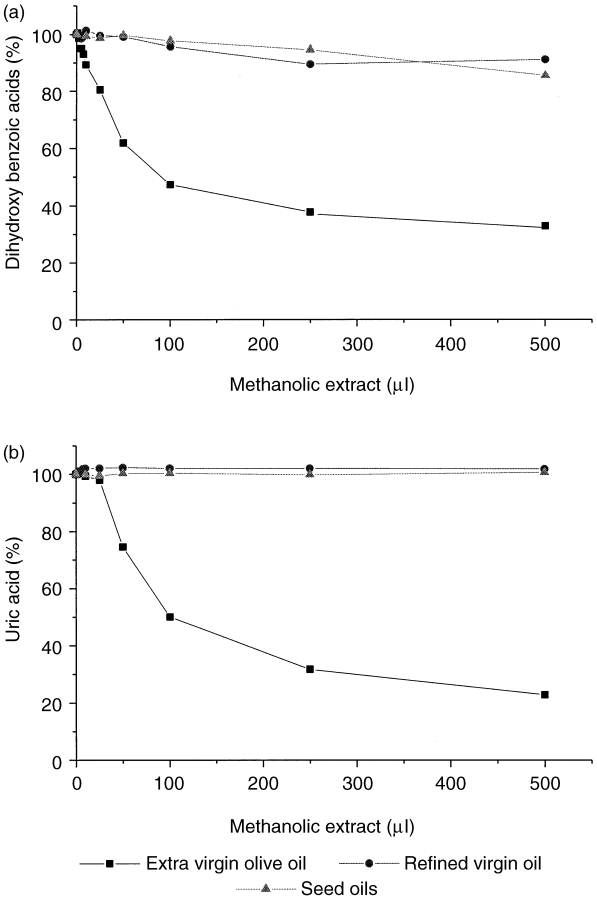

Fig. 16. (a) Attenuation of reactive oxygen species detection by extracts of oils in the hypoxanthine/xanthine oxidase assay. (b) Inhibition of xanthine oxidase activity by extracts of oils.

values for the secoiridoids as peak areas from HPLC chromatograms, but, made no effort to quantitate them on a mg/kg basis. Consequently, the values reported in this communication represent the first qualified attempt to assess their relative contribution to the antioxidant status and, therefore, health protecting properties of olive oils. In the current study, the levels of total secoiridoids and lignans detected in the olive oils (57.80±8.63) were approximately twice that of total simple phenols (33.79±4.48). Of the complex phenolics, while there was no significant difference between the concentrations of secoiridoids (SID-1 and SID-2), VOQ contained significantly higher ($P < 0.001$) levels of lignans than RVO. On a proportional basis, regardless of statistical significance, the levels of SID-1, SID-2 and lignans within VOQ and RVO were 4.8, 2.5 and 5.7:1, respectively.

Regarding all of the individual phenolic components quantitated by HPLC analysis, simple phenols, secoiridoids and lignans in VOQ represented 47% of total phenols. Therefore, approximately 50% of total phenols were not quantitated individually. A major proportion of these are undoubtedly represented by the aglycones of oleuropein and ligstroside but, due to non-homogeneity of the peaks in the HPLC chromatograms, they are difficult to evaluate definitively. In addition, a number of late eluting peaks in the chromatograms represent unknown compounds within olive oil and, these also may contribute significantly to antioxidant activity. Studies are in progress to isolate, identify and evaluate their antioxidant potential.

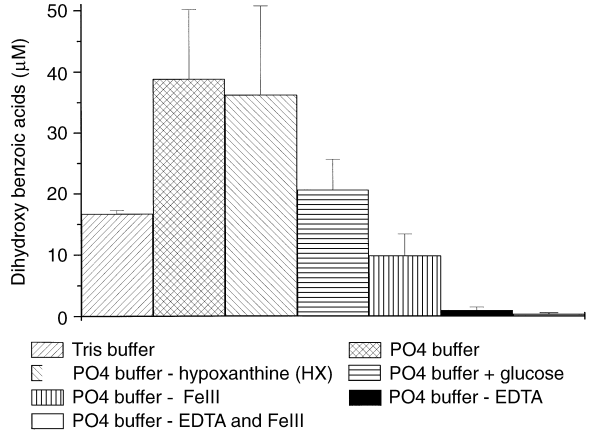

Fig. 17. Production of reactive oxygen species by the faecal matrix ($n = 4$) from adenoma patients under various conditions.

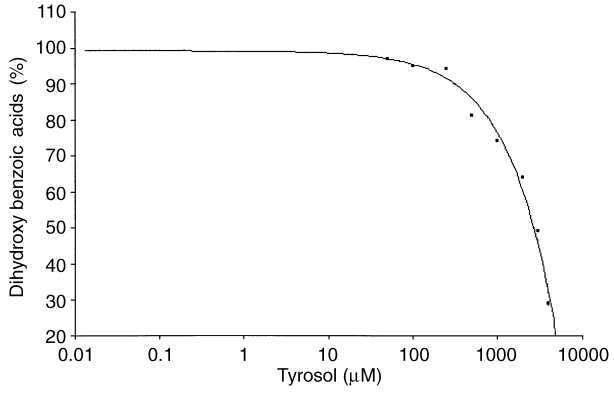

Fig. 18. Scavenging of reactive oxygen species generated in the faecal matrix by tyrosol ($IC_{50} = 2.69$ mM) isolated from olive oil.

Our data also shows that methanolic extracts of olive oils have far greater antioxidant capacities compared with seed oils. The assay used to assess this was the hydroxylation of salicylic acid by $HO^{\bullet}$, which has been described previously [13–16]. In this assay, antioxidants interfere with the hydroxylation of salicylic acid by either competing with it as substrate for $HO^{\bullet}$ attack, or by donating a proton to $HO^{\bullet}$ itself (representing the classical antioxidant activity of many phenolic compounds). A comprehensive screen of many of the phenolic compounds which have been described as components of olive oil, when tested in the system, all showed antioxidant properties.

Furthermore, extracts of olive oil containing a mixture of known and unknown phenolics, were effective at far lower concentrations than the compounds tested individually. While extracts of seed oils and RVO were virtually ineffective, extracts of VOQ were significant inhibitors of reactive oxygen species attack on salicylic acid. Indeed, extracts of VOQ, representing only 2.5 g of oil, inhibited this reaction significantly ($P < 0.0001$), when considering total phenols or phenyl alcohols plus secoiridoids and lignans.

Moreover, while similar concentrations of individual simple phenols, had no effect on xanthine oxidase activity (and thereby representing only proton donation), olive oil extracts appear to have a dual modality. In almost all cases, extracts of VOQ especially, while inhibiting the reaction of $HO^{\bullet}$ with salicylic acid, also had a profound effect on xanthine oxidase activity. At present, it is difficult to ascertain the predominant method for prevention of $HO^{\bullet}$ attack on salicylic acid. Obviously, inhibition of xanthine oxidase activity will reduce the amount of superoxide generated, and thereby the production of $HO^{\bullet}$ by Fenton chemistry in the HX/XO assay. In addition, the phenolic compounds themselves will donate a proton to $HO^{\bullet}$, again diminishing attack on salicylic acid and attenuating the signal. Despite this, some basic conclusions can be drawn from the general screen of olive oil extracts. Total phenols inhibit directly $HO^{\bullet}$ attack on salicylic acid, and, indirectly via inhibition of xanthine oxidase activity. Because, both the simple phenols hydroxytyrosol and tyrosol have no effect on xanthine oxidase activity when tested alone, it can be concluded that their mode of action is via proton donation. In contrast, both SID-1 and SID-2 appear to mediate their effects predominantly through inhibition of xanthine oxidase,

whereas lignans have a dual action, that is donation of a proton to $HO^{\bullet}$ and inhibition of xanthine oxidase.

The dual action of the major phenolics present in olive oil is of importance, in that not only will high dietary intakes increase defence in scavenging reactive oxygen species, but also, will suppress xanthine oxidase activity, a factor which is known to influence carcinogenesis. Recently Tanaka and colleagues [26], have shown that the xanthine oxidase inhibitor 1-acetoxychavicol, has a significant chemopreventive effect on azoxymethane-induced colonic aberrant crypt foci and 4-nitroquinoline-1-oxide-induced oral cancer in rats.

The identification of lignans as major antioxidant components of the phenolic fraction of olive oil [20] is also of considerable interest, because 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 [27,28]. In animal models consumption of flaxseed, a concentrated source of lignans, has been shown to inhibit the development of early biomarkers of mammary cancer risk [29,30] and mammary cancer itself [31]. Proposed mechanisms by which lignans may inhibit carcinogenesis include antiviral [32] and antioxidant (as shown in this and previous studies [33]) 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 effects in part as a result of anti-oestrogenic effects [34]. In fact, lignans have been shown to inhibit placental [34] and adipocyte [35] oestrogen synthesis, to inhibit oestradiol-induced proliferation of MCF-7 human breast carcinoma cells [36] and to stimulate sex hormone-binding globulin synthesis with a subsequent decrease in free oestradiol [37].

In conclusion, methods are described for the quantitative determination and measurement of the phenolic constituents present in olive oil. These have been assessed both individually, and in combination, for their antioxidant potential using a recently developed assay. Extra virgin oils contain significantly higher quantities of phenolic compounds than either RVO or SO, and this is reflected in their overall higher antioxidant activity. However, it should be noted that considerable variation also exists in the quantity of antioxidant phenolics present in VOQ. 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 study, not only between VOQ and RVO, but also the variation in antioxidant content of VOQ indicates that, in future epidemiological 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 which could lead to the formation of highly pro-mutagenic exocyclic DNA adducts [38]. The study of the inter-relationship between reactive oxygen species and dietary antioxidants is an area of great promise for elucidating mechanisms of colorectal carcinogenesis and possible future chemopreventive strategies.

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