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Phenolic composition and *in vitro* antioxidant capacity of four commercial phytochemical products: Olive leaf extract (*Olea europaea* L.), lutein, sesamol and ellagic acid

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ABSTRACT

Four commercially available natural products (olive leaf extract, lutein, sesamol and ellagic acid) were investigated for *in vitro* antioxidant properties using the DPPH$^{\cdot}$, ABTS$^{\cdot+}$, ferric reducing antioxidant capacity (FRAP), oxygen reducing antioxidant capacity (ORAC) and β-carotene-linoleic acid assays. Antioxidant potency followed the order: ellagic acid > sesamol > olive leaf extract > lutein for all antioxidant test methods. Total phenolic content of olive leaf extract was estimated as 1.60 mg gallic acid equivalents (GAE)/g dry weight of olive leaf extract using the Folin–Ciocalteu method. Qualitative and quantitative compositional analysis carried out using high performance liquid chromatography (HPLC) coupled with photo diode array detection (DAD) revealed six major polyphenolic compounds present in olive leaf extract: oleuropein, verbascoside, luteolin-7-O-glucoside, apigenin-7-O-glucoside, hydroxytyrosol and tyrosol. Lutein, sesamol, ellagic acid and olive leaf extract all showed great potential as natural antioxidants which would be useful in the prevention of diseases in which free radicals are implicated.

1. Introduction

Recently, the physiological effects of polyphenol-rich foods, such as fruits, vegetables, and beverages including fruit juices, wine, tea, coffee, chocolate, and olive oil have been receiving much attention as dietary sources of antioxidants that are valuable for human health (Yokozawa, Kim, Kim, Lee, & Nonaka, 2007). In addition, to their potential beneficial health effects, antioxidant compounds can increase shelf-life by delaying oxidative deterioration of substrates such as unsaturated lipids (lipid oxidation), which can lead to the development of off-flavours (Rhee, Anderson, & Sams, 1996) and is considered one of main causes of deterioration of food products during processing and storage.

Traditionally lipid oxidation in foods with high levels of unsaturated lipids such as cooked meats was controlled by the addition of powerful synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) (Monahan & Troy, 1997). However, new toxicological data on some synthetic antioxidants has led to caution regarding their use, as evidence has shown that they possess toxic, pathogenic and carcinogenic effects (Amarowicz, Naczk, & Shahidi, 2000; Peters, Rivera, Jones, Monks, & Lau, 1996). Hence, many recent investigations have been targeted at the identification of alternative novel antioxidants from natural sources which have similar properties (Hossain, Brunton, Barry-Ryan, Martin-Diana, & Wilkinson, 2008a; McBride, Hogan, & Kerry, 2007; McCarthy, Kerry, Kerry, Lynch, & Buckley, 2001). Other experimental studies demonstrated the potential of lutein, sesamol, ellagic acid and olive leaf extract by examining the benefits of these nutraceutical compounds as functional ingredients in processed meat products (Hayes, Stepanyan, Allen, O'Grady, & Kerry, 2010a; Hayes, Stepanyan, Allen, O'Grady, & Kerry, 2010b; Hayes et al., 2010). Ellagic acid, lutein and sesamol also exhibited cytoprotective and/or genoprotective effects as added ingredients in pork patties following cooking and digestion using an *in vitro* digestion and Caco-2 cell model system (Daly et al., 2010). Hence, further research on the antioxidant potential of these phytochemical compounds would prove beneficial and would contribute to the development of novel ingredients for use in functional foods.

There are many methods available for the measurement of *in vitro* antioxidant capacity and most researchers use one or more of the available assays as each method measures different antioxidant characteristics of the compound/extract. Examples of *in vitro* assays for determining antioxidant capacity include oxygen radical absorption capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC) (also known as 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS$^{\cdot+}$) assay), ferric reducing antioxidant capacity (FRAP) and 2,2-diphenyl-1-picrylhydrazul (DPPH$^{\cdot}$) and β-carotene bleaching assay. Previous studies in the literature on lutein, ellagic acid, sesamol and olive leaf extract do not discuss

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all of the assays reported in this study making comparisons of other research findings very difficult. Hence, analysing the *in vitro* antioxidant capacity of lutein, sesamol, ellagic acid and olive leaf extract using a wide variety of methods was a very useful piece of research and adds further scientific knowledge about these phytochemicals.

Lutein, an oxygenated carotenoid (xanthophylls), is abundantly present in dark green leafy vegetables and has been used as a dietary antioxidant for eye health. Research has shown that lutein significantly reduces the risk of age-related macular degeneration, atherosclerosis and UV damage (Mares-Perlman, Millen, Ficek, & Hankinson, 2002; O'Connell et al., 2008). Ellagic acid is a polyphenol with antioxidant properties found in numerous fruits and vegetables including; raspberries, strawberries and other plant foods. This compound has demonstrated a number of beneficial biological properties, including; antioxidant, anticancer, antimutagen, anti-inflammatory and cardioprotective activity (Ezdihar, Vodhanel, Holden, & Abushaban, 2006; Lei et al., 2003; Priyadarsini, Khopde, Kumar, & Mohan, 2002). Sesamol, a component of sesame oil, exhibits anticarcinogenic activity and inhibits atherosclerosis (Decker, 1995). Olive leaf extract has been shown to have a variety of biological activities including; antioxidative, antimicrobial, antiviral and anti-inflammatory agents, lipid stabilisers and blood pressure regulators in animals (Bouaziz, Fki, Jemai, Ayadi, & Sayadi, 2008; Micol et al., 2005; Visioli & Galli, 2002).

The main aim of the present work was to quantify the *in vitro* antioxidant activity of four phytochemicals derived from natural sources using a range of techniques and compare the efficiencies of the ABTS, DPPH, ORAC, FRAP, β-carotene bleaching assays in estimating their antioxidant activity. In addition, the main phenolic compounds present in olive leaf extract were identified and quantified to gain an insight into the compounds responsible for its antioxidant effect.

2. Materials and methods

2.1. Reagents

Lutein(4-[18-(4-Hydroxy-2,6,6-trimethyl-1-cyclohexenyl)-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaenyl]-3,5,5-trimethyl-cyclohex-2-en-1-ol), sesamol (1,3-benzodioxol-5-ol), ellagic acid (2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione) and olive leaf extract (*Olea europaea* L.) were obtained from Guinness Chemical (Ireland) Ltd. (Clonminam Industrial Estate, Portlaoise, Co. Laois, Ireland). Oleuropein, verbascoside, tyrosol, hydroxytyrosol, apigenin-7-O-glucoside, and luteolin-7-O-glucoside were obtained from Extrasynthése (Genay, France).

All solvents used in the experiments were HPLC grade and purchased from Lennox Laboratory Supplies Ltd. (Dublin, Ireland). Disodium ethyldiaminetetraacetate (EDTA), 3-(2-pyridyl)-5, 6-bis(4-phenly-sulphonic acid)-1,2,4-triazine (ferrozine), 2,2′-bipyridyl, 2,2-diphenyl-1-picryhydrazyl radical (DPPH), fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2.2′-azobis(2-amidino-propane) dihydrochloride (AAPH), 2,4,6-tripyridyl-*s*-triazine (TPTZ), linoleic acid, tween 40, β-carotene and butylated hydroxyanisole (BHA) were obtained from Sigma–Aldrich Ireland Ltd. (Dublin, Ireland). All chemicals used were of analytical grade.

2.2. Preparation of samples for in vitro antioxidant capacity measurements

For olive leaf extract, 1.25 g of extract powder was weighted and 25 ml of methanol was added. Following this step, samples were homogenised in a 50 ml test tube with an Ultra thorax T-25 tissue homogeniser (Jankle & Kunkel, IKA® Labortechnik, Saufen, Germany) for 1 min at 3870 g. Samples were then thoroughly mixed with a vortex (V400 Multitube vortexer, Alpha laboratories) for 20 min at 1050 rpm and then centrifuged for 10 min at 2218*g* (3000 rpm) (Sanyo MSE Mistral 3000i., UK). Samples (10 ml) of the supernatant was filtered through PTFE syringe filters (pore size 0.22 μm, Phenomenex, UK). For other pure phytochemicals, sample concentrations were prepared in methanol depending on *in vitro* assay and vortexed (V400 Multitube vortexer, Alpha laboratories) for 20 min at 1050 rpm followed by filtering. Finally, samples were stored at −20 °C in foil covered test tubes prior to analysis.

2.3. In vitro antioxidant capacity measurements

2.3.1. DPPH· radical scavenging activity

Antioxidant capacity was measured using the DPPH assay as described by Goupy, Hugues, Boivin, and Amiot (1999). Briefly, 500 μl of diluted sample and 500 μl of the DPPH (0.238 mg/ml) working solution were added to a micro-centrifuge tube. After vortexing, the tubes were left in the dark for 30 min at room temperature after which the absorbance was measured against methanol at 515 nm using a spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Milton Keynes, UK). Antioxidant activities were expressed as the IC_{50} i.e., the concentration of antioxidant required to cause 50% reduction in the original concentration of DPPH. For ease of interpretation antiradical power (ARP) was also calculated and defined as the inverse of the IC_{50} value. The scavenging activity of lutein, sesamol, ellagic acid and olive leaf extract were measured using concentrations ranging from 200 to 1000 ppm.

Radical scavenging activity was calculated as follows:

$$\text{Radical scavenging activity } (\%) = (1 - A_{515} \text{ of sample}/A_{515} \text{ of control}) \times 100$$

The reduction of DPPH· to DPPH-H is monitored by recording the decrease in its absorbance at a characteristic wavelength over a defined time period during the reaction. In its radical form, DPPH· absorbs at 515 nm, but upon reduction by an antioxidant (AH) or radical species (R·), the absorption disappears.

2.3.2. $ABTS^{\cdot+}$ radical scavenging activity

Antioxidant capacity of lutein, sesamol, ellagic acid and olive leaf extract was measured by degree of suppression of $ABTS^{\cdot+}$ radical cation produced by reaction of $ABTS^{\cdot+}$ (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) in comparison with the antioxidant activity of standard amounts of Trolox. The radical cation $ABTS^{\cdot+}$, produced by the ferrylmyoglobin radical generated from metmyoglobin (chromogen) and hydrogen peroxide is a blue/green chromogen with characteristic absorbance at 600 nm. The determination of the total antioxidant status (TAS) was carried out using the TAS RANDOX kit (Cat. No. NX 2332, Randox Laboratories Ltd., Co. Antrim, UK). Lutein, sesamol, ellagic acid and olive leaf extract (20 μl of 25–1000 μg/ml methanolic solutions) were added to 1 ml of chromogen solution previously incubated at 37 °C. The mixture was incubated for 2 min at 37 °C and the initial absorbance of a sample was measured at 600 nm on a spectrophotometer (Shimadzu UV-1700 Pharma Spec UV–visible spectrophotometer, Shimadzu Scientific Instruments, 7102 Riverwood Dr., Columbia, MD 21046, USA). A 200 μl volume of substrate (hydrogen peroxide in stabilised form, 250 μm/l) was added to the mixture, vortexed and the second absorbance measured exactly after 3 min of incubation at 37 °C. The reaction rate of the sample is compared to that of the Trolox standard (0.1–0.4 mM) and a blank to determine the antioxidant status of the sample. Results are expressed as g Trolox/100 g DW of sample. Each sample was analysed in triplicate.

2.3.3. Ferric reducing antioxidant capacity (FRAP)

The antioxidant potential of lutein, sesamol, ellagic acid and olive leaf extract was determined using the FRAP assay according to Stratil, Klejdus, and Kuban (2006). The assay was based on the reducing power of the compounds. A potential antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the latter forms a blue complex (Fe^{2+}/TPTZ), which increases the absorption at 593 nm. Briefly, the FRAP reagent was prepared by mixing acetate buffer (3.1 g sodium acetate and 20 ml acetic acid per litre, pH 3.6), a solution of 10 μM TPTZ in 40 μM HCl, and 20 mmol/L $FeCl_3 \cdot H_2O$ at 10:1:1 (v/v/v). The mixture was incubated at 37 °C for 8 min. A 900 μl volume of FRAP reagent (Fe^{3+} – TPTZ mixture) was added to 100 μl of each diluted sample, covered in tinfoil and incubated for 40 min. Absorbance readings of the coloured product [ferrous tripyridyltriazine complex] were measured at 593 nm. Fresh 1 mmol/L working solutions of $FeSO_4$ (dilution of 20 mmol/L stock solution) or 0.4 mmol/L working solutions of Trolox (dilution of 2 mmol/L) were used for calibration. The standard curve was linear between 0.1 and 0.4 mM Trolox or 0.1 and 1.0 mM ferrous sulphate. The results were corrected for dilution and expressed in g trolox or ferrous sulphate per 100 g dry weight (dw) of sample.

2.3.4. Measurement of oxygen radical absorbance capacity (ORAC)

The ORAC assay uses an automated plate reader (FLUOstar Optima microplate reader, BMG LABTECH) with 96-well plates. Antioxidant capacity of lutein, sesamol, ellagic acid and olive leaf extract was measured by inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of a biological relevant radical source, AAPH, as described in the method by Prior, Xianli, and Schaich (2005). In this study, samples had to be diluted 1:100 for lutein and olive leaf extract and 1:10,000 for sesamol and ellagic acid using methanol. To each well 25 μl of sample or 10 mM phosphate buffer (pH 7.4) (blank) or standard (Trolox, 20 μM) were added to 150 μl fluorescein solution (10 mM fluorescent salt ($C_{20}H_{10}Na_2O_5$)). Reactions were initiated by the addition of 25 μL of 240 mM AAPH reagent. Measurement temperature was set at 37 °C. The rate of fluorescence depression was measured against blank containing only fluorescein and AAPH solutions.

2.3.5. Inhibition of β-carotene-linoleic acid bleaching assay

Antioxidant activity of lutein, sesamol, ellagic acid and olive leaf extract was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation as described in the method of Emmons, Peterson, and Paul (1999). The compounds were dissolved with methanol to prepare various sample solutions at concentrations of 100, 200, 400, 600, 800, 1000 μg/ml. To prepare the β-carotene/linoleic acid emulsion, a 3 ml aliquot of β-carotene solution was added to 40 mg of linoleic acid and 400 mg of tween. The chloroform was removed under a stream of nitrogen for 10 min at 40 °C. A 100 ml volume of Milli-Q water was added and vortexed for 5 min. β-Carotene/linoleic acid emulsion. A 50 μl volume of methanolic solutions at concentration of 100–1000 μg/ml were added to 3 ml aliquots of β-carotene/linoleic acid emulsion. Initial absorbance of samples was measured after 1 min of vortexing at 470 nm on a spectrophotometer (Shimadzu UV-1700 Pharma, Shimadzu Scientific Instruments, 7102 Riverwood Dr., Columbia, MD 21046, USA). Samples were incubated for 60 min at 50 °C and the second absorbance was measured at 470 nm after 1 min of vortexing. Methanol (50 μl) mixed with 3 ml aliquots of β-carotene/linoleic acid emulsion served as a control. Antioxidant activity (AA) was calculated as follows: $AA = (DR_c - DR_s)/DR_c \times 100$) where, DR_c is the degradation rate of the reference sample = ln (a/b)/60, where a, b are absorbance at 0 and 60 min, respectively, of control. DR_s, is the degradation rate of the sample = ln (a/b) where, a, b = absorbance at 0 and 60 min.

2.4. HPLC determination of phenolic compounds in olive leaf extract

For the quantification of phenolics in the olive leaf extract (*O. europaea* L.), 1.25 g of extract powder was weighted and 25 ml of methanol was added. Following this step, samples were homogenised in a 50 ml test tube with an Ultra Turax T-25 tissue homogeniser (Janke and Kunkel, IKA®-Labortechnik, Saufen, Germany) for 1 min at 3870. The samples were vortexed with a V400 Multitube vortexer (Alpha laboratories, North New York, Canada) for 20 min at 1050 rpm and then centrifuged for 10 min at 717*g* (MSE Mistral 3000, Sanyo Gallenkamp, UK). A 10 ml volume of the sample was filtered through PTFE syringe filters (pore size 0.22 μm, Phenomenex, UK) and centrifuged again at 1612*g* and 4 °C for 15 min. All extracts were stored at −20 °C in foil covered test tubes for subsequent analysis.

Separations were conducted on a Zorbax SB C18, 5 μm, 150 × 4.6 mm column (Agilent Technologies, Dublin, Ireland). The gradient profile was based on a method of Tsao and Yang (2003). Acetic acid in 2 mM sodium acetate (final pH 2.55, v/v) was used as eluent A and 100% acetonitrile was used as eluent B. The column temperature was set at 37 °C and the flow rate was 1 ml/min. Chromatograms were recorded at a wavelength of 280, 320, 360, and 520 nm. The solvent gradient program was set as follows: initial conditions 100% A, 0% B; 0–45 min, 0–15% B; 45–60 min, 15–30% B; 60–65 min, 30–50% B; 65–80 min, 50–100% B. Prior to injection, sample extracts were filtered with PTFE syringe 0.22 μm filters (Phenomenex, UK). Phenolics in olive leaf extract were identified by comparison of their retention times with corresponding standards and by their UV spectra obtained with the diode array detector. Levels of oleuropein (50–1500 μg/ml), apigenin-7-O-glucoside (5–25 μg/ml), verbascoside (25–100 μg/ml), luteolin-7-O-glucoside (5–50 μg/ml) and tyrosol (5–30 μg/ml) in methanol were injected into HPLC at an injection volume of 10 μl. The linear regression equation for each standard curve was obtained by plotting the amount of standard compound injected against the peak area. The regression equation and the correlation coefficient (r^2) were calculated.

2.5. Determination of phenol content analysis of olive leaf extract

Phenol content in olive leaf extract was determined using Folin–Ciocalteu reagent according to the method of Singelton, Orthofer, and Lamuela-Raventos (1999). Methanolic extracts were prepared as described in Section 2.4 and 100 μl of methanol was added to 100 μl of methanolic extract and reacted with 100 μl 1 N Folin reagent and 700 μl of freshly prepared 20% sodium carbonate in 1.5 ml micro-centrifuge tubes and the samples were vortexed briefly. The tubes were left to incubate in the dark for 20 min at room temperature and then centrifuged (Eppendorf 5417R, Hamburg, Germany) at 9464*g* for 3 min. The absorbance of the sample was read at 735 nm using aqueous gallic acid (10–400 mg/ml) as a standard. Results were expressed as mg of gallic acid equivalent (GAE) per 100 g of dry weight of olive leaf extract.

2.6. Statistical analysis

All tests were carried out in triplicate and the results were presented as means ± standard deviation. Analysis of variance (ANOVA) was carried out using GenStat Release (10.1) Copyright 2007, Lawes Agricultural Trust (Rothamsted Experimental Station, Hertfordshire, UK). Differences at $P < 0.05$ were considered statistically significant. Correlations among data obtained were calculated using Pearson's correlation coefficient.

3. Results and discussion

3.1. DPPH radical scavenging activity of lutein, sesamol, ellagic acid and olive leaf extract

Radical scavenging activity of the four commercial natural products and a synthetic antioxidant BHA were assessed using the 2,2-diphenyil-picrylhydrazyl (DPPH·) assay are presented in Table 1. The antioxidant efficiency was in the order: ellagic acid > sesamol > BHA > olive leaf extract > lutein with higher ARP and lower IC_{50} indicating stronger antioxidant efficiency. The DPPH radical scavenging activity in terms of ARP of BHA 55.65 $(g/L)^{-1}$, respectively, indicating that sesamol and ellagic acid had higher ARP and lower IC_{50} compared to the synthetic antioxidant.

The potent radical scavenging capacity of ellagic acid is not surprising as this compound has four phenolic OH groups with a fused benzofuran structure. Ellagic acid has previously shown itself to have strong DPPH radical scavenging activities (Han, Lee, & Kim, 2006; Zafrilla, Ferreres, & Tomas-Barberán, 2001). Ellagic acid exhibits minimum solubility in water, however, its solubility increases in organic solvents such as methanol and dimethyl sulfoxide (DMSO). Priyadarsini et al. (2002) claimed that ellagic acid may act as a good lipophilic antioxidant due to its solubility.

Sesamol acts as a chain breaking antioxidant by scavenging the DPPH· and the hydroxyl radicals generated by the Fenton reaction (Joshi, Kumar, Satyamoorthy, Unnikrisnan, & Mukherjee, 2005). The antioxidant capacity of sesamol is derived from its phenolic group and a benzodioxole group in its molecular structure and previous research showed that 1,3-benzodioxole compounds exhibited 10-fold greater antioxidant activity than α-tocopherol with an IC_{50} of 2.9 μM compared to 28 μM of α-tocopherol (Tagashira & Ohtake, 1998). Additionally, its solubility in the lipid and aqueous phases makes it a useful and potent antioxidant (Joshi et al., 2005). In fact, the free radical scavenging and antioxidant properties of sesamol has been recently demonstrated by Suja, Jayalakshmy, and Arumugham (2004) in a DPPH system in which sesamol at concentrations of 4–320 μM was shown to have higher radical scavenging activity ($P < 0.05$) than the synthetic antioxidant BHT (butylated hydroxytoluene) and tocopherol. Using the DPPH assay, Kapadia et al. (2002) found the free radical scavenging activity of sesamol (IC_{50} = 6.0 μg/ml) was higher when compared to ascorbic acid (IC_{50} = 4.8 μg/ml) while Kanimozhi and Prasad (2009) found the IC_{50} value of sesamol and ascorbic acid to be 3.23 and 5.85 μg/ml, respectively. Some authors have commented that given sesamol's solubility in lipids and aqueous media and its ability to prevent the spoilage of oils, seasamol may protect the body from free radical damage (Nakagawa, Terokubota, Ikegami, & Tsuchihushi, 1994).

The antioxidant activity of phenolic hydroxyl compounds in olive leaf extract could be due to the presence of the hydroxyl groups in their structure such as oleuropein, hydroxytyrosol, and luteolin-7-O-glucoside acid (Benavente-García, Castillo, Lorente, Ortuño, & Del-Rio, 2000). A previous study suggested olive leaf extract (aqueous ethyl acetate solubles following extraction with hexane, ethyl acetate, methanol, and 80% aqueous methanol) at a concentration of 60 mg/kg could prevent oxidative damage in an animal system as a result of its ability to scavenge oxygen species such as hydroxyl radicals (Somova, Shode, Ramnanan, & Nadar, 2003).

The antioxidant activity of lutein may be attributed to its unique chemical structure (Fig. 3). Lutein not only has conjugated double bonds, which provides free radical quenching activity, but also has two phenolic hydroxyl groups on both ends of its chemical structure making it a stronger antioxidant when compared to other carotenoids (Miki, 1991). Carotenoids have been described as excellent antioxidants because of their ability to quench singlet oxygen and trap peroxyl radicals (Burton & Ingold, 1984). Carotenoids, such as lutein, can play a role as an antioxidant in lipid phases by trapping free radicals or physically quenching singlet oxygen which are believed to have roles in inhibiting lipid oxidation (Yanishlieva, Aitzetmuller, & Raneva, 1998). The antioxidant activity of lutein depends on the concentration of oxygen and the chemical structure of carotenoids (Krinsky, 1993).

Antioxidant activity usually depends on the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group (Rice-Evans, Miller, & Paganga, 1996; Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999). The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activity. Lutein, sesamol, ellagic acid and olive leaf extract have been shown to have almost zero iron chelating activity at concentrations of 200–1000 ppm, whereas EDTA exhibited 87.6–100% chelating activity (Hayes et al., 2009). Some phenolic compounds are able to chelate iron, while others which do not have a galloyl moiety do not (Alamed, Chaiyasit, McClements, & Decker, 2009; Andjelkovic et al., 2006).

In the present study, it is difficult to make general assumptions about structure–antioxidant activity relationships with the small number of antioxidant molecules under evaluation. The DPPH· scavenging data suggests that lutein, sesamol, ellagic acid and olive leaf extract are capable of scavenging free radicals at physiological pH, hence preventing the initiation and propagation of free-radical-mediated chain reactions which contribute to oxidative stress. This can be of valuable importance in preservation of foodstuffs, drug products and cosmetics, where free-radical-mediated chain reactions result in lipid oxidation and subsequent deterioration of these products. These natural compounds may also prove to have therapeutic potential, as free radicals are believed to be involved in the pathogenic cascade of events in many diseases (Dastmalchi et al., 2008).

3.2. $ABTS^{\cdot+}$ scavenging activity of lutein, sesamol, ellagic acid and olive leaf extract

The TEAC assay has been criticised as ABTS is not a physiological radical source and thus may not accurately represent *in vivo* effects (Prior et al., 2005). Antioxidant capacity as measured by the ABTS assay followed the order: ellagic acid > sesamol > BHA > olive leaf extract > lutein, with antioxidant capacity of 508.0, 347.9, 203.4, 37.9 and 36.5 g Trolox/100 g dw, respectively (Table 1). In a study

Table 1
Antioxidant activities of lutein, sesamol, ellagic acid and olive leaf extract obtained using a variety on *in vitro* assays.

Antioxidant	IC_{50}[a] (μg/ml)	ARP $(g/l)^{-1}$	ABTS g Trolox/100 g DW	FRAP g Trolox/100 g DW	ORAC g Trolox/100 g DW
Lutein	3768 ± 216.37	0.3 ± 0.001	36.54 ± 0.23	0.612 ± 0.01	0.931 ± 0.01
Sesamol	3.495 ± 0.12	290.0 ± 3.07	347.87 ± 0.55	385.13 ± 0.46	97.80 ± 2.34
Ellagic acid	2.012 ± 0.10	460.5 ± 3.50	508.03 ± 0.65	585.19 ± 0.10	151.77 ± 3.57
Olive leaf extract	34.58 ± 1.64	28.6 ± 1.38	37.93 ± 0.24	30.1 ± 0.1	17.43 ± 0.01
BHA	17.97 ± 0.78	55.7 ± 0.34	203.37 ± 0.97	207.31 ± 2.02	ND

[a] Results are expressed as mean ± standard deviation of three determinations. ND, not determined.

by Hossain et al., 2008a the antioxidant capacities of BHT and BHA as measured by the ABTS assay were 100.2 and 217.5 g Trolox/100 g dw, respectively, indicating that ellagic acid and sesamol also had higher antioxidant capacity relative to the synthetic antioxidants. Ellagic acid is the strongest antioxidant which is in agreement with the DPPH· assay results. Recent research shows that sesamol used in DPPH· and $ABTS^{\cdot+}$ radical-scavenging assays, exhibits high antioxidant activity and it has been comparable to the antioxidant potential of rosmarinic acid and carnosic acid (Erkan, Ayranci, & Ayranci, 2008). Benavente-García et al. (2000) found the $ABTS^{\cdot+}$ scavenging ability of olive leaf extract alone (1.58 mM TEAC) to be lower than the values reported in the present study. The reason for the difference in the TEAC values may lie in the different DMSO solvent extraction procedures of olive leaf extract, which would influence the quantity of polyphenol antioxidant compounds extracted. The higher TEAC value reported for olive leaf extract could be due to the higher content of phenolic substances that were extracted. Benavente-García et al. (2000) demonstrated that the relative abilities of the flavonoids from olive leaf to scavenge the $ABTS^{\cdot+}$ radical cation was influenced by the presence of functional groups in their structure, mainly the B-ring catechol, the 3-hydroxyl group and the 2,3-double bond conjugated with the 4-oxo function. For the other phenolic compounds present in olive leaf extract, their relative abilities to scavenge the $ABTS^{\cdot+}$ radical cation are mainly influenced by the number and position of free hydroxyl groups in their structure. Previous studies also found the antioxidant activities (TEAC) of vitamin E, C and lycopene to be 0.97, 0.99 and 2.9 mM, respectively (Van den Berg, Haenen, Van den Berg, & Bast, 1999), indicating that all the substances examined in the present study had higher antioxidant potential than these compounds.

3.3. Antioxidant capacity of lutein, sesamol, ellagic acid and olive leaf extract as measured using the FRAP assay

On the basis of FRAP values, the strength of peroxyl radical scavenging activity was in the order: ellagic acid > sesamol > BHA > olive leaf extract > lutein (Table 1). This study showed that trends were consistent with those obtained from DPPH· and $ABTS^{\cdot+}$ assays. Katalinic, Milos, Kulisic, and Jukic (2006) also found strong correlations between FRAP, DPPH· and $ABTS^{\cdot+}$ assays. The antioxidant capacity of synthetic antioxidants measured by the FRAP assay as reported by Hossain, Barry-Ryan, Martin-Diana, Wilkinson, and Brunton (2008b), compared to the antioxidant capacity of the nutraceutical compounds examined in this study, followed the order: ellagic acid > sesamol > PG > BHA > THQ > BHT > olive leaf extract > lutein. The antioxidant capacity of ellagic acid (585.2 g Trolox/100 g dw) and sesamol (385.1 g Trolox/100 g dw) were in fact higher than all tested synthetic antioxidants according to the FRAP assay as carried out by Hossain et al. (2008). The results suggest that ellagic acid and sesamol could potentially be used as natural alternatives to synthetic antioxidants in the prevention of oxidative damage in food systems.

3.4. Antioxidant capacity of lutein, sesamol, ellagic acid and olive leaf extract using ORAC assay

Unlike other popular antioxidant activity methods, the ORAC assay uses fluorescein as the fluorescent probe directly measures the antioxidant activities of chain-breaking antioxidants against peroxyl radicals (Ou, Hampsch-Woodill, & Prior, 2001). One of the advantages of the ORAC assay is that it uses biologically relevant free radicals. It also differs from the DPPH and FRAP assays in terms of the mechanism of chain breaking ability. The FRAP and DPPH assays operate on the basis of single electron transfer, whereas the ORAC assays is a hydrogen atom transfer-based reaction. On the basis of ORAC values, the strength of peroxyl radical scavenging activity was in the order: ellagic acid > sesamol > olive leaf extract > lutein (Table 1). A good correlation between radical scavenging activities as measured using FRAP, DPPH, ABTS and ORAC assays was observed (Table 3).

3.5. Analysis of lutein, sesamol, ellagic acid and olive leaf extract in β-carotene linoleate model system

β-Carotene bleaching is measured by the decrease in the initial absorbance at 470 nm (typical absorbance of β-carotene) and is slowed down in the presence of an antioxidant. If an antioxidant is present in the test solution it will compete competitively with the peroxyl radical. It is apparent from the results obtained in this study that percentage inhibition of linoleic acid oxidation increased with increasing concentration of lutein, sesamol, ellagic acid and olive leaf extract (Fig. 1). Antioxidant activity was in the order: ellagic acid > sesamol > olive leaf extract > lutein. As reported by Frankel and Meyer (2002), the order of antioxidant activity of the pure antioxidants maybe explained on the basis of their hydrophobicity and hence their solubility in linoleic acid emulsions in accordance with the observation that polar antioxidants are more active in bulk oil systems whereas non-polar antioxidants (hydrophobic) are more active in lipids suspended in aqueous systems. However, lutein is the most non-polar compound of those examined in this study; hence our results contradict this theory. Lutein exhibited the lowest antioxidant activity ranging from 5.0% to 10.2% at a concentration from 100 to 1000 ppm. Increasing concentrations (100–1000 ppm) of sesamol and olive leaf extract resulted in increased antioxidant activity from 27.6% to 70.7% and 2% to 16%, respectively. Bouzaiz and Sayadi (2005) also reported similar antioxidant activity of an olive leaf extract in the β-carotene linoleate model system after 60 min. Ellagic acid had highest antioxidant activity of up to 81% from concentrations ranging from 100 to 600 ppm, however, there was a reduction in antioxidant activity at 800 and 1000 ppm, which may be due to the solubility of ellagic acid at higher concentrations. When the structures of lutein, sesamol and ellagic acid are compared (Fig. 2), it is lutein, a known polar carotenoid, which is the most hydrophobic compound with two –OH groups at the terminal aromatic rings, followed by sesamol with a single –OH, followed by ellagic acid with four –OH groups. Hydrophobicity of the pure compounds based on their solubility in water followed the order: lutein > sesamol > ellagic acid. The strong antioxidant effect of the less polar compounds, sesamol and ellagic acid, and the plant extract, which

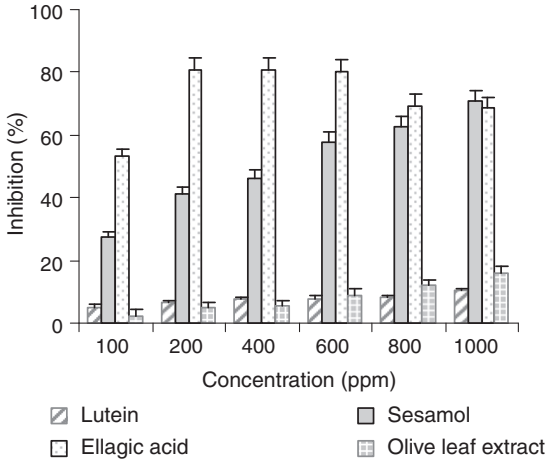

Fig. 1. Antioxidant activities of lutein, sesamol, ellagic acid and olive leaf extract as carried out by the β-carotene bleaching method.

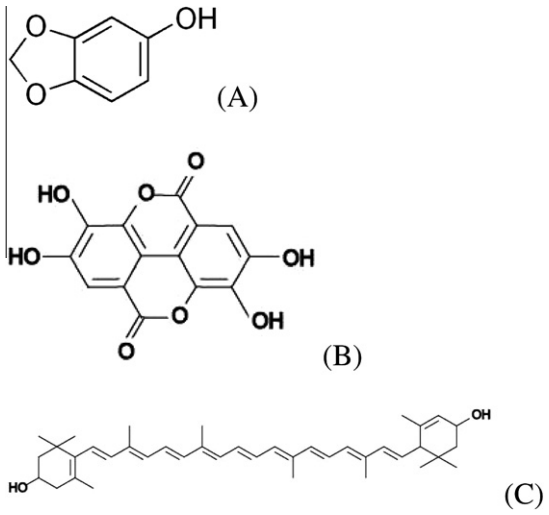

Fig. 2. Chemical structure of (A) sesamol, (B) ellagic acid and (C) lutein.

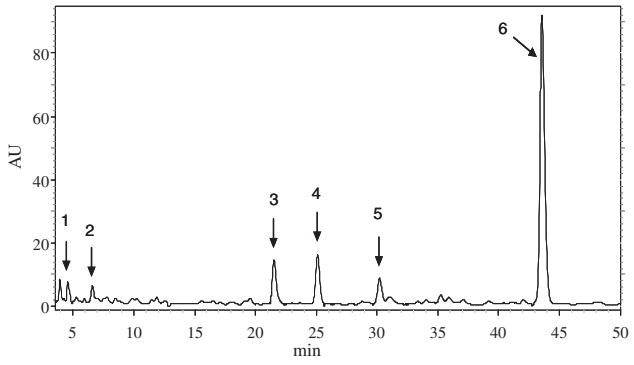

Fig. 3. HPLC phenolic profile of olive leaf extract. Detection at 280 nm. (1) Hydroxytyrosol; (2) tyrosol; (3) luteolin-7-O-glucoside; (4) verbascoside; (5) apigenin-7-O-glucoside; (6) oleuropein.

is of heterogeneous nature, may be explained by the 'polar paradox' phenomenon as described by Frankel, Huang, Kanner, and German (1994). The 'polar paradox' proposes that apolar antioxidants exhibit stronger antioxidant properties in oil in water emulsions, because they become concentrated at the oil–water interface, thus protecting the lipids from oxidation.

3.6. Phenolic composition of olive leaf extract

HPLC profiles of phenolic compounds present in olive leaf extract are shown (Fig. 3). Retention times and abundance of the main compounds present in olive leaf extract are also presented (Table 2). HPLC-DAD analysis of the olive leaf extract showed several peaks corresponding to different olive leaf polyphenols which were identified from their retention times and UV–Vis spectra as oleuropein and verbascoside (oleuropeosides); hydroxytyrosol and tyrosol (substituted phenols); apigenin-7-O-glucoside, and luteolin-7-O-glucoside (flavones) (Fig. 4). All these phenolic compounds have previously been reported to occur in olive leaf extract (Atiok, Bayçin, Bayraktar, & Ülkü, 2008; Bouzaiz & Sayadi, 2005; Briante et al., 2002; Pereria et al., 2007). Benavente-Garcia et al. (2000) quantified various polyphenols found in *O. europaea* L. leaves and also reported that oleuropein was the largest fraction present with 24.5% while other polyphenols like hydroxytyrosol (1.5%), luteolin-7-glucoside (1.4%), verbascoside (1.1%), tyrosol (0.7%), apigenin-7-O-glucoside (1.4%) were also isolated from the

Table 2
Retention times, abundance and quantification of the main phenolic compounds present in olive leaf extract.

Phenolics	Retention time (min)	% Absolute	μg/ml
Hydroxytyrosol	4.56	1.82	10.2 ± 0.1
Tyrosol	6.58	1.76	15.6 ± 0.1
Luteolin-7-O-glucoside	21.54	5.05	25.6 ± 0.6
Verbascoside	25.12	5.68	68.6 ± 0.8
Apigenin-7-O-glucoside	30.22	3.13	15.9 ± 0.7
Oleuropein	43.58	40.33	1151.5 ± 57.2

Values are means of three replicates ± standard deviation. A IC_{50} (mg/ml of nutraceutical) ± standard deviation is the concentration of antioxidant required to cause a 50% decrease in the initial concentration of DPPH.

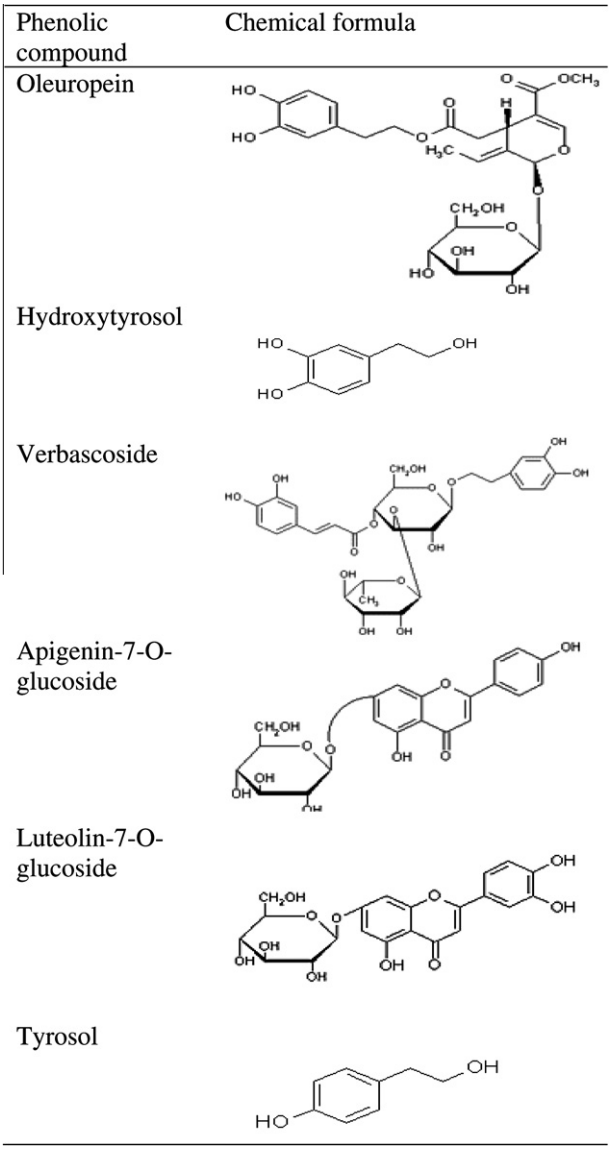

Fig. 4. Chemical structures of the most abundant phenolics in olive leaf extract.

leaves. Atiok et al. (2008) reported the abundance of oleuropein (29%) in an olive leaf crude extract. Pereria et al. (2007) also quantified oleuropein and lueolin-7-O-glucoside as the most abundant phenolic compounds present in a lyophilised olive leaf extract. Variations found in the phenolic compositions of the olive leaf extracts reported could be due to different methods applied and also

Table 3
Correlation coefficients between DPPH, ABTS, FRAP and ORAC assays.

	DPPH	ABTS	FRAP	ORAC
DPPH	–			
ABTS	0.996	–		
FRAP	0.999	0.998	–	
ORAC	0.998	0.990	0.997	–

the influence of sample origin. Previous studies have found that polyphenols also display a synergistic behaviour in their radical scavenging capacity when mixed, as occurs in olive leaf extract, with a high content of oleuropein and other active polyphenols compared to the individual phenolics alone (Benavente-García et al., 2000).

The content of polyphenols (free and total) determined by the Folin–Ciocalteu assay for olive leaf extract was 160.8 (2.9 mg GAE/100 g dry weight of olive leaf extract. As one of the most important antioxidant plant components, phenolic compounds are widely investigated in many medicinal plant and vegetables (Djeridane et al., 2006). Mylonaki, Kiassos, Makris, and Kefalas (2008) found the maximum theoretical yield of total polyphenol content to be 250.2 ± 76.8 mg GAE/100 g dry weight of olive leaf extract using optimal recovery techniques of polyphenols from olive leaves.

4. Conclusions

The ranking of four phytochemicals in terms of their *in vivo* antioxidant activity as measured by DPPH, FRAP, ORAC and ABTS assay was determined to be in decreasing order: ellagic acid > sesamol > olive leaf extract > lutein. The high correlations among the different assays indicated that the antioxidant capacity of lutein, sesamol, ellagic acid and olive leaf extract could be predicted by various assays. Because multiple reaction characteristics and mechanisms are likely involved, no single assay will accurately reflect all antioxidants in a mixed or complex system. Thus, to fully elucidate a full profile of antioxidant capacity of lutein, sesamol, ellagic acid and olive leaf extract, different antioxidant capacity assays (DPPH, ABTS, ORAC, FRAP, β-carotene linoleic acid) were used in this study. It is important to emphasise that the antioxidant assays mentioned in this research are strictly based on chemical reactions *in vitro* and do not necessarily correlate with their activity in biological systems, however, they do serve as useful indicators in suggesting how effective certain ingredients may reflect antioxidant activity and can be used for screening purposes. Results from this study also provide a better understanding of the antioxidant properties of the selected phytochemicals and will allow for the identification of phytochemicals with high antioxidant potential for further investigation and development into valued added foods and nutraceuticals.

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