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Selenium and sulphur derivatives of hydroxytyrosol: inhibition of lipid peroxidation in liver microsomes of vitamin E-deficient rats.

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ABSTRACT

Purpose: The objective of this study was to evaluate the capacity of modified phenols synthesized from hydroxytyrosol, a natural olive oil phenol, specifically those containing a selenium or sulphur group, to inhibit lipid peroxidation.

Methods: The compounds' abilities to inhibit lipid peroxidation in liver microsomes obtained from vitamin E-deficient rats were compared to hydroxytyrosol.

Results: All synthetic compounds had a significant higher ability to inhibit lipid peroxidation than hydroxytyrosol. Selenium derivates displayed a higher antioxidant activity than sulphur derivatives. In addition, the antioxidant activity increased with a higher number of heteroatoms in the hydroxytyrosol molecular structure.

Conclusion: The study shows, for the first time, the ability of synthetic compounds, derived from the most active phenol present in olives in free form (hydroxytyrosol), and containing one or two atoms of sulphur or selenium, to inhibit the lipid peroxidation of vitamin E-deficient microsomes. The antioxidant activity of five thioureas, a disulfide, a thiol, three selenoureas, a diselenide, and a selenonium were evaluated and the results showed a higher inhibition of lipid peroxidation than the natural phenol. Selenium and sulphur derivatives of hydroxytyrosol are novel antioxidants with the potential to supplement the lack of vitamin E in the diet as natural alternatives for the prevention of diseases related to oxidative damage.

Keywords: antioxidant, hydroxytyrosol, selenium, sulphur, lipid peroxidation, vitamin E, microsomes, Mediterranean diet.

1. Introduction

The Mediterranean diet is high in olive oil, fruits, vegetables, grains and legumes, and is associated with a lower rate of coronary heart disease [1] and a reduction in all-cause mortality [2]. In particular, the high olive oil intake of this diet seems to have a protective effect on total mortality [3]. The beneficial health effects of olive oil have been attributed to its high content in monounsaturated fatty acids (MUFA) and the presence of minor components, such as phenols, which contribute to the stability of the oil and provide additional benefits because of their antioxidant, anti-inflammatory and anti-atherosclerotic properties [4, 5]. In fact, a health claim for phenols in olives and olive oil was recently approved by the European Food Safety Authority (EFSA); concretely, that 5 mg of hydroxytyrosol (one of the most important phenols in olive) and its derivatives should be consumed daily.

Dietary phenols inhibit LDL oxidation and scavenge superoxide and other reactive oxygen species (ROS), increasing plasma antioxidant capacity [6]. The antioxidant effects of these dietary phenols, such as the reduction of the free radical-mediated oxidation of proteins, lipids and DNA, are associated with the prevention of many chronic diseases [7]. Moreover, dietary phenols were shown to inhibit the peroxidation of hepatic microsomal preparations from vitamin E-deficient rats [5, 8].

During the olive oil extraction process, the majority of phenolic compounds remain in the alperujo, a by-product of the modern two-phase olive oil extraction system [9]. The current management of alperujo by thermal treatment [10] permits the industrial extraction of hydroxytyrosol (HT), 3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol acetate, and polymeric phenolic fractions [11] from this otherwise considered “waste” material. Using a patented treatment system, HT can be commercialized from the olive

oil by-products with a purity level reaching as high as 99.6% [12]. Besides being a very rich source of antioxidant phenols, alperujo is also a source of other valuable components like oligosaccharides, sugars, functional oils, proteins, and cellulose, all of which can be solubilised in the oil and aqueous liquid phases of alperujo after its pre-treatment and the separation of liquid and solid phases [13].

In previous studies carried out by our research group, we showed the ability of certain phenols to inhibit lipid oxidation in microsomes [14]. The objective of this study was to evaluate the capacity of modified phenols, specifically those containing a selenium or sulphur group, to inhibit lipid peroxidation. Selenium is an essential element [15] that plays a vital role as a constituent of the antioxidant enzymes glutathione peroxidase [16], thioredoxin reductase, and iodothyronine deiodinase [17]. Moreover, low selenium in the diet may cause serious health effects as it is a critical factor in many cellular responses [18]. Diselenodivaleric acid and selenazine are currently used for the treatment of different diseases [19]. Organosulphur compounds are naturally occurring in plants in the form of diallyl sulfide, diallyl disulfide, diallyl trisulfide, S-allyl-L-cysteine sulphoxide, and sulphoraphane, which have antioxidant, anti-atherosclerotic, anti-proliferative, antibacterial, anti-platelet properties, as well as an ability to lower systolic blood pressure and reduce cholesterol levels [20]. Our hypothesis was that hydroxytyrosol components modified with selenium or sulphur molecules would have stronger antioxidant properties than pure hydroxytyrosol.

2. Material and Methods

2.1. Test compounds

The test compounds included compounds having a phenol structure with 1 or 2 sulphur atoms, such as thioureas (**1**-**5**), thiol (**6**) and disulfide (**7**), or 1 or 2 selenium atoms, such as 3 selenoureas (**8**-**10**), diselenide (**11**) and selenonium (**12**) (Figure 1). The procedure to synthesise these compounds has been described by us previously [20].

2.2. Inhibition of lipid peroxidation in vitamin E‑deficient microsomes

Microsomal lipid peroxidation was measured by the reaction of malondialdehyde as a product of lipid oxidation, with thiobarbituric acid to produce thiobarbituric acid reactive substances (TBARS). TBARS were quantified by high-pressure liquid chromatography (HPLC). Each extract was tested in triplicate.

The liver microsomes were obtained from male weanling rats of the Rowett Hooded Lister strain that had been fed on a vitamin E-deficient diet (less than 0.5 mg/kg) for 13 weeks, as previously described [21]. The protocol was approved by the Ethical Review Committee of Animal Studies at the Rowett Research Institute and was conducted in compliance with the United Kingdom's Animals (Scientific Procedures) Act, 1986.

Microsomes were extracted and washed with 0.15 M KCl from homogenized liver samples and diluted in potassium phosphate buffer 0.05 M pH 7.4. The protein and vitamin E concentrations in plasma were determined by the Biuret method and HPLC, respectively [14]. The effect of the test compounds on *in vitro* microsomal lipid peroxidation was determined using ethanol solutions and α-tocopherol as a control [22].

The concentration of TBARS was measured by HPLC (Alliance 2695) equipped with a fluorescence detector (Waters 2475). The column used was Phenomenex Luna 5u C18 (2) 100 A, 150 × 4.60 mm; the mobile phase was 60% KH_2PO_4 50 mM pH 7.0 and 40%

MeOH at a flow of 0.8 mL/min for 12 min, in isocratic mode. Inhibition of lipid peroxidation was calculated using the following equation:

$$\%Inhibition = \left(\frac{AUC(-VE) - AUC(-VE + Comp)}{AUC(-VE)} \right) x100$$

where AUC (−VE) is the area under the curve of microsomes from rats deficient in vitamin E and AUC (−VE + Comp) is the area under the curve of microsomes from rats deficient in vitamin E plus test compound. The inhibition of microsomal lipid peroxidation by each compound was compared with a negative control (no incubation) and two positive controls, i.e. microsomes from rats fed a vitamin E-adequate diet (100 mg α-tocopherol/kg) and effect in microsomes from rats fed a vitamin E-deficient diet supplemented with 100 mg/kg α-tocopherol.

2.3. Statistical analysis

Three replicates were performed for each assay. STATGRAPHICS® Plus software was used for statistical analysis. The correlation coefficients were determined using regression analysis at the 95% confidence level. Comparisons among samples were made with one-way analysis of variance (ANOVA) and the LSD method at the same confidence level.

3. Results

We screened the antioxidant properties of different HT compounds with sulphur or selenium molecules using an *in vitro* liver microsome model, with microsomes obtained from vitamin E-deficient rats. Previous studies have shown that a decreased membrane

concentration of α-tocopherol increased the rate of TBARS formation in all tissues, but this effect was specifically pronounced in adrenal mitochondria and microsomes [23]. We tested three different concentrations (0.05, 0.10 and 0.25 mM) of HT-derived compounds and the results were compared with data obtained for α-tocopherol at the same concentrations in microsomes from rats deficient in vitamin E (Table 1). For the positive standard, obtained in microsomes from rats whose diets were supplied with an appropriate amount of vitamin E, the average percentage (± SD) inhibition of lipid oxidation was 78.7 ± 2.6%. Generally, all HT compound derivatives decreased lipid peroxidation in a time-dependent and concentration-dependent manner (Figure 2).

The sulphur compound with the strongest ability to inhibit *in vitro* lipid peroxidation, i.e. at the lowest concentration, was the disulfide HT derivative (**7**). The thiol (**6**) showed the highest inhibition of lipid peroxidation at concentrations of 0.10 and 0.25 mM. Although all compounds had a stronger antioxidant capacity than hydroxytyrosol, none of the sulphur compounds were as effective as α-tocopherol in inhibiting lipid peroxidation.

The selenium compound with the strongest ability to inhibit *in vitro* lipid peroxidation at all concentrations tested was diselenide (**11**), showing the same antioxidant capacity as α-tocopherol. The three selenoureas (**8-10)** showed similar inhibition values for all concentrations tested. The selenonium compound (**12**) had the weakest antioxidant capacity of all the HT-selenium compounds tested, possibly because the bromine salt may cause an osmotic imbalance or even the different redox behaviour of the positively-charged selenium atom.

Therefore, using this lipid peroxidation system, the highest percentages of inhibition were achieved for the disulfide **(7)**, with a good percentage of inhibition at all

concentrations tested, and the diselenide **(11),** which presented values close to those observed for α-tocopherol. Also, the HT-selenium compounds presented a greater capacity of inhibition of the oxidation than the sulfide analogues. This effect was verified in two other pairs of compounds that have an analogous structure and only differ in the heteroatom they contain: For thiourea and selenourea, **(3**-**9**), a higher percentage of inhibition was observed for selenourea (60.7%) compared to thiourea (22.1%) at the 0.10 mM concentration. Whereas for the second pair, formed by thiourea and selenourea (**5**-**8**), the values were very similar for the lowest concentration; however for higher concentrations, a higher percentage of inhibition of lipid peroxidation was obtained for selenourea (**8**), 58% compared to 37% for the thiourea compound **5**.

Moreover, a certain influence of the substituent was observed on the synthetic compounds' capacity of inhibition. In the case of thioureas (**1**-**5**), compounds with a longer side chain showed a higher inhibition capacity. At concentrations of 0.25 mM, the inhibition capacity of thioureas (**1**-**5**) was **1** <**2** <**5** <**3** <**4,** relating to methyl <butyl <phenyl <tolyl <benzyl. Therefore, the function of the side chains and the change on electronic properties have a profound effect on the HT derivative's antioxidant capacity. A similar result was observed for the selenoureas **8** and **9**, which at 0.05 and 0.1 mM presented higher values of inhibition for the longer side chains, i.e. tolyl<benzyl.

The results obtained for the antioxidant capacity of sulphur and selenium derivatives of hydroxytyrosol are much higher than those obtained in the case of HT alone. Previous studies showed that for HT concentrations of 0.05, 0.1 and 0.25 mM, the observed inhibition of lipid peroxidation was 0, 4.8, and 5.5%, respectively. In a detailed study, Duthie & Morrice found that different flavonoids (i.e. quercetin, kaempherol, myricetin, galingin and fisetin) inhibited lipid peroxidation by 68–88% using a concentration of 0.25 and 0.5 mM [8], which is a fairly range of values to the results obtained in this

study. It was observed that of all the phytoestrogens tested, kaempherol had the greatest ability to inhibit lipid peroxidation in vitamin E-deficient microsomes, with an IC50 of 160 μM [24]. In comparison, the IC50 concentrations required to inhibit lipid peroxidation in liver microsomes was 31 μM for α-tocopherol and 124 μM for quercetin. The IC50 values for the isoflavones, chalcones, and coumestan were approximately 35, 22 and 16-fold higher than that of α-tocopherol, respectively [24].

4. Discussion

We established, for the first time, the ability of synthetic compounds, derived from HT and containing one or two atoms of sulphur or selenium, to inhibit the lipid peroxidation of vitamin E-deficient microsomes. Specifically, the antioxidant activity of five thioureas, a disulfide, a thiol, three selenoureas, a diselenide, and a selenonium were evaluated and the results were compared with the antioxidant activity of HT. Generally, all synthetic compounds showed a higher inhibition of lipid peroxidation than HT in our *in vitro* test system. The diselenide derivative presented a similar level of antioxidant protection as vitamin E and may therefore have the potential for development as a synthetic antioxidant compound with free radical scavenging capacity.

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Figure Captions

Figure 1. Molecular structures of hydroxytyrosol derivatives containing selenium or sulphur molecules.

Figure 2. The effect of pre-incubation of hepatic microsomal preparations from vitamin E-deficient rats with thioureas (**1**-**5**) on the production of thiobarbituric reactive substances (TBARS) following initiation of peroxidation with Fe/ADP.

Table 1

Table 1

Percentage inhibition of lipid peroxidation in hepatic microsomal preparations from vitamin E-deficient rats by α-tocopherol, hydroxytyrosol (HT) or different synthetic sulphur or selenium compounds

Compound	% Inhibition		
	− VE + 0.05 mM	− VE + 0.10 mM	− VE + 0.25 mM
α-toc	83.69 ± 0.01	86.48 ± 0.23	87.10 ± 0.18
HT[b]	n.d	4.84 ± 0.9	5.53 ± 1.1
Sulphur compounds			
1	n.d.[a]	n.d	n.d
2	9.02 ± 1.07	n.d	27.52 ± 1.14
3	26.57 ± 1.17	22.14 ± 0.14	50.77 ± 2.09
4	21.34 ± 1.20	29.45 ± 0.11	56.07 ± 1.99
5	16.05 ± 0.59	11.03 ± 0.13	37.23 ± 1.53
6	21.15 ± 0.71	64.61 ± 0.71	67.65 ± 1.10
7	50.78 ± 0.64	56.81 ± 0.97	59.17 ± 0.42
Selenium compounds			
8	17.13 ± 0.21	51.48 ± 0.16	58.0 ± 0.67
9	27.40 ± 0.45	60.69 ± 0.13	57.84 ± 0.65
10	15.02 ± 0.24	47.53 ± 0.22	58.55 ± 0.64
11	80.95 ± 0.09	84.20 ± 0.25	73.41 ± 0.47
12	n.d	4.16 ± 0.64	26.31 ± 0.33

[a]Activity not detected

[b]Bibliography date (Rubio-Senent et al. [14])

Figure 1

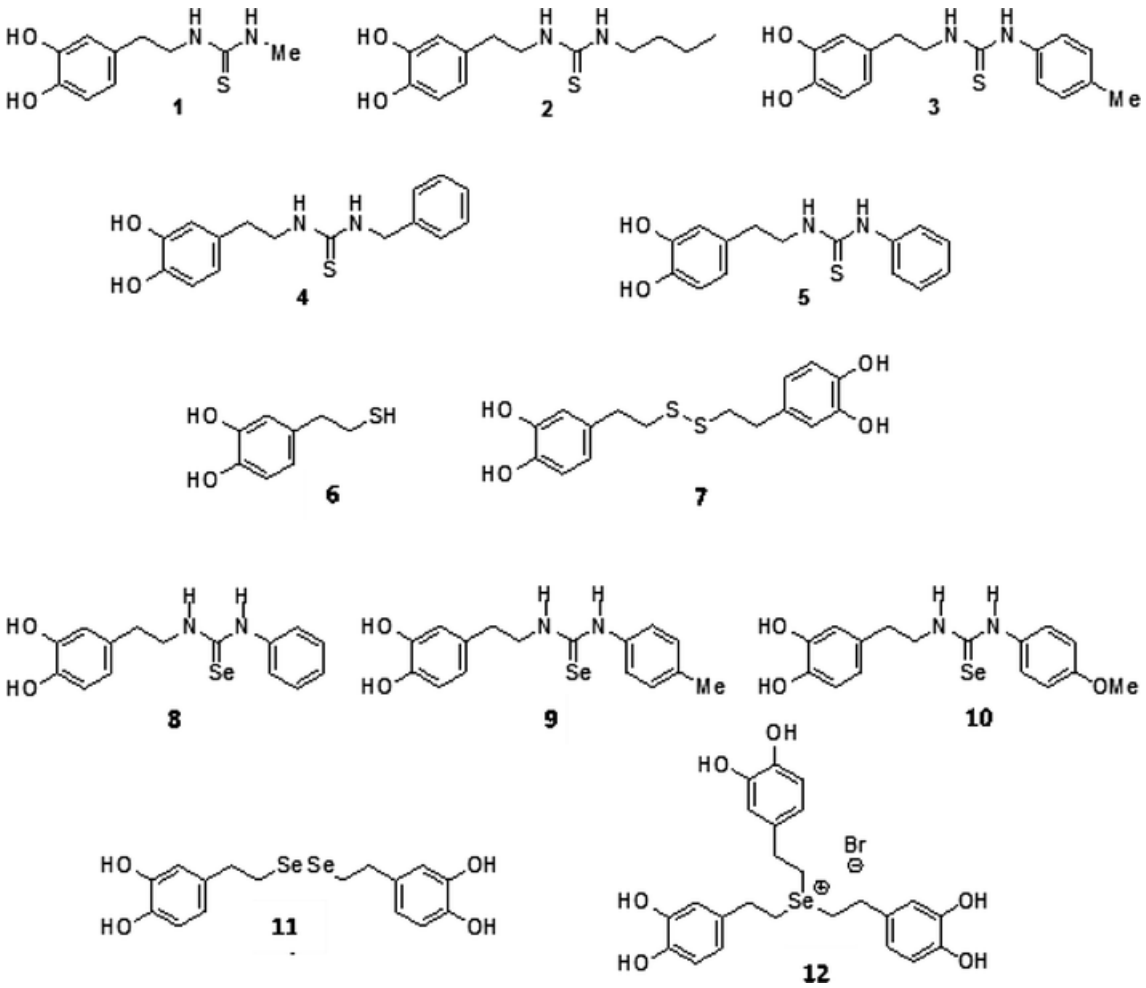
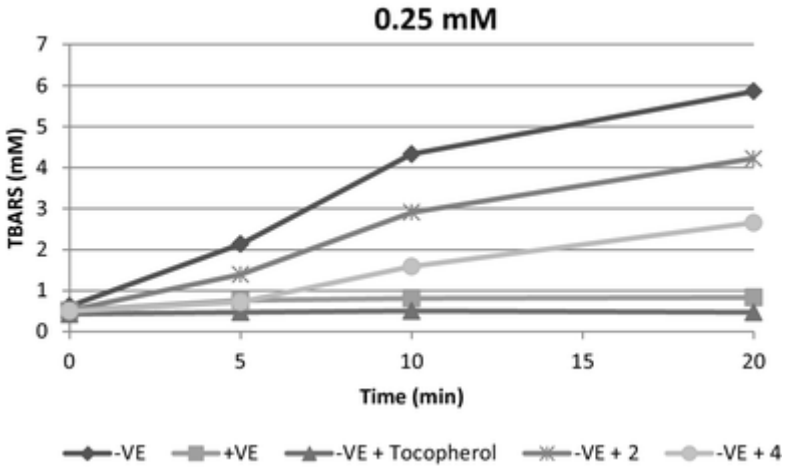


Figure 2


0.25 mM
TBARS (mM)
0
1
2
3
4
5
6
7
0
5
10
15
20
Time (min)
-VE
+VE
-VE + Tocopherol
-VE + 2
-VE + 4