Dietary antioxidant supplements: Benefits of their combined use

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ABSTRACT

Several dietary supplements claim medicinal benefits due to their composition in hydrophilic and lipophilic molecules, natural extracts or synthetic compounds with antioxidant properties. In the present work, the antioxidant activity of selected supplements taken in pills, capsules or infusions were studied either individually or combined. Linear discriminant analysis (LDA) was used to categorize the condensed formulations (pills and capsules), infusion bags and combined samples according with their antioxidant activity measured by radical scavenging activity, reducing power and lipid peroxidation inhibition using brain homogenates as models. AAF proved to have the highest antioxidant activity in all the assayed methods, either singly taken or included in mixtures. Furthermore, the mixtures containing this supplement revealed synergistic effects in 92% of the cases. The intake of antioxidant mixtures might provide some additional benefits.

1. Introduction

In living systems, Reactive Oxygen/Nitrogen Species (ROS/RNS) are produced primarily during normal aerobic metabolism (Halliwell and Gutteridge, 2007). At physiological levels, these intermediates participate in numerous metabolic processes including cell signaling, energy production, gene transcription and immune defense, among others (Seifried et al., 2007). However, decline of antioxidant defense mechanisms or exposure to environmental factors (smoke, pollution, ultraviolet radiation, high-fat diet, etc.) and pathological conditions (chronic infection, inflammation, etc.) can lead to increased ROS/RNS production, resulting in oxidative stress (Valko et al., 2007). Oxidative stress can damage key organic substrates such as DNA, lipids and proteins, compromising cells physiological function (Nordberg and Arnér, 2001). This condition has been associated to the ageing process in general, and to the initiation and progression of a variety of chronic conditions related to it, such as cardiovascular disease and cancer (Valko et al., 2007).

Protection against ROS/RNS-induced damage is provided by complex antioxidant defense systems, comprising endogenous enzymatic and non-enzymatic antioxidants (e.g., superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) and exogenous antioxidants (e.g., vitamin C, vitamin E, carotenoids and polyphenols), the latter provided mainly by the diet (Young and Woodside, 2001). Indeed, numerous epidemiological and clinical studies have linked high intake of fruits, vegetables, whole grains, and beverages of plant origin, which are rich in antioxidants, with lower incidence and mortality rates of chronic diseases including diabetes, atherosclerosis, rheumatoid arthritis, neurodegenerative and coronary diseases and cancer (Cerhan et al., 2003; de Kok et al., 2010; Esposito et al., 2002; Ford and Mokdad, 2001; Hertog et al., 1993; Kris-Etherton et al., 2002). These potential physiological benefits of dietary antioxidants have lead, in recent years, to a dramatic growth of the market of functional foods and dietary supplements claiming “antioxidant power”, and to the widespread consumption of these products.

Antioxidant dietary supplements are sold as isolated substances or as mixtures, from natural or synthetic origin, and are presented in a variety of forms including tablets, pills, capsules, powders, drinks and supplement bars. Antioxidant formulations use a plethora of ingredients, including antioxidant vitamins (tocopherols, ascorbic acid), bioactive compounds of plant origin (polyphenols and carotenoids), plant and algae extracts, fruits and vegetables concentrates, enzymes, minerals (selenium, zinc, manganese), polysaccharides, organosulfur compounds, etc.

The antioxidant activity of foodstuffs as well as the purified bioactive compounds to be used in supplement formulations, has been intensely researched (Barreira et al., 2008; Borges et al., 2010; Gorinstein et al., 2011; Müller et al., 2011; Stratil et al., 2007; Tabart et al., 2009). However, data regarding antioxidant activity of formulations already on the market is scarce. These products are promoted has antioxidant boosters but labels often lack information regarding effective antioxidant capacity values.

Therefore, the present study aimed to evaluate the antioxidant activity of different commercial antioxidant dietary supplements available in Portuguese market, by three in vitro assays: scavenging
activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, reducing power, and inhibition of lipid peroxidation using TBARS in brain homogenates. Moreover, some of the samples were mixed and further assayed in search of synergistic effects.

2. Materials and methods

2.1. Standards and reagents

2.2. Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Standards trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and α-tocopherol were purchased from Sigma (St. Louis, MO, USA). Methanol and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples and samples preparation

Samples were dietary supplements commercially available and labeled with antioxidant potential. In order to confirm and compare their antioxidant activity, the samples were prepared using the formulation available: pill, capsule or bag (Table 1). Each formulation was weighted and dissolved in 200 mL of distilled water in order to obtain the concentration of the stock-solution. Pills and the inner part of the capsules were dissolved in distilled water, while bags were used to prepare infusions. Several dilutions of each sample were prepared to perform the antioxidant activity assays.

Some of the samples were mixed and further assayed in search of synergistic effects. Four mixtures were prepared: AAF + Res + EMCO (stock-solution 4.56 mg/C24 L) and methanolic solution (270 μL) containing DPPH radicals (6 × 10−3 mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm (Guimarães et al., 2010). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

\[
RSA = \left( \frac{A_{0} - A_{S}}{A_{0}} \right) \times 100
\]

2.3. Antioxidant activity assays

2.3.1. General

The antioxidant activity of the individual and mixed samples was evaluated by DPPH radical-scavenging activity, reducing power and inhibition of lipid peroxidation using TBARS in brain homogenates. The sample concentrations providing 50% inhibition of TBARS (EC50) were calculated from the graphs of antioxidant activity percentages (DPPH and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations. The concentrations range was defined in order to allow percentages of antioxidant activity from 10% to 100% (stock-solution and successive dilutions). Trolox and α-tocopherol were used as standards.

2.3.2. DPPH radical-scavenging activity

This methodology was performed using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc). The reaction mixture on 96 wells plate consisted of a solution of well of the different samples concentrations (30 μL) and methanol solution (270 μL) containing DPPH radicals (6 × 10−3 mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm (Guimarães et al., 2010). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

\[
RSA = \left( \frac{A_{0} - A_{S}}{A_{0}} \right) \times 100
\]

2.3.3. Reducing power

The different concentrations of the samples solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% v/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, as also denoised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in the Microplate Reader described above (Guimarães et al., 2010).

2.3.4. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Brains were obtained from pig (Sus scrofa), dissected, and homogenized with a Polytron in ice cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate which was centrifuged at 3000g for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different concentrations of the samples solutions (0.2 mL) in the presence of FeSO4 (10 mM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL). The mixture (1 mL) was then incubated at 37 °C for 1 h. A portion of the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm (Ng et al., 2000). The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A0 – B)/A0] × 100, where A and B were the absorbance of the control and the sample solution, respectively.

2.4. Statistical analysis

All the assays were carried out in triplicate in three different samples of each single supplement. The results are expressed as mean value ± standard deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with α = 0.05, coupled with Welch's statistic. The homoscedasticity of distribution was checked through Levene's test.

In addition, a linear discriminant analysis (LDA) was used as a supervised learning technique to classify the assayed antioxidant dietary supplements according to their antioxidant activity results. A stepwise technique, using the Wilks’ λ method with the usual probabilities of F (3.84 to enter and 2.71 to remove), was applied for variable selection, verifying which canonical discriminant functions were significant. To avoid overoptimistic data modulation, a leaving-one-out cross-validation procedure was carried out to assess the model performance.

Moreover, the sensitivity and specificity of the discriminant model were computed from the number of individuals correctly predicted as belonging to an assigned group (López et al., 2009; Rencher, 1995). Sensitivity was calculated by dividing the number of samples of a specific group correctly classified by the total number of samples belonging to that specific group. Specificity was calculated by dividing the number of samples of a specific group classified as belonging to that group by the total number of samples of any group classified as belonging to that specific group. LDA statistical analysis and the other statistical tests were performed at a 5% significance level using the SPSS software, 18.0 (SPSS Inc.).

3. Results and discussion

The composition of the assayed dietary supplements is described in Table 1. Their selection was based in the different components included in the available formulations, either as single active components or in different combinations. The antioxidant components comprise lipophilic (e.g. vitamin E and β-carotene) and hydrophilic (e.g. vitamin C and polyphenols) molecules, natural extracts (e.g. Ginkgo biloba and Mentha spicata) or synthetic compounds (e.g. sodium selenite and zinc sulfate).

A wide range of methods have been used to screen the in vitro antioxidant capacity of foods and dietary supplements (Antolovich et al., 2002; Dávalos et al., 2003; Prior and Cao, 2000; Moon and Shibamoto, 2009). Standard procedures regarding antioxidant capacity methods have been recommended (Dávalos et al., 2003; Frankel and Meyer, 2000; Frankel and Finley, 2008; Prior and Cao, 1999), but this issue is still matter of debate.

Herein, three in vitro assays: scavenging activity against DPPH radicals, reducing power, and inhibition of lipid peroxidation using TBARS in brain homogenates were applied to evaluate the antioxidant activity of dietary supplements commercialized in Portugal. Table 2 gives the results obtained for the antioxidant activity of individual and combined samples. As expected, results regarding antioxidant activity of the different commercial dietary antioxidant supplements show great variability, reflecting their diverse composition and concentrations.

Considering DPPH scavenging activity, AAF (0.052 ± 0.001 mg/mL) and SACE (0.12 ± 0.02 mg/mL) were the most powerful substances; regarding TBARS inhibition, AAF (0.032 ± 0.003 mg/mL), VB (0.047 ± 0.002 mg/mL), Pyc (0.047 ± 0.001 mg/mL), AA (0.049 ± 0.004 mg/mL), Res (0.051 ± 0.001 mg/mL) and BAPN (0.071 ± 0.005 mg/mL) achieved the best results; in the case of reducing power, SACE (0.0337 ± 0.0004 mg/mL), AAF (0.042 ± 0.005 mg/mL), VB (0.0436 ± 0.0005 mg/mL) and AA (0.0614 ± 0.0005 mg/mL) revealed the highest antioxidant activity. In general, the obtained results confirmed the antioxidant potential of the assayed supplements, and some EC50 values are similar to those obtained with reference standards like α-tocopherol or trolox (Table 2).

The net effect of dietary antioxidants on health depends on intake levels, bioavailability, ability to scavenge ROS/RNS and...
<table>
<thead>
<tr>
<th>Sample</th>
<th>Composition</th>
<th>Formulation</th>
<th>Recommended daily dose</th>
<th>Stock solution (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SACE</td>
<td>Disodium selenium (0.056%), vitamins A (retinol acetate: 0.74%), C (L-ascorbic acid: 22.5%) and E (α-tocopherol: 15%)</td>
<td>Pill (397 mg)</td>
<td>1 pill</td>
<td>1.98</td>
</tr>
<tr>
<td>S200</td>
<td>Selenium: 200 µg, brewer's yeast</td>
<td>Pill (614 mg)</td>
<td>1 pill</td>
<td>3.07</td>
</tr>
<tr>
<td>VB</td>
<td>Vitamins A, C (L-ascorbic acid) and E (α-α-tocopherol succinate), broccoli sprouts powder, red fruit (grape, blueberry, cranberry, cherry, strawberry and raspberry) combined extract, selenium (yeast).</td>
<td>Pill (1058 mg)</td>
<td>1 pill</td>
<td>5.29</td>
</tr>
<tr>
<td>BAPN</td>
<td>Vitamins A (retinol: 864 µg), B1 (thiamine: 1.8 mg), B2 (riboflavine: 2.8 mg), B3 (nicotinamide), B5 (pantothenic acid: 7.5 mg), B6 (pyridoxine), B7 (biotin: 100 µg), B11 (folacin: 200 µg), B12 (cyanocobalamin: 4.5 µg), D (calcipherol: 2.5 µg) and E (α-α-tocopherol: 30 mg), magnesium (75 mg), zinc (7.5 mg), Selenium (L-selenomethionine: 62.5 µg), chromium (yeast: 50 µg), manganese (2.5 mg), copper (1 mg)</td>
<td>Pill (578 mg)</td>
<td>1 pill</td>
<td>2.89</td>
</tr>
<tr>
<td>LLSC</td>
<td>Soy isoflavones, vitamin C, Lycopersicon esculentum extract, lactoproteins, soy lecitin, Lacto-licopene</td>
<td>Pill (737 mg)</td>
<td>2 pills</td>
<td>3.68</td>
</tr>
<tr>
<td>KAG</td>
<td>Aged garlic extract, Silybum marianum extract, green tea (powder), vitamnins A (β-carotene), C (L-ascorbic acid) and E (α-α-tocopherol succinate), grape seed extract, pine bark extract, selenium (L-selenomethionine)</td>
<td>Capsule (431 mg)</td>
<td>4 capsules</td>
<td>2.16</td>
</tr>
<tr>
<td>SZCEA</td>
<td>Sodium selenite (0.02%), zinc sulfate (4.8%), vitamins A (β-carotene: 7.5%), C (calcium β-ascorbate: 12%) and E (α-α-tocopherol acetate: 12%)</td>
<td>Capsule (374 mg)</td>
<td>1 capsule</td>
<td>1.87</td>
</tr>
<tr>
<td>AAF</td>
<td>Vitamins A (β-carotene: 4.5 mg), C (calcium L-ascorbate: 500 mg) and E (α-α-tocopherol succinate: 134 mg and other tocopherols: 20 mg), α-cysteine chloride; food based antioxidants: powdered extracts of green tea (7.5 mg of polyphenols), red wine (4.5 mg of polyphenols) and Pycnogenol (3 mg of proanthocyanidins), zinc glycinate (10 mg), taurine (50 mg), l-glutathione (50 mg), manganese glycinate (4 mg);powdered active plant base (Spirulina, G. biloba, G. marianum and Gotu kola extracts), selenomethionine (50 µg), copper lysinate (1 mg) and riboflavin-5-phosphate (6 mg)</td>
<td>Capsule (744 mg)</td>
<td>2 capsules</td>
<td>3.72</td>
</tr>
<tr>
<td>PyC</td>
<td>Pycnogenol (Pinus maritima bark extract): 30 mg</td>
<td>Capsule (247 mg)</td>
<td>1–2 capsules</td>
<td>1.24</td>
</tr>
<tr>
<td>Res</td>
<td>Resveratrol (Polygonum cuspidatum root extract): 200 mg</td>
<td>Capsule (605 mg)</td>
<td>1–2 capsules</td>
<td>3.48</td>
</tr>
<tr>
<td>GC</td>
<td>Coffea arabica seeds (whole cryogrinded powder): 1% caffeine</td>
<td>Capsule (288 mg)</td>
<td>2 capsules/day</td>
<td>1.44</td>
</tr>
<tr>
<td>AA</td>
<td>Vitamin C (L-ascorbic acid) and E (α-α-tocopherol: 50%), green tea powder, rosemary leaf powder, grape extract, propolis alcoholic extract, Pinus albicaulis</td>
<td>Capsule (220 mg)</td>
<td>2 capsules</td>
<td>1.10</td>
</tr>
<tr>
<td>GBGT</td>
<td>Vitamin A (retinol acetate: 0.083%), C (L-ascorbic acid: 13.9%) and E (α-α-tocopherol: 5.6%), Lycopersicum esculentum fruit: 6.9%, Gingko biloba leaves (6.9%), Camellia sinensis (green tea): 1.9%; β-carotene: 0.7%.</td>
<td>Capsule (650 mg)</td>
<td>2 capsules</td>
<td>3.25</td>
</tr>
<tr>
<td>GM</td>
<td>Mangosteen 10:1 (Garcinia mangostana)</td>
<td>Capsule (848 mg)</td>
<td>2 capsules</td>
<td>4.24</td>
</tr>
<tr>
<td>VRFR</td>
<td>Vitis vinifera (red vine leaves: 35%), Hibiscus sabdariffa (flowers: 25%), Pyrus malus (fruit: 16%), orange and red fruits natural flavors</td>
<td>Bag (1500 mg)</td>
<td>1–3 teacups</td>
<td>7.50</td>
</tr>
<tr>
<td>EA</td>
<td>Equisetum arvense</td>
<td>Bag (1300 mg)</td>
<td>2–3 teacups</td>
<td>6.5</td>
</tr>
<tr>
<td>EMCO</td>
<td>E. arvense (30%), Olea europaea (30%), Crataegus laevigata (20%), Mentha piperita (20%)</td>
<td>Bag (1300 mg)</td>
<td>2–3 teacups</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* Pills and the inner part of the capsules were dissolved in 200 mL of distilled water; bags were used to prepare infusions using the same volume of distilled water.
The results were also analyzed through LDA to evaluate if the intake of antioxidant mixtures might provide some additional benefits. Furthermore, the mixtures containing AAF + Res demonstrated an additive effect. The theoretical values were obtained considering additive contributions of the individual species.
benefits, since the same antioxidant activity can be achieved with lower amounts of the chemical compounds included in the pills or capsules. For the assayed combinations, the synergistic interaction was the main observed effect. Regarding LDA, the assayed dietary antioxidant supplements proved to have distinctive features, derived from being condensed (pills or capsules) or bags (infusions) formulas. Furthermore, it is relatively clear that the tested combinations retain an antioxidant profile highly similar to the presented by the condensed formulas included in those mixtures.

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References


Fig. 1. Canonical analysis of antioxidant dietary supplements (condensed – pills and capsules, infusion bags and combined – mixed samples) based on antioxidant activities (DPPH radical scavenging activity, reducing power, and TBARS formation inhibition). The two significant functions are plotted.


