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.

Keywords: Dietary Supplements; Antioxidant activity; Synergistic Effects; Linear Discriminant Analysis

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 signalling, energy production, gene transcription and immune defence, among others (Seifried et al., 2007). However, decline of antioxidant defence 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 defence 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., 2003). 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/mL) and the corresponding binary combinations: AAF+Res (stock-solution 3.60 mg/mL), AAF+EMCO (stock-solution 5.11 mg/mL) and Res+EMCO (stock-solution 4.99 mg/mL).

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% of antioxidant

activity or 0.5 of absorbance (EC_{50}) 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 ~90% (sock-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 by well of the different samples concentrations (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} 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 discolouration using the equation: % RSA = $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

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 ºC for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, as also deionised 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 3000*g* for10 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 $FeSO_4$ (10 mM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloro acetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA,2%, w/v, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the colour 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 (%) = [(A - B)/A] × 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 $\alpha = 0.05$, 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 sensibility and specificity of the discriminant model were computed from the number of individuals correctly predicted as belonging to an assigned group (López et al., 2008; Rencher, 1995). Sensibility 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 sulphate).

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., 2005; Prior and Cao, 2000; Moon and Shibamoto, 2009). Standard procedures regarding antioxidant capacity methods have been recommended (Dávalos et al., 2005; 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 supplements; 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 EC_{50} 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 synergistic effects (Liu, 2004; Manach, et al., 2005). It´s well accepted that a cooperative mix of antioxidants in a balanced form appears to be more effective than high levels of one or a few antioxidants (Liu, 2004; Wang et al., 2011). Therefore, four mixtures were assayed in search of synergistic effects: AAF+Res+EMCO and the corresponding binary combinations: AAF+Res, AAF+EMCO and Res+EMCO. Since some of the assayed supplements already contain several components, it was decided to include an infusion to avoid the unique use of pharmaceutical formulations. Besides, infusions are apparently better accepted by the consumers. EMCO was chosen due to its highest antioxidant activity among infusions. Furthermore, the sample that showed the highest antioxidant activity (AAF) was also included. Res was chosen in order to evaluate the influence of an antioxidant supplement which is composed mainly by a single molecule.

The types of interactions (synergistic, additive or antagonist) observed in the antioxidant activity of the dietary supplements mixtures are given in **Table 3**. For DPPH radical scavenging activity, as well as for reducing power assays, the mixtures were always synergistic (increase of antioxidant capacity). The synergistic effect predominated also in the TBARS inhibition assay, being observed in 75% of the mixtures; AAF+Res demonstrated an additive effect.

The results were also analyzed through LDA to evaluate if the observed differences were sufficient to differentiate the condensed dietary supplements (pills and capsules) from the bags formulations (infusions). All independent variables selected by the stepwise procedure of the discriminant analysis were statistically significant according to the Wilks'λ test ($P < 0.05$). The stepwise LDA was performed considering the results obtained in all the assayed antioxidant methods, ensuing in a discriminant model with

two significant ($P < 0.001$ for the Wilks' λ test) discriminant functions. These functions explained 100.0% of the variance of the experimental data (the first explained 71.2% and the second 28.8%. The first function (**Figure 1**) revealed to be more powerfully correlated with DPPH scavenging activity, while TBARS inhibition was the most important variable for function 2. The model showed a satisfactory classification performance allowing to correctly classifying 70.8% of the samples for the original groups as well as for the cross-validation procedure. This separation is clearer in the case of infusion bags, demonstrating that the assayed combinations permitted to obtain mixtures with antioxidant potential more related with the condensed formulas (pills and capsules) than with the included infusion samples (bags).

Overall, AAF proved to have the highest antioxidant activity in all the assayed methods, providing the best results, 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, 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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Table 1. Composition of the dietary supplements and concentrations of the stock-solution of each sample.

Sample	Composition	Formulation	Recommended Daily Dose	Stock solution[a] (mg/mL)
SACE	Disodium selenium (0.056%), vitamins A (retinol acetate: 0.74%), C (L-ascorbic acid: 22.5%) and E (α-tocopherol: 15%)	Pill (397 mg)	1 pill	1.98
S200	Selenium: 200 μg, brewer's yeast	Pill (614 mg)	1 pill	3.07
VB	Vitamins A, C (L-ascorbic acid) and E (D-α-tocopherol succinate), broccoli sprouts powder, red fruit (grape, blueberry, cranberry, cherry, strawberry and raspberry) combined extract, selenium (yeast).	Pill (578 mg)	1 pill	2.89
BAPN	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), C (ascorbic acid: 200 mg), D (calcipherol: 2.5 μg) and E (D-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)	Pill (1058 mg)	1 pill	5.29
LLSC	Soy isoflavones, vitamin C, *Lycopersicon esculentum* extract, lactoproteins, soy lecitin, Lacto-licopene	Pill (737 mg)	2 pills	3.68
KAG	Aged garlic extract, *Sylibum marianum e*xtract, green tea (powder), vitamins A (β-carotene), C (L-ascorbic acid) and E (α-tocopherol succinate), grape seed extract, pine bark extract, selenium (L-selenomethionine)	Capsule (431 mg)	4 capsules	2.16
SZCEA	Sodium selenite (0.02%), zinc sulphate (4.8%), vitamins A (β-carotene: 7.5%), C (calcium L-ascorbate: 12%) and E (D-α-tocopherol acetate: 12%)	Capsule (374 mg)	1 capsule	1.87
AAF	Vitamins A (β-carotene: 4.5 mg), C (calcium L-ascorbate: 500 mg) and E (D-α-tocopherol succinate: 134 mg and other tocopherols: 20 mg), L-cysteine chloridrate; 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 procyanidins), zinc glycinate (10 mg), taurine (50 mg), L-glutathione (50 mg), manganese glycinate (4 mg),powdered	Capsule (744 mg)	2 capsules	3.72

	active plant base (Spirulina, *Ginkgo biloba*, *Sylibum marianum* and *Gotu kola* extracts), selenomethionine (50 μg), copper lysinate (1 mg) and riboflavin-5-phosphate (6 mg)			
Pyc	Pycnogenol (*Pinus maritima* bark extract): 30 mg	Capsule (247 mg)	1-2 capsules	1.24
Res	Resveratrol(*Polygonum cuspidatum* root extract): 200 mg	Capsule (695 mg)	1-2 capsules	3.48
GC	*Coffee arabica* seeds (whole cryogrinded powder): 1% caffeine	Capsule (288)	2 capsules/day	1.44
AA	Vitamin C (L-ascorbic acid) and E (α-tocopherol: 50%), green tea powder, rosemary leaf powder, grape extract, propolis alcoholic extract, *Pinus albicaulis*	Capsule (220 mg)	2 capsules	1.10
GBGT	Vitamin A (retinol acetate: 0.083%), C (L-ascorbic acid: 13.9%) and E (D-α-tocopherol: 5.6%), *Lycopersicum esculentum* fruit: 6.9%, *Gingko biloba* leaves (6.9%), *Camelia sinensis* (green tea): 1.9%; β-carotene: 0.7%.	Capsule (650 mg)	2 capsules	3.25
GM	Mangosteen 10:1 (*Garcinia mangostana*)	Capsule (848 mg)	2 capsules	4.24
VRFR	*Vitis vinifera* (red vine leaves: 35%), *Hibiscus sabdariffa* (flowers: 25%), *Pyrus malus* (fruit: 16%), orange and red fruits natural flavors	Bag (1500 mg)	1-3 teacups	7.50
EA	*Equisetum arvense*	Bag (1300 mg)	2-3 teacups	6.5
EMCO	*Equisetum arvense* (30%), *Olea europaea* (30%), *Crataegus laevigata* (20%), *Mentha piperita* (20%)	Bag (1300 mg)	2-3 teacups	6.5

[a]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.

Table 2. Antioxidant activity of individual and combined samples of dietary supplements. In each column different letters mean significant differences ($p<0.05$).

Sample	DPPH scavenging activity EC_{50} (mg/mL)	Reducing power EC_{50} (mg/mL)	TBARS inhibition EC_{50} (mg/mL)
SACE	0.12±0.02 l	0.0337±0.0004 i	1.9±0.1 b
S200	2.9±0.1 c	2.96±0.05 b	2.8±0.1 a
VB	0.36±0.02 jk	0.0436±0.0005 i	0.047±0.002 j
BAPN	1.03±0.02 f	0.145±0.001 hi	0.071±0.005 j
LLSC	0.62±0.04 hi	0.352±0.004 f	1.55±0.04 e
KAG	0.76±0.05 gh	0.290±0.005 fgh	0.14±0.01 i
SZCEA	0.20±0.04 kl	0.42±0.04 ef	1.82±0.02 c
AAF	0.052±0.001 l	0.042±0.005 i	0.032±0.003 j
Pyc	0.46±0.05 ij	0.171±0.001 ghi	0.047±0.001 j
Res	1.6±0.1 e	0.68±0.01 d	0.051±0.001 j
GC	0.84±0.05 fg	0.355±0.003 f	0.9±0.1 g
AA	0.21±0.04 kl	0.0614±0.0005 i	0.049±0.004 j
GBGT	0.18±0.05 kl	0.556±0.002 de	1.70±0.04 d
GM	3.7±0.1 a	3.61±0.01 a	1.43±0.05 f
VRFR	3.2±0.4 b	0.98±0.02 c	0.46±0.01 h
EA	1.8±0.2 d	0.88±0.01 c	0.51±0.02 h
EMCO	0.73±0.02 gh	0.636±0.003 d	0.5±0.1 h

AAF+Res	0.069±0.001 l	0.0568±0.0002 i	0.043±0.003 j
AAF+EMCO	0.089±0.004 l	0.0690±0.0005 i	0.056±0.002 j
Res+EMCO	0.844±0.004 fg	0.31±0.01 fg	0.086±0.001 ij
AAF+Res+EMCO	0.14±0.02 l	0.0738±0.0003 i	0.07±0.01 j
Standard Trolox	0.041±0.00 l	0.032±0.00 l	0.0043±0.0002
Standard α-Tocopherol	0.040±0.002	0.0695±0.0005	0.0050±0.0006

EC_{50}: sample concentration providing 50% of antioxidant activity in DPPH and TBARS assays, or 0.5 of absorbance at 690 nm in reducing power assay.

Table 3. Theoretical[a] *versus* experimental values of antioxidant activity of the combined samples of dietary supplements.

Mixtures	DPPH scavenging activity EC_{50} (mg/mL)			Reducing power EC_{50} (mg/mL)			TBARS inhibition EC_{50} (mg/mL)		
	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect
AAF+Res+EMCO	0.78±0.04	0.14±0.02	S	0.18±0.03	0.07±0.01	S	0.452±0.002	0.0738±0.0003	S
AAF+Res	0.81±0.05	0.069±0.001	S	0.041±0.001	0.043±0.003	A	0.361±0.001	0.0568±0.0002	S
AAF+EMCO	0.39±0.01	0.089±0.004	S	0.25±0.04	0.056±0.002	S	0.339±0.002	0.0690±0.0005	S
Res+EMCO	1.15±0.05	0.844±0.004	S	0.26±0.04	0.086±0.001	S	0.658±0.004	0.31±0.01	S

[a]The theoretical values were obtained considering additive contributions of the individual species.

A - Additive effect: theoretical and experimental values reveal differences lower than 5%. S - Synergistic effect: experimental values are more than 5% lower for EC_{50} when compared with theoretical values. AN-Antagonist effect: experimental values are more than 5% higher for EC_{50} then compared with theoretical values.

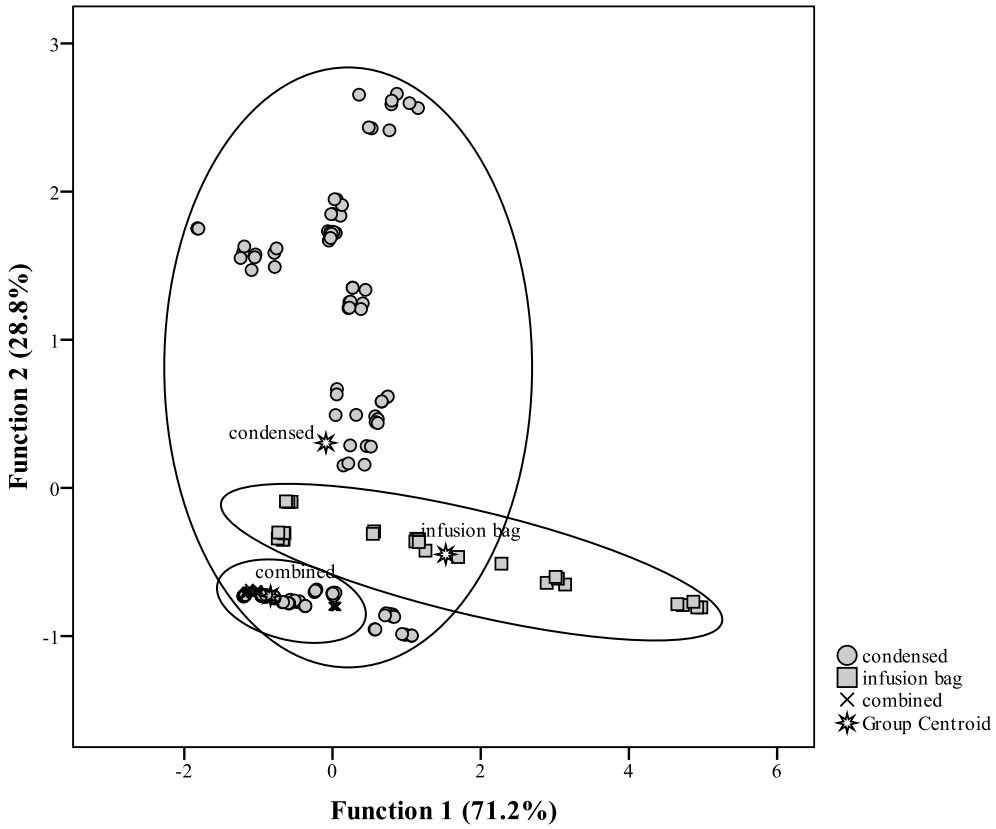

Figure 1. Canonical analysis of antioxidant dietary supplements (condensed- pills and capsules, infusion bags and combined- mixed samples) based on antioxidant activities (DPPH radical scavenging activity, reducing power, and TBARS formation inhibition). The two significant functions are plotted.