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In Vitro Analyses of Antioxidant Activity of Food Supplements GE Kids® and GE 132+ Natural®

Nevena Todorović*, Neda O. Đorđević, Snežana B. Pajović

Laboratory of Molecular Biology and Endocrinology, Institute of Nuclear Sciences "Vinča", University of Belgrade, Belgrade, Serbia
*Corresponding author: nevenat@vin.bg.ac.rs

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Abstract Nutrition related research, including the studies focused on the natural dietary antioxidants are getting more and more attention. Functional foods, that contain known or unknown biologically active compounds, promote health and wellbeing beyond dietary needs. Exogenous originating reducing compounds such as vitamin C, vitamin E, carotenoids and polyphenols, play important role in many antioxidant mechanisms in living organisms. There are a great number of synthetic antioxidant products in the market, whereby tendency is to replace synthetic antioxidants by natural ones. Here we examined the antioxidant properties of GE kids® and GE 132+ natural®, food supplements designed for maintenance and improvement of immune response in children and adults, respectively. Antioxidant properties were analyzed *in vitro* by testing their ability to scavenge free radical species and their ferric reducing antioxidant power. GE kids® and GE 132+ natural® showed antioxidant activity against physiologically relevant hydroxyl and superoxide radical, as well as against artificial radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH·) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$), commonly used for *in vitro* analyses of antioxidant properties. Interestingly, both food supplements showed the highest efficacy in scavenging hydroxyl radical, free radical which can be neutralized only by non - enzymatic systems. Accordingly, anti-hydroxyl radical activity was equivalent to activity of 533.3 ± 6.1 mg of ascorbic acid (AA) per GE kids® sachet and 172.6 ± 12.3 mg of AA per GE 132+ natural® capsule. Results suggest that GE kids® and GE 132+ natural® may contribute maintaining the physiological levels of free radicals and therefore the oxido-redox balance in the organism.

Keywords: *food supplements, antioxidant activity, radical scavenging, reducing power*

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1. Introduction

A food supplement, also known as a nutritional or dietary supplement, is a preparation intended to provide nutrients such as vitamins, minerals, fibers, amino or fatty acids that are, either missing, or not consumed in sufficient amounts in person's diet. Dietary supplements, as well as, functional food ingredients are important for health promotion and disease risk reduction. Functional foods deliver additional or enhanced benefits beyond basic nutrition. It's well known that health giving properties of functional foods are partially due to their ability to maintain the physiological levels of reactive species [1,2]. There is growing interest in the research of natural dietary antioxidants. Their beneficial impact on health due to direct antioxidant effects by radical scavenging, or by effecting the cell signaling and gene expression, were shown previously [3]. The redox balance is believed to be critical in maintaining healthy biological systems. The imbalance between the reactive oxygen species (ROS) and antioxidant defenses, in favor of the ROS production, leads to an oxidative stress, and consequently to the oxidation of biologically relevant macromolecules. The 'antioxidant hypothesis' proposes that vitamin C, vitamin E, carotenoids, and polyphenols produced in those plant foods provide protection against oxidative damage. Namely, dietary antioxidants are believed to play an important role in the human body defense system. Thus, they protect against oxidative damage induced by ROS, which is involved in the pathogenesis of many degenerative diseases such as cardiovascular diseases and cancers, as well as in the process of aging [4]. It is well established that functions of the human immune system depend on the intake of micronutrients with antioxidant properties [5,6]. Thus, adequate intake of vitamins and other antioxidant elements seems to be essential for an efficient function of the immune system [7]. The intake of food rich in naturally-occurring antioxidants, including vitamins and polyphenols, has been suggested to be beneficial for health promotion. Humans are not capable of synthesizing these antioxidant compounds *de novo*, so they rely on the diet as the source of these compounds. Plant food (e.g., apples, tomatoes, broccolis, onions, etc.) represents the natural source of many antioxidants [4,8]. Besides, there are a large number of synthetic antioxidant products in the market. But emerging tendency is to replace

synthetic antioxidants by natural ones, due to presumably increased safety and higher acceptance by the consumer.

According to the manufacturer, GE kids® is a food supplement composed of seven biologically active components, designed for children and young people with weakened immune system, like those who suffer from recurrent infectious diseases. Lower respiratory tract infections are a substantial public health problem, whereby children younger than 5 years old are worst affected [9]. Active components of GE kids® are extracts of *Ganoderma lucidum*, curcumin, rosehip and tomato (lycopene 5%), royal jelly as well as β-glucan 1.3/1.6D, and resveratrol extracted from skins and seeds of red grapes. On the other hand, GE 132+ natural® is the antioxidant formula for adults containing extracts of *Ganoderma lucidum*, grape seed (resveratrol), tomato (lycopene 7%) and broccoli sprout (sulforaphane), and royal jelly. As indicated by the manufacturer, both products are completely natural preparations designed to maintain and improve immune response.

This study aimed to investigate the antioxidant properties of GE kids® and GE 132+ natural® by testing their ability to scavenge physiologically relevant hydroxyl and superoxide radical, as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH·) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$), artificial radical species which are the most often applied on plant extracts. In addition, ferric reducing antioxidant power (FRAP) of examined preparations was determined.

2. Materials and Methods

2.1. Dietary Supplements and Chemicals

GE kids® and GE 132+ natural® were produced by Alphacaps GmbH, Augustdorf, Germany and provided by International Health d.o.o., Belgrade, Serbia. Nitro blue tetrazolium (NBT); 1,10-phenanthroline; β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH); phenazine methosulfate (PMS); $ABTS^{\cdot+}$; DPPH; 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) and ascorbic acid (AA) were purchased from Sigma-Aldrich (Steinheim, Germany). Trolox was purchased from Acros Organics (Geel, Belgium).

2.1.1. Preparation of Dietary Supplements Solutions

GE kids® sachets and GE 132+ natural® capsules were dissolved in distilled water with the aid of ultrasound (30 min). Both solutions were filtered through Whatman No. 42 filter paper and used for further analyses.

2.2. Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging assay was carried out using the method described by Li et al. [10] with some modifications. Reaction mixture contained 200 µL of sample/standard at various concentrations, 200 µL of 3 mM 1,10-phenanthroline (dissolved in 20 mM phosphate buffer, pH 7.4), 200 µL of 3 mM $FeSO_4$ (dissolved in water) and 200 µL of 0.02% H_2O_2. Four different dilutions of both analyzed samples were made. Each dilution was made in triplicate. The mixture was incubated at 37°C for 60 min, and the absorbance was measured at 536 nm. Synthetic ascorbic acid (AA, Vitamin C), the well-known standard with strong antioxidant activity, was used as positive control. Results were determined using the following equation:

$$\textit{Hydroxyl radical scavenging activity}\ (\%) = \left[\left(A_S - A_C\right)/\left(A_0 - A_C\right)\right]\times 100$$

whereby A_S is the absorbance of the sample/standard; A_C, absorbance of control solution containing 1,10-phenanthroline, $FeSO_4$ and H_2O_2; A_0, absorbance of blank solution containing 1,10-phenanthroline and $FeSO_4$. The scavenging activities of samples are expressed as mg of AA that have the same activity as one sachet or capsule of GE kids® and GE 132+ natural®, respectively.

2.3. Anti-Superoxide Radical Formation and Free Radical Scavenging Activities

The superoxide anion scavenging activity was measured using the method described by Liu et al. [11]. Superoxide radicals were generated in 20 mM Tris-HCl, pH 8.3 containing 50 µM NBT, 78 µM NADH and different dilutions of the GE kids® or GE 132+ natural® complex dissolved in water. Each dilution was made in triplicate. The reaction was initiated by the addition of 10 µM PMS solution to the mixture. The reaction mixture was incubated at 25°C for 5 minutes, and the blue chromogen, formed due to NBT reduction, was read at 560 nm. The absorbance was measured against the corresponding blank solution (reaction mixture without PMS). Synthetic AA and the water-soluble vitamin E analogue Trolox were used as the positive controls. Results were calculated as percentage of inhibition of superoxide radicals, using the following formula:

$$\textit{Superoxide radical scavenging activity}\ (\%) = \left(A_C - A_S\right)/A_C \times 100$$

whereby A_C is the absorbance of control solution containing 50 µM NBT, 78 µM NADH and 10 µM PMS in 20 mM Tris-HCl, pH 8.3; A_S, absorbance of the mixture with sample/standard. The anti-superoxide radical activities of samples are expressed as mg of AA or Trolox that have the same activity as one sachet or capsule of GE kids® and GE 132+ natural®, respectively.

2.4. Anti-$ABTS^{\cdot+}$ Radical Activity

Anti-$ABTS^{\cdot+}$ radical activity was assayed by the method described by Re et al. [12]. The method is based on measuring capacity of tested compounds to scavenge the $ABTS^{\cdot+}$ radical cation ($ABTS^{\cdot+}$) compared to a standard antioxidants (AA and Trolox) in a dose-response curve. The radical cation was prepared by mixing 7 mM $ABTS^{\cdot+}$ stock solution with 2.4 mM potassium persulfate (1/1, v/v). The mixture was left in dark for 16 hours. The $ABTS^{\cdot+}$ solution was diluted with methanol to an absorbance ~ 0.700 at 734 nm. The reaction mixture contained 1 mL of diluted $ABTS^{\cdot+}$ solution and 100 µL of different concentrations of sample/standard. Each concentration was run in triplicate. Measurements were

taken at 734 nm after 6 min. The anti-ABTS$^{\cdot+}$ activity of tested samples were calculated by determining the decrease in absorbance at different concentrations by using the following equation:

$$ABTS^{\bullet+}\ scavenging\ activity\ (\%) = (A_C - A_S) / A_C \times 100$$

whereby A_C is the absorbance of control solution containing ABTS$^{\cdot+}$ solution and methanol; A_S, absorbance of the mixture containing ABTS$^{\cdot+}$ solution and sample/standard. The anti-ABTS$^{\cdot+}$ radical activities of samples are expressed as mg of AA or Trolox that have the same activity as one sachet or capsule of GE kids® and GE 132+ natural®, respectively.

2.5. Anti-DPPH· Radical Activity

Anti-DPPH· radical activity was measured according to the method described by Blois [13], with slight modifications. The reaction mixture contained 100 µL of five different concentrations of sample/standard, 500 µL of DPPH· dissolved in methanol and 400 µL of 50 mM phosphate buffer, pH 7.0. Each dilution was made in triplicate. After incubation in dark, at room temperature for 30 min, the absorbance was recorded at 517 nm. DPPH· radical scavenging activity was calculated using the following equation:

$$DPPH^{\bullet}\ scavenging\ activity(\%) = (A_C - A_S) / A_C \times 100$$

whereby A_C is the absorbance of control solution containing DPPH· solution and buffer; and A_S is the absorbance of the mixture containing sample/standard in the reaction mixture. The anti-DPPH· activities of samples are expressed as mg of AA or Trolox that have the same activity as one sachet or capsule of GE kids® and GE 132+ natural®, respectively.

2.6. Ferric Reducing Antioxidant Power - FRAP

FRAP assay was performed according to the method described by Benzie and Strain [14], with slight modifications. The FRAP reagent contained 20 mM $FeCl_3$, 10 mM TPTZ and 0.3 M acetate buffer, pH 3.6 in a ratio 1:1:10. Freshly prepared FRAP reagent was warmed to 37°C. The reaction mixture contained 950 µL of FRAP reagent and 50 µL of sample/standard. After incubation at 37 °C for 10 min, the absorbance was measured at 593 nm. FRAP values of samples were extrapolated from standard curves obtained by using AA and Trolox standard solutions at various concentrations ranging from 10-100 µg/mL and 20-150 µg/mL, respectively. All measurements were done in triplicate.

3. Results and Discussion

Results showed that both GE kids® and GE 132+ natural® showed antioxidant activities in *in vitro* conditions. In order to quantify antioxidant properties, the anti-radical activities of tested preparations were expressed as mg of AA or Trolox which scavenge the same amount of tested radical species as one sachet of GE kids® and one capsule of GE 132+ natural®.

Hydroxyl radical and superoxide radical are two major ROS in organisms that are being continuously formed in a process of reduction of oxygen to water [15]. Results showed that anti-hydroxyl radical activity of one GE kids® sachet corresponds to activity of 533.3 ± 6.1 mg of AA, while one capsule of GE 132+ natural® had the same activity as 172.6 ± 12.3 mg of AA (Figure 1). The fact that hydroxyl radical, the most reactive and dangerous ROS, may be neutralized only by non-enzymatic systems [16], highlights the importance of anti-hydroxyl activity shown by both tested supplements, but in greater extend by the GE kids®. Having in mind that one GE kids® sachet contains 6.7 mg of AA (as indicated on the packaging), the observed anti-hydroxyl radical activity can be attributed not only to AA, a well known antioxidant, but to the other components as well. Namely, resveratrol is the best known scavenger of hydroxyl radicals. Similar action is exerted by ferulic acid that belongs to the family of hydroxycinnamic acid with the chemical structure similar to curcumin [15]. It was also shown that β-glucan extracted from barley exerts significant antioxidant activity, in addition to the various biologic activities previously described [17]. Thus, it is reasonable to assume that resveratrol, curcumin and β-glucan, which are the active compounds of GE kids® preparation, contribute to its high anti-hydroxyl radical activity. In addition, determined anti-hydroxyl radical activity is probably the result of synergistic effects of different antioxidant components present in the preparation [18,19].

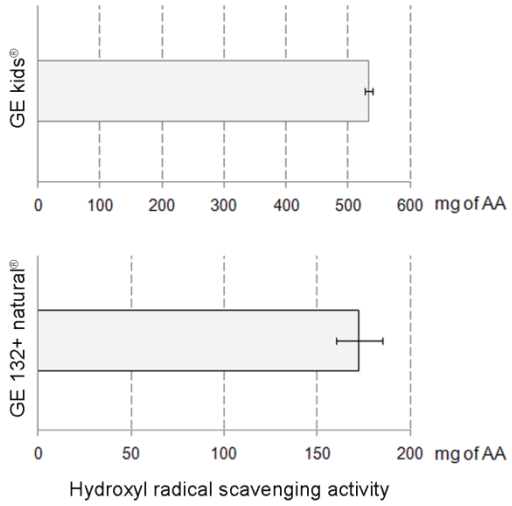

Figure 1. Hydroxyl radical scavenging activity of GE kids® and GE 132+ natural® complex expressed as mg of ascorbic acid (AA) that have the same activity as one sachet or capsule of GE kids® and GE 132+ natural®, respectively. Values are mean ± SD of three replicates

With regard to superoxide anion radical, scavenging activity of one GE kids® sachet corresponded to 17 ± 0.6 mg of AA, or 645.9 ± 22.6 mg of Trolox, while one capsule of GE 132+ natural® had the same activity as 1.4 ± 0.1 mg of AA, or 55.2 ± 0.2 mg of Trolox (Figure 2).

Ganoderma lucidum extract, constituent of both tested preparations, was previously shown to have antioxidant activity against superoxide, hydroxyl, DPPH· and ABTS$^{\cdot+}$ radical, as well as against hydrogen peroxide. Its reducing power was also detected [21]. In line with this, both examined food supplements showed scavenging activity against tested artificial radicals ABTS$^{\cdot+}$ and DPPH·. The

ABTS$^{\cdot+}$ assay, also known as Trolox equivalent antioxidant capacity, is commonly used method for *in vitro* determination of antioxidant activities of plant extracts, natural compounds in foods and beverages [12]. Accordingly, the obtained results showed that one GE kids® sachet scavenged the same amount of ABTS$^{\cdot+}$ radical as 94.7 ± 0.9 mg of AA, or 85.5 ± 0.8mg of Trolox, while one capsule of GE 132+ natural® had the anti-ABTS$^{\cdot+}$ radical activity of 8.4 ± 0.1 mg of AA, or 7.4 ± 0.1 mg of Trolox (Figure 3).

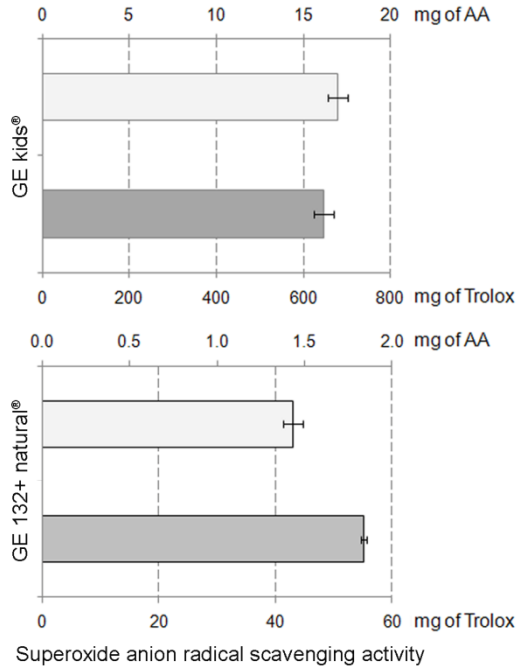

Figure 2. Superoxide anion radical scavenging activity of GE kids® and GE 132+ natural® complex expressed as mg of ascorbic acid (AA) or Trolox that have the same activity as one sachet or capsule of GE kids® and GE 132+ natural®, respectively. Values are mean ± SD of three replicates

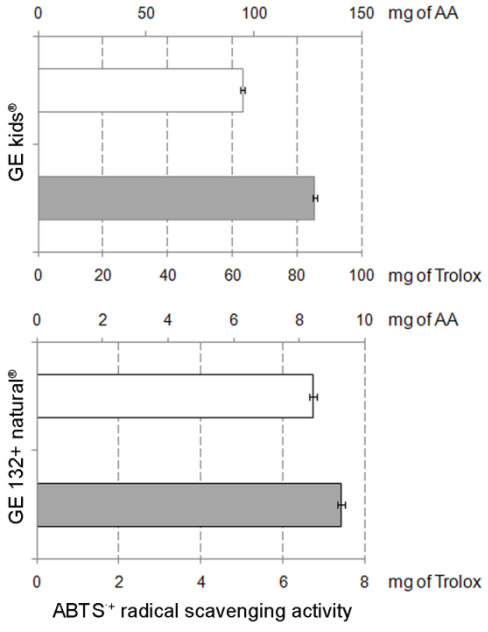

Figure 3. ABTS$^{\cdot+}$ radical scavenging activity of GE kids® and GE 132+ natural® complex expressed as mg of ascorbic acid (AA) or Trolox that have the same activity as one sachet or capsule of GE kids® and GE 132+ natural®, respectively. Values are mean ± SD of three replicates.

In addition to ABTS$^{\cdot+}$, antioxidant activity of analysed food supplements was tested against DPPH$^{\cdot}$, the most often applied artificial radical [22]. Results showed that scavenging activity of one GE kids® sachet corresponded to activity of 19.1 ± 1.0 mg of AA, or 24.4 ± 1.2 mg of Trolox, while one capsule of GE 132+ natural® had the same activity as 61.2 ± 1.3 mg of AA, or 78.1 ± 1.7 mg of Trolox (Figure 4). Besides extract of *Ganoderma lucidum*, resveratrol, a component also present in both supplements, was previously shown to have anti-ABTS$^{\cdot+}$ and anti-DPPH$^{\cdot}$ activity [23]. The similar results were found in the case of curcumin extract, constituent of GE kids® [24], as well as for broccoli seed extracts, ingredient of GE 132+ natural® which represents the source of sulforaphane [25]. Interestingly, broccoli seed extracts showed strong free radical scavenging activities, but sulforaphane itself showed much lower activity, indicating that this isothiocyanate is not the main contributor to the radical scavenging capacity of broccoli [25]. This highlights the importance of the synergistic effects of the antioxidant components from various natural products. The synergism in the antioxidant activity of natural compounds is well documented in food products. Namely, synergistic antioxidant effects of different natural, as well as synthetic antioxidants were found previously by the number of studies [18,19,20].

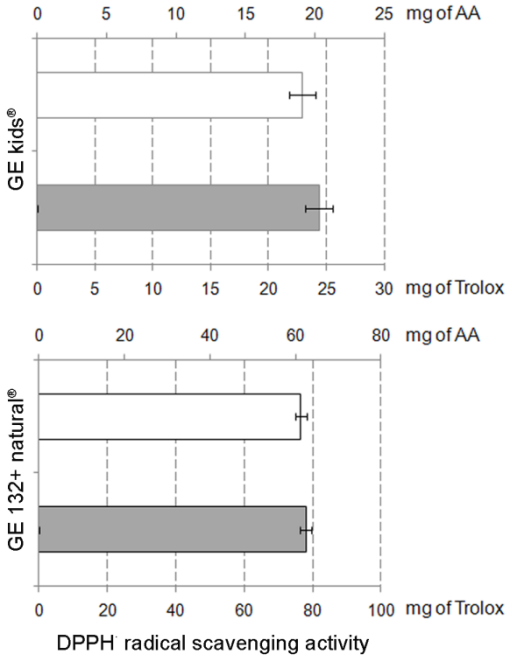

Figure 4. DPPH$^{\cdot}$ radical scavenging activity of GE kids® and GE 132+ natural® complex expressed as mg of ascorbic acid (AA) or Trolox that have the same activity as one sachet or capsule of GE kids® and GE 132+ natural®, respectively. Values are mean ± SD of three replicates

The reducing capacity of natural products may serve as a significant indicator of their potential antioxidant activity [26]. Thus, in addition to radical scavenging activity, reducing power of GE kids® and GE 132+ natural® was examined by FRAP assay. The one GE kids® sachet showed the same reducing power as 9.9 ± 0.6 mg of AA, or 13.1 ± 0.9 mg of Trolox, while one capsule of GE 132+ natural® had the activity equivalent to activity of 51.4 ± 2.0 mg of AA, or 61.2 ± 2.9 mg of Trolox

(Figure 5). Various antioxidant compounds in food and beverages display strong antioxidative properties by forming complexes with the metal ions, particularly iron and copper which can be toxic if present in excess. For example, high antioxidant capacities, including ferrous ion chelating ability of brocolli seed extracts were found previously [27]. Interestingly, reducing power of GE 132+ natural®, which contains brocolli sprout extract, was higher compared to GE kids®.

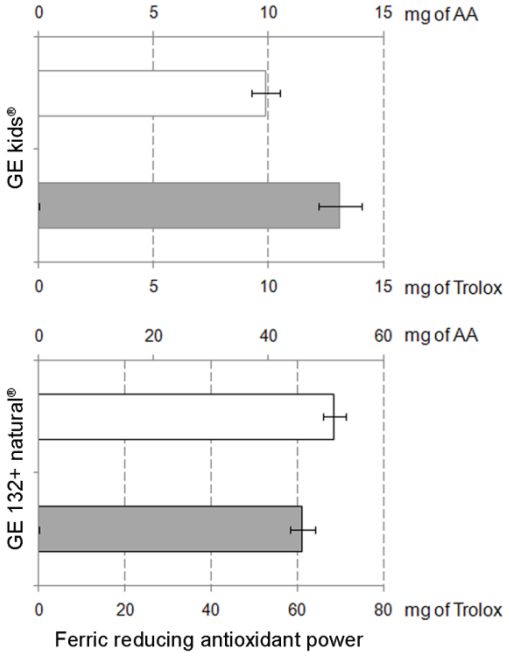

Figure 5. Ferric reducing antioxidant power of GE kids® and GE 132+ natural® complex expressed as mg of ascorbic acid (AA) or Trolox that have the same reducing power as one sachet or capsule of GE kids® and GE 132+ natural®, respectively. Values are mean ± SD of three replicates

4. Conclusion

In summary, both GE kids® and GE 132+ natural® showed antioxidant activity against physiologically relevant hydroxyl and superoxide radical, as well as artificial radicals $ABTS^{\cdot+}$ and $DPPH^{\cdot}$, commonly used for *in vitro* analyses of antioxidant properties. Maintenence of the physiological levels of free radicals is necesary for homeostasis, in both children and adults. Namely, growth and differatiation of young organisms, as well as exposure to stress which is the characteristic of modern lifestyle, are accompanied with increased free radical production. Thus, balanced intake of dietary antioxidants is of great importance. Both examined food supplements were shown to be the most efficient in scavenging hydroxyl radical which is significant since threre is no enzymatic system responsible for its neutralization. Based on the obtained results it can be concluded that GE kids® and GE 132+ natural® may contribute maintaining the physiological levels of free radicals, and thus the oxido-redox balance in the organism. In line with this, natural antioxidant supplementation may improve immune response and, therefore, may be useful for health preservation, in both children and adults.

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