



A Natural Formulation Increases Brain Resistance to Oxidative Stress

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Authors' contributions

This work was carried out in collaboration between all authors. Authors LM, LB and MV defined experimental protocols and the interpretations of the results with statistical analysis. Authors LL and GP managed the cell culture and biochemical analyses. Authors CF and GO performed in vivo test and measurements. Authors LR, RS and AC performed synaptosome test and RIA. All the authors have read and approved the final manuscript.

Original Research Article

Received 26th September 2013

Accepted 26th October 2013

Published 5th December 2013

ABSTRACT

Aims: Oxidative stress is an imbalance in the pro-oxidant/antioxidant homeostasis, characterized by excess accumulation of reactive oxygen/nitrogen species (ROS/RNS) and free radicals that can be toxic for cells by initiating disruptive peroxidation reactions on cellular substrates such as proteins, lipids, and nucleic acids. Neurons have a high content of unsaturated fatty acids which are easily peroxidable by the elevated levels of ROS and RNS produced by brain oxygen metabolism, yielding isoprostanes among which 8-iso-PGF_{2α} derived from arachidonic acid represents a stable marker of lipoperoxidation, *in vivo*. Numerous findings pointed to the protective role of natural products against oxidative stress in the brain.

Methodology: In the present work we evaluated the effects of a natural formula containing bacopa extract, vitamin E, astaxanthin and phosphatidylserine on lipoperoxidation in rat brain cortex, both *in vivo* and *in vitro*.

Results: The results demonstrate that the natural formula could reduce basal and

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hydrogen peroxide- and amyloid β peptide-induced oxidative stress, as evidenced by the reduction of 8-iso-PGF_{2 α} and ROS/RNS production in the rat brain.

Conclusion: Results could account for a rational use of herbal products in the treatment of conditions characterized by increased burden of oxidative stress and defective antioxidant mechanisms, such as aging and neurodegenerative disorders.

Keywords: Oxidative stress; *Bacopa*; Astaxanthin; vitamin E; phosphatidylserin; hydrogen peroxide; amyloid β -peptide.

1. INTRODUCTION

Oxidative/nitrosative stress plays a key role in the onset of age-related cellular degeneration, particularly in the brain, where the increase of highly peroxide-modified unsaturated fatty acids is coupled to elevated oxygen consumption and a significant deficiency of antioxidant systems [1-2].

The adult brain has a high content of unsaturated fatty acids which are easily peroxidable by the elevated levels of reactive oxygen/nitrogen species (ROS/RNS) produced by brain oxygen metabolism [3]. One such group of unsaturated fatty acids are F2-isoprostanes, non-enzymatic oxidative derivatives of arachidonic acid esterified to membrane phospholipids, which play a master role both as cellular mediators and as markers of lipoperoxidation *in vivo* [4]. Amongst F2-isoprostanes, 8-iso-prostaglandin F_{2 α} (8-iso-PGF_{2 α}) has been studied more extensively as an index of oxidative stress [5-6]. We have previously shown that oxidative stimuli such as hydrogen peroxide or amyloid beta-peptide increase 8-iso-PGF_{2 α} production in rat brain synaptosomes [7-8].

On the other hand, clinical findings and laboratory data point to a protective effect of antioxidants naturally present in food or supplemented in the diet of rats subjected to oxidative brain damage [9]. We have previously found that the alleged neuroprotective effects of both ginkgo and garlic extracts could be partially related to inhibition of brain 8-iso-PGF_{2 α} production in young as well as aged rats [10-11]. Natural products such as astaxanthin, vitamin E, phosphatidylserine and *Bacopa monnieri*, an Indian traditional nootropic medicinal plant, are increasingly reputed as neuroprotective agents [2,12-15]. They were also reported to be active after peripheral administration in the animal model [15-18]. According to these findings, the aim of the present work is to explore the potential benefits related to the association of these natural products, in order to explore new pharmacological tools against neural oxidative damage, deeply involved in the onset of neurodegenerative disorders. In our study, we evaluated the antioxidant effect of herbal formula Illumina® in the rat, in both *in vivo* and *in vitro* experimental models. The results support a rationale use of the commercial formula as antioxidant agent.

2. MATERIALS AND METHODS

2.1 Animals and Drugs

Thirty-six male adult Wistar rats (200-250 g) were housed in plexiglas cages (40 cm × 25 cm × 15 cm), one rat per cage, in climatized colony rooms (22±1°C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00 – 19:00 h), with free access to tap water and food, 24 h/day throughout the study, with no fasting periods. Rats were fed a standard laboratory diet

(3.5% fat, 63% carbohydrate, 14% protein, 19.5% other components without caloric value; 3.20 kcal/g). Housing conditions and experimentation procedures were strictly in accordance with the European Community ethical regulations on the care of animals for scientific research.

Illumina® is formulated as a mixture (in weight) of astaxanthin (dry extract, 31%), *Bacopa monnieri* (leaf dry extract 43%, containing bacoside, 20%), vitamin E (13%) and phosphatidylserine (13%). Quantity and standardized quality of each ingredient were confirmed by specific phytochemical analysis certificate.

2.2 *In vivo* Studies

Twenty-four rats were randomized in two groups (n=12) and orally treated for one week, once a day at 9 a. m., with either 1 ml vehicle, or with Illumina® 150 mg/rat diluted in 1 ml saline. The selected dose of the natural formula was chosen on the basis of previous works performed in rats [19-22].

Twenty-four hours after the last treatment, rats were sacrificed by decapitation and the frontal and the parietal cortex quickly dissected for isoprostane and nitrite determinations. Tissue isoprostane extraction was performed as previously reported [23]. Immediately after sacrifice, tissue fragments were incubated in Dubnoff shaking bath at 37°C, for 1 h, in Krebs-Ringer buffer additioned with calcium-ionophore A23187. Perfusionates were collected and isoprostane levels, expressed as pmol/mg wet tissue, were measured by radioimmunoassay (RIA). Regarding nitrite determination, immediately after sacrifice, tissue fragments were homogenized in 0.32 M saccharose solution and centrifuged at 4,000 g for 10 min. Therefore, nitrites were determined on supernatant fraction by Griess assay, as previously reported [24]. Nitrite concentration was expressed as μmol/mg wet tissue.

2.3 *In vitro* Studies

2.3.1 Cell culture

C2C12 cells were cultured in DMEM (Euroclone) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1.2% (v/v) penicillin G/streptomycin in 75 cm² tissue culture flask (n=5 individual culture flasks for each condition). The cultured cells were maintained in humidified incubator with 5% CO₂ at 37°C [25].

2.3.2 Viability test

To assess the basal cytotoxicity, a viability test was performed on 96 microwell plates, using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) test. C2C12 were incubated with natural products (ranging concentration 0.01-100 μg/ml) for 24 h. About 10 μL of MTT (5 mg/mL) was added to each well and incubated for 3h. The Formosan dye formed was extracted with dimethyl sulfoxide and absorbance recorded as previously described [18]. Effects on cell viability were evaluated in comparison to untreated control group.

2.3.3 Synaptosome preparation

Synaptosomes were prepared from a pool of frontal and parietal cortex, and have long been employed as a valuable *in vitro* experimental model to study the antioxidant effects of drugs

on free radical-mediated oxidative damage to brain cells [7-8,10-11]. Briefly, twelve male rats were sacrificed by decapitation, the frontal and parietal cortex quickly dissected, homogenized in 0.32 M saccharose solution and centrifuged, first at 4,000 g for 10 min, and then at 12,000 g for 20 min, to isolate neuronal endings from cell nuclei and glia. The purified synaptosomes were suspended at 37°C, under O₂/CO₂ 95%/5%, pH 7.35–7.45, in Krebs-Ringer buffer (mM: NaCl 125, KCl 3, MgSO₄ 1.2, CaCl₂ 1.2, Tris-HCl 10, glucose 10). Then, the synaptosome suspension was divided in 20 fractions (each containing 100 mg of tissue in 3 ml medium) that were differently treated for ROS and isoprostane determination. Regarding 8-iso-PGF_{2α} analysis, each fraction (n=10) was incubated at 37°C, under agitation for 30 min (incubation period), and treated with a pharmacological stimulus as follows: (1) Krebs-Ringer buffer (vehicle); (2) vehicle plus oxidant stimulus [either hydrogen peroxide 1 mM or amyloid β peptide (1-40) (1μM)]; (3) vehicle plus natural formula (0,01-10 μM); (4) vehicle plus oxidant stimulus and natural formula (0,01-10 μM). Finally, the synaptosome fractions were subjected to a perfusional study for the determination of 8-iso-PGF_{2α} release, as index of lipoperoxidation. Regarding ROS determination, in the incubation period, each synaptosome fraction (n=10) was incubated at 37°C, under agitation, with either vehicle or vehicle plus natural formula (0,01-10 μM) for 30 min. After the incubation period, synaptosomes were treated for ROS determination.

2.3.4 Synaptosome perfusion

For lipoperoxidation determination, the synaptosome fractions (n=10) were layered onto 0.8 μm Millipore filters, placed into 37°C water-jacketed superfusion chambers (50 mg of tissue per chamber, 18 different chambers for each experiment), and perfused with Krebs-Ringer buffer, collecting medium in 6 min fractions, as previously described [7]. Superfusion was started at a rate of 0.6 ml/min and synaptosomes were perfused for 8 min to allow stable release (equilibration period), after which perfusion was switched to 0.3 ml/min with Krebs-Ringer buffer (vehicle), oxidative stimulus, natural formula, alone or in combination, for 30 min (stimulation period). Finally, calcium ionophore A23187 (10 nM) was added in the perfusion medium to activate phospholipase A2 and cleave 8-iso-PGF_{2α} from synaptosomal membrane phospholipids. Perfusates were immediately frozen at -80°C and then lyophilized. The dry residue was suspended in distilled water (0.25 ml) and 8-iso-PGF_{2α} levels, expressed as pg/ml, were measured by RIA. In a previous work, we found that exposure of synaptosomes to calcium ionophore A23187 (10 nM) or to oxidant did not modify 8-iso-PGF_{2α} release, when the substances were used alone; on the other hand, when calcium ionophore A23187 (10 nM) was given after exposure to oxidative stimulus; it significantly increased 8-iso-PGF_{2α} release [7].

2.3.5 Nitrite measurement

Nitrites are stable nitric oxide end products, whose determination is commonly used as index of nitric oxide production, *in vivo*. Briefly, nitrite production was determined by mixing 50 μl of the assay buffer with 50 μl of Griess reagent (1.5% sulfanilamide in 1 M HCl plus 0.15% N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water, v/v). After 10 min incubation at room temperature, the absorbance at 540 nm was determined and nitrite concentrations were calculated from a sodium nitrite standard curve. All measurement were performed in triplicate.

2.3.6 Radioimmunoassay (RIA)

8-iso-PGF_{2α} quantification was performed as previously reported [26]. Briefly, specific anti-8-iso-PGF_{2α} was developed in the rabbit; its cross-reactivity with 8-iso-PGE₂ is 7.7%, whereas that with other prostanoids is <0.3% [20]. 100 µL of prostaglandin standard or sample were incubated overnight at 4°C with the ³H-prostaglandin (3000 cpm/tube; NEN) and antibody (final dilution: 1:120000; kindly provided by Prof. G. Ciabattoni), in a volume of 1.5 ml of 0.025 M phosphate buffer. Free and antibody-bound prostaglandins were separated by the addition of 100 µL 5% bovine serum albumin and 100 µL 3% charcoal suspension, followed by centrifuging for 10 min at 4000 × g at 5°C and decanting off the supernatants into scintillation fluid (Ultima Gold™, Perkin Elmer) for β emission counting. The detection limit of the assay method is 0.6 pg/ml. The IC50 is 39.8 pg/ml. The intraassay and interassay coefficients of variation are ± 2.0% and ±2.9% at the lowest concentration of standard (2 pg/ml) and ± 3.7% and ± 9.8% at the highest concentration of standard (250 pg/ml).

2.3.7 ROS generation

ROS generation was assessed using a ROS-sensitive fluorescence indicator, DCFH-DA. When DCFH-DA is introduced to viable cells, it can penetrate the cell and become deacetylated by intracellular esterases to form 2',7'-dichlorodihydrofluorescein (DCFH), which can react quantitatively with ROS within the cell, and be converted to 2',7'-dichlorofluorescein (DCF), which is detected by a fluorescence spectrophotometer. To determine intracellular effects on ROS production, synaptosomes were seeded in a black 96-well plate (1.5 x 10⁴ cells/well) in medium containing scalar concentration of extracts. Immediately after seeding, the synaptosomes were stimulated for 1 h with either amyloid β peptide (1-40) (1 µM) or H₂O₂ (1 mM). After the cells were incubated with DCFH-DA (20 µM) for 30 min, the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, using a fluorescence microplate reader.

2.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). *In vivo* data, concerning isoprostane and nitrite determinations, were collected from each of the 24 animals used in the experimental procedure and means ± S.E.M. were determined for each experimental group and analyzed by unpaired t-test (two-tailed *P* value). *In vitro* data, concerning isoprostane and ROS determinations, represent the means ± S.E.M. of 5 experiments performed in quadruplicate and were analyzed by analysis of variance (ANOVA), followed by Newman-Keuls comparison multiple test. Statistical significance was set at *P*<0.05.

3. RESULTS AND DISCUSSION

Our study showed that 1 week oral administration of Illumina® led to a significant reduction in basal lipoperoxidation, *in vivo*, as revealed by isoprostane determination, in rat brain (Fig. 1). This could be at least in part explained by the radical scavenging properties of the natural formula that was able to reduce brain nitrite content (Fig. 2). We cannot exclude that the beneficial effects of Illumina®, *in vivo*, could be the consequence of adaptative stress responses to moderate levels of hydrogen peroxide. It is well known that antioxidants in the liquids could exert pro-oxidative effects, by generating hydrogen peroxide and thus activating adaptative responses of cells to mild oxidative stress [27].

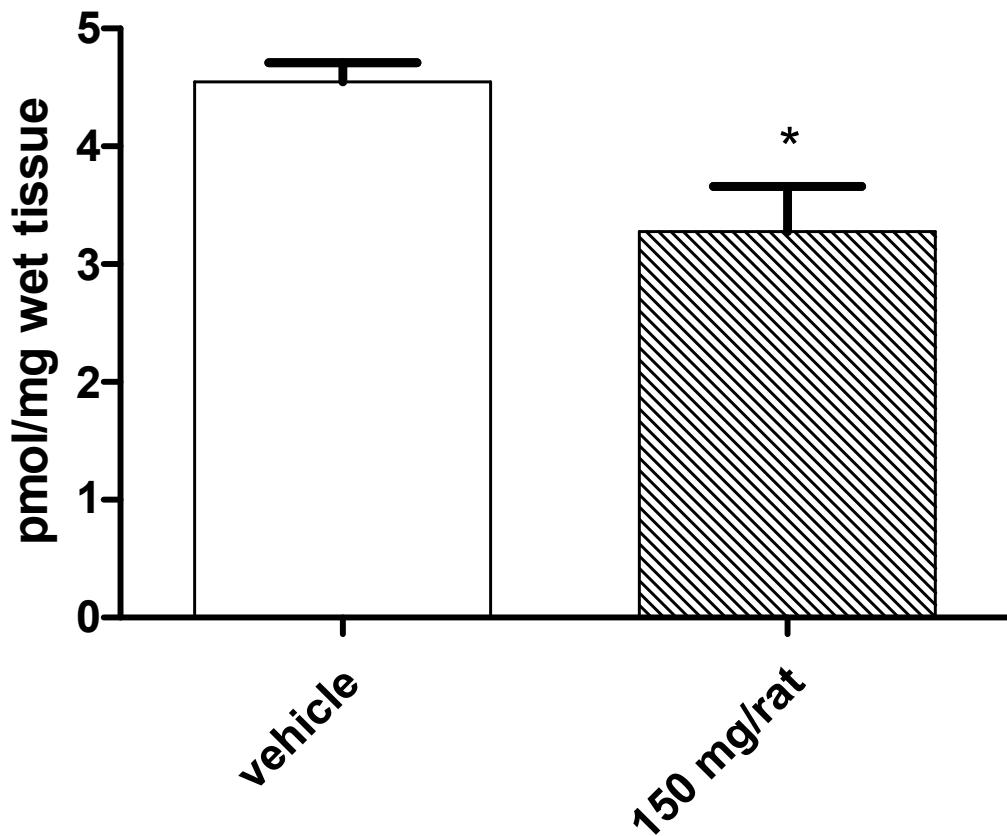


Fig. 1. Effects of Illumina® (150 mg/rat) on 8-iso-prostaglandin (PG)F_{2α} levels in brain homogenate

Data were collected from each of the 24 rats used in the experimental procedure. Means \pm S.E.M. were determined for each experimental group and analyzed by unpaired t-test. *P<0.05 vs. vehicle.

MTT test revealed no significant effects of extracts on cell viability up to 100 µg/ml (data not shown). In order to exclude possible interferences, a maximum dose of 10 µg/ml was chosen for biochemical assays, *in vitro*.

When we tested Illumina® on cortex synaptosomes, we confirmed its antioxidant properties, evaluated by isoprostane and ROS determinations (Figs. 3-4), both in basal and oxidative stress conditions induced by either hydrogen peroxide or amyloid β peptide stimulation. These results indicate a potential use of Illumina® as preventive and curative agent against brain oxidative damage. In this experimental model, the oxidant and antioxidant are co-administered and is not possible to exclude direct interactions between treatments present in the perfusion medium.

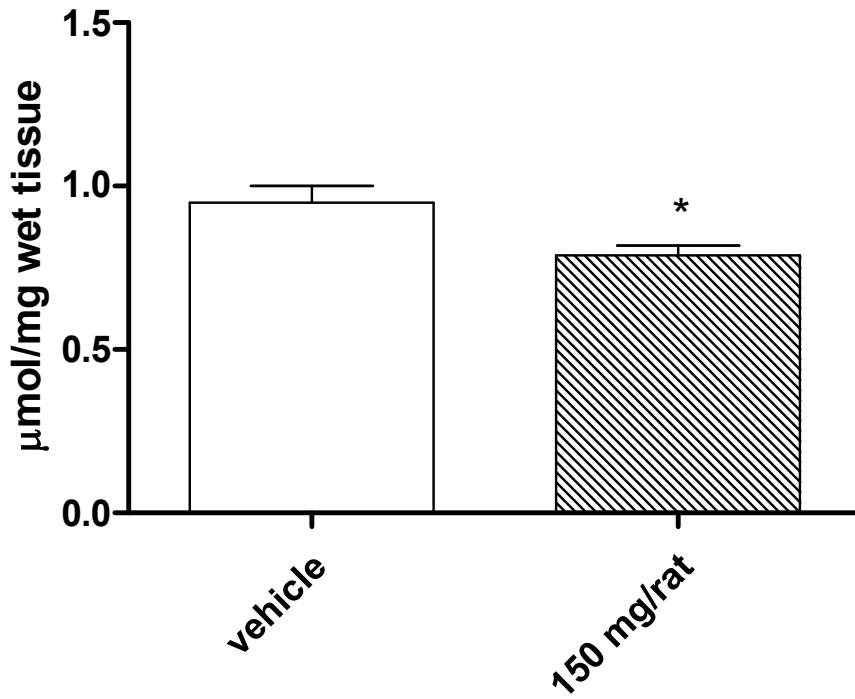


Fig. 2. Effects of Illumina® (150 mg/rat) on nitrite levels in brain homogenate
Data were collected from each of the 24 rats used in the experimental procedure. Means \pm S.E.M. were determined for each experimental group and analyzed by unpaired t-test. * $P < 0.05$ vs. vehicle.

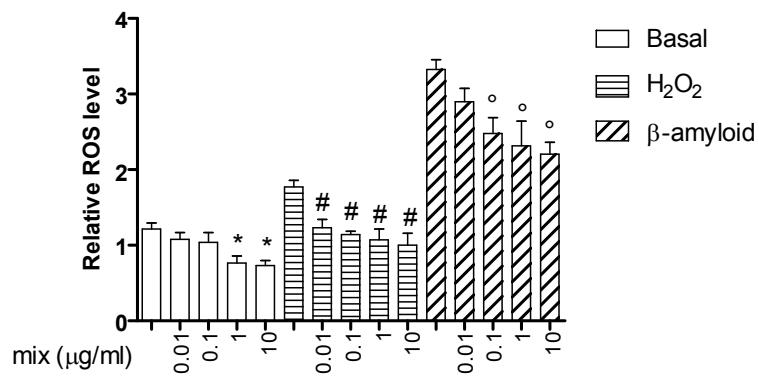


Fig. 3. Effects of Illumina® (0.01-10 $\mu\text{g/ml}$) on basal (Krebs-Ringer buffer) or oxidative stress- [either hydrogen peroxide (1 mM) or amyloid β peptide (1-40) (1 μM)] stimulated reactive oxygen species production from brain synaptosomes, obtained from 12 rats
Each bar indicates the mean \pm SEM of 4 experiments performed in quadruplicate. ANOVA: $P < 0.05$; post-hoc *, #, ° $P < 0.05$ vs. respective vehicle.

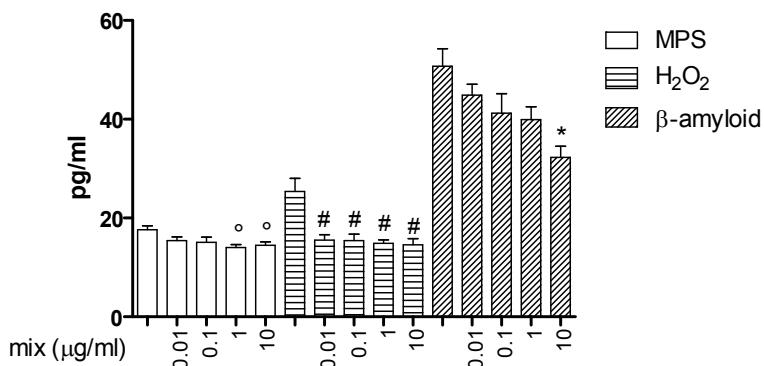


Fig. 4. Effects of Illumina® (0.01-10 µg/ml) on basal (Krebs-Ringer buffer) or oxidative stress- [either hydrogen peroxide (1 mM) or amyloid β peptide (1-40) (1µM)] stimulated 8-iso-prostaglandin(PG)F_{2α} production from brain synaptosomes, obtained from 12 rats

Each bar indicates the mean ± SEM of 4 experiments in which vehicle and Illumina® perfusion lines were performed in quadruplicate. ANOVA: P<0.0001; post-hoc *, #, *P<0.05 vs. respective vehicle.

Oxidative stress is an imbalance in the pro-oxidant/antioxidant homeostasis, characterized by excess accumulation of reactive oxygen/nitrogen species (ROS/RNS) and free radicals that can be toxic for cells by initiating disruptive peroxidation reactions on cellular substrates such as proteins, lipids, and nucleic acids [1]. Neurons have a high content of unsaturated fatty acids which are easily peroxidizable by the elevated levels of ROS and RNS produced by brain oxygen metabolism [4], yielding isoprostanes among which 8-iso-PGF_{2α} deriving from arachidonic acid represents a stable marker of lipoperoxidation, *in vivo* [24]. Our experiments demonstrate that 8-iso-PGF_{2α} production is reduced in rat, after 1 week of natural formula administration, and this could be partially explained by the radical scavenging activity of the product, as revealed by nitrite assay on brain homogenate. Furthermore, when we tested the effects of Illumina® on cortex synaptosomes we observed that the natural formula is effective in blunting ROS and isoprostanate production both in the basal state and after oxidative stress. Hydrogen peroxide is a common byproduct of aerobic metabolism, which plays a key role in membrane lipid peroxidation, yielding compounds such as isoprostanes, which are linked to neurodegenerative processes [28-30]. Previous works have also indicated the stimulatory effects of amyloid β peptide on lipoperoxidation [31-32]. Amyloid β peptide could quickly accumulate near the plasma membrane and shuttle redox active metals, such as iron or copper, with consequent production of hydrogen peroxide [33]. This results in a early and sharp increase in membrane oxidative-injury. The natural formula reduce the oxidative stress induced by either hydrogen peroxide and amyloid β peptide (1-40), as confirmed by reduction of ROS and isoprostanate levels in brain synaptosome, thus further supporting the antioxidant properties of the active principles of the natural formula. Both epidemiological and experimental data point to antioxidants contained in food for the prevention and therapy of neurodegenerative disorders, as well as in relieving neuronal damage induced by oxidative stress [34].

The reported memory enhancing and neuroprotective effects of *Bacopa monnieri* are consistent with free radical scavenging activities [35-38].

Astaxanthin is a naturally occurring carotenoid widely distributed in various living organisms, such as plants, algae, and seafoods and is a more powerful antioxidant than other

carotenoids [39]. The neuroprotective effect of astaxanthin is suggested to be dependent upon its antioxidant potential and mitochondrial protection; therefore, it is strongly suggested that treatment with astaxanthin may be effective for oxidative stress-associated neurodegeneration and can be considered a potential candidate for natural brain supplement [40]. The mechanisms responsible for the neuroprotective effects of astaxanthin are, however, not fully clarified, although it has been reported that this beneficial effect is related to its antioxidant, anti-inflammatory and anti-apoptotic effects [41-42]. Vitamin E is the major lipophilic antioxidant in the brain and low concentrations of this antioxidant have been related to the development of Alzheimer disease [2]. Furthermore, in a clinical trial, daily supplementation of vitamin E in Alzheimer disease patients slowed functional deterioration [43]. Finally, phosphatidylserine is a component of mammalian cell membranes and plays important roles in biological processes such as apoptosis, cell signaling and brain development [12].

4. CONCLUSION

Considering the role played by the single components of Illumina® as antioxidant and neuroprotective agents, the present *in vivo* finding support the supplement in the prevention of brain oxidative stress. Furthermore, considering the promising results obtained *in vitro*, where the natural formula revealed effective in reversing brain oxidative damage induced by both hydrogen peroxide and amyloid β peptide (1-40), our experimental data support a possible use of Illumina® in condition of both increased burden of oxidative stress and defective antioxidant mechanisms, strongly related to physiological aging as well as in neurodegenerative disorders. Since each technique measures something different and has its own inherent limitations, further investigations, comparing different analytical methods and experimental paradigms for detection and quantification of oxidative stress are required for an accurate evaluation of *in vivo* activity

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

The work was supported by Italian Ministry of Education grant.

COMPETING INTERESTS

Authors declare that no competing interests exist.

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