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Comparative analysis of synthetic and nutraceutical antioxidants as possible neuroprotective agents

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Damage caused by oxidative stress in cases of neurodegenerative diseases such as Alzheimer's or Parkinson's has been proven to be irreversible. However, diet supplementation of antioxidants can be beneficial to the total antioxidant status of the human body. Given that oxidative stress is a principal cause of neurodegenerative disease, effective natural antioxidants could provide therapeutic options for these disorders. Therefore we investigated the antioxidant properties of two natural extracts and five synthetic compounds in lipid rich environment of liposomes with oxidation induced by Fenton's reagent. Antioxidant activity has been quantified using the Klein peroxidation index and oxygen uptake. Both natural extracts from Acai berry and Ginseng as well as all synthetic compounds – N-acetyl-L-cysteine, tocopherol, huperzines, caffeic acid and lipoic acid displayed good antioxidants properties, which confirm their suitability as neuroprotective agents.

1. Introduction

Under some pathological conditions oxidative and free radical–mediated reactions in degenerative processes related to aging and chronic disease conditions are important. Free radicals in the form of reactive oxygen species (ROS) are important in numerous diseases such as inflammation, metabolic disorders, reperfusion damage, atherosclerosis, and carcinogenesis (Kogan et al. 2008; Babincová et al. 2002). Brain tissue is not only susceptible to oxidative attack because of its high oxygen consumption, but also because of its anatomical structure. Neural tissue contains large amounts of unsaturated fatty acids susceptible to oxidation and iron - a metal that can take part in many redox reactions, i.e. Fenton's reaction (Singh et al. 2004). Most of the antioxidant activity in brain is maintained by superoxide dismutase and ascorbic acid. Oxidative stress plays a role in many neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, multiple sclerosis and amylotropic lateral sclerosis (Uttara et al. 2009), but it is yet to be determined whether it is the first initiator or a byproduct of changes induced by another antagonist. Nevertheless ROS contribute to development of neurodegeneration by targeting biomacromolecules resulting in production of their oxidized form and further oxidation products some of which (4-hydroxy-2-nonenal, protein carbonyl, malondyaldehyde) may be harmful to neural tissue. Since oxidative damage is one of the major characteristics of neurodegenerative diseases, implementations of strategies to fight against it seem to be the thing to do. There is evidence that neuroprotection may be achieved by use of antioxidants that are harmless to the human body and abundant in different kinds of foods. Based on the premise that oxidative stress underlies a number of neurodegenerative diseases, the identification of novel antioxidants as potential therapeutics is a prolific area of neuroscience research.

The purpose of the present study was to measure several different synthetic compounds and two natural extracts for their ability to act as antioxidants in lipid rich environment of liposomes with oxidative stress induced by Fenton's reaction. As nutraceuticals extracts from Acai and Ginseng were used, and the synthetic compounds were lipoic acid, tocopherol, caffeic acid, N-acetyl-L-cysteine and mixture of huperzines A and B.

Acai (*Euterpe oleracea*) is a palm tree that grows in South America. The plant has small dark colored berries, with relatively large seeds which are not consumed. The pulp of the berry is widely used for juice preparation and also as food. Rich in polyphenols, high levels of unsaturated fatty acids and low levels of sugar, acai fruit has been reportedly to passess one of the highest antioxidant activities of all fruits and vegetables (Schauss et al. 2006).

Ginseng (*Panax ginseng*) is a plant that grows in Asia and North America. For medicinal purposes mainly its roots, berries or, alternatively, leaves are used. Ginseng is composed of a mixture of glycosides, essential oils, a variety of complex carbohydrates and phytosterols, amino acids and trace minerals. The main active ingredients are believed to be a mixture of triterpenoid saponins commonly referred to as ginsenosides (Kitts et al. 2000).

α-Lipoic acid (LA) is a coenzyme found in both prokaryotic and eukaryotic microorganisms, animals and plants, that plays important role in energy metabolism (Navari-Izzo et al. 2002). It is readily absorbed from diet and can cross into brain (Packer et al. 1996) and is rapidly converted to its reduced form – dihydrolipoic acid (DHLA). LA and DHLA act as redox pair and both have antioxidant activities such as metal chelation and quenching of different ROS species.

Caffeic acid belongs to the family of hydroxycinnamates, phenylpranoid metabolites that occur in plants and plant products and have bioactive capabilities. It exhibits in vitro antioxidant activity, can protect α-tocopherol in LDL (Gulcin, 2005), and inhibit formation of hydroxyl radicals in Fenton's reaction by chelating iron ions (Mori and Iwahashi 2009).

N-Acetyl-L-cysteine (NAC) is an acetylated cysteine residue and can serve as a cysteine donor for glutathione synthesis (Kersick and Willoughby 2005). Thanks to its SH group it can interact with oxidants such as hydrogen peroxide, and has been proven to have *in vivo* antioxidant capabilities separate from serving only as glutathione precursor (Zhang et al. 2011).

Huperzines A and B are selective acetylcholine-esterase inhibitors that are mainly examined as potential treatments from Alzheimer's disease (Wang et al. 2001). Both are isolated from the Chinese herb, *Huperzia serrata.* Huperzine A has been proven to attenuate cognitive dysfunction induced by amyloid β-protein even when this dysfunction is not confined to the cholinergic system. There has been evidence that it acts as antioxidant, anti-apopototically and improves the function of mitochondria (Zhang et al. 2008).

2. Investigations, results and discussion

Peroxidation of brain tissue lipids is a measure of damage to the neuronal membrane caused by the attack of reactive oxygen species. Inhibition of lipid peroxidation by any external agent is often used to quantify its antioxidant capacity. Peroxidation of fatty acids of phospholipids occurs via the free radical chain mechanism. Formation of the lipid free radicals is initiated by abstraction of a hydrogen atom from the lipid chain. Most susceptible to degradation are lipids containing double bonds, since unsaturation permits delocalization of the remaining unpaired electrons along the lipid chain. Polyunsaturated lipids are thus particularly prone to oxidative degradation. In the presence of oxygen, the process further proceeds via formation of the hydroperoxides, which degrade spontaneously to form aldehydes with concomitant fission of fatty acid chain. Natural phospholipids contain only non-conjugated double bonds and, therefore, have a UV absorbance peak at a very short wavelength (200-205 nm). Removal of a hydrogen atom from the methylene group located between two double bonds spreads the unsaturation over five carbon atoms and results in the formation of a conjugated diene which is energetically more favourable than the two isolated double bonds. As a result, the second absorbance maximum at 233 nm appears, which is rationale for Klein peroxidation index given as the ratio the absorbance values A_{233}/A_{215} (Klein 1970). We have studied lipid peroxidation induced by $^{\cdot}OH$ radicals produced via Fenton's reaction, which is a standard method in the investigation of the capabilities of $OH^{\cdot}$ radical scavengers. The values of Klein peroxidation index for all studied antioxidants are shown in Fig. 1. The peroxidation index of non-peroxidated liposomes is 0.53 whereas the peroxidation index of peroxidated liposomes that do not contain any antioxidant is 1.06, which means that the lower the Klein peroxidation index gets the better are the antioxidant properties of a compound. Both Acai berries and Ginseng extract have decreasing values of Klein peroxidation index with increasing concentration of used extract. In Fig. 1 peroxidation indexes for concentrations of studied extracts and compounds are shown, when this value achieved a plateau and did not further change (3.5 mmol.l^{-1} - huperzine, 1.5 mmol.$^{-1}$ - α-tocopherol, 10 mmol.$^{-1}$ - caffeic acid. 10 mmol.$^{-1}$ - lipoic acid, and 30 mmol.$^{-1}$ - N-acetyl-L-cysteine). The comparison of the influence of all antioxidants on oxygen uptake is shown in Fig. 2. The higher the amount of dissolved oxygen is in the sample the better are the antioxidant abilities of a given compound.

As may be clearly seen all antioxidants studied performed very well in both these tests, although the mechanism of their action is different, e.g caffeic acid is a Fe^{2+} chelator, N-acetyl-L-cysteine and lipoic acid are $^{\cdot}OH$ radical scavengers. Particularly potent antioxidant is huperzine, effective at very low concentrations, with capabilities comparable with other known free radical scavengers, e.g α-tocopherol, considered to be a "gold standard" for lipid membrane protection.

The results of our study revealed the important role of these compounds and nutraceuticals in the protection of experimental biological membranes against adverse effects of free radicals mediated mostly through their scavenging. Since it is now known that free radicals play detrimental role in the processes of ageing and impairment of the human cells, as well as in some neurodegenerative and neoplastic diseases, implementation of preparations based on them may be advantageous in the therapy of neurodegenerative diseases involving free radicals. The nutraceutical antioxidants Acai berry and Ginseng may be the best options for these patients in the short term since they are subject to fewer regulations than traditional pharmaceuticals and therefore could be made available to patients much more rapidly than new prescription drugs.

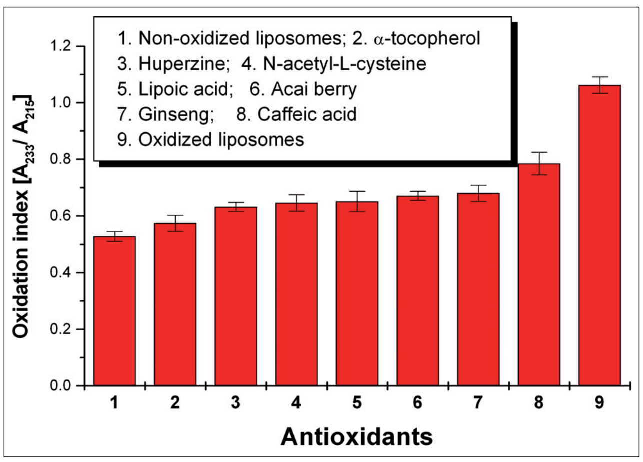

Fig. 1: Comparison of Klein peroxidation indexes of all studied antioxidants

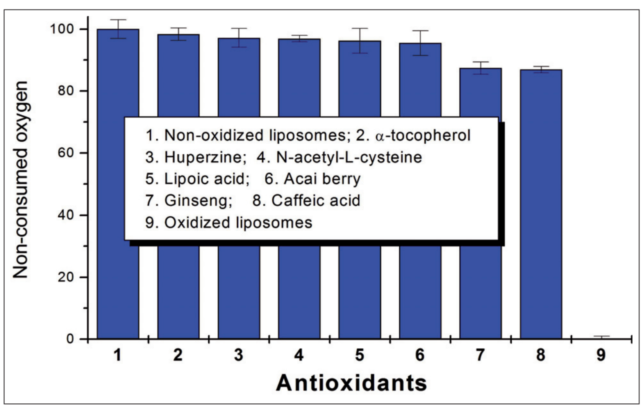

Fig. 2: Comparison of the amount of non-consumed oxygen in peroxidated liposomes at the presence of all studied antioxidants

3. Experimental

3.1. Liposome preparation

Lipid soy-bean phosphatidylcholine (Sigma, St. Louis, USA) was dissolved in organic solvents (a mixture of chloroform and methanol 2:1 v/v) together with caffeic acid, huperzine, tocopherol, or lipoic acid all obtained from Sigma, St. Louis, USA). The lipid solution was evaporated in vacuum at a rotary evaporator. After evaporation of the solvent, Tris buffer (pH 7.4, obtained from Sigma, St. Louis, USA) with desired concentrations of N-acety-L-cysteine, Acai or Ginseng extracts were added into the glass vessel with lipid film, and the solution was shaken up mechanically. The suspension was then sonicated with Labsonic 2000 sonicator (Braun Biotech, Gottingen, Germany) at 80 W for 15 min under nitrogen in an ice-bath in order to obtain a clear suspension of liposomes with phosphatidylcholine concentration 3.5 mM.

3.2. Preparation of acai berry and ginseng extracts

Both extracts were prepared in deionized water. Ginseng 5 g was suspended in 50 ml of 90 °C water and then left to infuse for 4 hours. After filtering this extract was used as the highest concentration for encapsulation into liposomes and diluted to make all the smaller concentrations. Acai berries (1g) were suspended in 50 ml of 90 °C water and then left to infuse for 4 hours. As in case of Ginseng, this extract was filtered, used as the highest concentration and then diluted with deionized water to obtain desired concentrations.

3.3. Induction of lipid peroxidation by Fenton reaction

To generate the hydroxyl radical $^{\cdot}OH$ in order to test the antioxidant efficacy of the prepared compounds, the Fenton (Haber–Weiss) reaction was used. Ferrous sulphate reacts with hydrogen peroxide in the following manner:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + HO^{-}$$

Fenton reaction was initiated by the addition of H_2O_2 and $FeCl_2$ in final concentrations of 100 mM and 2 mM, respectively, to a liposome suspension containing varying concentrations of antioxidants.

3.4. Determination of oxidation index

Absorption spectra of the conjugated dienes were recorded in the wavelength range 215-320 nm using a UV MINI 1240 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). The increase of the absorption at 233 nm was considered as an evidence of the formation of the conjugated dienes, and the oxidation index was calculated from the ratio of the absorbance values (A_{233}/A_{215}) (Klein 1970; Babincová and Sourivong 2001).

3.4. Measurement of oxygen consumption

Oxygen consumption in liposomes was measured using a Clark-type electrode using inoLab® Laboratory Oxygen Meter Oxi 740, (WTW Measurement Systems Inc., Troy, MI, USA). The highest value c_N was obtained for non-peroxidated liposomes (4.43 mg/ml) because oxygen is non-consumed, and the lowest value c_P (0.22 mg/ml) for fully peroxidated liposomes after application of Fenton reagent because oxygen is depleted during peroxidation reaction (Schronerova et al. 2007). The actual value

c_A in the presence of a given antioxidant can be used for calculation of non-consumed oxygen $(c_A - c_P)/(c_N - c_p) \times 100$ %, which has been used as a useful measure of an antioxidant capability against liposome peroxidation.

3.5. Statistical analysis

The experiments were performed at least for three times. Data were expressed as the mean±standard deviation (SD). Statistical correlation of data was checked for significance by ANOVA and Student's t test. $P < 0.05$ was considered to indicate a statistically significant difference.

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Conflicts of interest: None declared.

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