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Original research article

Inhibition of lipid oxidation in foods and feeds and hydroxyl radical-treated fish erythrocytes: A comparative study of *Ginkgo biloba* leaves extracts and synthetic antioxidants

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ABSTRACT

This study explored the effects of butylated hydroxytoluene (BHT) and ethoxyquin (EQ) and ethyl ether extracts, ethyl acetate extracts (EAE), acetone extracts, ethanol extracts and aqueous extracts of *Ginkgo biloba* leaves (EGbs) on lipid oxidation in a linoleic acid emulsion, fish flesh and fish feed and in hydroxyl radical (·OH)-treated carp erythrocytes. The linoleic acid, fish flesh and fish feed were incubated with BHT, EQ and EGbs at 45°C for 8 d, respectively, except for the control group. The lipid oxidation in the linoleic acid emulsion, fish flesh and fish feed was then measured by the ferric thiocyanate method or thiobarbituric acid method. The carp erythrocytes were treated with BHT, EQ or EGbs in the presence of 40 μmol/L $FeSO_4$ and 20 μmol/L H_2O_2 at 37°C for 6 h, except for the control group. Oxidative stress and apoptosis parameters in carp erythrocytes were then evaluated by the commercial kit. The results showed that BHT, EQ and EGbs inhibited lipid oxidation in the linoleic acid emulsion, fish flesh and fish feed and ·OH-induced phosphatidylserine exposure and DNA fragmentation (the biomarkers of apoptosis) in carp erythrocytes. Furthermore, BHT, EQ and EGbs decreased the generation of reactive oxygen species (ROS), inhibited the oxidation of cellular components and restored the activities of enzymatic antioxidants in ·OH-treated carp erythrocytes. Of all examined EGbs, EAE showed the strongest effects. The effects of EAE on lipid oxidation in the linoleic acid emulsion and on superoxide anion and malonaldehyde levels, catalase activity and apoptosis in ·OH-treated carp erythrocytes were equivalent to or stronger than those of BHT. Moreover, these results indicated that the inhibition order of EGbs on the generation of ROS and oxidation of cellular components in fish erythrocytes approximately agreed with that for the food and feed materials tested above. And, the antioxidative and anti-apoptotic effects of EGbs were positively correlated with their flavonoid content. Taken together, these results revealed that the fish erythrocyte system can be used as an experimental model to evaluate lipid oxidation in food and feed ingredients. The EAE can be used as a potential natural antioxidant or apoptosis inhibitor. The inhibition effects of EGbs on lipid oxidation and apoptosis may be due to the presence of flavonoid compounds.

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1. Introduction

Lipid oxidation is a common problem in foods and feeds (Fritsche and Johnston, 1988; Wqsowicz et al., 2004). Lipids can undergo peroxidation in a chain reaction with reactive oxygen species (ROS), including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) from cells (German, 1999). Cells produce

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O_2^- and H_2O_2, resulting in unsaturated fatty acid peroxidation in animal tissues after slaughter (Kanner, 1994). Lipid oxidation is propagated under the catalytic actions of iron and other redox metal ions (Morrissey et al., 1998). However, the cytosol contains antioxidants that can suppress lipid oxidation in food and feed ingredients (Girotti, 1985). This process is particularly similar to the metabolism of ROS in human erythrocytes. Erythrocytes continuously produce O_2^- by the autoxidation of haemoglobin (Cimen, 2008). The dismutation of O_2^- generates H_2O_2 that can initiate lipid peroxidation in erythrocytes. Hydrogen peroxide reacts with heme Fe^{2+} to produce ·OH that can strengthen the process (Puppo and Halliwell, 1988). Our previous study demonstrated that exposure to $FeSO_4$ and H_2O_2 triggers lipid oxidation in fish erythrocytes (Li et al., 2013). Similar to human erythrocytes, fish erythrocytes contain high concentrations of haemoglobin that can continuously produce ROS and a high content of unsaturated fatty acids that be easily oxidized by ROS in membranes (Li et al., 2016). The mechanisms of antioxidant defences in fish cells are similar to those in mammal cells (Winston and Giulioz, 1991). Moreover, fish erythrocytes retain the nucleus, mitochondria and other organelles, which are mostly similar to those of animal tissue cells in structure (Rothmann et al., 2000). Thus, it is possible that the fish erythrocyte system could be used as a model of lipid oxidation in food and feed ingredients.

Lipid oxidation leads to the breakdown of nutritional ingredients, change in taste, scent and colour, the development of toxic metabolites and a decrease in the shelf life of foods and feeds (Błaszczyk et al., 2013; Smet et al., 2008). Diets with oxidized lipid can result in decreased animal health, performance and quality (Chen et al., 2013; Han et al., 2012; Zhang et al., 2011). Thus, it is essential to expand our knowledge of how to protect foods and feeds against lipid oxidation. Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and ethoxyquin (EQ), have been used for many years to stop lipid oxidation in foods and feeds (Błaszczyk et al., 2013). However, studies indicated that BHT and EQ are carcinogenic and toxic to animals (Ito et al., 1985; Nakagawa et al., 1994). Therefore, there is growing interest in replacing synthetic antioxidants with natural ingredients (Aksoy et al., 2013). *Ginkgo biloba* L. (Gb), a native plant of China, is now cultivated as an ornamental plant throughout the world. The standard extract of Gb leaves is well known as a natural antioxidative drug (Mahadevan and Park, 2008). However, a large amount of Gb leaves are treated as rubbish in many cities of China in the autumn and winter (Briancon-Scheid et al., 1983). Hence, it is possible to develop the extract of Gb leaves (EGbs) as an inhibitor of lipid oxidation in foods and feeds. However, information regarding the protective effects of the EGbs on food and feed materials is scarce.

In our previous study, ·OH induced apoptosis in fish erythrocytes, which provided a good model of oxidative damage in fish cells (Li et al., 2013). In this study, the apoptosis was induced in the same manner. We explored the effects of BHT and EQ and of ethyl ether extracts (EEE), ethyl acetate extracts (EAE), acetone extracts (AE), ethanol extracts (EE) and aqueous extracts (AQE) of EGbs on lipid oxidation in a linoleic acid emulsion, fish flesh and fish feed and ·OH-treated carp erythrocytes. The purpose was to evaluate the protective effects of EGbs against lipid oxidation in food and feed ingredients for comparison with the effects of BHT and EQ. The results may provide a basis for applications of fish erythrocytes as a model of lipid oxidation and the use of EGbs as a natural antioxidant in foods and feeds.

2. Materials and methods

2.1. Chemicals

The BHT (analytical standard), heparin sodium (≥99%) and dimethyl sulfoxide (DMSO, ≥99.7%) were purchased from Sigma–Aldrich Co., LLC (St. Louis, MO, USA). The EQ (≥90%) was obtained from Shanghai PuZhen Biotech. Co., LTD (Shanghai, China). Ethyl ether, ethyl acetate, acetone and ethanol were of analytical grade and purchased from the Chengdu Kelong Chemical Reagent Factory (Chengdu, China). Aqueous solutions of H_2O_2 (30%) and $FeSO_4$ (analytical grade) were obtained from the Shanghai Chemical Reagent Factory (Shanghai, China). Physiological carp saline (PCS) which contained (in mmol/L) 141.10 NaCl, 1.43 KCl, 0.99 $CaCl_2$, 2.64 $NaHCO_3$, and 6.16 glucose, was modified to obtain a total osmolarity of 280 mOsm/L and pH of 7.9 and was prepared in our laboratory. All other chemicals were analytical grade.

2.2. Preparation of EGbs

Leaves of Gb were collected in November from the trees growing near Neijiang Normal University (Neijiang, Sichuan, China). Botanical identification was performed in the Herbarium of the College of Life Sciences, where voucher samples were assigned a reference number and deposited. Prior to extraction following the methods of Wojcikowski et al. (2007), the dried leaves were ground to a powder (max particle size of 0.32 mm) using a Chinese medicine mill (Ronghao RHP-2000A, Zhejiang, China). Next, 50 g of the powder was extracted with 500 mL of ethyl ether, ethyl acetate, acetone, or ethanol or water at 20°C for 8 h using an Agitator (Dalong OS40-S, Beijing, China). The extraction using each solvent was repeated 3 times under the same conditions. After filtration, the solutions were removed and dried in vacuo by a rotary evaporator (Jinye RE-52CS, Shanghai, China) until a constant mass was achieved. The extraction process was repeated 4 times under the same conditions. The EEE, EAE, AE, EE and AQE were kept in sealed bottles in the dark and stored at −80°C until use.

2.3. Determination of total flavonoid content (TFC)

The TFC of EGbs was estimated using an aluminium chloride colorimetric assay (Zou et al., 2004).

2.4. Measurement of lipid oxidation in a linoleic acid emulsion

A mixture of 4 mL of absolute ethanol (control), EGbs, BHT or EQ (2 mg/mL), 4.1 mL of 2.51% linoleic acid in absolute ethanol, 8.0 mL of 0.02 mol/L phosphate buffer (pH 7.0), and 3.9 mL of distilled water in covered test tubes was placed in an oven at 45°C in the dark. After incubation for 24 h, the lipid oxidation in the emulsion was determined by measuring the absorbance of the resulting mixture at 500 and 532 nm based on the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods, respectively (Sharma and Vig, 2014). The manoeuvre described above was repeated every 24 h until the absorbance of the control reached the maximum level. Four replicates were prepared for each treatment.

2.5. Measurement of lipid oxidation in fish flesh

Lipid oxidation in fish flesh was measured using the method described by Movileanu et al. (2013) with slight modifications. Healthy carp (100 to 110 g) obtained from local fisheries was anaesthetized and killed. Next, the head, fins and visceral organs were removed from the fish. The residual carcasses were homogenized in a meat grinder (Deming DM-JRJ10, Hangzhou, China) and extruded through a 3 mm die. The samples of fish flesh were individually blended and reground in a grinder (Ronghao RHP-2000A, Zhejiang, China) for 3 min and then extruded through a 1 mm die after 1.00 g/kg of dried BHT, EQ or EGbs in their original form was incorporated directly into the grinder, except for the control. Each sample (20 g) of fish flesh was placed in a separate