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Lotus seed epicarp extract as potential antioxidant and anti-obesity additive in Chinese Cantonese Sausage

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ABSTRACT

The antioxidative activities of a lotus seed epicarp extract in different concentrations (6.25, 12.5, 25, 50 and 100 μg.mL^{-1}) in pork homogenates representative of Chinese Cantonese Sausage were evaluated using three methods: thiobarbituric acid-reactive substances (TBARS) values, peroxide values (POVs) and acid values (AVs). Also the cytotoxic and anti-obesity effects of the lotus seed epicarp extracts were evaluated using an *in vitro* 3T3-L1 preadipocyte cell model. Results showed that the lotus seed epicarp extracts were non-toxic and effective in inhibiting preadipocyte differentiation. Supplementation of pork homogenate with lotus seed epicarp extracts was effective in retarding lipid oxidation. Moreover, the antioxidative and preadipocyte differentiation inhibition effects of the lotus seed epicarp extracts were dose-dependent. Thus, the lotus seed epicarp extract might be a good candidate as an antioxidant and anti-obesity natural additive in Chinese Cantonese Sausage.

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1. Introduction

Chinese Cantonese Sausage (CCS) is a popular Chinese-style semi-dry sausage famous for its unique texture, flavor and taste (Du & Ahn, 2001; Sun, Zhao, Zhao, Zhao, & Yang, 2010). To achieve these qualities, the common ingredients in CCS include large quantities of wine, sugar (4%–9%) and fat (the ratio of lean meat to fat meat is about 7 to 3). The high levels of sugar and fat meat content could result in at least two health problems. One is accelerated lipid peroxidation which would lead to deterioration in meat quality (flavor, color, texture and nutritional value) and production of cytotoxic and genotoxic compounds (Kanner, German, & Kinsella, 1987; Kanner, 1994; Lapidot, Granit, & Kanner, 2005). The other is the risk of obesity due to high levels of sugar and fat intake. Obesity is associated with many serious afflictions such as cardiovascular disease, cancer, and diabetes (Carlotti et al., 2004). It would be useful if an additive in meat products could prevent obesity as well as retard lipid oxidation. One of the common strategies for retarding lipid oxidation is the use of antioxidants. Various plant materials containing phenolic compounds have been demonstrated to be effective antioxidants in meat products (Mielnik, Olsen, Vogt, Adeline, & Skrede, 2006). Flavonoids, a group of important phenolic compounds have shown their abilities not only as potent antioxidants (Shahidi & Wanasundara, 1992; Pietta, 2000) but also as potent anti-obesity compounds (Kuppusamy & Das, 1992; Ono, Hattori, Fukaya, Imai, & Ohizumi, 2006; Kwon et al., 2007).

Lotus, an aquatic plant (*Nelumbo nucifera*, Gaertn.), is widely cultivated in Asia, Oceania and America. Until now, the studies on lotus mainly focus at its edible parts, such as the seeds (Rai, Wahile, Mukherjee, Saha, & Mukherjee, 2006), leaves (Deng et al., 2009), stamens (Jung, Kim, Chung, & Choi, 2003) and roots (Wahida, Abderrahman, & Nabil, 2007). In China, thousands of tons of lotus seed epicarp are discarded during the manufacture of lotus seeds (Kredy et al., 2010). Studies on the components and properties of the lotus seed epicarp are very scarce. To our knowledge, there are only three articles on the topic (Huang et al., 2009; Kredy et al., 2010; Zhou, Gao, Yang, Chen, & Mai, 2011). The antioxidative effects of the flavonoids extracted from the lotus seed epicarp have been reported and this is the first study of the use lotus seed epicarp extract in meat product. The objectives were to evaluate the antioxidative effect of the lotus seed epicarp extract in CCS and the *in vitro* cytotoxicity and anti-obesity effects of the extract and thus evaluate its potential as an antioxidant and anti-obesity natural additive in CCS.

2. Materials and methods

2.1. Lotus seed epicarp extract

Lotus seed epicarp was ground using a micro-grinder (Model BEM-6B, Jinan, China). Ten grams of ground lotus seed epicarp powder were placed in a 500 mL flask and refluxed with 200 mL of 50% ethanol for 2 h in a 60 °C water bath. The residual was removed by filtration and the filtrate concentrated under vacuum and then freeze dried to give a brownish solid extract.

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2.2. Analysis the total flavonoid content in the lotus seed epicarp extract

Total flavonoid content analysis was carried out using an aluminum nitrate colorimetric method (Moreno, Isla, Sampietro, & Vattuone, 2000). In brief, 0.1958 g lotus seed epicarp extract was dissolved in 50% ethanol to prepare an extract solution of 250 mL. Three hundred microliters of the solution was added to a 10 mL colorimetric cylinder and diluted with 50% ethanol to 5 mL. Then, 0.3 mL of 5% $NaNO_2$ (Xilong, Guangzhou, China) solution was added, mixed and left standing for 6 min. Afterwards, 0.3 mL of 5% $Al(NO_3)_3$ (Jinjie, Tianjin, China) was added, mixed and left for 6 min. Finally, 4 mL of 4% NaOH solution was added and diluted with 50% ethanol to 10 mL, mixed and left for 12 min. Sample absorbance was read at 504 nm in a Tu-1810 UV–vis spectrophotometer (PUXI, Beijing, China) and interpolated into a rutin (Sigma) standard curve.

2.3. Cytotoxicity of the lotus seed epicarp extract

3T3-L1 (mouse fibroblast, preadipocyte), NIH3T3 (mouse fibroblast, embryo), L-02 (normal hepatocyte) cells were obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (SIBS, CAS, China). Cells were cultured in RPMI 1640 medium (NIH3T3, L-02) or Dulbecco's Modified Eagle's Medium (DMEM) (3T3-L1) supplemented with 10% bovine calf serum and 1% L-glutamine/penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator. The cytotoxic effects induced by the lotus seed epicarp extracts were determined using Alamar Blue (also called resazurin, Sigma, USA) Assay (O'Brien, Wilson, Orton, & Pognan, 2000) which was based on the detection of metabolic activity. In brief, 1×10^4 cells were seeded in a 96-well cell culture plate and incubated for 8 h at 37 °C in a humidified 5% CO_2 incubator, afterwards, the lotus seed epicarp extract solutions were added and incubated with the cells for 48 h and 72 h. The final concentrations of the extract solutions were 6.25, 12.5, 25, 50 and 100 $\mu g.mL^{-1}$. Non-treated cells were used as control and the wells without cells were used as blanks. At the conclusion of treatment, the medium was removed and the cells were washed with Phosphate Buffered Saline (PBS) once, then 200 μL of Alamar Blue solution (2 $mg.mL^{-1}$ prepared in DMEM or in RPMI 1640) was added to each well and incubated for another 6 h at 37 °C in a humidified 5% CO_2 incubator. Fluorescence was read in a microplate reader (530 nm excitation and 584 nm emission). The relative viability was expressed as a percentage $[OD_{sample} - OD_{blank}]/[OD_{control} - OD_{blank}]$. Each experiment was performed in triplicate.

2.4. Effect of the lotus seed epicarp extract on preadipocyte differentiation

Mature adipocyte is round-shaped with many large cytoplasmic vesicles containing lipid droplets. These lipid droplets are formed with increasing lipid synthesis of adipocyte during the late stage of differentiation. The amount of cytoplasmic triglyceride can reflect the level of differentiation. Thus, the quantitative and qualitative analysis of cytoplasmic triglyceride can be the assay for adipocyte differentiation. Oil Red O staining (Reed & Lane, 1980; Cornelius, MacDougald, & Lane, 1994) of cytoplasmic triglyceride was employed as a cell-based assay to evaluate the change in cytoplasmic triglyceride of 3T3-L1 cells after incubation with the extract solutions. In brief, 3T3-L1 preadipocytes were seeded in a 48-well cell culture plate and were induced to differentiate into adipocytes. Adipocyte conversion was induced by treating 2-day postconfluent cultures with DMEM containing 1 μm dexamethasone (Sigma) and 10 $\mu g.mL^{-1}$ bovine insulin (Sigma) (induction media I) for 2 days and then with DMEM containing 10 $\mu g.mL^{-1}$ bovine insulin (induction media II) for 4 days. Afterwards, the culture medium was refreshed every 2 days. Full differentiation of adipocytes was routinely obtained. Cells cultured in this way were used as control. To evaluate the effects of the lotus seed epicarp extract solutions on 3T3-L1 preadipocyte differentiation, the extract solutions of five different concentrations, 6.25, 12.5, 25, 50 and 100 $\mu g.ml^{-1}$ were supplemented when inducing the cells with induction media I and II. At the conclusion of incubation, the cells monolayer was fixed with 10% formalin for 15 min. A 0.6% Oil Red O (Sigma)-isopropanol solution was added to the cells for 1 h, after which the monolayer was washed several times with distilled water. The stained cytoplasmic triglycerides were visualized under a microscope. After morphology observation, the stained cells were destained with isopropanol and the OD of the destaining isopropanol was measured at 510 nm using a Biophotometer (Eppendorf). Finally, the ratio of differentiation was counted by the following formula: Differentiation (%) = $[OD_{sample}/OD_{control}]\times100\%$. The differentiation rate of the control was set as 100%. Each experiment was performed in triplicate.

2.5. Preparation of the pork homogenate representing the typical formula and manufacturing process of CCS

Pork homogenate representing the typical formula and manufacturing process of CCS was made as follows: lean pork and lard were separately chopped in 1 cm^2 cubes, then, hundred gram pork batters were formulated with 56 g lean pork, 24 g lard, 1.6 g sodium chloride, 4 g sugar, 2.4 g wine and 12 g double distilled water. Lotus seed epicarp extracts were added to the pork batters at 0.05%, 0.1% and 0.2% of the weight ratio and mixed. Pork homogenate without lotus seed epicarp extract addition was used as blank. Treated samples and the blank were heated in a 60 °C oven for half an hour. After cooling, the treated samples and blank were stored at 4 °C before use.

2.6. Antioxidative effects of the lotus seed epicarp extracts on pork homogenate

Thiobarbituric acid-reactive substances (TBARS) values, peroxide values (POVs) and acid values (AVs) were used to evaluate the antioxidative effects of the lotus seed epicarp extracts in pork homogenates. The TBARS values were determined by the extraction method (Sørensen & Jørgensen, 1996) with some modification. For extraction, 10 g pork homogenate was mixed with 50 mL extraction solution containing 7.5% trichloroacetic acid (TCA) and 0.1% EDTA and shaken vigorously. After filtration and grease removing, 10 mL extract was mixed with 10 mL of 0.02 $mol.L^{-1}$ thiobarbituric acid (TBA) solution. The samples were incubated at 90 °C for 40 min in a water bath. After cooling and centrifugation, 5 mL chloroform was added to the supernatant. Absorbance of the supernatant was measured at 532 nm by an UV–vis spectrophotometer (TU-1810, PU XI, Beijing, China). Results were expressed as milligrams malondialdehyde (MDA) per kilogram meat sample ($mg.kg^{-1}$) according to a standard curve of MDA. The POVs were determined according to Pegg (2001) with minor modification. Fifty milliliter of a glacial acetic acid and isooctane mixture (v/v = 3:2) was added to a 5 g meat homogenate and shaken vigorously. The following steps were the same as the method described except that 0.01 $mol.L^{-1}$ sodium thiosulphate was used as titrant. Results were expressed as milliequivalents (meq) per kilogram meat sample ($meq.kg^{-1}$). The AVs were determined according to AOCS official method Cd 3d-63 (1997) with some modification: in brief, 5 g of pork homogenate was mixed with 50 mL of neutralized ethyl alcohol and 0.5 mL phenolphthalein indicator. The mixture was heated in a water bath and then titrated with 0.1 $mol.L^{-1}$ sodium hydroxide. Results were expressed as milligram sodium hydroxide per gram meat sample ($mg.g^{-1}$). For all the experiments, a blank test was performed under the same conditions.

2.7. Statistical analysis

Data are reported as the mean ± standard deviations (SD) of three independent experiments. Statistical analyses were performed using

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