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Antioxidant activities of novel galloyl phytosterols evaluated by human erythrocytes with the aid of confocal microscopy imaging

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ABSTRACT

Notable protective effect of the novel galloyl phytosterol antioxidant on human erythrocytes was disclosed in this study. 2,2′-Azo-bis (2-amidinopropane) hydrochloride (AAPH) induced haemolysis of erythrocytes, and depletion of cellular glutathione were effectively inhibited by the galloyl phytosterol antioxidant. It was revealed that the level of glutathione was a more sensitive indicator than haemolysis for cellular oxidative stress. The morphology of erythrocytes obtained through laser scanning confocal microscopy (LSCM) imaging provided structural details for haemolysis and glutathione investigation and was more sensitive than the level of glutathione. The liposome model exhibited that the novel antioxidant preferred to anchor in the membrane and had better performance for membrane protection than gallic acid. The strategy of integrated use of biochemical and visual methods developed in this study is valuable for investigation on mechanistic behaviours of antioxidants.

1. Introduction

Phytosterols are widespread plant-derived natural compounds that share structural similarity with cholesterol. Numerous investigations have unveiled that consumption of phytosterols is strongly associated with enhancement of serum lipid profiles and reduction of cardiovascular disease (Kendall & Jenkins, 2004) and cancers such as breast and prostate cancers (Awad, Downie, Fink, & Kim, 2000; Awad, Fink, Williams, & Kim, 2001; Ju, Clausen, Allred, Almada, & Helferich, 2004). Owing to their established beneficial effects, there are growing interest in analysis, preparation, and derivation of phytosterols (Ostlund, 2002; de Jong, Plat, & Mensink, 2003; St-Onge & Jones, 2003; Moruisi, Oosthuizen, & Opperman, 2006; Ellegard, Andersson, Normen, & Andersson, 2007; Van Horn et al., 2008; Grundy, 2005; Food and Drug Administration, 2002; Liu & Ruan, 2013). Antioxidants are important food additives and extensively applied in foods to protect against lipid oxidation, off-flavour development, and oxidative stress. As representative antioxidants, gallic acid and its derivatives are abundant natural antioxidants and ubiquitously present in various foods especially wines and green tea (Bertelli & Das, 2009; Birosová, Mikulásová, & Vaverková, 2005; Butt & Sultan, 2009; Giftson, Jayanthi, & Nalini,

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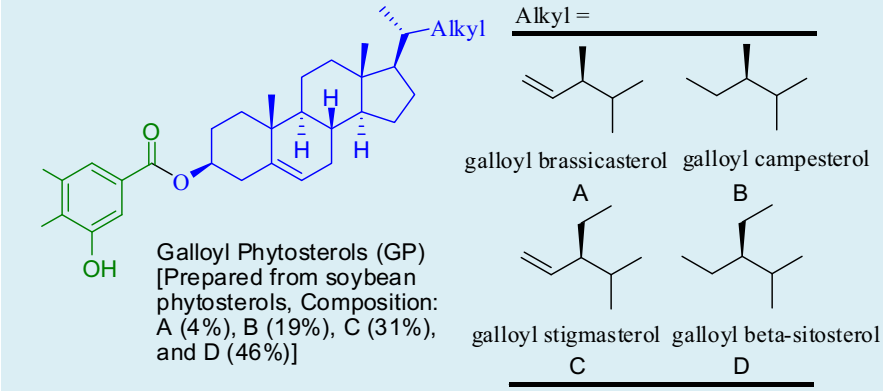

Fig. 1 – The structure and composition of the novel galloyl phytosterol antioxidant (GP).

2009; Kang, Oh, Kang, Hong, & Choi, 2008; Kim et al., 2006a; Paixão, Pereira, Marques, & Cãmara, 2008). Compared with gallic acid, its ester derivatives usually exhibit improved activities (Kim et al., 2006b; Kubo, Fujita, & Nihei, 2002; Ortega et al., 2003).

Generally phytosterols themselves have very limited antioxidant capacity. Due to growing concerns about the safety of the synthetic antioxidants, food scientists are forced to seek naturally derived alternatives. Considering the beneficial effects of phytosterols and excellent antioxidant activity of gallic acid, it will be valuable to combine them together and develop novel phytosterol-derived antioxidants through esterification of phytosterols with gallic acid. Unfortunately, the complication of phenolic hydroxyls result in great synthetic challenge in esterification of phytosterols with phenolic acids, and only few studies have achieved synthesis of phenolic phytosterols with enzymatic approaches (Tan & Shahidi, 2012, 2013a, 2013b). Very recently we successfully developed a straightforward chemical method for esterification of phytosterols with gallic acid without prior protection and activation (Fig. 1) (Fu et al., 2014). The simple operation and good selectivity of this method facilitate preparation of phenolic phytosterols. Evaluation of the synthesized novel galloyl phytosterol antioxidant with radical scavenging and Rancimat methods revealed its excellent antioxidant activities in edible oils.

Oxidative stress resulting from accumulation of excessive reactive oxygen species (ROS) in the body is believed to cause toxic effects and contribute to the development of chronic diseases including cancer, Alzheimer's disease, and heart failure (Halliwell, 2007a; Singh, Dhalla, Seneviratne, & Singal, 1995; Valko et al., 2007). Generally antioxidants are very effective in reduction of oxidative stress through scavenging excessive ROS. Therefore, it will be beneficial to further evaluate ROS scavenging activity of the novel galloyl phytosterol antioxidant since the excellent antioxidant activities in edible oils have been uncovered. One of the most frequently applied models for determination of ROS scavenging activity is the erythrocyte that is an *ex vivo* model for examination of protective effect of antioxidants on cell membranes and cellular glutathione (Liu, 2010). Because of the extraordinary capability of laser scanning confocal microscopy (LSCM) to obtain high resolution images of cells, application of LSCM will help visualization of dynamic progress of haemolysis of erythrocytes and monitor protective effect of antioxidants (Pawley, 2006).

Therefore, protective effect of the novel galloyl phytosterols on human erythrocytes aided by LSCM imaging was probed in this study. The notable protective effect of the novel galloyl phytosterols was observed and LSCM imaging disclosed interesting morphological details of human erythrocytes during the study. Herein the details of this study were described as follows.

2. Materials and methods

2.1. *Chemicals*

Phytosterols (composed of 32% stigmasterol, 5% brassicasterol, 21% campesterol and 42% β-sitosterol) from soybean were purchased from Jiatian Biotechnology (Xian, China). Sodium bicarbonate, trisodium citrate dehydrate, disodium hydrogen phosphate, sodium hydroxide, sorbic acid, anhydrous magnesium sulfate, dimethyl sulfoxide (DMSO), and tetrahydrofuran were obtained from Sinopharm Chemical Reagent (Shanghai, China). N,N′-dicyclohexylcarbodiimide (DCC), gallic acid, 2,2′-azo-bis (2-amidinopropane) hydrochloride (AAPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione (GSH), methyl gallate, DL-α-tocopherol (VE), Tween-80, trichloroacetic acid were obtained from Aladdin Reagent (Shanghai, China). Phosphate-buffered saline buffer (PBS, pH 7.2–7.4) was purchased from Shanghai Dingjie Biotechnology (Shanghai, China). Cyanohemoglobin assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All chemicals were of analytical grade.

2.2. *Preparation of galloyl phytosterols*

To a solution of 510.4 mg of gallic acid and 412.7 mg of phytosterols in 5 mL tetrahydrofuran (THF) was added a solution of DCC (617.0 mg) in 5 mL THF. After the solution was stirred for 1.5 h, the white precipitate was removed by filtration. The solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate and washed successively with saturated aqueous sodium bicarbonate solution, dried over anhydrous magnesium sulfate, and evaporated under reduced pressure. Purification over a silica gel chromatography eluted