Research in Veterinary Science 97 (2014) 358–364

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Protection of *Clitoria ternatea* flower petal extract against free radical-induced hemolysis and oxidative damage in canine erythrocytes

Wathuwan Phrueksanan [a,b], Sirinthorn Yibchok-anun [a,b], Sirichai Adisakwattana [b,c,*]

[a] Department of Pharmacology, Faculty of Veterinary Sciences, Chulalongkorn University, 10330, Thailand
[b] Research Group of Herbal Medicine for Prevention and Therapeutic of Metabolic Diseases, Chulalongkorn University, 10330, Thailand
[c] Department of Nutrition and Dietetics, Faculty of Allied Health Sciences, Chulalongkorn University, 10330, Thailand

ARTICLE INFO

Article history:
Received 3 March 2014
Accepted 29 August 2014

Keywords:
Anthocyanin
Antioxidant
Erythrocytes
Clitoria ternatea flower petal
Free radical
Hemolysis
Oxidative damage

ABSTRACT

The present study assessed the antioxidant activity and protective ability of *Clitoria ternatea* flower petal extract (CTE) against *in vitro* 2,2′-azobis-2-methyl-propanimidamide dihydrochloride (AAPH)-induced hemolysis and oxidative damage of canine erythrocytes. From the phytochemical analysis, CTE contained phenolic compounds, flavonoids, and anthocyanins. In addition, CTE showed antioxidant activity as measured by oxygen radical absorbance capacity (ORAC) method and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. CTE (400 μg/ml) remarkably protected erythrocytes against AAPH-induced hemolysis at 4 h of incubation. Moreover, CTE (400 μg/ml) reduced membrane lipid peroxidation and protein carbonyl group formation and prevented the reduction of glutathione concentration in AAPH-induced oxidation of erythrocytes. The AAPH-induced morphological alteration of erythrocytes from a smooth discoid to an echinocytic form was effectively protected by CTE. The present results contribute important insights that CTE may have the potential to act as a natural antioxidant to prevent free radical-induced hemolysis, protein oxidation and lipid peroxidation in erythrocytes.

1. Introduction

Oxidative stress is an imbalance between the production of reactive oxygen species and antioxidant defense mechanisms. Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion, and hydroxyl radical are normally generated under aerobic metabolic pathways in the human body. Excessive generation of ROS causes oxidative damage to the cellular biomolecules including DNA, protein, nucleic acid, and membrane lipids. The increased oxidative stress of cellular physiology has been implicated in the pathogenesis of many diseases such as cancer, chronic kidney disease, and neurodegenerative diseases (Dimakopoulos and Mayer, 2002; Macotpet et al., 2013; Silva et al., 2013). Recent studies reveal that oxidative stress may play a significant role in the initiation and regulation of cardiomyocyte apoptosis in a variety of cardiac diseases (Corcoran et al., 2004).

Erythrocytes, also known as red blood cells (RBCs), have a unique shape and inner components that allow them to efficiently transport oxygen and direct the elimination of carbon dioxide. Besides their specific roles as oxygen carriers, they are also highly susceptible to endogenous oxidative damage. Especially, the polyunsaturated fatty acids (PUFAs) of the erythrocyte membrane and the redox active proteins of hemoglobin are key targets for free radical-induced hemolysis and oxidative damage (López-Revuelta et al., 2006). The alteration can cause changes in their shape and the loss of functional membrane integrity, leading to the onset of acute and chronic diseases (Iyer et al., 2013).

Numerous studies suggest that antioxidants have gained worldwide popularity for the prevention of oxidative stress-related diseases (Esfahani et al., 2011; Landete, 2013). It has recently been reported that antioxidant supplementations exert a marked protective effect on damage to DNA in dogs (Heaton et al., 2002). Recently, edible plants containing antioxidants have become a major area of scientific research because they have greater health benefits with various pharmacological activities. *Clitoria ternatea L.* (family: Fabaceae), commonly known as “Butterfly pea”, is widely cultivated in the Caribbean area, Central America, Africa, and Southeast Asia. The flower petal of this plant is recognized as a good source of dietary anthocyanins and used as a natural blue colorant in a variety of foods (Mukherjee et al., 2008). The flower petal of *Clitoria ternatea* contains ternatins, a group of delphinidin glycosides. The six major ternatins were isolated from the flower and characterized as A1, A2, B1, B2, D1, and D2 (Terahara et al., 1996, 1998). In addition, other phytochemical compounds including triterpenoids, flavonol glycosides, and steroids have been isolated from *Clitoria ternatea* Linn. The *Clitoria ternatea* extract possesses a wide range of pharmacological activities including anti-inflammatory,

Abbreviations: AAPH, 2,2′-azobis-2-methyl-propanimidamide dihydrochloride; CTE, *Clitoria ternatea* flower petal extract; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MDA, malondialdehyde; GSH, glutathione

* Corresponding author. Tel.: +66 2 2181067; fax: +66 2 2181076.
E-mail address: Sirichai.a@chula.ac.th (S. Adisakwattana).

http://dx.doi.org/10.1016/j.rvsc.2014.08.010
0034-5288/

anti-diabetic, anti-microbial, and antioxidant activities (Kamkaen and Wilkinson, 2009; Mukherjee et al., 2008). Although pharmacological activities of *Clitoria ternatea* extract were well investigated, studies regarding its protective effects against free radical-induced hemolysis and oxidative damage have not been undertaken. The aim of this study was to evaluate the effect of *Clitoria ternatea* flower petal extract (CTE) against AAPH-induced hemolysis and oxidative damage in dog erythrocytes. In addition, the phytochemical analyses of *Clitoria ternatea* flower petal and its antioxidant capacities were also determined.

2. Materials and method

2.1. Chemicals

Thiobarbituric acid (TBA), 2,2-diphenyl-1-picrylhydrazyl, trolox, trichloroacetic acid (TCA), 2,2′-azobis-2-methyl-propanimidamide dihydrochloride (AAPH), 2,4-dihydrophenylhydrazine, potassium cyanide, guanidine hydrochloride, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), malondialdehyde (MDA), potassium ferricyanide, and trichloroacetic acid (TCA) were from Sigma-Aldrich Co. (St. Louis, MO, USA). All chemicals and solvents used were analytical grade.

2.2. Animals

Five healthy male dogs (5–10 years) were obtained from Chulalongkorn University Small Animal Hospital. This study has been reviewed and approved by Certification of Institutional Animal Care and Use Committee (IACUC) in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Proposes edited by the National Research Council of Thailand (Approval no. 12310041, 30/4/2011).

2.3. Plant materials

The dried flower petals of *Clitoria ternatea* were obtained from a local market in Thailand. The flower petals (0.5 kg) were boiled in 3 l of distilled water for 2 h. Then the solution was filtrated through Whatman 70 mm filter paper. Finally, the aqueous solution was dried using spray dryer SD-100 (Eyela World, Tokyo Rikakikai Co., LTD, Japan). Spray drying conditions were inlet/outlet temperature (90 °C/178 °C), blow rate (0.8 m^3/min), and atomizing (80–90 kPa). The dried extract of *Clitoria ternatea* was stored under refrigeration (4 °C) until used for further analysis.

2.4. The phytochemical analysis

The measurement of total phenolic content in CTE was modified according to a previously published method (Mäkynen et al., 2013). The extract was dissolved with distilled water. A sample (20 µl) was mixed with 100 µl of the Folicin–Ciocalteu reagent (previously diluted 10-fold with distilled water), followed by 80 µl of aqueous Na_2CO_3 (60 g/l). The absorbance was then measured at 725 nm after incubation for 90 min. Total phenolic content was expressed as milligram gallic acid equivalents/gram dry weight of extract. Total flavonoid content in CTE was measured using the aluminum chloride colorimetric assay (Adisakwattana et al., 2012). The sample solution (100 µl) was added to 30 µl of $AlCl_3$ solution (10% w/v), 30 µl of $NaNO_2$ (15% w/v), 400 µl of 4% NaOH, and 440 µl of distilled water. After incubation at room temperature for 10 min, the absorbance was measured immediately at 510 nm. Total flavonoid content was calculated from a calibration curve of catechin and expressed as milligram catechin equivalents/gram dry weight of extract. The total anthocyanin content in CTE was analyzed by a pH differential method (Moldovan et al., 2012). The absorption of the samples developed through pH 1 and pH 4.5 buffers was measured in terms of cyanidin-3-glucoside at 510 and 700 nm for 15 min at room temperature. The monomeric anthocyanin pigment concentration was calculated according to the following equation: Monomeric anthocyanin pigment (mg/l) = $(A \times MW \times DF \times 1000)/(\varepsilon \times 1)$ where $A = (A_{510} - A_{700})$ pH 1.0 – $(A_{516} - A_{700})$ pH 4.5, MW is the molecular weight of cyanidin-3-glucoside (449.2), ε is the molar absorptivity (26,900), and DF is the dilution factor. The total anthocyanin content was expressed as milligram cyanidin-3-glucoside equivalents/gram dry weight of extract.

2.5. Antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was measured according to a previously described method (Mäkynen et al., 2013). Briefly, the sample (500 µl) was added to 500 µl DPPH solution (0.2 mM in ethanol) and incubated for 30 min at room temperature. The decrease in the solution absorbance was measured at 515 nm and the percent inhibition was calculated using the following formula: The percent inhibition = $(A_0 - A_{sample})/A_0 \times 100\%$ where A_0 is the absorbance of the control (blank) and A_{sample} is the absorbance of tested extract. The DPPH radical scavenging activity was expressed as IC_{50} and calculated from the plot of the percent inhibition against sample concentration. L-ascorbic acid was used as a positive control.

The oxygen radical absorbance capacity (ORAC) assay was measured according to a previously published method (Mäkynen et al., 2013). Briefly, 25 µl of the extract was mixed with 150 µl of 48 nM fluorescein solution and placed in the wells of a microplate. The mixture was preincubated for 10 min at room temperature. A free radical generator solution (2,2′-azobis-2-methyl-propanimidamide dihydrochloride; AAPH; 25 µl; 64 mM) was added into the solution. The fluorescent intensity was recorded every 2 min for 60 min with emission and excitation at 535 and 485 nm, respectively. A standard curve was generated with a trolox concentration range from 0.024 to 3.125 µM. The ORAC value was calculated as the area under the curve (AUC) and expressed as micromoles of trolox equivalent (TE) per gram of dry extract.

2.6. Preparation of erythrocytes suspension

Fresh whole blood (5 ml) was obtained from healthy dogs *via* cephalic venipuncture. The whole blood was centrifuged at 1500 *g* for 10 min at 4 °C using a refrigerated centrifuge. The separated erythrocytes were washed three times in 10 mM phosphate buffer saline (PBS), pH 7.4. After centrifugation, the supernatant and the buffy coat were carefully removed with each wash. Washed erythrocytes were finally re-suspended to the desired hematocrit level in 10 mM PBS. The erythrocytes were stored at 4 °C and used within 2 h of sample preparation.

2.7. Erythrocyte hemolysis assay

The inhibition of free radical-induced erythrocyte hemolysis was performed according to a previously published method (Wang et al., 2009). The erythrocyte hemolysis was induced by thermal decomposition of AAPH. Briefly, 10% of erythrocyte suspension in PBS was preincubated for 5 min at 37 °C with CTE (50–400 µg/ml) and trolox (100 µg/ml), followed by incubation with or without 50 mM AAPH solution for 6 h with gentle shaking. At 60 min intervals, aliquots of the reaction mixture (50 µl) were taken out and diluted with 1000 µl of PBS. After centrifugation at 2000 *g* for 10 min, the absorbance (*A*) of the supernatant was measured at 540 nm using a spectrophotometer. The reference values were determined by using the same amount of erythrocyte in distilled water to obtain a