Potential Activity of Thai Shallot (*Allium ascalonicum L.*) Extract on the Prevention of Hemolysis and Glutathione Depletion in Human Erythrocyte from Oxidative Stress

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ABSTRACT

Thai shallot (Allium ascalonicum L.) is an economic vegetable from which few commercial products are produced for health promotion. The tendency of shallot and the possible modified processing as medical plant are very interesting. The aim of this study was to test the protective effects of shallot extract on red cell hemolysis, lipid oxidation (malondialdehyde; MDA) and keeping the glutathione (GSH) in erythrocyte from oxidation, compared with garlic extract. Three Thai shallot extracts were prepared by directly crushing and pressing (crude), water- or hexane-extraction and then freeze dried. The protective effect on hemolysis was performed, using erythrocyte H_2O_2-lysis test. For lipid peroxidation protection and GSH maintenance activities demonstrated by oxidation with 2,2'-Azobis (2-amidino-propane) dihydrochloride (AAPH) and the levels of MDA and GSH were detected by TBARs and DTNB reactions. The results showed that hexane-extract shallot inhibited erythrocyte hemolysis after incubation with H2O2 whereas crude and water-extract had lower activity. The hexane-extract shallot could better protect lipid peroxidation and maintain GSH from AAPH oxidation compared with crude and water-extract. This study indicated that hexane- extract shallot had very high activity on protecting the human erythrocyte from radicals and is possible to be modified for medical plants or commercial product in the future.

Key words: Shallot, Garlic, Oxidative stress, Erythrocyte, Glutathione, Malondialdehyde

INTRODUCTION

Most of Thai herbs have antioxidant property which are active compounds such as different phenolic compounds. Excessive free radical production in our body is related to the disease progression, both direct and indirect to biological component destructions i.e., lipid, protein or DNA. Oxidative stress involves many physiological and pathological phenomena. Because of their susceptibility to oxidation, red blood cells (RBCs) have been used as a model to investigate oxidative damage in biomembranes (Koster and Slee, 1983) due to exposure of RBCs to free-radical-induced lipid peroxidation, reduction in deformaliity (Kurata et al., 1994) and hemolysis (Miki et al., 1989; Vissers et al., 1994).

Most of challenging researches on herbal antioxidative effects are aimed at protecting and reducing the oxidation process in vitro or in vivo models such as for *Gingko*. Onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) in the family Alliaceae demonstrate the significant antioxidant as well as anti-inflammation activity that is useful in the protection of various diseases such as respiratory and nervous diseases. Water-soluble compounds in garlic such as Sñ allyl cysteine (SAC) and S-allyl-mercaptocystein (SAMC) were reported to have antioxidant activity (Imai et al., 1994), involving glutathione detoxification (Prasad et al., 1996). It was also shown to have anti-inflammation property by regulating the expression of nuclear factor kappa B (NF-B) in T-lymphocyte (Zhaohui et al., 1997). Shallot (*Allium ascalonicum* L.), also belonging to the Family Alliaceae, is one of the promising plants but only few studies on it had been carried out. We investigated the antioxidant property of Thai shallot, compared with well-known garlic extracts, and the activity on the protection of erythrocyte hemolysis from hydrogen peroxide. Studies on the activity of shallot and garlic extracts on the protection of lipid peroxidation and depletion of glutathione in AAPH-oxidized erythrocyte were performed. Moreover, the antioxidant stability of shallot was also studied.

MATERIALS AND METHODS

Materials and reagents

The glasswares used in this study was cleaned by heating overnight in conc. HNO_3, washing with distilled water and heat-drying. Solutions were made up in distilled water and purified by passing through a four-stage Milli Q system. 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), dimethyl sulfoxide (DMSO) and thobarbituic acid-reactive (TBAR) were purchased from Sigma (St.Louis.MO,USA). H_2O_2 was from Carlo Eeba (Rodano)(Milano, Italy). (2,2'-azobis(2-amindinopropane)-dihydrochloride; AAPH), malonaldehyde bis(dimethyl acetal) (99%) were purchased from Aldrich company.Inc (USA).

Preparation of crude shallot extract

Shallot bulbs from local agricultural farm approximately one-month old after harvesting and raw garlic bulbs from Chiang Mai local market were used. Three lyophilized materials were processed by directly crushing and pressing or extracting. Crushing and pressing of fresh bulbs was performed by using a mortar. Extraction process was performed by mixing fresh bulbs with solvents, water and hexane in an equal ratio of weight to volume(V;W), then homogenized with electrical blender. The portion of water-extract was filtered and lyophilized. The mixture from hexane-extract was allowed to settle overnight and then filtered. Hexane layer separated was evaporated before freeze-drying and lyophilizing.

In the assays, three lyophilized materials; crude (crushing and pressing), water- and hexane-extracts were reconstituted with deionised distilled water and DMSO respectively, then centrifuged at 14,000 rpm for 5 min. Clear supernatant was used for the experiments.

Biochemical assays

H_2O_2 red cell lysis assay

Heparinized blood was collected from healthy subject with criteria of Hemoglobin 14.1 (10-16 g/dl), Hematocrit 42 (40-50%), WBC 5,700 (5,000-10,000/cu.mm), ESR 6 (0-15 mm/hr), MCV 89 (80-95 fL), MCH 29.2 (27-32 pg), MCHC 32.8 g/dl (32-36 g/dl), Hb typing (HbA2 = 2.5%, HbF = 0.6%), and G-6-PD negative. The protocol for determination of hemolysis was modified from Jha's protocol (Jha et al., 1984). Heparinized blood was centrifuged and the plasma and buffy coat were removed. The RBCs were washed three times with isotonic buffer solution (pH 7.4), containing 8.1 mmol/L $Na_2PO_4.2H_2O$, 1.5 mmol/L KH_2PO_4, and 137 mmol/L NaCl, before adding H_2O_2 (0.5%). Inhibitory activity was studied by addition of extract in the system. An equal volume of isotonic buffer was used as a control. Samples were incubated for 30 min at 37°C, then centrifuged at 3,000 rpm for 5 min and supernatant was collected. The red blood cells hemolysis was determined by reading absorbance of haemoglobin at 405 nm (A405) in the supernatant fraction compared with 100% hemolysis of RBCs plus H_2O_2.

AAPH preincubation

A 1.0 mL of blood treated with AAPH in the presence or absence of shallot or garlic extracts at 1 mg/ml was incubated for up to 6 hours at 37°C. Negative control was run along with an equivalent volume of isotonic buffer solution, which will not significantly change the contents of total glutathihone and thobarbituic acid-reactive substances (TBARs) in RBCs within 6 hours. After incubation for 4 h, centrifuged at 3,000 rpm for 5 min, total glutathione (GSH) level in erythrocyte and malondialdehyde (MDA) in plasma were detected with DTNB (Beutler et al., 1963) and TBARs (Chirico, 1994) reactions, respectively.

Total GSH determination

The 0.4 ml of erythrocytes was mixed with 1.6 ml distilled water and 3.0 ml of precipitant solution (glacial *m*-phosphoric acid, EDTA and NaCl). The supernatant was separated by filtering through Whatman No.1. One ml of clear supernatant was added to 4.0 ml phosphate buffer (pH 7.4) and 0.5 ml DTNB reagent. The absorbance was read at 412 nm within 5 min. The total glutathione concentrations were obtained by comparing with standard GSH (Sigma) at 20– 100 mg/ml.

Malondialdehyde (MDA) determination

One ml of plasma was mixed with 0.75 ml 0.44 M of H_3PO_4, kept in room temperature for 10 min and added 0.25 ml of 0.6% TBA. Then, the mixture was incubated in water bath (90°C) for 30 min. The pink color developed was read at 532 nm after stopping the reaction with cold water. The MDA concentration was calculated by comparing with standard malondialdehyde (bis) acetate (Aldrich).

Data analysis

Data were presented as mean and standard derivation. The one-way ANOVA in SPSS 10 program was used for statistical significance analysis ($p< 0.05$).

RESULTS

Protective effects on hemolysis in H2O2-lysis test.

From this study, 1% red blood suspension and 0.5% of H_2O_2 gave optimized hemolysis after incubation for 4 h. High concentration of all extracts at 1 mg/ml represented the inhibitory effects after incubation for 3- 4 h. The hexane-extract shallot showed highest activity, compared with crude and water-extraction (Figure1).

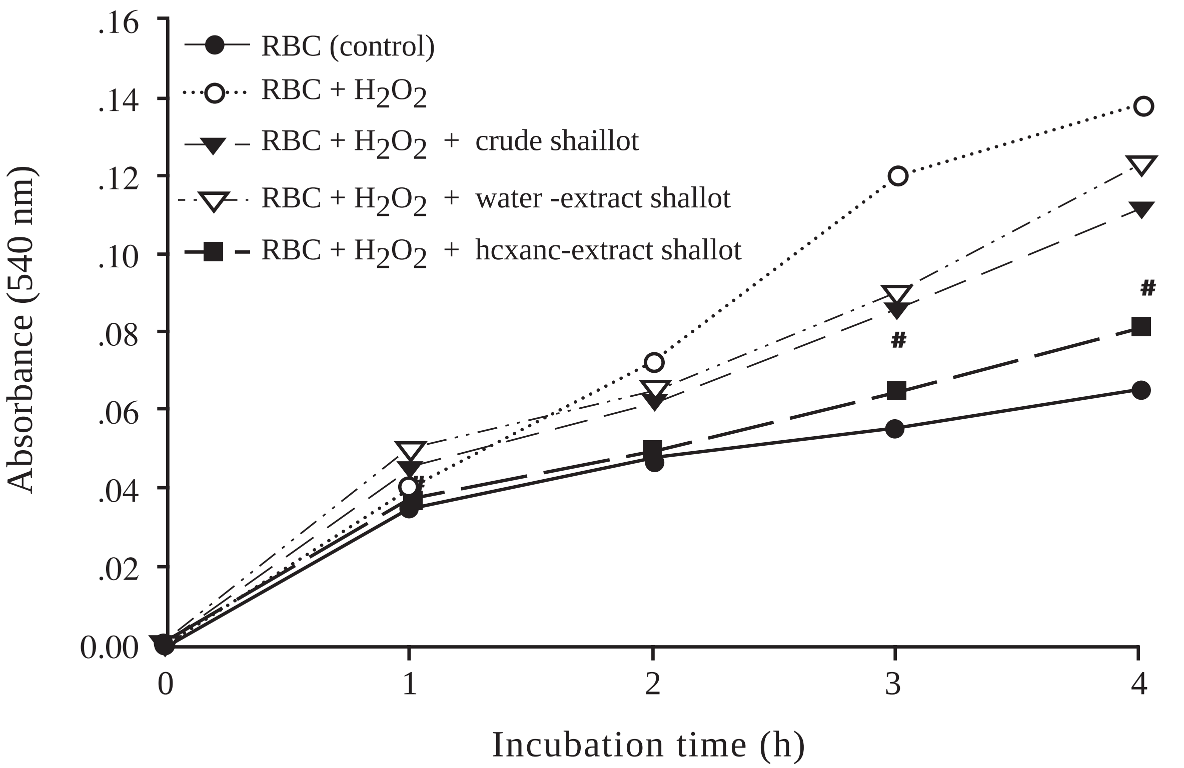

Figure 1. Changes in absorbance at 450 nm of erythrocyte hemolysis by three shallot extracts; crude, water, and hexane-extraction (1 mg/ml) compared with positive (RBC in the presence of H_2O_2) and negative control (RBC only). (# , $P < 0.05$ compared with other extracts at the same incubation time and positive controls).

Protective effects on Lipid peroxidation and Glutathione depletion in AAPH-oxidized erythrocytes

The 10 mM of AAPH was able to oxidize and induce erythrocyte hemolysis after incubation at 37°C for 6 h. Figure 2 and 3 showed an increase in the lipid peroxidation and a decrease in glutathione. At low concentration of 100 and 200 μg/ml, the significant inhibition of lipid peroxidation (Figure 2) and GSH depletion (Figure 3) in hexane-extract shallot, compared with other extracts or controls, were demonstrated. The activities of shallot on protection of lipid peroxidation and GSH depletion from AAPH oxidation were also found after storage for 3 months in the dark in sealed bottle with anti-moisture (Figure 4, 5).

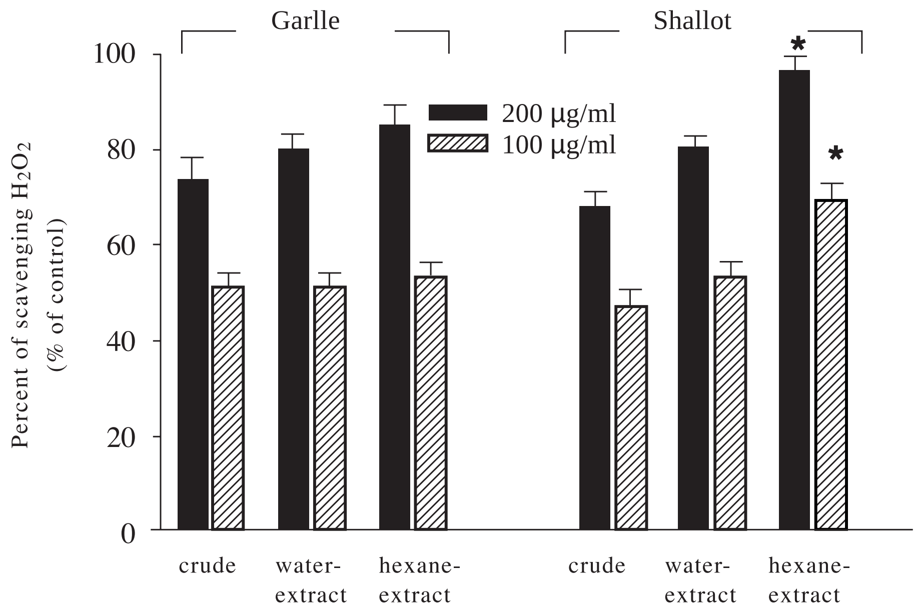

Figure 2. The malondialdehyde (MDA) concentration in plasma after incubation of red blood cells (RBCs) with (10 mM) AAPH in the presence or absence of garlic and shallot extracts 100 or 200 µg/ml at 37°C for 6 h. Each bar represented the mean and standard deviation from five samples. (*, $P < 0.05$ compared with other extracts).

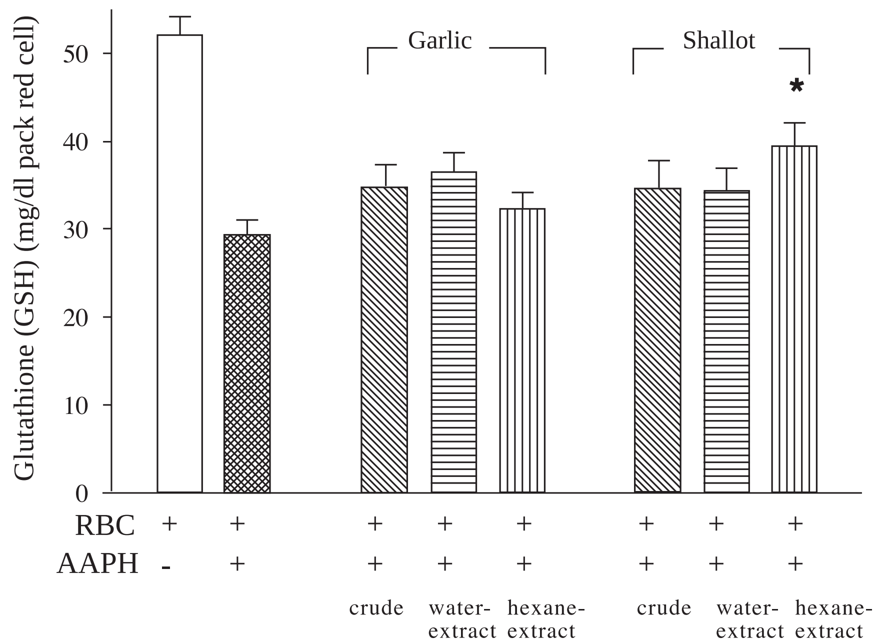

Figure 3. The glutathione (GSH) concentration in red blood cells after incubation of red blood cells (RBCs) with (10 mM) AAPH in the presence or absence of garlic and shallot extracts (200 µg/ml) at 37°C for 6 h. Each bar represented the mean and standard deviation from five samples. (*, $P < 0.05$ compared with other shallot and garlic extracts).

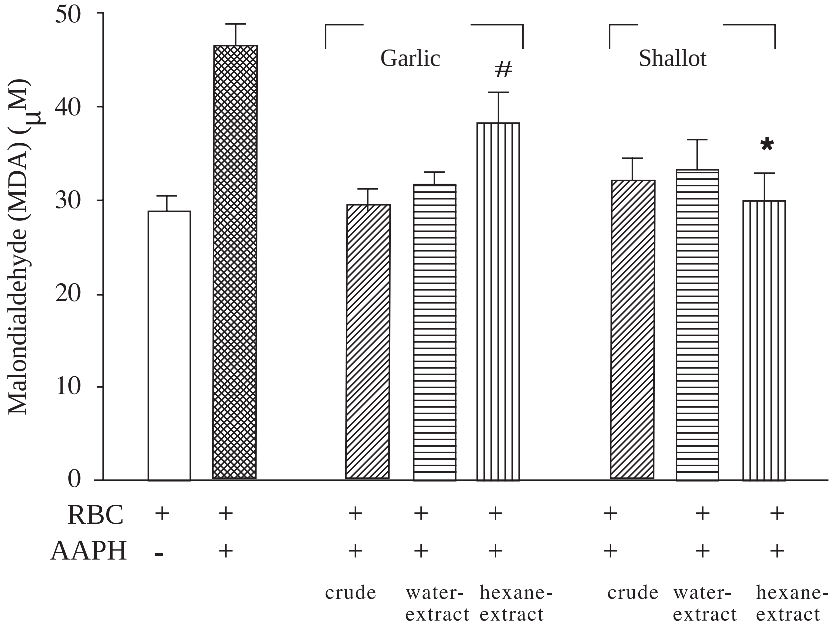

Figure 4. The malondialdehyde (MDA) concentration plasma after incubation of red blood cells (RBCs) with (10 mM) AAPH in the presence or absence of garlic and shallot extracts (200 µg/ml) at 37°C for 6 h. Each bar represented the mean and standard deviation from five samples. (*, $P < 0.05$ compared with other garlic and shallot extracts).

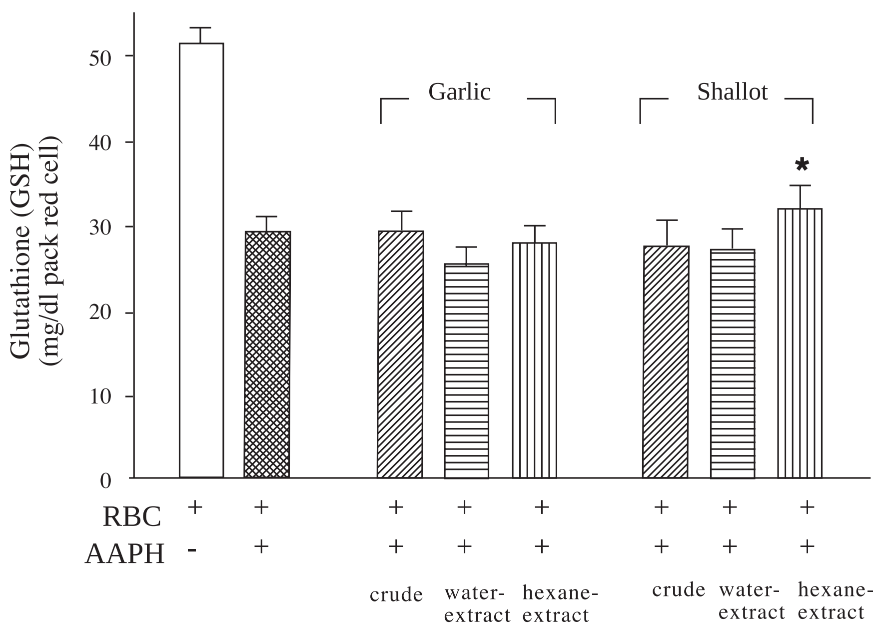

Figure 5. The glutathione (GSH) concentration in red blood cells after incubation of red blood cells (RBCs) with (10 mM) AAPH in the presence or absence garlic and shallot extracts (200 µg/ml) at 37°C for 6 h. Each bar represented the mean and standard deviation from five samples. (*, $P < 0.05$ compared with other garlic and shallot extracts).

DISCUSSION

There are lots of evidences which show high potential capacity of aged garlic extract (AGE) to inhibit inflammation, bacterial growth and cancer cell progression by active sulfur compound, allyl sulfide or S-allyl cysteine (Imai et al., 1994). Some evidence showed that AGE had scavenging activity on H_2O_2 (Prasad et al., 1996) and could protect lipid peroxidation in low density lipoprotein (LDL) particles by chelating the copper ion (Cu^{2+}) (Dillon et al., 2003). Our results revealed the higher antioxidant activity of shallot when compared with garlic extracts (Figure 1, 2), especially, shallot preparation by hexane extraction which was able to protect the erythrocyte hemolysis from radical such as hydrogen peroxide.

Previous studies reported the polyphenols in green tea as epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and gallic acid (GA) were able to suppress the erythrocyte hemolysis from AAPH-oxidation (Lanping et al., 2000). In our study, protective effect on lipid peroxidation from AAPH and depletion of glutathione in erythrocyte of shallot extracts were obviously demonstrated.

The potential protection on lipid peroxidation and glutathione depletion in AAPH-oxidized erythrocytes, were also studied after keeping the shallot extracts for 3 months at different temperatures; room temperature, cold room (-80°C) and high temperature (40°C). The results also supported the previous study on ability of shallot extracts and garlic extracts on the protection of protein and amino acid hydroperoxide formation from irradiation and glutathione decomposition by protein hydroperoxide in vitro (Leelarungrayub et al., 2004). GSH depletion is important in human erythrocytes in order to control thiol proteins and reduce free radical In other vital organs such as brain, it is also found that the increase of GSH can protect the organ against hydroxyl free radical-induced protein oxidation in vivo (Pocernich et al., 2000).

The important activity compounds in Alliaceae family such as onion, garlic or shallot are different but they mainly contain total phenolic compound that show the – OH group (Cao et al., 1997). In addition, most of phenolic compounds such as furostane saponins and high level of quercetin, isorhametin and other glycosides are presented in shallot (Fattorusso et al., 2002). Previous report showed various concentrations of total phenolics in edible part of various onions and different cloves of garlic (Nuutila et al., 2003). In co-experiment to identify any compound as total phenolic and sulfur compound between garlic and shallot extracts, the results showed that hexane-extract shallot contained the total phenolics as much as in hexane-extract garlic (5,413.62± 62.97, 5,477.53± 33.49 GAE mg/kg) whereas the total phenolics in water-extract found in shallot were significantly higher (4,599.01± 88.32 GAE mg/kg) than in garlic (3,804.77± 77.70 GAE mg/kg) ($p< 0.01$). In part of crude extract shallot from crushing and pressing (4,086.37± 84.54 GAE mg/kg), the total phenolics present were similar as in the garlic extract (4,102.53± 193.44 GAE mg/kg). Moreover, the garlic also showed various sulfur compounds that represented antioxidant properties in parts of polar and non-polar compounds (Yin et al., 2002). We proved in part of sulfur compound that standard allyl disulfides were measured by high performance liquid chromatography (HPLC) and confirmed with gas chromatography-mass spectrum (GC/MS) analysis. The study showed diallyl monosulfide eluting at 4.62± 1.12 min, a major disulfide peak at 5.15± 2.60 and a minor at 8.58± 1.20 min and trisulfide at 6.6± 1.0 min. The dominant diallyl disulfide in hexane-extract shallot was 325.5± 42.17 mg per gram of extract, with the slightly lower value of 283.5± 28.12μg/g for garlic extract. The levels of other sulfides in μg per gram of hexane-extract garlic were diallyl mono-sulfide, 169.5± 21.56 and tri-sulfide 261.57± 12.98. In previous study, the concentration of diallyl mono-, di-, and tri-sulfides in garlic product from steam-distillation were higher than in this study (87± 64, 1,134± 94, and 810± 96μg/g of product respectively) (Lawson et al., 1991).

From this study, we can conclude that Thai shallot give an activity on protection of human erythrocytes that is possible to be affected from external or internal radicals such as H2O2 or peroxyl radical such as AAPH. Especially it is able to inhibit lipid peroxidation and glutathione depletion in erythrocytes. This study suggested that the Thai shallot extracts have protective effect on the GSH deterioration in vitro from protein hydroperoxide (PrOOH) or hydroxyl radical from gamma irradiation (Leelarungrayub et al., 2004a). Thai shallot extracts can also protect and scavenge the protein and lipid hydroperoxide (LOOH) formation in vitro study (Leelarungrayub et al., 2003). In addition, we also found the interested activity of Thai shallot extract from crude powder (crushing and pressing) increasing the total glutathione synthesis and inhibited the hydroperoxide and nitric oxide production in monocytic cell line (U937) from either lipoplysaccharide(LPS) or H_2O_2 stimulation (Leelarungrayub et al., 2004b). Thus Thai shallot extracts either hexane-extraction or crude showed an anti-oxidant and anti-inflammatory activity that possible used for promotion human health or clinical treatment in the future.

CONCLUSION

From this study, we conclude that Thai shallot gives an activity on protection of human erythrocyte lysis, affected from external or internal radicals such as H_2O_2 or peroxyl radical such as AAPH. It also inhibits lipid peroxidation and glutathione depletion in erythrocytes.

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