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Full Length Research Paper

Antioxidant effects of anthocyanins-rich extract from black sticky rice on human erythrocytes and mononuclear leukocytes

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The present study was aimed at determining the optimum dose of anthocyanins-rich extract (ARE) from Thai pigmented rice for its antioxidant effects. Three different methods of antioxidant analysis [Oxygen Radical Absorbance Capacity (ORAC) assay, Folin Cioculteau Phenol (FCP) assay and Vanillin assay] were used and the results obtained were excellently correlated (r > 0.98). Among red rice, black rice and black sticky rice, black sticky rice had the highest antioxidant level within the range 1368.34 ± 16.85 TE mM /kg dry mass, 922.03 ± 3.84 mM GE/kg dry mass and 218.97 ± 0.74 CE mM/kg dry mass, respectively. ARE with the highest antioxidant level was used in the following tests: Hemolysis test and Heinz body formation, which were used to assess the antioxidant activities of ARE on human erythrocytes, respectively. Comet assay was also used to assess the antioxidant activities of ARE on mononuclear leukocytes and it was found that the optimum dose of ARE (600 mg/L) inhibited hemolysis induced by 2,2'-Azobis(2-amidinopropane) dihydrochloride and Heinz body formation induced by N-acetylphenylhydrazine. Moreover, 200 - 1000 mg/L of ARE was able to inhibit hydrogen peroxide-induced genotoxicity in mononuclear leukocytes in a dose-dependent manner. Nevertheless, high dose of ARE (≥ 800 mg/L) induced cytotoxicity and genotoxicity by itself. The results strongly suggested that optimum dose of ARE was beneficial for health promotion by reducing oxidative stress in cellular model.

Key words: Antioxidant, pigmented rice, comet assay, hemolysis, Heinz body.

INTRODUCTION

Rice (*Oryza sativa* L. indica) is a major rice cereal crop in the developing world and important in Asian countries, especially Thailand, the world's largest rice exporter. Pigmented rice, an enriched source of flavonoids, is widely used for therapeutic purposes in traditional and folk medicine and gradually becoming popular in Thailand.

The major flavonoids of pigmented rice are anthocyanins. Two anthocyanins, cyanidin 3-glycoside and peonidin 3-glycoside, have been identified as the major polyphenolic compounds extracted from black rice (Zhang et al., 2006). Experimental and clinical studies have reported a positive correlation between cardiovascular health status and consumption of black rice and/or black rice pigments. This particular rice possesses protective effects through many mechanisms, including antioxidant, anti-inflammatory, anti-proliferation, lipid lowering effects. Antioxidant properties of black rice have

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been found to exert though not only the induction of superoxide dismutase, catalase (Chiang et al., 2006) and glutathione peroxidase activities (Auger et al., 2002), but also the suppression of reactive oxygen species (ROS) and nitric oxide radicals in clinical and biological model systems (Hu et al., 2003). Supplementation of black rice fraction has been shown to result in numerous advantages including improvement of antioxidant and anti-inflammatory status in patients with coronary heart disease, reduction of oxidative stress and inflammation (Wang et al., 2007), decrease in plasma lipid levels and alleviation of atherosclerotic lesions in animal models (Ling et al., 2002). However, certain flavonoids have been found to act differently depending on their doses, particularly cytoprotective effect at low dose, while cytotoxic effect and DNA strand breakage at high dose (Wätjen et al., 2005).

The present investigation aimed to determine the optimum dose of anthocyanins-rich extract (ARE) from Thai pigmented rice for its antioxidant effects. Human fresh blood cells were used to test antioxidant effect as *in vitro* models.

MATERIALS AND METHODS

Chemicals and reagents

N-Acetylphenylhydrazine (APHZ), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), hydrogen peroxide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, catechin, Folin Ciocalteu's phenol reagent, fluorescien, ethidium bromide and Titan-X 100 were purchased from Sigma-Aldrich, St. Louis, MO, USA. Low temperature melting point agarose gel and normal temperature melting point agarose gel were purchased from Invitrogen Co., Carlsbad, CA, USA. All other basic reagents were of analytical grade.

Preparation of ARE from Thai pigmented rice

ARE fractions were prepared from three groups of pigmented rice including; 7 kinds of red rice, 7 kinds of black rice and 7 kinds of sticky rice. They were bought from supermarkets in Bangkok. In brief, 100 g of rice was milled and extracted in 2000 ml of 80% methanol in water. This crude extract was refluxed at 75 - 78°C for 3 h, shaken in ultrasonic bath for 30 min, cooled, stored in the dark at 4 - 8°C for 7 days and removed the sediment by centrifugation at 1006 × g for 15 min. The extracts were concentrated using vacuum rotary evaporator at 50°C, freeze drying and kept in deep freezer at -80°C until used.

Preparation of fresh whole blood

Human blood samples were obtained from The National Blood Centre, Thai Red Cross Society, Bangkok, Thailand. Fresh blood was collected in heparinised tubes and centrifuged at 252 × g for 3 min. Most of plasma was carefully removed by aspiration in order to obtain a hematocrit of approximately 85% (packed red blood cells; RBCs) for hemolysis test and Heinz body formation, and fresh whole blood was used in Comet assay. The blood was stored at 4°C and used within 3 h.

Determination of antioxidants in pigmented rice extracts

To investigate antioxidant levels in herb, vegetable and fruit, ORAC assay and FCP assay are the standard methods for determination of antioxidant properties as described by Huang et al. (2005) and add one more method, Vanillin assay.

Oxygen radical absorbance capacity assay (ORAC assay)

The ORAC assay was used to measure the oxidative degradation of the fluorescent molecules after being mixed with free radical generators, AAPH. Antioxidant is able to protect the fluorescent molecules from the oxidative degradation. This assay was performed with some modification (Davalos et al., 2004). Briefly, AAPH 153 mM in 75 mM phosphate buffer (pH 7.4) was freshly made daily. A fluorescein stock solution (40 µM) was made in 75 mM phosphate buffer (pH 7.4) and stored at 4°C. Immediately prior to used, the stock solution was diluted 1:1000 with 75 mM phosphate buffer (pH 7.4). To a 96 well plate, in addition blank wells received 25 µl of 75 mM phosphate buffer (pH 7.4) while standards received 25 µl of Trolox solution (10, 20, 30,40, 60, 80 µM/ml) and sample received 25 µl of diluted sample. Then add 150 µl of working sodium fluorescein solution, mixed 5 s. The plate was equilibrated by incubating for a minimum of 30 min at 37°C in the Wallac 1420 VICTOR^2 Multilabel Counter (Perkin Elmer Life and Analytical Sciences, Finland). Reaction was initiated by 25 µl of AAPH solution and followed by shaking for 10 s. The fluorescence was then monitored kinetically with data taken every minute by using 485 nm and 535 nm for excitation and emission, respectively. The plate reader 1420 VICTOR^2 was controlled by Wallac 1420 Software Version 3.00. The fluorescence of each well was measured every minute for 35 min. ORAC values were calculated by using area under curve (AUC). The AUC and the Net AUC of the standards and samples were determined using Wallac 1420 Software with the equations:

$$\text{Net AUC}_{\text{Standard}} = \text{AUC}_{\text{Standard}} - \text{AUC}_{\text{blank}}$$

$$\text{Net AUC}_{\text{sample}} = \text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}$$

The loss in fluorescence can be assessed by measuring the area under the curve (AUC) of the kinetic plot for each concentration. The greater the extent of florescent decay, the smaller the expected AUC value would be. When Net AUC were calculated from these kinetic curves and plotted against Trolox concentration, a linear relationship is observed. The results were expressed as Trolox equivalents (TE) mM/kg of dry mass.

Folin cioculteau phenol assay (FCP assay)

Modified Folin Cioculteau Phenol assay (Singleton et al., 1999) is used to determine reducing properties of phenolics contents. Briefly, 500 µl of samples or standards was mixed with 500 µl of 10% FCP reagent, let stand for 20 min, add 10 mM Na_2CO_3 350 µl, let stand for 20 min for the solution to turned blue color and then measured

the absorbance at 750 nm by using gallic acid 1.562, 3.125, 6.25, 12.5, 25 and 50 µg/ml as the standards. The results were expressed as Gallic acid equivalents (GE) mM/kg of dry mass. This method was carried out by using Shimadzu UV 1601 Spectrophotometer.

Vanillin assay

Proanthocyanins content in pigmented rice was measured by using Vanillin method (Sun et al., 1998). It is the method of depolymerization with concentrate sulfuric acid, based on the transformation of proanthocyanins into anthocyanidins. Briefly; mixed 1 ml of sample or standards with 2 ml vanillin reagent (1 g of vanillin in 100 ml of 70% H_2SO_4), and incubated at 20°C. After exactly 15 min, the absorbance of pink solution was measured at 500 nm by using catechin 2, 5, 10, 20, and 40 µg/ml as the standards. The results were expressed as catechin equivalent (CE) mM/kg of dry mass. This method was carried out by using Shimadzu UV 1601 Spectrophotometer.

Determination of AAPH-induced oxidative damage of erythrocyte membrane

Since a peroxyl radical initiator, AAPH, has been proved to cause an oxidation of cell membrane proteins and lipids resulting in lysis of red blood cells (RBCs), oxidative hemolysis induced by AAPH was used as a tool in the present study according to the previously described method (Niki et al., 1988) with some modification. Briefly, 100 µl of fresh RBCs was mixed with 1.0 ml of incubated medium [phosphate buffer saline (PBS), pH 7.4, supplemented with ARE (0 - 1000 mg/L as 0 = control)] either in the presence or absence of 100 mM AAPH. Then, the reaction mixtures were incubated for 4 h at 37°C with gentle shaking under aerobic condition. During incubation period, 0.1 ml of the reaction mixture was withdrawn to 1.5 ml of ice-cold PBS at 0, 30, 60, 90, 120, 150, 180, 210 and 240 min and subsequently centrifuged at 1006 × g for 10 min. The extent of hemolysis was determined by measuring the absorbance of hemolysate at 540 nm with Shimadzu UV 1601 Spectrophotometer.

Determination of APHZ-induced Heinz body formation in cytosol of erythrocytes

APHZ was used as a free radical initiator inside RBCs to oxidize proteins mostly hemoglobin based on the modification of protocol described previously (Sangkitikomol et al. 2001; Calabrese et al., 1982). Briefly, 100 µl of RBCs was mixed with 1.0 ml of reaction mixture [2 g/L of glucose in phosphate buffer (1.3 parts of 1/15 M of KH_2PO_4 and 8.7 parts of 1/15 M of Na_2HPO_4, pH 7.4)] supplemented with ARE (0 - 1000 mg/L as 0 = control). Thereafter, addition of 1 ml of reaction mixture with the presence and absence of 2 g/L of APHZ was performed, and incubation in the dark was carried out for 2 h at 37°C with gentle shaking under aerobic condition. Finally, Heinz bodies in RBCs were examined by staining with crystal violet solution [3 drops of 10 g/L of crystal violet in 0.73% of normal saline] using 3 drops of RBCs suspension, and incubating for 5 min at room temperature. After blood smears on glass slides were made and air dried, they were counted for RBCs with Heinz body inside/1000 of RBCs using light microscope at 1000 magnifications. The results were reported in % of Heinz body formation. If it was found hemolysis, the reaction mixture would turn brown color because of oxidized hemoglobin leakage.

Determination of H_2O_2-induced DNA damage of mononuclear leukocytes using Comet assay

To study H_2O_2-induced DNA oxidative damage in human mononuclear leukocytes, Comet assay or single cell gel electrophoresis (Singh et al., 1988) was used. Human mononuclear leukocytes in 80 µl of fresh whole blood was mixed with 1.0 ml of phosphate buffered saline (PBS) supplemented with ARE (0 - 1000 mg/L as 0 = control), either in the presence or absence of oxidant agent (10 mM H_2O_2) and incubated in the dark for 2 h at 4°C. The cells were washed with ice-cold PBS, and they were subjected to single cell gel electrophoresis. Briefly, microscopic slides were covered with 150 µl of 1% high melting point agarose in Ca^{2+}- and Mg^{2+}-free PBS and kept at room temperature to allow the agarose to solidify. The cells were mixed with 100 µl of 1% low melting point agarose (LMA) at 40°C, rapidly pipetted onto the first agarose layer, spread out with a cover slip and maintained at 4°C. Upon solidification of the second layer and removal of the cover slip, 120 µl of 1% LMA at 40°C was added and spread out with a cover slip. The slides were then immersed in cold lysing solution [2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris buffer, pH 10 with 1% Triton X-100 and 10% DMSO added just before use] for at least 1 h in the dark at 4°C. Slides were rinsed for 20 min in three changes of an alkaline rinsing solution (10 N NaOH, 200 mM EDTA, pH > 13) to allow unwinding of DNA. These slides were placed in chamber filled with fresh alkaline rinsing solution, and subjected to electrophoresis at 300 mA for 25 min. The slides were rinsed 3 times with neutralization buffer (0.4 M Tris buffer, pH 7.5), drained and stained with 65 µl of 20 mg/L ethidium bromide for image analysis. The nuclei were examined under the fluorescence microscope. Randomly selected 200 nuclei per 2 slides were analyzed. Nuclei images were appeared after electrophoresis liked a comet shape and the longer tail implied more severe damage of DNA.

Statistical analysis

The data represented the mean ± SEM. The values of antioxidant in 3 different methods; ORAC assay, FCP assay and Vanillin assay were compared by using one-way ANOVA and student t-test. The Comet assay result is presented as percentage of each class and analyzed with the chi-square test. P value was two tailed, and $P < 0.05$ was considered statistically significant when comparing between data sets.

RESULTS

Antioxidant levels in pigmented rice extracts

Among 3 different methods for antioxidant determination, the correlation analysis (r) between each method was calculated to be 0.983 for ORAC assay and FCP assay, 0.984 between ORAC assay and Vanillin assay, and 0.992 between FCP assay and Vanillin assay. Antioxidants of ARE as measured by ORAC assay, FCP assay and Vanillin assay varied considerably with the varieties of rice. Pigmented rice extracts with the highest to the

Table 1. Comparison of total antioxidants of 3 groups of pigmented rice extracts among 3 methods of analysis (ORAC assay, FCP assay and Vanillin assay).

Pigmented rice extracts (ARE)	ORAC assay (TE mM/Kg dry mass) (Mean ± SEM)	FCP assay (GE mM/Kg dry mass) (Mean ± SEM)	Vanillin assay (CE mM/Kg dry mass) (Mean ± SEM)
Group 1. Black rice			
No. 1	564.84 ± 14.87	347.46 ± 0.67	57.70 ± 0.92
No. 2	476.02 ± 25.16	282.96 ± 2.70	35.44 ± 0.83
No. 3	454.04 ± 20.00	240.07 ± 0.78	30.57 ± 0.38
No 4	427.84 ± 14.20	199.56 ± 0.98	16.77 ± 0.03
No. 5	422.85 ± 15.37	215.77 ± 1.84	21.32 ± 0.63
No. 6	419.65 ± 17.87	240.82 ± 0.26	21.84 ± 0.24
No. 7	405.94 ± 22.31	226.81 ± 4.19	22.14 ± 0.31
Mean	446.22 ± 20.98	250.50 ± 18.91	29.40 ± 8.40
Group 2. Red rice			
No. 1	724.66 ± 21.03	475.03 ± 2.22	102.88 ± 0.11
No. 2	658.22 ± 22.46	516.83 ± 3.00	94.19 ± 0.11
No. 3	529.89 ± 10.69	393.88 ± 0.91	72.22 ± 0.02
No. 4	513.75 ± 26.94	364.69 ± 1.54	71.06 ± 0.46
No. 5	504.30 ± 4.38	359.51 ± 2.16	65.30 ± 0.26
No. 6	491.31 ± 20.18	357.07 ± 4.77	53.89 ± 0.73
No. 7	371.18 ± 11.07	233.38 ± 2.23	34.77 ± 0.07
Mean	525.43 ± 46.33	385.77 ± 34.64	70.62 ± 8.72
Group 3. Black sticky rice			
No. 1	1368.34 ± 16.85	922.03 ± 3.84	218.97 ± 0.74
No. 2	1254.01 ± 13.05	864.68 ± 3.09	210.96 ± 1.80
No. 3	1199.93 ± 30.27	737.12 ± 0.80	191.11 ± 0.78
No. 4	1095.09 ± 34.15	723.99 ± 2.20	163.54 ± 0.75
No. 5	985.19 ± 17.40	646.19 ± 4.43	144.23 ± 0.52
No. 6	898.13 ± 31.46	551.34 ± 3.68	126.42 ± 0.11
No. 7	722.96 ± 6.33	471.88 ± 2.38	111.96 ± 0.97
Mean	1074.56 ± 83.95	702.46 ± 60.82	166.74 ± 15.75

Every sample was done in triplicate analyses. The values of antioxidant contents were compared among three methods. The results were significantly correlated:
1. Comparison between ORAC assay and FCP assay, $r = 0.983$, p value < 0.01.
2. Comparison between ORAC assay and Vanillin assay, $r = 0.984$, p value< 0.01.
3. Comparison between Vanillin assay and FCP assay, $r = 0.992$, p value < 0.01.

lowest antioxidant levels were black sticky rice, red rice and black rice, respectively (Table 1). Black sticky rice extract with the highest antioxidant within the range 1368.34 ± 16.85 TE mM/kg dry mass, 922.03 ± 3.84 GE mM/kg dry mass and 218.97 ± 0.74CE mM/kg dry mass, respectively was used to study the antioxidant effect of ARE on lipids, proteins and DNA oxidation in fresh blood cells.

The effects of ARE on oxidative damage to lipids and proteins of erythrocytes

The protective effect of ARE on oxidative damage to lipids and proteins induced by free radical initiators, AAPH and APHZ, was studied. ARE in the 100 - 600 mg/L concentration range could inhibit oxidative damage to proteins and lipids induced by AAPH according to the hemolysis

test (Figure 1A). Upon incubation of RBCs with 800 -1000 mg/L of ARE, moderate induction of AAPH-mediated hemolysis was found, while the same concentration range of ARE resulted in severe hemolysis in the absence of AAPH (Figures 1A and B). Heinz body formation in RBCs induced by APHZ was completely inhibited by 600 mg/L of ARE, whereas, inhibition in the presence of 100 - 500 mg/L of ARE was not found. The presence of 700 mg/L of ARE could induce partial hemolysis and completely inhibit Heinz body formation at the same time. At this high concentration, ARE had cytotoxic effect on cell membrane and also had antioxidant effect by obstructing the Heinz body formation inside RBCs (sample 14, Table 2). ARE in the high concentration range (800 - 1000 mg/L) induced complete hemolysis in both treated and untreated RBCs with APHZ (Table 2), and these concentrations resulted in the similar results when using AAPH as an initiator (Figure 1).

The effect of ARE on H_2O_2-induced DNA oxidative damage of mononuclear leukocytes

The distribution of different grades of DNA damage in mononuclear leukocytes treated with H_2O_2 and ARE was analyzed (Table 3). The presence of H_2O_2 significantly increased DNA damage in mononuclear leukocytes, showing 2% undamaged cells and 98% damaged cells. The number of damaged nuclei significantly reduced when the cells were treated with ARE. Pretreated cells with 200, 400, 600, 800 and 1000 mg/L of ARE significantly increased the numbers of undamaged cells to 54.5, 62.5, 69, 76, 82.5%, respectively ($P < 0.05$). Comet assay showed antioxidant effect of ARE on DNA oxidative damage in mononuclear leukocytes in a dose-dependent manner. However, ARE in the 800 - 1000 mg/L concentration range generated slight damage to DNA by itself. These findings suggested that ARE in the high concentrations might not be appropriated for health-promoting purposes.

DISCUSSION

Determination of antioxidant contents in ARE with 3 different methods, ORAC assay, FCP assay and Vanillin assay, the values of antioxidant contents in ARE showed varied widely in antioxidant levels. Nevertheless, there was excellent relationship between the results from the FCP assay and Vanillin assay which gave a good measure of the capacity of a wide range of antioxidants. The relationship between antioxidants in ORAC assay and FCP assay and between those in ORAC assay and Vanillin assay were very good. To study the effect of ARE against oxidative damage to lipids and proteins in human RBCs, free radical initiators, AAPH and APHZ, were employed for inducing oxidative stress. AAPH was a source of free radical formation capable of inducing oxidation of lipid and protein structurally located on erythrocyte membrane (Chaudhuri et al., 2007), while APHZ was a source of free radical formation inside cytosol of RBCs leading to induction of proteins mostly hemoglobin and lipid bilayer membrane oxidation (Sangkitikomol et al., 2001). The results showed that RBCs were resistant to AAPH-induced oxidation when ARE in the 100 - 600 mg/L concentration range were supplemented. Flavonoids may regulate cell metabolism by modulating the bilayer state since they have been demonstrated to control cell signal pathways by targeting receptors on the cell surface or by intercalating the lipid bilayer of membranes (Tarahovsky et al., 2008). In addition, interactions of flavonoids at the surface of bilayer through hydrogen bonding have been shown to be crucial in reducing the accessibility of oxidants, thus protecting the structure and function of membranes (Oteiza et al., 2005). That might be the reason to explain the capability of appropriate concentration range of ARE for inhibiting AAPH-induced hemolysis. Nonetheless, ARE in the 100 - 500 mg/L concentration range could not inhibit Heinz body formation induced by APHZ. This finding suggested that the process of proteins and lipids oxidation was still taking place as a result of insufficiency of absorbed ARE inside RBCs to inhibit oxidation, while ARE in the higher concentration like 600 mg/L was sufficient to completely inhibit the Heinz body formation. ARE in the concentration of 700 mg/L appeared to be too high since they induced partial and complete hemolysis of RBCs in APHZ-treated and APHZ-untreated RBCs, respectively. This concentration of ARE had both toxic effect on cell membranes (samples 14 and 6, Table 1) and antioxidant effect on hemoglobin oxidation induced by APHZ. ARE at higher concentrations (800 - 1000 mg/L) induced hemolysis in treated and untreated RBCs with either AAPH (Figure 1) or APHZ (Table2). However, the severity of hemolysis in AAPH-treated RBCs was less than that of AAPH-untreated ones. The correlation between an increased antioxidant activity and a protective effect has not been positively proved because various factors such as formation of ROS, antioxidant localization and other mediators may also influence the cellular activity (Williams et al., 2004).

Regarding the effect of ARE on H_2O_2-induced DNA damage in mononuclear leukocytes, it was found in the present study that ARE had antioxidant effect at low concentrations (200 - 600 mg/L), and that particular effect was dependent on a concentration of ARE. However,

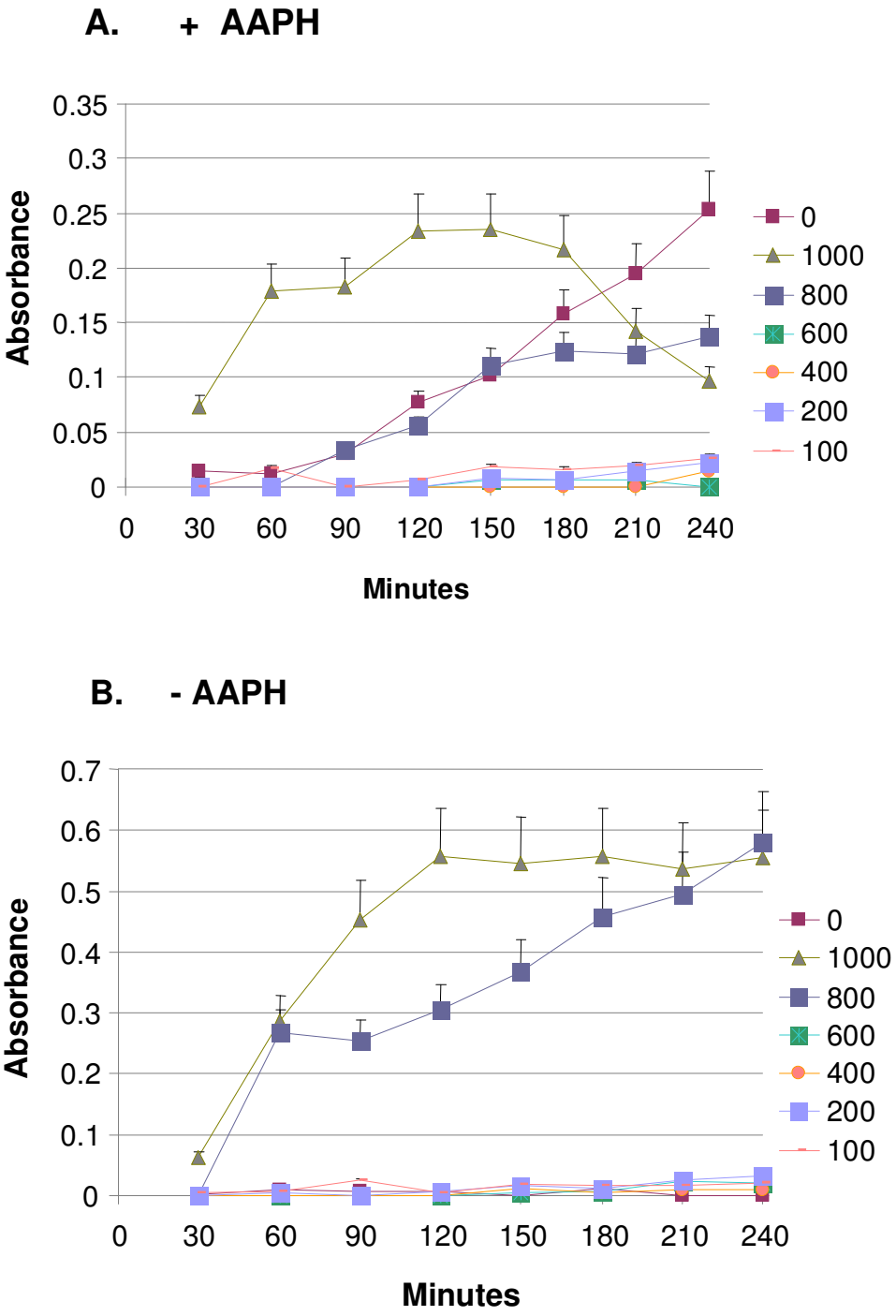

Figure 1. The absorbance of hemoglobin leaking from RBCs supplemented with different concentrations of ARE (0 - 1000 mg/L, 0 = control) either in the presence (A) or absence of 100 mM AAPH (B) at the end of experimental period. Optimum concentrations of ARE (100 - 600 mg/L) could inhibit lipid and protein oxidation by showing undetectable to low amount of hemoglobin when comparing to positive control (RBCs treated with AAPH), (A). High concentrations of ARE (800-1000 mg/L) showed cytotoxic effect on RBCs due to an induction of hemolysis (A and B). Values are means with their standard error of means (SEM) depicted by vertical bars. All experiments were performed in triplicate (n = 3).

Table 2. The antioxidant effect of ARE on inhibition of Heinz body formation in erythrocytes induced by N-acetylphenylhydrazine and cytotoxic effect on induction of hemolysis.

Sample treatment	Total number of RBCs count (1000 cells)	% RBCs with Heinz bodies
1. ARE 0 mg/L (Negative control)	1000	0
2. ARE 100 mg/L	1000	0
3. ARE 200 mg/L	1000	0
4. ARE 400 mg/L	1000	0
5. ARE 600 mg/L	1000	0
6. ARE 700 mg/L	0 (hemolysis)	No intact RBCs
7. ARE 800 mg/L	0 (hemolysis)	No intact RBCs
8. ARE 1000 mg/L	0 (hemolysis)	No intact RBCs
9. ARE 0 mg/L + APHZ (Positive control)	1000	100
10. ARE 100 mg/L + APHZ	1000	100
11. ARE 200 mg/L + APHZ	1000	100
12. ARE 400 mg/L + APHZ	1000	100
13. ARE 600 mg/L + APHZ	1000	0
14. ARE 700 mg/L + APHZ	100 (partial hemolysis)	0
15. ARE 800 mg/L + APHZ	0 (hemolysis)	No intact RBCs
16. ARE 1000 mg/L + APHZ	0 (hemolysis)	No intact RBCs

Each sample was evaluated in triplicate. Sample = Red blood cells suspension in glucose phosphate buffer; RBCs = red blood cells; ARE = anthocyanins-rich extract; APHZ = N-acetylphenylhydrazine.

Table 3. The effect of ARE on H_2O_2-induced DNA oxidative damage of mononuclear leukocytes (Comet assay).

Samples	Percent of nuclei in class				
	No damaged nuclei Class 0 (%)	Damaged nuclei			
		Class 1 (%)	Class 2 (%)	Class 3 (%)	Class 4 (%)
1. Cells (Negative control)	100	0	0	0	0
2. Cells + ARE 200 mg/L	100	0	0	0	0
3. Cells + ARE 400 mg/L	100	0	0	0	0
4. Cells + ARE 600 mg/L	100	0	0	0	0
5. Cells + ARE 800 mg/L	96.5	0.5	1.5	0.5	1
6. Cells + ARE 1000 mg/L	97.5	0.5	1	0.5	0.5
7. Cells + H_2O_2 (Positive control)	2	18	25.5	20.5	34
8. Cells + ARE 200 mg/L + H_2O_2	54.5	10	13.5	9	13
9. Cells + ARE 400 mg/L + H_2O_2	62.5	7	4.5	6	20
10. Cells + ARE 600 mg/L + H_2O_2	69	7	5	7.5	11.5
11. Cells + ARE 800 mg/L + H_2O_2	76	5.5	4	6	8.5
12. Cells + ARE 1000 mg/L + H_2O_2	82.5	7	2.5	3.5	4.5

Cells = Mononuclear leukocytes, ARE = anthocyanins rich extract. The Cells were supplemented with different concentrations of ARE (0 - 1000 mg/l) either in the presence or absence of oxidant agent (10 mM H_2O_2) at the end of experimental period. Two hundreds nuclei were examined in each treatment. DNA damage was classified into 5 classes; 0 = no damaged nucleus, 1 = low damage, 2 = moderate damage, 3 = high damage, and 4 = severe damage. Data are presented as percentage.

ARE in the concentrations ≥ 800 mg/L enhanced the DNA oxidative damage. Oxidative stress of RBCs (enucleated cells) in hemolysis test and oxidative stress of mononuclear leukocytes (nucleated cells) in Comet assay have broadly been acknowledged as good approaches for testing ARE because of their simplicity with regard to

experimental approach and data interpretation. Therefore, the toxic effect of ARE on normal RBCs and mononuclear leukocytes may be due to their inhibitory or stimulatory actions via kinase signaling pathways which are likely to affect cellular function by altering the phosphorylation state of target molecules (Williams et al., 2004). Other studies indicated that lower concentrations of flavonoids (nM to low µM) could lead to antioxidant response element-mediated gene expression, including that of phase II detoxifying enzymes. In contrast, higher concentrations of flavonoids may sustain activation of mitogen-activated protein kinases or stress activated protein kinases, which could induce apoptosis (Chen et al., 2000; Kong et al., 2000).

In conclusion, the results strongly suggested that optimum dose of ARE was beneficial for health promotion by reducing oxidative stress in cellular model. The right dose of ARE may be good for development of a functional food.

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