Inhibitory Effects of Some Thai Plant Extracts on AAPH-induced Protein Oxidation and Protein Glycation

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Received 19 September 2006; accepted 26 March 2007

Abstract

Oxidants can cause damage to biomolecules and alter cellular metabolism. Keys among the targets for oxidative damage are structural proteins and enzymes. It is expected that several constituents from Thai plants may be able to prevent such oxidative damages. The present study was carried out to investigate the inhibitory activity on 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced protein oxidation and nonenzymatic protein glycation of Thai medicinal plant extracts, including the extracts of *Phyllanthus emblica* Linn., *Kaempferia parviflora*, and *Globba wintii* C.H. Wright. The plant extracts were investigated for their inhibitory activities on AAPH-induced protein oxidation to allophycocyanin. The loss of allophycocyanin fluorescence was measured and interpreted as a half-life of the protein. The half-life of allophycocyanin in the presence of 1.0 μg/ml of *P. emblica*, *K. parviflora* and *G. wintii* C.H. Wright extracts were 21.24, 11.31, and 11.39 minutes, respectively. The assay of protein glycation at 37 ^{o}C for 2 weeks was also performed. The percent inhibition of protein glycation by the plant extracts was reported. *P. emblica* achieved 88.09 % inhibition against protein glycation formation at 10.0 μg/ml whereas, *K. parviflora* and *G. wintii* C.H. Wright achieved 28.10 % and 15.74 % inhibition at 50 μg/ml, respectively. Among the tested extracts, *P. emblica* Linn. extract had higher oxidative protein damage protection and higher protein glycation inhibitory effect than *K. parviflora* and *G. wintii* C.H. Wright extracts. Thus, it has a higher potential use as anti-aging therapy.
Keywords: Glycation; Protein oxidation; Medicinal plant; AAPH

Introduction

Several oxidants such as reactive oxygen species (hydrogen peroxide, superoxide radical and etc.) and reactive nitrogen species are implicated in mediating a wide array of human diseases including atherosclerosis, cancer, diabetes, parkinson and neurodegenerative diseases. Oxidants contribute to disease processes by causing damage to biomolecules and altering cellular metabolism. Keys among the targets for oxidative damage are structural proteins and enzymes.

The aldehyde or ketone groups of reducing sugars react nonenzymatically with the free amino groups of proteins, lipids and nucleic acids leading to the formation of advanced glycation endproducts (AGEs) (Basta et al., 2004). It is an important biochemical abnormality that accompanies diabetes mellitus. The formation and accumulation of AGEs are characteristic features of aged or diabetic tissue and these products also have been strongly implicated in the pathogenesis of diabetic micro- and macrovascular complications. AGEs structure is a covalently cross-linked formation. Proteins affected by this process are usually stable and long lived, such as collagen. The chemistry behind the cross-link formation is complex and not fully understood. The pathological cross-link formation induced by AGEs lead to increased stiffness of the protein matrix, hence blocking function as well as increasing resistance to removal by proteolytic enzymes, and in turn affecting the process of tissue remodeling. These changes with advancing age are accelerated in diabetes. AGEs may generate atherogenesis by oxidizing low density lipoproteins (LDL) and causing changes in the collagen. Proteins as well are the major target biomolecules of such reactive free radical species. Inhibition of AGEs formation and the protective effect of protein oxidation by the natural compounds, therefore, bring great attention to the scientists.

Several plant extracts and compounds could inhibit the protein glycation and free radical-induced protein oxidation as reported by several researchers (Jedsadayanmata, 2005;

Lahet et al., 2003; Nakagawa et al., 2002; Yokozawa & Nakagawa, 2004). In Thailand, traditional practitioners also used several medicinal plants for anti-aging therapy. Among those plants, *Phyllanthus emblica* Linn., *Kaempferia parviflora* and *Globba wintii* C.H. Wright are well-known.

K. parviflora Wall. Ex Baker (Kra-Chai-Dam), the rhizomes have been known as health-promoting for longevity. It was also used for the treatment of colic disorder and duodenal ulcer (Rujjanawate et al., 2005; Yenjai et al., 2004). Nowadays, the fermented juices containing *K. parviflora* are popular in Thailand because it was believed to help relieve impotent symptoms.

P. emblica Linn. (Ma-Kham-Pom) is of great importance in traditional Asiatic medicine. In Buddha Scripture, it was used as a medicine of the monks for several symptoms. It is also used for the health promotion of ordinary people. It is one of the richest sources of natural ascorbic acid. In Ayurvedic (ancient Indian system of medicine), it has been used for treatment of several disorders such as common cold, scurvy, cancer and heart diseases (Cherallier, 1996; Summanen, 1999). In Thailand, *P. emblica* is traditionally used as an expectorant, antipyretic, diuretic, antidiarrheal and antiscurvy. This fruit is widely used by local Thais to quench the thirst when walking in the forest.

The leaves of the *G. wintii* C.H. Wright enriched with volatile oil. It was used as the anti-inflammatory during Thai massage. It can relieve muscle tension and pain. It can also alleviate the mood of the users.

In this study, the *P. emblica* Linn., *K. parviflora* and *G. wintii* C.H. Wright extracts were tested and discussed for the protein glycation and protein oxidation inhibitory effects.

Materials and Methods

Extract preparation

All of plant materials were purchased from a traditional plant market, Chiangdoew district, Chiang Mai, Thailand. The dried power of *P. emblica* (fruits, 1.5 kg) and *K. parviflora* (tuberous roots, 1.5 kg) were separately extracted with 95 % ethanol (2 L) for 10 hours by soxhlet apparatus. While the fresh leaves of *G. wintii* C.H. Wright (1.5 kg) were macerated with 95 % ethanol (2.5 L). The ethanol filtrate of each plant was evaporated under reduced pressure to give a syrupy mass. The percent yield of *P. emblica*, *K. parviflora*, and *G. wintii* C.H. Wright were 0.67 %, 0.84 %, and 0.33 %, respectively. All of sample extracts were kept at 4 ^{o}C until use.

Measurement of 2,2'-Azobis (2-amidinopropane) dihydrochloride-(AAPH) induced protein oxidation

The test was carried out using the modified method of Courderot-Masuyer et al. (1999). The reaction mixtures containing 37.5 nM allophycocyanin, 3 mM AAPH and various concentrations of the plant extracts in 75 mM phosphate buffer (pH 7.0) were incubated at 37 ^{o}C. The fluorescence intensity measured just prior to the addition of AAPH (radical generator) was used as the 100 % value prior to initiation of oxidative reaction for that sample. Loss of allophycocyanin fluorescent intensity was measured at 5, 10, 20 and 30 minutes on a fluorescence spectrophotometer (Shimadzu, RF-5300PC) with an excitation wavelength of 598 nm and an emission wavelength of 651 nm. Results were reported as the percentage of the initial fluorescence intensity and as a half-life of allophycocyanin, defined as the time required for 50 % loss of the observed protein fluorescence intensity.

Measurement of nonenzymatic protein glycation

Experiments were performed as described by Vinson and Howard (Vinson & Howard, 1996). Briefly, bovine serum albumin (BSA, 10 mg/ml) in phosphate buffer (50 mM, pH 7.4) containing 0.02% sodium azide was preincubated with plant extracts at various concentrations

at room temperature. Glucose (25 mM) and fructose (25 mM) solutions were added to the reaction mixture and incubated at 37 ^{o}C for two weeks. Fluorescent reaction products were then determined using a fluorescence spectrophotometer with an excitation wavelength of 350 nm and an emission wavelength of 450 nm. All incubations were repeated five times. Results were expressed as percentage inhibition of the formation of glycated proteins.

Statistical analysis

Data are presented as mean±S.E. of five determinations. Where appropriate, data were subjected to statistical analysis using ANOVA and post hoc comparison was done using Duncan multiple range test. All calculations were performed with the SPSS software. A p-value of less than 0.05 was considered statistically significant.

Results

Allophycocyanin test

Peroxyl radicals in this test were generated from the reaction of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) with oxygen in the atmosphere. When the different final concentrations of *P. emblica* Linn., *K. parviflora* and *G. wintii* C.H. Wright (at 1 and 5 µg/ml) were tested, the decrease in fluorescence of allophycocyanin was observed and shown in Figures 1, 2 and 3, respectively. In the absence of tested extracts (blank), a rapid loss of fluorescence intensity was observed, the value decreasing to nearly 0 % after about 30 minutes.

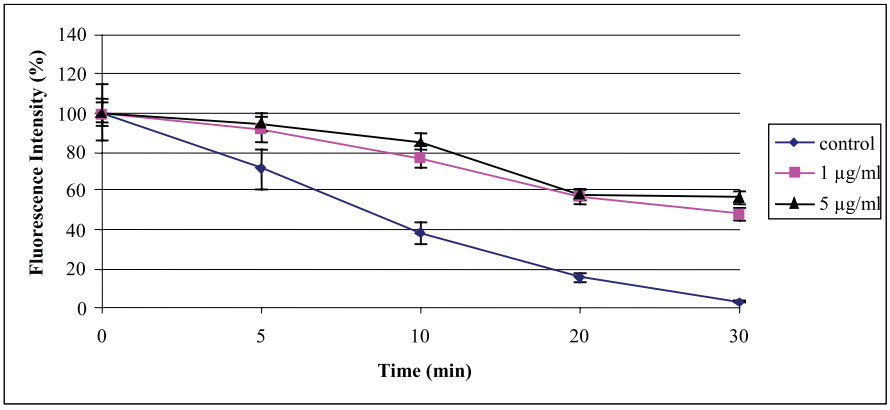

Figure 1. The time course of AAPH-induced a decrease in allophycocyanin fluorescence intensity in the presence of *P. emblica* Linn. extracts at 1 and 5 µg/ml.

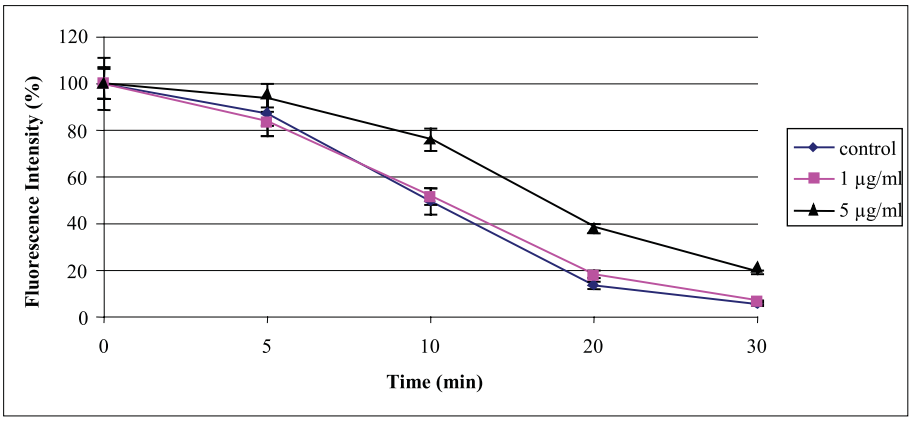

Figure 2. The time course of AAPH-induced a decrease in allophycocyanin fluorescence intensity in the presence of *K. parviflora* extracts at 1 and 5 µg/ml.

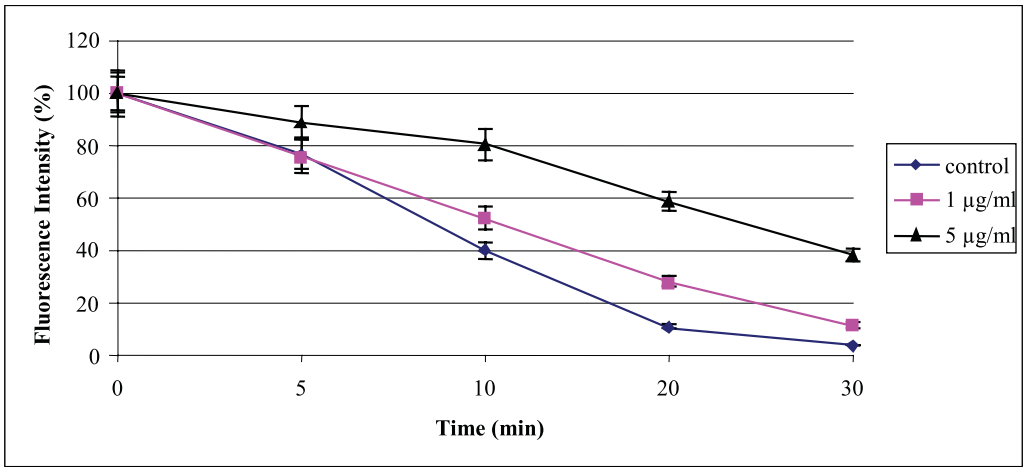

Figure 3. The time course of AAPH-induced a decrease in allophycocyanin fluorescence intensity in the presence of *G. wintii* C.H. wright extracts at 1 and 5 µg/ml.

For further clarification, the half-life of allophycocyanin defined as the time required for the 50 % loss of the initial fluorescence intensity was estimated. A longer half-life reflected the higher protective effect of the sample on oxidative stress. We found that the half-life of allophycocyanin in the absence of any tested extracts was about 8 minutes. However, the half-life became longer in the presence of sample extracts at various concentrations (Table 1). The extracts of *G. wintii* C.H. Wright at the concentration of 1 µg/ml was as potent as the extracts of *K. parviflora* at the same concentration. A higher concentration of *G. wintii* C.H. Wright (5 µg/ml) has a higher protective effect on AAPH-induced allophycocyanin damage than the *K. parviflora* extract at the same concentration. This might be due to the higher polyphenol content in the *G. wintii* C.H. Wright extract than that of the *K. parviflora* extract (unpublished data). The *P. emblica* Linn. extract showed the strongest protective effect on allophycocyanin damage at any concentration compared to the other two extracts. Thus, *P. emblica* Linn. has higher free radical scavenging ability and oxidative protein damage protection than *G. wintii* C.H. Wright and *K. parviflora* based on the assay used in this study.

Table 1. The half-life of allophycocyanin observed in various reaction mixtures

Sample	Concentration (µg/ml)	Allophycocyanin Half-life (min) (mean±SE)	p-value	
Control (no plant extract)	-	8.76±0.10	-	-
Phyllanthus emblica Linn.	1	21.24±0.16	0.00*	0.00**
	5	31.00±0.45	0.00*	
Kaempferia parviflora	1	11.31±0.19	0.00*	0.00**
	5	16.73±0.16	0.00*	
Globba wintii C.H. Wright	1	11.39±0.20	0.00*	0.00**
	5	24.34±0.39	0.00*	

*compared with the control group
**compared within groups

Inhibition of protein glycation

The protective effects of sample extracts on AGEs formation were determined from the decrease in fluorescence intensity of the reaction mixtures containing these extracts. Less fluorescence intensity indicated less AGEs formation. The percentage of fluorescence intensity compared to the blank (without sample extracts) was used to evaluate the protective effect of the exrtacts on AGEs formation as shown in Table 2. In comparison to the control, all of the extracts showed significant inhibition on AGEs formation by the ANOVA test at $p<0.05$. According to Table 2, the inhibitory activity of the extracts on AGEs formation showed a concentration-dependent effect. The higher concentration of the sample extracts exerts the higher inhibition on AGEs formation. Among all of the tested compounds, *P. emblica* Linn. showed higher percentage inhibitions than the *K. parviflora* and the *G. wintii* C.H. Wright extracts. The values of the % inhibition on AGEs formation of the extracts were shown in Table 2. *P. emblica* Linn. was able to achieve more than 88.09 % inhibition against AGEs formation *in vitro* at the concentration of 10 µg/ml. Its effect was significantly higher than those of *K. parviflora* and *G. wintii* C.H. Wright at the same concentration and even at the higher concentrations of those latter two extracts.

Table 2. The inhibitory activities of sample extracts on AGEs formation

Sample extracts	Concentration (µg/ml)	%Inhibition (mean±SE)	p-value
Phyllanthus emblica Linn.	1	6.42±0.42	0.00
	2.5	15.26±1.11	
	5	54.93±1.13	
	10	88.09±0.42	
Kaempferia parviflora	10	1.69±0.28	0.00
	25	11.34±1.21	
	50	28.10±0.96	
Globba wintii C.H. Wright	10	4.03±0.23	0.00
	25	10.46±1.04	
	50	15.74±1.21	

Discussion

Allophycocyanin test

Allophycocyanin, as an organic part of some marine algae, is characterized by its natural fluorescence. In the presence of free radicals, its structural alteration takes place and fluorescence activity gradually decreases (Courderot-Masuyer et al., 1999; Lahet et al., 2003). The damage effects to this protein reflects the presence of oxidative stress. It is, therefore, used for the evaluation of the protective effects of sample extracts against oxidative damage of proteins caused by peroxyl radicals.

The fluorescence intensities of all the reaction mixtures containing the plant extracts were higher than the blank fluorescence intensity. It indicated that all of the plant extracts scavenged the free radical species generated by AAPH. With increasing concentration of each extract, the decomposition of allophycocyanin was delayed in a concentration-dependent manner. *P. emblica* Linn. showed the strongest scavenging ability as compared to the other sample extracts. The half-life of allophycocyanin, therefore, is a very useful parameter in determining oxidative stress protection on AAPH-induced allophycocyanin damage.

Inhibition of protein glycation

AGEs are a complex and heterogeneous group of compounds that have been implicated in several complications of diabetes such as diabetic nephropathy. Reducing sugars such as glucose and fructose, react non-enzymatically with amino groups in proteins, lipids and nucleic acids through a series of reactions forming Schiff bases and Amadori products to produce AGEs (Basta et al., 2004; Vinson & Howard, 1996). This process occurs over a period of weeks, thereby affecting long life proteins. In this study, we used BSA as a model because the most abundant protein in serum is albumin. Glucose and fructose when incubated with protein formed AGEs, which can be estimated in total by using their fluorescence intensity. In the present study, *P. emblica* Linn. extract showed the strongest inhibition on protein glycation as compared to the other plant extracts. Ascorbic acid was not found in the extract of *P. emblica* Linn. after being processed through refluxing in this experiment. Therefore, the higher polyphenol contents of the extracts of *P. emblica* Linn. (unpublished data) might play an important role for the higher inhibitory effect on protein glycation than the other extracts in this experiment.

Conclusion

Based on the present study, it was observed that the *P. emblica* Linn. extract has higher free radical scavenging ability and oxidative protein damage protection than *G. wintii* C.H. Wright and *K. parviflora* extracts. In addition, the *P. emblica* Linn. extract had a higher inhibitory effect on AGEs formation than *K. parviflora* and *G. wintii* C.H. Wright extracts. Further works will be conducted to clarify the fraction and compounds possessing such activities.

Acknowledgements

This work was supported by Grants-in-Aid of the National of Science and Technology Development Agency, Thailand. The authors would like to thank Mr. Prasit Suwannalert for assistance on editing manuscript. C. C. also thanks Faculty of Pharmacy, Chiang Mai University for supporting the research facilities.

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