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Protection from oxidative damage using *Bidens pilosa* extracts in normal human erythrocytes

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Abstract

Bidens pilosa (*B. pilosa*) is well known in Taiwan as a traditional Chinese medicine. The purpose of this study was to evaluate the ability of both the ethanol (EtOH) and ethylacetate/ethanol (EA/EtOH) extracts from the whole *B. pilosa* plant, to protect normal human erythrocytes against oxidative damage in vitro. It was determined that the oxidative hemolysis and lipid/protein peroxidation of erythrocytes induced by the aqueous peroxyl radical [2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)] were suppressed by both EtOH (50–150 μg/ml) and EA/EtOH (25–75 μg/ml) extracts of *B. pilosa* in concentration- and time-dependent manners. *B. pilosa* extracts also prevented the decline of superoxide dismutase (SOD) activity and the depletion of cytosolic glutathione (GSH) and ATP in erythrocytes. These results imply that *B. pilosa* may have protective antioxidant properties.

Keywords: *Bidens pilosa*; Antioxidants; Hemolysis; Glutathione; Superoxide dismutase

1. Introduction

Bidens pilosa (*B. pilosa*), a Asteraceae plant, widely distributed in almost all tropical and subtropical countries, is used in traditional medicine for the treatment of a variety of ailments, including diabetes (Dimayuga and Agundez, 1986; Winkelman, 1989; Lin, 1992), inflammation (Zamora-Martinez and de Pascual Pola, 1992), and hepatitis (Winkelman, 1989). The methanol extract of *B. pilosa* is effective for preventing increases in blood pressure induced by salt loading in rats (Dimo et al., 1999, 2002). Furthermore, *B. pilosa* is used as a remedy for treating stomach disorders such as peptic ulcer (Alvarez et al., 1999). Bioactive compounds isolated from *B. pilosa* reportedly possess antibiotic and antimalarial properties, and inhibit prostaglandin synthesis (Geissberger and Sequin, 1991; Rabe and van Staden, 1997). An extract of *B. pilosa* also exhibited significant DPPH free radical scavenging activity (Chiang et al., 2004). It has been reported that the hot-water extract of *B. pilosa* inhibited four leukemic cells (L1210, P3MR1, Raji, and K562) with IC_{50} values of below 200 μg/ml (Chang et al., 2001). A

Abbreviations: *B. pilosa*, *Bidens pilosa*; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; SOD, superoxide dismutase; GSH, glutathione; ROS, reactive oxygen species; MDA, malondialdehyde; HMW, high molecular weight; LMW, low molecular weight.

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recent investigation indicates that caffeic acid ethyl ester, a natural phenolic compound isolated from *B. pilosa*, inhibits LPS induction of cytokine, iNOS and COX-2 expression by blocking NF-κB activation in RAW 264.7 macrophages (Chiang et al., 2005).

Increasing evidence suggests that oxidative damage to cell components may play an important pathophysiological role in many types of human diseases (Ames et al., 1993; Edgington, 1994). Several reports have shown that erythrocyte attack of reactive oxygen species (ROS) is the key event in β-thalassemia, sickle cell anemia, glucose-6-phosphate dehydrogenase deficiency, and other hemoglobinopathies (Sadrzadeh et al., 1984; Rice-Evans et al., 1986; Vives Corrons et al., 1995). Erythrocytes, potentially powerful promoters of oxidative processes, are extremely susceptible to oxidative damage as a result of the high polyunsaturated fatty acid (PUFA) content of their membranes and the high cellular oxygen and hemoglobin (Hb) concentrations (Clemens et al., 1987; Scott et al., 1993). Malondialdehyde (MDA), the well-characterized product of the lipid peroxidation of erythrocytes, is a highly reactive and bifunctional molecule, which has been shown to cross-link erythrocyte phospholipids and proteins to impair a variety of the membrane-related functions, which ultimately lead to diminished erythrocyte survival (hemolysis) (Jain and Hochstein, 1980; Chiu et al., 1989; Hebbel et al., 1990; Sugihara et al., 1991). Further, erythrocyte lipid peroxidation may be involved in normal cell aging, and it has been associated with a variety of pathological events. Oxidants also produce alterations in erythrocyte membranes as manifested by a decreased cytoskeletal protein content (low-molecular-weight, HMW), and production of high-molecular-weight (HMW) proteins (Snyder et al., 1985; Flynn et al., 1983), which can lead to abnormalities in erythrocyte shape and disturbances in the microcirculation (Somer and Meiselman, 1993).

B. pilosa has been widely used in Taiwan as a traditional Chinese medicine and as a major ingredient of herbal tea (Chih et al., 1996). It has been claimed that the tea prevents inflammation and cancer. There is relatively little information, however, with respect to its antioxidant activity. Given the interesting biological properties and potential clinical applications of *B. pilosa*, in our research, ethanol (EtOH) and ethylacetate/ethanol (EA/EtOH) extracts from the whole plant of *B. pilosa* were used to study its antioxidant properties. In this paper, the two *B. pilosa* extracts were used to inhibit the oxidative hemolysis induced by aqueous peroxyl radicals [2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)] and lipid/protein peroxidation of human erythrocytes.

2. Materials and methods

2.1. Chemicals

The following reagents were obtained from Sigma Chemical Co. (ST. Louis, MO): sodium citrate, sodium chloride (NaCl), sodium phosphate dibasic (anhydrous) (Na_2HSO_4), bovine serum albumin (BSA), 2-thiobarbituric acid (TBA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), glutathione (GSH), and ethylenediaminetetraacetic acid (EDTA). Additionally, phosphoric acid (H_3PO_4), AAPH, and trichloroacetic acid were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Ammonium persulfate, bisacrylamide (30%), Coomassie Brilliant Blue R-250, sodium dodecyl sulfate (SDS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and dye reagent concentrate were obtained from the Bio-Rad Co. (Hercules, CA). Drabkin's reagent and SOD kit were obtained from Randox Laboratories Ltd. (Crumlin, UK).

2.2. Sample preparation

Fresh *B. pilosa* Linn. Var. *radiata* (Asteraceae) was harvested from the environs of the Academia Sinica campus in Taipei, Taiwan. A voucher specimen (No. 0211943) was deposited at the Herbarium of National Taiwan University, Taiwan. Two methods of *B. pilosa* extraction were used: (i) the whole *B. pilosa* plant was finely chopped and then percolated with 70% ethanol (EtOH) at 25 °C for 3 days, which yielded an EtOH extract; (ii) the EtOH extracts (1 l) partitioned with ethylactetate (EA) (1 l), yielding an EA/EtOH extract. The EtOH and EA/EtOH extracts were then subjected to evaporation and lyophilization before being pulverized into a fine powder. The yields of both EtOH and EA/EtOH extracts were 5% and 0.9%, respectively, of the whole *B. pilosa* plant. For preparation of the stock solution, two powder samples were shaken in isotonic phosphate saline buffer (PBS) [154 mM NaCl and 10 mM phosphate buffer at pH 7.4] or 0.1% DMSO at 25 °C. The stock solution was stored at −20 °C before analysis of the antioxidant properties. The HPLC profile of EA/EtOH extracts from *B. pilosa* was performed using a RP-18 column [Phenomenex Luna 3μ C18 (2), 150 × 4.6 mm] at a flow rate of 1.0 ml/min, detected at UV 274 nm. The solvent gradient for HPLC was 0.05% TFA/acetonitrile (B) in 0.05% TFA/H_2O (A): 10–45% B from 0 to 30 min and 45–100% B from 30 to 35 min.

2.3. Preparation of erythrocytes suspensions

Blood (10–20 ml) was obtained from normal volunteers via venapuncture after informed consents were obtained. Human erythrocytes from citrated blood were isolated by centrifugation at 3000*g* for 10 min and washed four times with PBS, and then re-suspended using the same buffer to the desired hematocrit level. Cells stored at 4 °C were used within 6 h of sample preparation. In order to induce free-radical chain oxidation in erythrocytes, aqueous peroxyl radicals were generated by thermal decomposition of AAPH (an azo compound) in oxygen. An erythrocyte suspension at 5% hematocrit was incubated with PBS (control) and pre-incubated with the EtOH and EA/EtOH fractions of *B. pilosa* (50–150 μg/ml and 25–75 μg/ml, respectively) and ethyl caffeic acid from the EA/EtOH extracts of *B. pilosa* (0.5–2 μg/ml) separately at 37 °C for 30 min, followed by incubation with and without 25 mM AAPH in PBS at pH 7.4. This reaction mixture was shaken gently while being incubated for a fixed interval at 37 °C.

2.4. Hemolysis assay

The reaction mixture (200 μl) was removed and centrifuged at 3000*g* for 2 min, with absorbance of the supernatant determined at 540 nm. Reference values were determined using the same volume of erythrocytes in a hypotonic buffer (5 mM phosphate buffer at pH 7.4; 100% hemolysis). The hemolysis percentage was calculated using the formula: absorbance of sample supernatant/reference value ×100.

2.5. Measurement of lipid peroxidation of erythrocytes

Lipid peroxidation was assessed indirectly through measurement of the thiobarbituric acid (TBA) reaction. One-hundred microlitres of H_3PO_4 (0.44 M), and 250 μl (0.67%) thiobarbituric acid were added to our 1 ml reaction mixture, and incubated at 95 °C for 1 h, this was then cooled in an ice bath for 10 min before 150 μl trichloroacetic acid (20%) was added.

After centrifugation at 13,000 rpm for 10 min, the peroxide content of the supernatant obtained was assayed using a TBA reaction with the molar extinction coefficient (OD_{532}) of malondialdehyde (MDA). Tetraethoxypropane was used as the standard (Yagi, 1984). MDA values were expressed as pmol/g Hb. An aliquot of lysate was also used for determination of the hemoglobin content using colorimetry. Briefly, 8 μl of lysate was added into a final volume of 2 ml in Drabkin's solution, and the absorbance of samples was measured against a reagent blank at 540 nm. Hemoglobin was expressed as g/ml.

2.6. Analysis of erythrocyte membrane proteins using SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of erythrocyte ghosts was prepared from the reaction mixture using hypotonic lysis in 30 volumes of 5 mM NaH_2PO_4 (pH 7.4) as previously described (Dodge et al., 1962). Hemolysate preparations were washed six times with the above buffer, and centrifuged at 12,000*g* for 30 min. Protein concentrations of the erythrocyte-ghost pellets were determined using BSA and fraction V as the standards. Erythrocyte-ghost pellets were dissolved to a concentration of 2 mg protein/ml (12.5 μl) in SDS sample buffer and brought to a total volume of 17 μl. The ghost was incubated at 95 °C for 5 min and stood on ice for 5 min, then centrifuged at 3000 rpm for 3 min. SDS-PAGE was performed on a slab gel 1.5 mm thick using 3% and 8% gels for condensation and separation, and then stained with Coomassie Brilliant Blue (Arduini and Strn, 1985). The molecular weight determination of the gel system was calibrated by measuring migration of the standard proteins.

2.7. Determination of erythrocyte GSH content

The intracellular glutathione (GSH) level was determined by DTNB titration as described previously (Ven den Berg et al., 1992). After centrifugation of the reaction mixtures (2 ml), 0.6 ml of water was added to the erythyrocyte pellets in order to lyse the cells. Then, 0.6 ml of the lysate was precipitated by the addition of 0.6 ml metaphosphoric acid solution [1.67 g metaphosphoric acid, 0.2 g EDTA (disodium salt), and 30 g NaCl in 100 ml water]. After 5 min, the protein precipitate was isolated from the remaining solution by centrifugation at 18,000*g* for 10 min. We then combined 0.45 ml of the solution with 0.45 ml of 300 mM Na_2HPO_4, and the absorbance at 412 nm was read against a blank consisting of 0.45 ml solution plus 0.45 ml water. Continuously, 100 μl DTNB solution (20 mg DTNB in 100 ml of 1% citrate solution) was added to the blank and the sample. The absorbance of the sample was read against the blank at 412 nm. The GSH values were expressed as μmol/g Hb.

2.8. Measurement of erythrocyte ATP content

The procedure for measurement of the ATP content was based on the reactions described by Adams (1963). One milliliter of the reaction mixture and 1 ml TCA (12%) was placed into an eppendorf tube, mixed well and then cooled for 5 min (ice bath), before centrifugation at about 800 g for 10 min to obtain a clear supernatant. The supernatant (0.5 ml), 0.3 mg NADH (reduced form of α-nicotinamide adenine dinucleotide), and 1.0 ml H_2O were added into 1.0 ml of phosphoglyceric acid (PGA) buffer. Glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglyceric phosphokinase (GAPD/PGK) enzyme mixture (0.04 ml) was combined with the above solution. After 10 min, absorbance was measured against the blank at 340 nm. By ascertaining the absorbance decrease at 340 nm caused by oxidation of the NADH to NAD, the original quantity of ATP could be gauged. These calculations were then used to determine blood ATP concentration, with ATP expressed as μmol/g Hb.

2.9. Measurement of SOD activity in erythrocytes

This procedure is a variation of the classical NBT method (Spitz and Oberley, 1989). The reaction mixture (100 μl) was centrifuged at 3000 rpm for 2 min and removed the supernatant. Then, 35 μl of ice water was added for breaking of the erythrocytes. Afterwards, a superoxide dismutase (SOD) kit from Randox Laboratories was used to measure SOD activity. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazoliun chloride (INT) to form a red formazan dye. SOD activity is measured by the degree of inhibition of this reaction. The calculation was performed according to the RANSOD instructions, with SOD activity expressed as U/mg Hb.

2.10. Statistics

The salient data are presented as mean ± SEM, with analysis of variance (ANOVA) followed by Dunnett's test for pairwise comparison. Statistical significance was defined as $p < 0.05$.

3. Results

In this study, normal human erythrocytes were used to investigate the capability of the EtOH and EA/EtOH extracts of *B. pilosa* (50–150 and 25–75 μg/ml, respectively) to protect erythrocytes against oxidative damage in vitro and elaborate the molecular mechanism.

3.1. Effects of B. pilosa on AAPH-induced hemolysis in erythrocytes

Fig. 1 shows the human erythrocyte hemolysis induced by AAPH with the addition of EtOH and EA/EtOH extracts of *B. pilosa*. When erythrocytes were incubated in air at 37 °C as a 5% suspension in phosphate buffered saline (PBS), they were stable with little hemolysis observed for 6 h (6.7 ± 1.5%). When a water-soluble radial inhibitor, AAPH (25 mM), was added to the aqueous suspension of erythrocytes, hemolysis induction was time dependent. The hemolysis is lagged, indicating that endogenous antioxidants in the erythrocytes can trap radicals to protect them against free-radical-induced hemolysis, as described previously (Zou et al., 2001). Both the EtOH (at 0, 50, 100, and 150 μg/ml) and EA/EtOH (at 0, 25, 50, and 75 μg/ml) extracts of *B. pilosa* suppressed AAPH-induced hemolysis in a concentration- and time-dependent manner (Fig. 1A and B). Further, a dose-dependent correlation can be demonstrated between lag time and *B. pilosa* concentration. When the cells were incubated with EtOH (150 μg/ml) or EA/EtOH (75 μg/ml) extracts of *B. pilosa* alone, hemolysis was maintained at a background level similar to that in the AAPH-untreated samples (data not shown).

3.2. Effects of B. pilosa on AAPH-induced lipid peroxidation in erythrocytes

The AAPH-induced lipid peroxidation of erythrocytes is reflected in the MDA generation that occurs with addition of EtOH and EA/EtOH extracts of *B. pilosa* (Fig. 2). MDA levels for the control-group erythrocytes were 2.0 ± 0.0, 2.4 ± 0.3, and 3.3 ± 0.7 pmol/g Hb, respectively, at 2, 4, and 6 h, increasing to 13.0 ± 1.8,

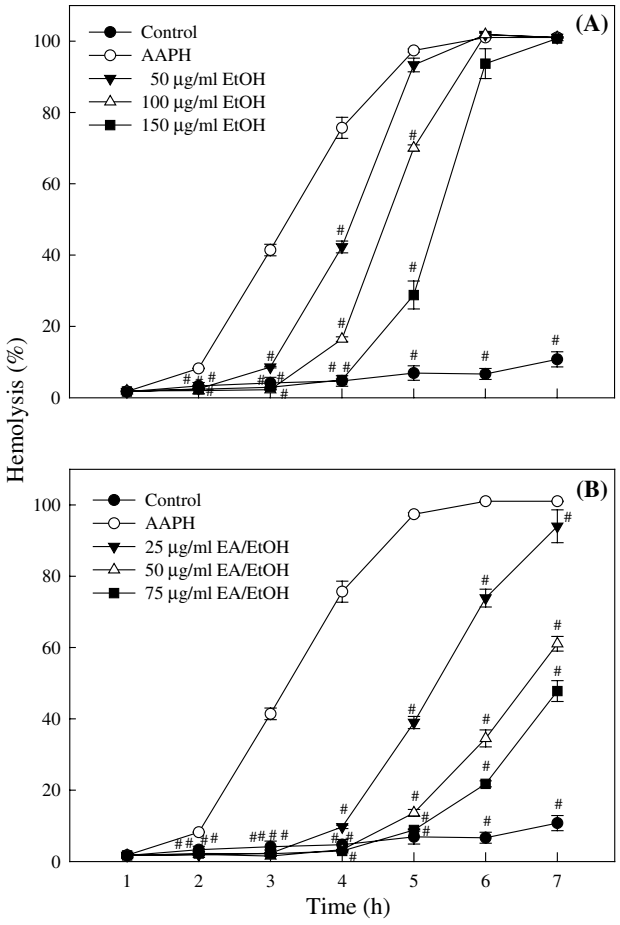

Fig. 1. Effects of *B. pilosa* on AAPH-induced hemolysis in erythrocytes. Erythrocyte suspension at 5% hematocrit was incubated with PBS (control) or preincubated with EtOH and EA/EtOH extracts of *B. pilosa* at the indicated concentrations for 30 min. The product was then incubated with 25 mM AAPH for 6 h at 37 °C. Values are expressed as the mean ± SEM of three experiments. # indicates a significant difference from AAPH group ($p < 0.05$).

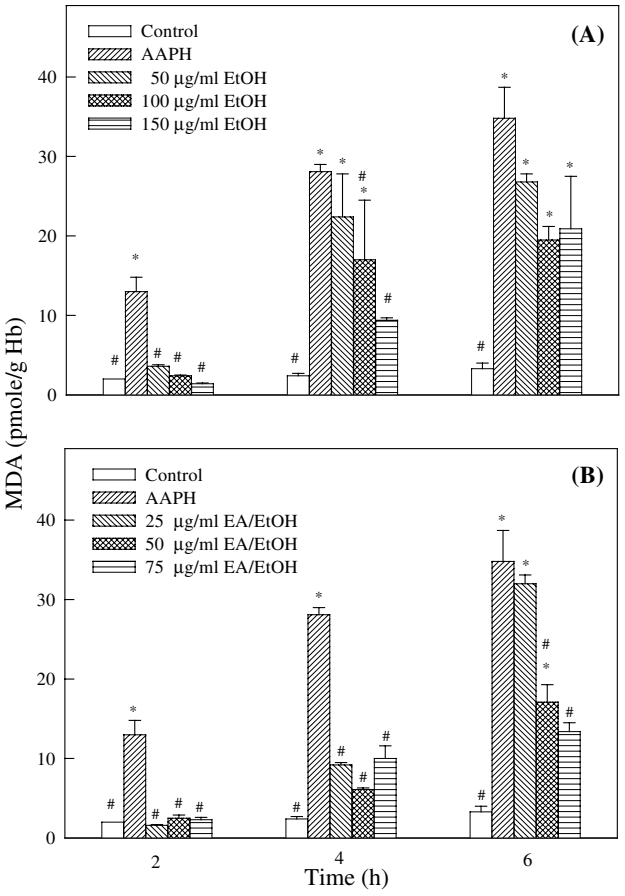

Fig. 2. Effects of *B. pilosa* on AAPH-induced changes in erythrocyte MDA. Erythrocyte suspension at 5% hematocrit was incubated with PBS (control), or preincubated with EtOH and EA/EtOH extracts of *B. pilosa* at the indicated concentrations for 30 min. Then it was incubated with 25 mM AAPH at 2, 4, and 6 h at 37 °C. MDA values were expressed as pmol/g Hb. Values are expressed as the mean ± SEM of three experiments. * indicates a significant difference from control group ($p < 0.05$); # indicates a significant difference from AAPH group ($p < 0.05$).

28.1 ± 0.9, and 34.8 ± 3.9 pmol/g Hb, respectively, after incubation with 25 mM AAPH. The addition of AAPH caused time-dependent lipid peroxidation of erythrocytes. Further, both EtOH and EA/EtOH extracts of *B. pilosa* inhibited AAPH-induced MDA formation (Fig. 2A and B). When the cells were incubated with EtOH (150 μg/ml) or EA/EtOH (75 μg/ml) extracts of *B. pilosa* alone, MDA formation was maintained at a background level similar to that in the AAPH-untreated samples (data not shown).

3.3. Effects of B. pilosa on AAPH-induced changes in erythrocyte-membrane proteins

The membrane proteins of erythrocytes are basically composed of band 1 and 2 (spectrins), band 3, band 4.1, band 4.2 and other accessory proteins. Oxidants produce alterations in erythrocyte membranes as manifested by a decreased cytoskeletal protein content (LMW proteins), and production of HMW proteins (Snyder et al., 1985; Flynn et al., 1983). The SDS-PAGE results for the erythrocyte ghosts prepared from the reaction mixtures are presented in Fig. 3. After treatment of the human erythrocytes with AAPH for 6 h, the level of HMW proteins increased and that of the LMW proteins between Band 5 and the front decreased, as described previously (Yamamoto et al., 1985; Miki et al., 1987). Both EtOH and EA/EtOH extracts of *B. pilosa* inhibited AAPH-induced changes in the erythrocyte-membrane proteins (Fig. 3). When the cells were incubated with EtOH (150 μg/ml) or EA/EtOH (75 μg/ml) extracts of *B. pilosa* alone, the level of in erythrocyte-membrane proteins was maintained at a background level similar to that in the AAPH-untreated samples (data not shown). As shown in Table 1, densitometric analysis of LMW proteins revealed that the EtOH and EA/EtOH extracts of *B. pilosa* inhibited AAPH-induced changes in the amount of erythrocyte-membrane proteins.

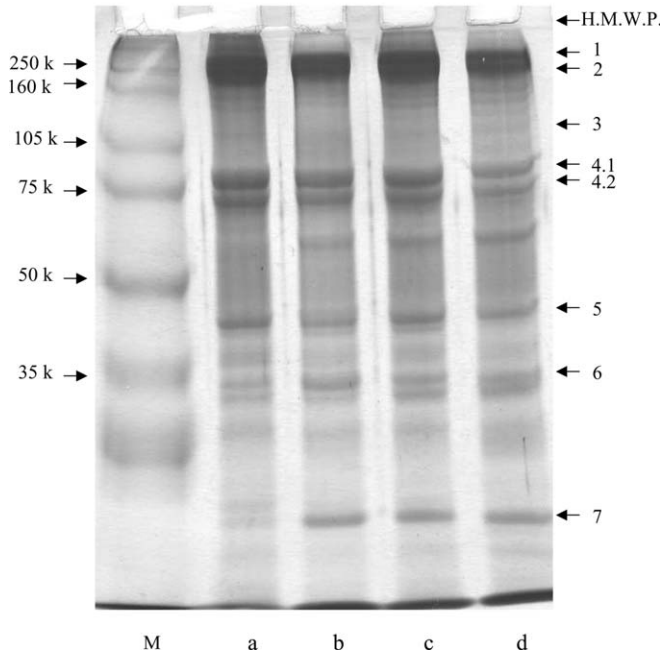

Fig. 3. Effects of *B. pilosa* on AAPH-induced changes in erythrocyte membrane proteins analyzed using SDS-PAGE. Erythrocyte suspension at 5% hematocrit was incubated with PBS (control), or preincubated with EtOH and EA/EtOH extracts of *B. pilosa* for 30 min. The product was incubated with 25 mM AAPH for 6 h at 37 °C. Lane a: erythrocytes oxidized with 25 mM AAPH. Lane b: intact erythrocyte membrane proteins. Lanes c and d: erythrocytes preincubated with EtOH and EA/EtOH extracts of *B. pilosa* at 150 and 75 μg/ml. The amount of layered protein was 25 μg in each case. This experiment was repeated three times with similar results achieved. HMWP: represents high-molecular-weight proteins.

3.4. Effects of B. pilosa on AAPH-induced changes in erythrocyte GSH content

In the control group, the GSH in the erythrocytes was 3.95 ± 0.04, 3.94 ± 0.07 and 3.33 ± 0.21 μmol/g Hb, respectively, at 2, 4, and 6 h (Table 2). Incubation with 25 mM AAPH caused significant time-dependent consumption of cytosolic GSH, with the GSH content decreasing to 1.94 ± 0.37, 0.93 ± 0.14, and 0.55 ± 0.04 μmol/g Hb, respectively, at 2, 4, and 6 h. Both EtOH and EA/EtOH extracts of *B. pilosa* inhibited AAPH-induced consumption of the cytosolic GSH (Table 2). When the cells were incubated with EtOH (150 μg/ml) or EA/EtOH (75 μg/ml) extracts of *B. pilosa* alone, GSH level was maintained at a background level similar to that in the AAPH-untreated samples (data not shown).

Table 2
Effect of *B. pilosa* on AAPH-induced changes in GSH, and ATP content of erythrocytes

Experimental condition	Time (h)	GSH (umol/g Hb)	ATP (umol/g Hb)
Control	2	$3.95 \pm 0.04^{\#}$	$4.61 \pm 0.07^{\#}$
AAPH		$1.94 \pm 0.37^{*}$	$3.17 \pm 0.19^{*}$
+50 μg/ml EtOH		$2.40 \pm 0.13^{*}$	$3.97 \pm 0.18^{*,\#}$
+100 μg/ml EtOH		$2.53 \pm 0.26^{*,\#}$	$3.77 \pm 0.17^{*,\#}$
+150 μg/ml EtOH		$2.68 \pm 0.11^{*,\#}$	$3.94 \pm 0.00^{*,\#}$
+25 μg/ml EA/EtOH		$2.16 \pm 0.07^{*}$	$4.33 \pm 0.12^{\#}$
+50 μg/ml EA/EtOH		$2.66 \pm 0.11^{*,\#}$	$4.40 \pm 0.23^{\#}$
+75 μg/ml EA/EtOH		$2.21 \pm 0.14^{*}$	$4.48 \pm 0.21^{\#}$
Control	4	$3.94 \pm 0.07^{\#}$	$4.76 \pm 0.09^{\#}$
AAPH		$0.93 \pm 0.14^{*}$	$3.13 \pm 0.02^{*}$
+50 μg/ml EtOH		$1.10 \pm 0.08^{*,\#}$	$3.79 \pm 0.15^{*}$
+100 μg/ml EtOH		$1.12 \pm 0.10^{*}$	$3.81 \pm 0.15^{*}$
+150 μg/ml EtOH		$1.16 \pm 0.04^{*}$	$3.83 \pm 0.57^{*}$
+25 μg/ml EA/EtOH		$0.84 \pm 0.01^{*}$	$4.31 \pm 0.42^{\#}$
+50 μg/ml EA/EtOH		$0.91 \pm 0.03^{*}$	$4.36 \pm 0.02^{\#}$
+75 μg/ml EA/EtOH		$1.11 \pm 0.11^{*}$	$4.53 \pm 0.04^{\#}$
Control	6	$3.33 \pm 0.21^{\#}$	$4.63 \pm 0.04^{\#}$
AAPH		$0.55 \pm 0.04^{*}$	$2.44 \pm 0.01^{\#}$
+50 μg/ml EtOH		$0.56 \pm 0.02^{*}$	$2.92 \pm 0.38^{*}$
+100 μg/ml EtOH		$0.40 \pm 0.03^{*}$	$3.38 \pm 0.03^{*,\#}$
+150 μg/ml EtOH		$0.47 \pm 0.01^{*}$	$3.68 \pm 0.30^{*,\#}$
+25 μg/ml EA/EtOH		$0.38 \pm 0.03^{*}$	$4.44 \pm 0.07^{\#}$
+50 μg/ml EA/EtOH		$0.35 \pm 0.03^{*}$	$4.45 \pm 0.64^{\#}$
+75 μg/ml EA/EtOH		$0.36 \pm 0.03^{*}$	$4.49 \pm 0.25^{\#}$

Erythrocyte suspension at 5% hematocrit was incubated with PBS (control), or preincubated with *B. pilosa* for 30 min. Then it was incubated with 25 mM AAPH for 2, 4, and 6 h at 37 °C. The GSH, and ATP content were measured as described in Methods. Values are expressed as the mean ± SEM of three experiments.

* Significant difference from control group ($p < 0.05$).

Significant difference from AAPH group ($p < 0.05$).

3.5. Effects of B. pilosa on AAPH-induced changes in erythrocyte ATP content

The ATP level in the control-group erythrocytes was 4.61 ± 0.07, 4.76 ± 0.09, and 4.63 ± 0.04 μmol/g Hb, respectively, at 2, 4 and 6 h (Table 2). Incubation with 25 mM AAPH produced significant time-dependent consumption of cytosolic ATP, with the ATP content

Table 1
Effect of *B. pilosa* on AAPH-induced relative change in erythrocyte membrane proteins by densitometric analysis

Treatment	Band 1 and 2	Band 3	Band 4.1	Band 4.2	Band 5	Band 6	Band 7
+25 mM AAPH	87 ± 7	$74 \pm 5^{*}$	86 ± 7	82 ± 4	84 ± 4	$55 \pm 5^{*}$	$12 \pm 3^{*}$
+150 μl/ml EtOH	87 ± 6	$83 \pm 2^{*}$	88 ± 7	85 ± 4	90 ± 2	$86 \pm 3^{\#}$	$96 \pm 4^{\#}$
+75 μl/ml EA/EtOH	95 ± 2	$91 \pm 4^{\#}$	91 ± 4	83 ± 6	92 ± 3	$95 \pm 2^{\#}$	$98 \pm 2^{\#}$

Erythrocyte suspension at 5% hematocrit was incubated with PBS (control), or preincubated with EtOH and EA/EtOH extracts of *B. pilosa* for 30 min. Then it was incubated with 25 mM AAPH for 6 h at 37 °C. Expressed as percent of control (0 μg) taking control as 100%. Values are means ± SEM of three experiments.

* Significant difference from control group ($p < 0.05$).

Significant difference from AAPH group ($p < 0.05$).

decreasing to 3.17 ± 0.19, 3.13 ± 0.02, and 2.44 ± 0.01 μmol/g Hb, at 2, 4, and 6 h, respectively. Both EtOH and EA/EtOH extracts of *B. pilosa* inhibited AAPH-induced consumption of cytosolic ATP (Table 2). When the cells were incubated with EtOH (150 μg/ml) or EA/EtOH (75 μg/ml) extracts of *B. pilosa* alone, ATP level was maintained at a background level similar to that in the AAPH-untreated samples (data not shown).

3.6. Effects of B. pilosa on AAPH-induced changes in erythrocyte SOD activity

The erythrocyte SOD activity for the control group was 1043 ± 37, 847 ± 92, and 795 ± 109 U/mg Hb, respectively, at 2, 4 and 6 h (Fig. 4). After incubation with 25 mM AAPH, this caused a significant, time-dependent inhibition in SOD activity, which decreased to 734 ± 43, 250 ± 57, and 128 ± 6 U/mg Hb, respectively, at 2, 4, and 6 h. Both EtOH and EA/EtOH extracts of *B. pilosa* prevented AAPH-induced decline in erythrocyte SOD activity (Fig. 4). When the cells were incubated with EtOH (150 μg/ml) or EA/EtOH (75 μg/ml) extracts of *B. pilosa* alone, SOD activity was maintained at a background level similar to that in the AAPH-untreated samples (data not shown).

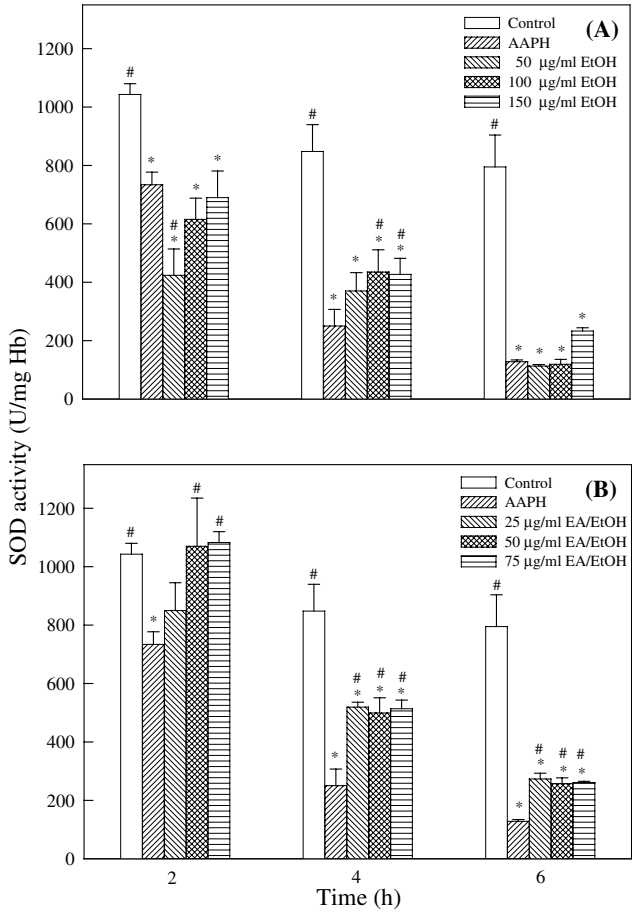

Fig. 4. Effects of *B. pilosa* on AAPH-induced changes in erythrocyte SOD activity. Erythrocyte suspension at 5% hematocrit was incubated with PBS (control), or preincubated with EtOH and EA/EtOH extracts of *B. pilosa* for 30 min. Then it was incubated with 25 mM AAPH for 2, 4, 6 h at 37 °C. SOD activity was expressed as U/mg Hb. Values are expressed as the mean ± SEM of three experiments. * indicates a significant difference from control group ($p < 0.05$); # indicates a significant difference from AAPH group ($p < 0.05$).

3.7. HPLC metabolite profiling of EA/EtOH extracts from B. pilosa

The HPLC profile of EA/EtOH extracts from *B. pilosa* was performed using a RP-18 column (Fig. 5). Major compounds were isolated from the extracts and their structures were identified as 3,4-di-*O*-caffeoylquinicacid (1), 3,5-di-*O*-caffeoylquinicacid (2), 4,5-di-*O*-Caffeoylquinic acid (3), ethyl caffeate (4), 2-β-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (5), 3-β-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (6), and 1,3-dihydroxy-6(*E*)-tetradecene-8,10,12-triyne (7) which have been isolated from the same sources (Chang et al., 2004; Chiang et al., 2004; Wu et al., 2004; Chiang et al., 2005).

4. Conclusion

Erythrocytes, which are the most abundant cells in the human body and possess desirable physiological and morphological characteristics, are exploited extensively in drug delivery (Hamidi and Tajerzadeh, 2003). Oxidative damage to the erythrocyte membrane (lipid/protein) may be implicated in hemolysis associated with some hemoglobinopathies, oxidative drugs, transition metal excesses, radiation, and deficiencies in some erythrocyte antioxidant systems (Ko et al., 1997). Lipid peroxidation is one of the consequences of oxidative damage, and it has been proposed as a general mechanism for cell injury and death (i.e., hemolysis) (Miki et al., 1987). In this study, therefore, we first tested the efficacy of 2 *B. pilosa* (EtOH and EA/EtOH) extracts as inhibitors of AAPH-induced erythrocyte hemolysis and lipid/protein peroxidation. AAPH, a water-soluble azo compound, can be used as a free-radical resource for erythrocyte attack, as the generation rate of free radicals from AAPH decomposition can be easily controlled at physiological temperatures (Constantinescu et al., 1994; Beauseigneur et al., 1996). Our results indicate that AAPH-induced lipid peroxidation and oxidative hemolysis of erythrocytes were suppressed by the extracts of *B. pilosa* in vitro, and therefore, that it may have valuable antioxidant properties which may be used for applications in food and drug products.

It has been reported that AAPH-induced oxidation of erythrocyte membrane proteins was accompanied by the formation of HMW proteins and a decrease in LMW protein content between Band 5 and the front decreased (Yamamoto et al., 1985; Miki et al., 1987). The hydrophilic radicals generated from AAPH may attack membranes from the outside of erythrocyte membranes. This is in agreement with the experimental result that band 1 and 2

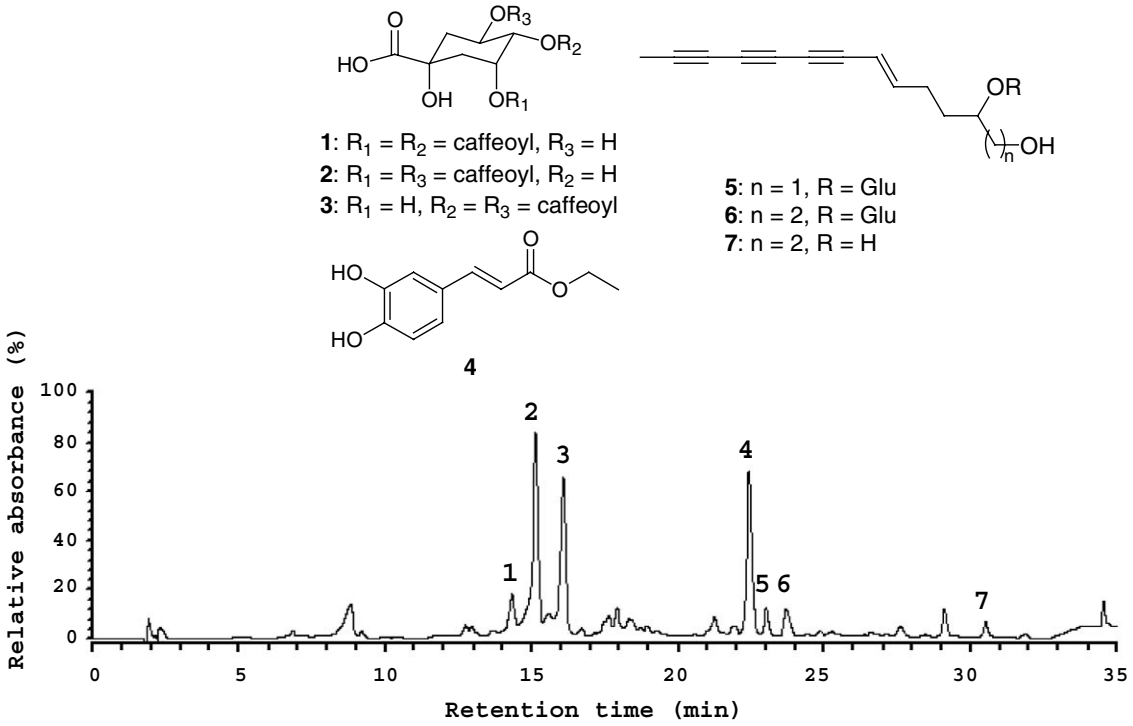

Fig. 5. The HPLC profile of EA/EtOH extracts from *B. pilosa*. The chemical profile of EA/EtOH extracts was performed using a RP-18 column and detected at UV274 nm (see Materials and methods).

(spectrins) in the SDS-PAGE did not change during the oxidation (Miki et al., 1987). The HMW proteins are formed by direct cross-linking and interaction between the LMW proteins and the oxidized lipids, which can lead to abnormalities in erythrocyte morphology and disturbances in microcirculation (Sato et al., 1998, 1999). Our results show that *B. pilosa* extracts inhibits formation of HMW proteins, and produces a concomitant decrease in LMW proteins in AAPH-challenged erythrocytes. The present findings suggest that *B. pilosa* extracts not only suppressed radical-induced erythrocyte lipid peroxidation and hemolysis but also prevented erythrocyte protein oxidation, and therefore, *B. pilosa* may act as a radical scavenger in erythrocytes.

GSH appeared to provide the primary antioxidant defense for the stored erythrocytes while providing an increase in the oxidative modification of membrane lipids and proteins, which may destabilize the membrane skeleton thereby compromising erythrocyte survival (Yamamoto et al., 1985). Oxidant scavenging at the intracellular level within the cytosol appears to rely on GSH and glutathione peroxidase for elimination of low micromolar levels of hydrogen peroxides and lipid hydroperoxides (Suttorp et al., 1986). GSH oxidation, which can be the result of direct radical attack, can also occur indirectly through GSH-requiring repair processes such as the reduction of oxidized membrane-protein thiol groups (Ko et al., 1997). Results suggest that *B. pilosa* prevents AAPH-induced erythrocyte hemolysis and lipid/protein peroxidation while modulating AAPH-related depletion of erythrocyte GSH.

ATP is used by erythrocytes to maintain membrane shape, control deformation, and maintain osmotic stability (Suttorp et al., 1986). The ATP depletion indicates a possible relationship between changes in erythrocyte shape and alterations in the submembrane skeletal-network proteins, which reportedly results in decreased filterability, deformations and increased blood viscosity (LaCelle, 1970; Rendell et al., 1992). These changes contribute to microvascular occlusion, local tissue ischemia, and consequent tissue damage (Somer and Meiselman, 1993). The results suggest that *B. pilosa* prevents AAPH-induced changes in erythrocyte protein while modulating AAPH-induced depletion of erythrocyte ATP.

The antioxidant enzymes, e.g., superoxide dismutase (SOD), widely distributed in all cells, are present in high amounts in erythrocytes (Speranza et al., 1993). SOD, usually found in aerobic systems, is a principal defense against oxygen toxicity (Fridorich, 1978; Frank et al., 1980). SOD protects cell against O_2^- by dismutation of the highly reactively superoxide anion to O_2 and to a less reactive species, H_2O_2 (McCord, 2000; Zima et al., 1996). Our results support the hypothesis that *B. pilosa* prevents AAPH-induced erythrocyte hemolysis and lipid/protein peroxidation while modulating AAPH-related decline in SOD activity.

In recent years, plants have become an increasingly important source of biologically active natural products (Vinson et al., 1995; Liu et al., 2000). Scientific interest in these active compounds such as polyphenols isolated from plants has recently been aroused due to evidence indicating antioxidant, antiinflammatory, antimutagenic,

and anticarcinogenic properties (Mizuno et al., 1990; Liu et al., 1997; Wasser and Weis, 1999; Zhu et al., 1999; Rao and Gurfinkel, 2000). Further, it has been shown that these active compounds present in a number of plants may produce beneficial effects for cardiovascular diseases such as hypertension, atherosclerosis, and coronary artery disease (Fitzpatrick et al., 1995). In this study, a number of compounds, including caffeoyl derivatives, flavonoids, alkaloids, and terpene derivatives were isolated from EtOH and EA/EtOH extracts of the whole *B. pilosa* plant as determined by HPLC (Fig. 5), and these compounds are suggested to possess significant antioxidant activity in vitro and in vivo (Chiang et al., 2005; Arora et al., 1998; Morand et al., 1998; Rose and Kasum, 2002; Simonetti et al., 2001). In addition to the above compounds, compounds such as oligosaccharides, polysaccharides, denatured proteins, and nucleotides were isolated from the EtOH extracts of *B. pilosa* also using HPLC (data not shown). These results imply that natural *B. pilosa* extracts in *B. pilosa* possibly act as a chemopreventive agent with respect to inhibition of oxidative damage in erythrocytes. However, full identification and characterization of possible compounds which may account for the antioxidant activity of *B. pilosa* require further study.

In summary, *B. pilosa* supplementation reduced AAPH-induced erythrocyte hemolysis, lipid/protein peroxidation, and cell damage in our study. These findings suggest that *B. pilosa* may have protective antioxidant, making it suitable for application in food and drug products. Further investigation of its in vitro and in vivo activity is warranted, however.

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