



Original Research Article

The cytotoxicity and protective effects of *Astragalus membranaceus* extracts and butylated hydroxyanisole on hydroxyl radical-induced apoptosis in fish erythrocytes



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ABSTRACT

Erythrocytes play an essential role in transporting O₂ and CO₂ for respiration in fish. However, erythrocytes continuously suffer from reactive oxygen species (ROS)-induced oxidative stress and apoptosis. Thus, it is essential to expand our knowledge of how to protect erythrocytes against ROS-induced oxidative stress and apoptosis in fish. In this study, we explored the cytotoxicity and the effects of butylated hydroxyanisole (BHA), ethyl ether extracts, ethyl acetate extracts, acetone extracts (AE), ethanol extracts, and aqueous extracts of *Astragalus membranaceus* (EAm) on hydroxyl radical ([•]OH)-induced apoptosis in carp erythrocytes. The rat hepatocytes and carp erythrocytes were incubated with different concentrations of BHA or EAm (0.125 to 1 mg/mL). The toxicity in rat hepatocytes and carp erythrocytes was then measured using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and a haemolysis assay, respectively. The carp erythrocytes were treated with BHA or EAm in the presence of 40 μmol/L FeSO₄ and 20 μmol/L H₂O₂ at 37 °C, except for the control group. Oxidative stress and apoptosis parameters in the carp erythrocytes were then evaluated using the commercial kit. The results indicated that at high concentrations, BHA and EAm could induce toxicity in rat hepatocytes and fish erythrocytes. However, BHA was more toxic than EAm at the same concentrations. Moreover, the toxicity order of BHA and EAm in the fish erythrocytes approximately agreed with that for the rat hepatocytes. Butylated hydroxyanisole and EAm suppressed the [•]OH-induced phosphatidylserine exposure and DNA fragmentation (the biomarkers of apoptosis) by decreasing the generation of ROS, inhibiting the oxidation of cellular components, and restoring the activities of antioxidants in carp erythrocytes. Of all of the examined EAm, the AE showed the strongest effects. The effects of AE on superoxide anion, H₂O₂, met-haemoglobin and reduced glutathione levels, as well as glutathione reductase activity and apoptosis were equivalent to or stronger than those of BHA. These results revealed that the AE of *Astragalus membranaceus* could be used as a potential natural antioxidant or apoptosis inhibitor in fish erythrocytes.

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1. Introduction

Erythrocytes play an essential role in transporting O₂ and CO₂ for respiration in fish (Kulkeaw and Sugiyama, 2012). However, erythrocytes may be continuously exposed to both endogenous and exogenous sources of reactive oxygen species (ROS), including the superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radical ([•]OH). Erythrocytes continuously produce O₂^{•-}

through the autoxidation of haemoglobin (Hb) in humans (Cimen, 2008). The dismutation of $O_2^{\cdot-}$ generates H_2O_2 that can react with heme Fe^{2+} to produce $\cdot OH$ (Cimen, 2008). Our previous study demonstrated that $\cdot OH$ triggers the oxidation of lipids and proteins resulting in the apoptosis of fish erythrocytes (Li et al., 2013). Studies indicated that many pollutants initiate the generation of ROS in fish (Livingstone, 2001). The ROS can activate erythrocyte oxidative stress and subsequent apoptosis (Cimen, 2008), which can lead to a wide variety of diseases or clinical conditions (Lang et al., 2008). Thus, it is essential to expand our knowledge of how to protect erythrocytes against oxidative stress and apoptosis in fish. Because ROS are an important factor leading to oxidative stress and apoptosis (Cimen, 2008), it is possible that chemicals or drugs that can scavenge ROS could inhibit oxidative stress and apoptosis in fish erythrocytes. It has been showed that butylated hydroxyanisole (BHA), a synthetic antioxidant, can prevent the production of ROS in cerebral glioma cells (Ansari et al., 2014). However, studies indicated that BHA is carcinogenic and toxic to animals (EFSA, 2011). Thus, there is growing interest in replacing BHA with natural ingredients. *Astragalus membranaceus* Bunge (Am) is a well-known Chinese medicinal herb with multifunctional pharmacological activities (Monograph, 2003). It has been shown that the extracts of Am (EAm) have inhibitory effects on oxidative stress in mice (Mao et al., 2013). Therefore, it is possible to use EAm as a feed ingredient to inhibit oxidative stress and apoptosis of erythrocytes in fish. However, there are few studies on the protective effects of EAm in fish erythrocytes. It has been reported that the mammal non-nucleated erythrocytes system provides a very useful model for studying cytotoxicity caused by chemicals and drugs (Pagano and Faggio, 2015). Thus, it is possible that the fish erythrocyte system could be used as a model for assessing cytotoxicity in feed ingredients. However, there are few papers describing the cytotoxicity caused by feed ingredients in fish erythrocytes.

In our previous study, $\cdot OH$ induced apoptosis in fish erythrocytes, which provided a good model of oxidative stress in fish cells (Li et al., 2013). In this study, we hypothesised that EAm could inhibit $\cdot OH$ -induced apoptosis in fish erythrocytes. We explored the toxicity and the effects of BHA and EAm on $\cdot OH$ -induced apoptosis in carp erythrocytes. The purpose was to evaluate the cytotoxicity and protective effects of EAm against oxidative stress and apoptosis compared with BHA. The results may provide the basis for using fish erythrocytes as a model of cytotoxicity in feed ingredients and for using EAm as an inhibitor of oxidative stress and apoptosis in fish erythrocytes.

2. Materials and methods

2.1. Chemicals

Heparin sodium ($\geq 99\%$) and dimethyl sulfoxide (DMSO $\geq 99.7\%$) were purchased from Sigma–Aldrich Co., LLC (St. Louis, MO, USA). Butylated hydroxyanisole (analytical standard) was obtained from Shanghai Puzhen Biotech. Co., LTD (Shanghai, China). Ethyl ether, ethyl acetate, acetone, and ethanol were analytical grade and purchased from the Chengdu Kelong Chemical Reagent Factory (Chengdu, China). Aqueous solutions of H_2O_2 (30%) and $FeSO_4$ (analytical grade) were obtained from the Shanghai Chemical Reagent Factory (Shanghai, China). Physiological carp saline (PCS), which contained (in mmol/L) 141.10 NaCl, 1.43 KCl, 0.99 $CaCl_2$, 2.64 $NaHCO_3$, and 6.16 glucose, was prepared in our laboratory and modified to obtain a total osmolarity of 280 mOsm/L and a pH of 7.9. All of the other chemicals were analytical grade.

2.2. Preparation of EAm

Root of Am was obtained from the Chengdu Pharmaceuticals market of China (Chengdu, Sichuan, China). Botanical identification was performed in the Herbarium of the College of Life Sciences, where voucher samples were assigned a reference number and deposited. Prior to sequential extraction following the methods of Wojcikowski et al. (2007), the dried roots were ground to a powder (with a maximum particle size of 0.32 mm) using a Chinese medicine mill (Ronghao RHP-2000A, Zhejiang, China). Next, 50 g of the powder was sequentially extracted with 500 mL of ethyl ether, ethyl acetate, acetone, and ethanol and water at 20 °C for 8 h using an Agitator (Dalong OS40-S, Beijing, China), respectively. The extraction using each solvent was repeated 3 times under the same conditions. After filtration, the solutions were removed and dried in a vacuum using a rotary evaporator (Jinye RE-52CS, Shanghai, China) until a constant mass was achieved. The dry ethyl ether extracts (EEE), ethyl acetate extracts (EAE), acetone extracts (AE), ethanol extracts (EE), and aqueous extracts (AQE) of orange colour were kept in sealed bottles in the dark and stored at -80 °C until use.

2.3. Cytotoxicity assays

2.3.1. Three-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The MTT assay was used to evaluate the toxicity of all of the agents in isolated rat hepatocytes (Shi et al., 2016; Thetsrimuang et al., 2011). In brief, the hepatocytes were seeded in 96 well culture plates at a density of 1×10^6 cells/mL in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 with 10% foetal bovine serum for 10 h. All of the test samples were dissolved in the culture medium containing 0.1% DMSO (vol/vol) and then added to the plates at a final concentration of 0, 0.125, 0.25, 0.5, 0.75, or 1 mg/mL. After incubating at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h, the MTT reagent was added and further incubated under the same conditions for 4 h. Then, the plates were read at 490 nm using a microplate reader (Thermo, USA). Cell viability (%) was expressed as a percentage of the absorbance in each treatment with sample with that in the treatment without sample. Four replicates were prepared for each treatment. An equal number of control replicates without hepatocytes were used for each treatment.

2.3.2. Haemolysis assay

The procedures for isolating carp erythrocytes were based on those described by Li et al. (2015a). The haemolysis ratio is used to evaluate the cytotoxicity of all test agents (Pagano and Faggio, 2015). In brief, all of the test samples were dissolved in PCS containing 1% erythrocytes (vol/vol) and 0.1% DMSO (vol/vol) to obtain final concentrations of 0, 0.125, 0.25, 0.5, 0.75, and 1 mg/mL. After incubation at 37 °C for 9 h, the erythrocyte suspension was centrifuged for 3 min ($1,000 \times g$, 4 °C), and the absorbance of the supernatants was measured using the microplate reader at 540 nm. The haemolysis ratio is expressed as the percentage of the absorption in each treatment with that in complete haemolysis (Sopjani et al., 2008). Four replicates were prepared for each treatment. Each replicate had a corresponding replicate without erythrocytes that served as the control.

2.4. Cytoprotection assays

2.4.1. Experimental procedure

The experimental procedures were based on those described by Li et al. (2016a and 2016c), with slight modifications. Butylated

hydroxyanisole or the EEE, EAE, AE, EE or AQE of Am was dissolved in PCS containing 1% erythrocytes (vol/vol) and 0.1% DMSO (vol/vol) to obtain a final concentration of 0.25 mg/mL. For the positive and control groups, BHA and EAm was not added to the PCS, but the PCS did contain 1% erythrocytes (vol/vol) and 0.1% DMSO (vol/vol). After all of the above treatments were pre-incubated at 37 °C for 3 h, FeSO₄ and H₂O₂ were added at a final concentration of 40 and 20 μmol/L for the induction of apoptosis, respectively, except for the control group. After incubation at 37 °C for 6 h, the samples were centrifuged for 3 min (1,000 × g, 4 °C). The erythrocytes were collected to measure the levels of O₂⁻, H₂O₂, met-haemoglobin (Met-Hb), malonaldehyde (MDA), protein carbonyl (PC), and reduced glutathione (GSH). In addition, the activities of glutathione reductase (GR) and glutathione S-transferase (GST) were measured, as well as phosphatidylserine (PS) exposure and DNA fragmentation. The experiment was performed with 4 replicates per treatment and the control.

All of the procedures above were approved by the Institutional Animal Care and Use Committee of the Neijiang Normal University in accordance with the Institutional Ethics Committee of the Chinese Institute of Chemical Biology guidelines.

2.4.2. Biochemical analysis

The contents of O₂⁻, Met-Hb, PC, and GSH in the erythrocytes were measured as described by Li et al. (2013). The levels of H₂O₂ (Fan et al., 2015) and MDA (Li et al., 2015b) in the erythrocytes were determined by assay kits according to the manufacturer's instructions (Nanjing Jiancheng Technology LTD., China) (Yin et al., 2015). The activities of GR and GST were determined using the method described by Li et al. (2014) and Wen et al. (2015). The protein concentration was evaluated using the method described by Li et al. (2016b).

2.4.3. Measurement of apoptosis

The PS exposure and DNA fragmentation in fish erythrocytes were assessed using the Annexin V-FITC Apoptosis Detection Kit and TdT-mediated dUTP nick end labelling (TUNEL) Apoptosis Assay Kit (Beyotime, Nantong, China), respectively, as described previously (Li et al., 2016c).

2.5. Statistical analysis

The data are expressed as the means ± standard deviation (SD). The data were subjected to one-way analysis of variance (ANOVA). Duncan's multiple range test was used to determine significant differences. The significance level was 95% ($\alpha = 0.05$). The concentration required for 50% inhibition of cell survival (IC₅₀) was determined using the probit analysis. The statistical analysis was performed using SPSS 13.0 for Windows (Chicago, IL, USA) (Tan et al., 2010).

3. Results

3.1. The toxicity of BHA and EAm in rat hepatocytes and carp erythrocytes

We examined the effects of BHA and EAm on cell viability in primary cultured rat hepatocytes using an MTT assay. The results showed that the cell viability gradually decreased with increasing concentrations of BHA and EAm ($P < 0.05$) (Fig. 1A). Butylated hydroxyanisole at a concentration of 0.125 mg/mL caused a dramatic toxic response, and approximately 80% of the cells lost their viability within 24 h of BHA treatment. Increasing the BHA concentration to 0.25 mg/mL caused a more vigorous response, and over 90% of the cells lost viability after incubating for the same

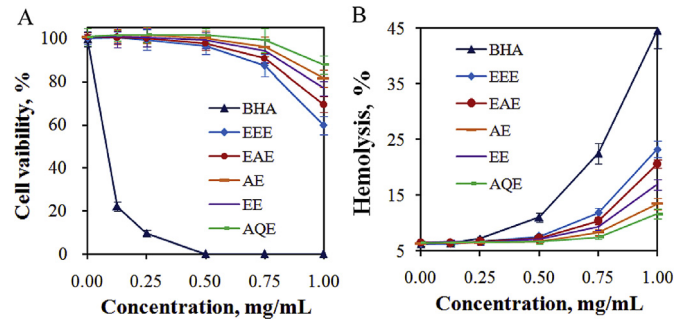


Fig. 1. The cytotoxicity of butylated hydroxyanisole (BHA) and of ethyl ether extracts (EEE), ethyl acetate extracts (EAE), acetone extracts (AE), ethanol extracts (EE), and aqueous extracts (AQE) of *Astragalus membranaceus* root by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (A) and the haemolysis assay (B). The data represent the means ± SD of 4 replicates.

period of time. In contrast, no apparent toxic effect was observed for EAm at 0.125, 0.25, or even 0.5 mg/mL ($P < 0.05$). Furthermore, EAm at a concentration of 0.75 mg/mL only slightly reduced hepatocyte viability. The rank order of IC₅₀ found using the MTT assay was AQE > AE = EE > EAE > EEE > BHA ($P < 0.05$) (Table 1).

The haemolysis of erythrocytes incubated with different concentrations of BHA and EAm (0.125 to 1.00 mg/mL) is presented in Fig. 1B. The results were similar to those for the rat hepatocytes. The rank order of IC₅₀ found using the haemolysis assay was AQE > AE > EE > EAE > EEE > BHA ($P < 0.05$) (Table 1). There was a significant increase in haemolysis caused by BHA and EAm at 0.50 and 0.75 mg/mL, respectively ($P < 0.05$). Thus, 0.25 mg/mL was the maximal treatment concentration of BHA and EAm that did not cause a significant increase in haemolysis. Thus, 0.25 mg/mL was selected as a non-toxic therapy concentration for BHA and EAm, and this concentration was used for the experiments using carp erythrocytes.

3.2. The effects of BHA and EAm on the •OH-treated carp erythrocytes

As shown in Fig. 2, the levels of annexin binding and TUNEL-positive cells were significantly increased in the carp erythrocytes exposed to •OH alone ($P < 0.05$), which suggests an increase in the PS exposure and DNA fragmentation in the control group. However, treatment with BHA and EAm significantly decreased the levels of annexin binding (Fig. 2A) and the number of TUNEL-positive (Fig. 2B) cells ($P < 0.05$), which suggests a decrease in the PS exposure and DNA fragmentation in •OH-treated carp erythrocytes. In particular, when the erythrocytes were treated with AE in the

Table 1

The concentration required for 50% inhibition of cell survival (IC₅₀) of butylated hydroxyanisole (BHA) and of ethyl ether extracts (EEE), ethyl acetate extracts (EAE), acetone extracts (AE), and ethanol extracts (EE), and aqueous extracts (AQE) of *Astragalus membranaceus* root on rat hepatocytes and carp erythrocytes.¹

Extracts	Rat hepatocytes	Carp erythrocytes
BHA	0.07 ± 0.01 ^a	1.36 ± 0.06 ^a
EEE	1.12 ± 0.07 ^b	4.69 ± 0.31 ^b
EAE	1.24 ± 0.06 ^c	7.15 ± 0.44 ^c
AE	1.39 ± 0.09 ^d	69.60 ± 2.67 ^e
EE	1.35 ± 0.06 ^d	15.28 ± 1.11 ^d
AQE	1.60 ± 0.09 ^e	423.18 ± 24.18 [*]

^{a-e} Within a same column, values with different superscripts are significantly different ($P < 0.05$).

^{*} Within a same column, values with asterisks indicate significant differences (t -test, $P \leq 0.05$).

¹ The data represent the means ± SD of 4 replicates.

presence of $\cdot\text{OH}$, the levels of annexin binding and TUNEL-positive cells were estimated to be the minimum value for all of the examined compounds. The level of PS exposure and DNA fragmentation in the $\cdot\text{OH}$ -treated erythrocytes treated with EEE and EAE were lower than those for the BHA treatments ($P < 0.05$) (Fig. 2).

The effects of BHA and EAm on $\text{O}_2^{\cdot-}$, H_2O_2 , Met-Hb, MDA, and PC in $\cdot\text{OH}$ -treated carp erythrocytes are presented in Table 2. Exposure to $\cdot\text{OH}$ significantly increased the levels of $\text{O}_2^{\cdot-}$, H_2O_2 , Met-Hb, MDA, and PC in the erythrocytes relative to those in the untreated control ($P < 0.05$). However, treatment with BHA and EAm effectively prevented the increase in $\text{O}_2^{\cdot-}$, H_2O_2 , Met-Hb, MDA, and PC levels in the erythrocytes that had been exposed to $\cdot\text{OH}$ ($P < 0.05$). In particular, the $\cdot\text{OH}$ -treated erythrocytes that were also treated with BHA showed the minimum values of $\text{O}_2^{\cdot-}$, H_2O_2 , Met-Hb, MDA, and PC for all of the examined compounds. The levels of $\text{O}_2^{\cdot-}$, H_2O_2 , and Met-Hb for the AE treatment were equivalent to those for the BHA treatment. Additionally, the levels of MDA and PC for the AE treatment were estimated to be the minimum value for all of the examined extracts.

As presented in Table 3, the levels of GSH and the activities of GR and GST were markedly decreased in carp erythrocytes exposed to $\cdot\text{OH}$ alone ($P < 0.05$). However, for the BHA and EAm treatments,

the decreases were effectively inhibited in the erythrocytes that had been exposed to $\cdot\text{OH}$ ($P < 0.05$). The levels of GSH and the activities of GR and GST in the $\cdot\text{OH}$ -treated carp erythrocytes that were also treated with BHA were the highest values compared with those of the examined compounds. The levels of GSH and the activities of GR for the AE treatment were estimated to be equivalent to those of the BHA treatment. The GST activity for the AE treatment was estimated to be 162.40 U/mg proteins, which is the maximum value for the examined extracts.

4. Discussion

4.1. Butylated hydroxyanisole was more cytotoxic than EAm in rat hepatocytes and carp erythrocytes

The nucleated hepatocyte system provides a very useful model for studying cell damage caused by chemicals and drugs (Liu and Zeng, 2009). The MTT assay in rat hepatocytes has been used to investigate the cytotoxicity of chemicals and drugs (Fotakis and Timbrell, 2006). In the present study, BHA caused a dramatic toxic response in a concentration-dependent manner at the concentrations ranging from 0.125 to 0.50 mg/mL based on the MTT assay. In contrast, no apparent toxic effect was observed for EAm at the same concentrations. This is in good agreement with the reports that BHA was cytotoxic in Vero cells (Labrador et al., 2007). No reports have been published on the cytotoxicity of EAm in hepatocytes.

Haemolysis in the mammal non-nucleated erythrocytes represents a good model for studying cytotoxicity caused by organic, inorganic, natural, or synthetic compounds (Pagano and Faggio, 2015). The present study showed that BHA and EAm at high concentrations led to an increase of haemolysis in fish erythrocytes. However, at the same concentrations, BHA is more toxic than EAm in fish erythrocytes. This is in line with the above-mentioned results in rat hepatocytes. Moreover, this study indicated that the toxicity order of BHA and EAm in fish erythrocytes was similar to that observed in rat hepatocytes. Similar to mammal erythrocytes, fish erythrocytes contain high concentrations of Hb and unsaturated fatty acids (Li et al., 2016c). Fish erythrocytes retain the nucleus, mitochondria and other organelles, which are mostly similar to those of mammal tissue cells in structure (Rothmann et al., 2000). Moreover, studies suggested that the mechanisms of antioxidant defence and apoptosis in fish erythrocytes are similar to those in mammalian cells (Li et al., 2013, 2016c). In our experience, fish erythrocytes are easy to harvest and culture *in vitro* compared with rat hepatocytes (Li et al., 2016c). Thus, the fish erythrocyte system can be used as an experimental model to evaluate the cytotoxicity of chemicals and drugs.

4.2. Butylated hydroxyanisole and EAm inhibited $\cdot\text{OH}$ -induced apoptosis in carp erythrocytes

Phosphatidylserine exposure and DNA fragmentation are biomarkers of apoptosis (Li et al., 2015a). In this study, BHA and EAm effectively inhibited PS exposure and DNA fragmentation induced by $\cdot\text{OH}$ in carp erythrocytes. These results confirmed that BHA and EAm could protect against $\cdot\text{OH}$ -induced apoptosis in fish erythrocytes. In particular, the inhibitory effects of EAE and AE on apoptosis were stronger than those of BHA. This finding is consistent with the reports that BHA and AQE of Am abrogated H_2O_2 -induced apoptosis in mouse hepatocytes (Hwang et al., 2015). However, data on the anti-apoptosis properties of the other extracts of Am in animal cells are scarce.

There may be a positive correlation between the anti-apoptotic effects of BHA and EAm and ROS in fish erythrocytes. It has been

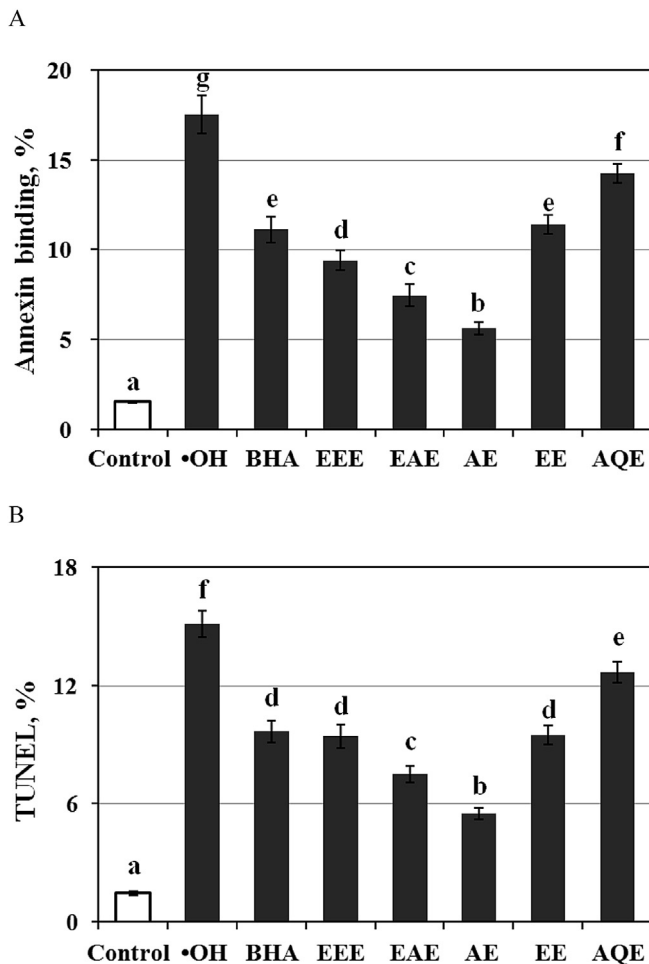


Fig. 2. The effects of butylated hydroxyanisole (BHA) and of ethyl ether extracts (EEE), ethyl acetate extracts (EAE), acetone extracts (AE), ethanol extracts (EE), and aqueous extracts (AQE) of *Astragalus membranaceus* root on the levels of annexin binding (A) and TUNEL (B) in $\cdot\text{OH}$ -treated carp erythrocytes. The data represent the means \pm SD of 4 replicates. ^{a–g}Bars with different superscripts are significantly different ($P < 0.05$).

Table 2
The effects of butylated hydroxyanisole (BHA) and of ethyl ether extracts (EEE), ethyl acetate extracts (EAE), acetone extracts (AE), ethanol extracts (EE) and aqueous extracts (AQE) of *Astragalus membranaceus* root on the levels of superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), met-haemoglobin (Met-Hb), malonaldehyde (MDA), protein carbonyl (PC) in $\cdot OH$ -treated carp erythrocytes.¹

Treatment	$O_2^{\cdot-}$, U/g protein	H_2O_2 , $\mu mol/g$ protein	Met-Hb, g/L	MDA, nmol/mg protein	PC, nmol/mg protein
Control	28.24 \pm 2.04 ^a	42.46 \pm 2.00 ^a	1.56 \pm 0.06 ^a	1.77 \pm 0.11 ^a	0.99 \pm 0.04 ^a
$\cdot OH$	67.68 \pm 2.76 ^g	114.59 \pm 6.40 ^f	3.46 \pm 0.22 ^f	3.79 \pm 0.25 ^g	2.72 \pm 0.13 ^g
$\cdot OH$ + BHA	33.22 \pm 1.62 ^b	52.24 \pm 3.90 ^b	1.85 \pm 0.10 ^b	2.08 \pm 0.13 ^b	1.15 \pm 0.06 ^b
$\cdot OH$ + EEE	47.26 \pm 1.88 ^d	74.87 \pm 3.77 ^c	2.44 \pm 0.16 ^c	3.14 \pm 0.14 ^e	1.94 \pm 0.12 ^d
$\cdot OH$ + EAE	41.90 \pm 3.20 ^c	72.61 \pm 4.43 ^c	2.22 \pm 0.12 ^c	2.75 \pm 0.14 ^d	1.83 \pm 0.10 ^d
$\cdot OH$ + AE	35.66 \pm 1.93 ^b	56.31 \pm 3.83 ^b	1.83 \pm 0.14 ^b	2.40 \pm 0.11 ^c	1.43 \pm 0.10 ^c
$\cdot OH$ + EE	52.15 \pm 2.91 ^e	81.87 \pm 4.28 ^d	2.84 \pm 0.13 ^d	3.17 \pm 0.19 ^e	2.19 \pm 0.09 ^e
$\cdot OH$ + AQE	58.96 \pm 3.08 ^f	91.00 \pm 4.35 ^e	3.10 \pm 0.22 ^e	3.43 \pm 0.08 ^f	2.48 \pm 0.17 ^f

^{a–g} Within a same column, values with different superscripts are significantly different ($P < 0.05$).

¹ The data represent the means \pm SD of 4 replicates.

Table 3
The effects of butylated hydroxyanisole (BHA) and of ethyl ether extracts (EEE), ethyl acetate extracts (EAE), acetone extracts (AE), ethanol extracts (EE), and aqueous extracts (AQE) of *Astragalus membranaceus* root on the levels of reduced glutathione (GSH) and the activities of glutathione reductase (GR) and glutathione S-transferase (GST) in $\cdot OH$ -treated carp erythrocytes.¹

Treatment	GSH, $\mu mol/g$ protein	GR, U/g protein	GST, U/mg protein
Control	6.41 \pm 0.23 ^g	5.53 \pm 0.41 ^g	201.80 \pm 7.32 ^g
$\cdot OH$	1.71 \pm 0.07 ^a	0.95 \pm 0.04 ^a	99.29 \pm 5.79 ^a
$\cdot OH$ + BHA	5.48 \pm 0.30 ^f	4.97 \pm 0.39 ^f	177.07 \pm 10.63 ^f
$\cdot OH$ + EEE	3.91 \pm 0.27 ^d	3.55 \pm 0.20 ^d	147.75 \pm 9.39 ^d
$\cdot OH$ + EAE	4.45 \pm 0.24 ^e	4.32 \pm 0.24 ^e	159.97 \pm 6.64 ^{de}
$\cdot OH$ + AE	5.17 \pm 0.25 ^f	5.01 \pm 0.25 ^f	162.40 \pm 10.20 ^e
$\cdot OH$ + EE	3.28 \pm 0.16 ^c	2.71 \pm 0.12 ^c	134.59 \pm 7.82 ^c
$\cdot OH$ + AQE	2.31 \pm 0.10 ^b	1.91 \pm 0.08 ^b	118.60 \pm 9.05 ^b

^{a–g} Within a same column, values with different superscripts are significantly different ($P < 0.05$).

¹ The data represent the means \pm SD of 4 replicates.

reported that ROS can trigger apoptosis in animal cells (Wang et al., 2016). In the present study, BHA and EAm effectively decreased the levels of $O_2^{\cdot-}$ and H_2O_2 in carp erythrocytes exposed to $\cdot OH$. Among the examined EAm, AE showed the strongest inhibitory effects, which was almost equivalent to that of BHA. This finding suggested that BHA and EAm could decrease the generation of ROS in fish erythrocytes, which is consistent with the reports showing that BHA prevents the production of ROS in cerebral glioma cells (Ansari et al., 2014), and EE of Am inhibited the pentylenetetrazol-induced increase in ROS *in vitro* (Aldarmaa et al., 2010). These results demonstrated that BHA and EAm could protect fish erythrocytes against apoptosis by inhibiting the generation of ROS.

The generation of $O_2^{\cdot-}$ was accompanied by the oxidation of Hb to Met-Hb, which does not bind or transport O_2 in erythrocytes (Cimen, 2008). In the present study, BHA and EAm effectively prevented the increase in Met-Hb levels in the erythrocytes exposed to $\cdot OH$. Particularly, the inhibitory effects of AE on Met-Hb levels were almost equivalent to that of BHA in carp erythrocytes. This finding suggested that BHA and EAm could inhibit the oxidation of Hb in the erythrocytes. No prior study has addressed the effects of BHA and EAm on the oxidation of Hb in erythrocytes. These results demonstrated that BHA and EAm could maintain the function of erythrocytes by preventing the oxidation of Hb.

The anti-apoptotic effects of BHA and EAm may be closely associated with the oxidation of cellular components in fish erythrocytes. The oxidative products of lipids and proteins play an important role in the induction of apoptosis in mammalian cells (Li et al., 2016c). Hydroxyl radical can oxidise cellular components, such as lipids and proteins, leading to the formation of MDA and PC (Li et al., 2013). In this study, BHA and EAm markedly decreased the MDA and PC levels in $\cdot OH$ -treated carp erythrocytes. Among all of the examined extracts, AE showed the strongest effects on MDA

and PC. This finding suggested that BHA and EAm could decrease the oxidation of lipids and proteins in $\cdot OH$ -treated carp erythrocytes. This result is in agreement with the reports showing that BHA decreases lipid peroxidation in cerebral glioma cells (Ansari et al., 2014) and that EE of Am inhibited the pentylenetetrazol-induced oxidation of lipids and proteins *in vitro* (Aldarmaa et al., 2010). These studies demonstrated that BHA and EAm could protect fish erythrocytes from apoptosis by preventing the oxidation of cellular components.

The cytosol contains antioxidants that can scavenge intracellular ROS and suppress lipid oxidation in cells (Surai et al., 2016; Wu et al., 2011). Reduced glutathione is the major non-enzymatic antioxidant (Yin et al., 2016) and plays an important role in inhibiting apoptosis (Li et al., 2013). The intracellular GSH are usually kept at high levels through the reduction of oxidised GSH to GSH by GR (Wen et al., 2015). Glutathione S-transferase also plays an antioxidant role by conjugating the cleavage products of lipid peroxides (Wen et al., 2015). Both GR and GST have a protective role against oxidative stress-induced apoptosis in human cells (Kim et al., 2010; Rao and Shaha, 2000). Thus, the anti-apoptotic activity of BHA and EAm may be related to the antioxidants in fish erythrocytes. In the present study, BHA and EAm restored the levels of GSH and the activities of GR and GST in carp erythrocytes, which were decreased following $\cdot OH$ exposure. Among all of the examined extracts, AE showed the strongest effects. Moreover, the protective effects of AE on GSH and GR were almost equivalent to those of BHA. This finding is in agreement with the report showing that BHA can maintain the levels of GSH in cerebral glioma cells (Ansari et al., 2014). No reports have been published on the effects of EAm on antioxidants in animal cells. These results revealed that in addition to quenching ROS and preventing the oxidation of cellular components, BHA and EAm may affect apoptosis by elevating the activity of antioxidant. The AE of Am showed the most anti-oxidative and anti-apoptotic effects among all of the examined extracts. Thus, it is possible that the AE is used as a feed ingredient for inhibiting oxidative stress and apoptosis in fish erythrocytes.

The EAm maybe be digested by enzymes or fermented by microflora in the gut of fish. No reports have been published demonstrating that these extracts could directly enter the blood circulation and inhibit apoptosis in fish erythrocytes. However, studies have demonstrated that dietary AQE of Am preserves the activities of apoptosis-inhibitory factors, such as superoxide dismutase (SOD), catalase (CAT) (Sulowska et al., 2005), glutathione peroxidase (GPx) (Franco and Cidlowski, 2009), and GR (Kim et al., 2010) in the myocardium tissue of mice (Mao et al., 2013). Orally administrated EE of *Astragalus* decreased the levels of apoptosis-inducing factors (Li et al., 2016c), such as MDA, PC, and ROS in the brain of rats (Aldarmaa et al., 2010). However, information regarding the effect of dietary EAm on fish is scarce. The protective

effect of EAm may be closely associated with their constituents. The main active ingredients of Am include polysaccharides, saponins, and flavonoids (Monograph, 2003). It has been shown that the dietary polysaccharides of *Astragalus* ameliorate the increase in toxicity and MDA levels and decrease the activity of SOD and CAT as well as GSH levels in the liver of mice (Liu et al., 2014). The saponins of *Astragalus* inhibited the formation of lipid peroxides in the myocardium of patients (Monograph, 2003). A few clinical studies have pointed out that flavonoids protect against oxidative stress and damage by scavenging apoptosis-inducing factors (Li et al., 2016a), such as metal ions, ROS, and oxidised lipids in patients (Nijveldt et al., 2001). Moreover, studies suggested that flavonoids in the aglycone form (flavonoid aglycones) could be absorbed directly in the intestine of humans. Unabsorbed flavonoids in the glycosidic form (flavonoid glycosides) may be metabolised by bacterial enzymes in the colon, and then absorbed and transported to the liver (Mahadevan and Park, 2008). However, little is known about the effect of these ingredients in fish *in vivo*.

Most flavonoid aglycones and sugars combine into flavonoid glycosides (Corradini et al., 2011; Kumar and Pandey, 2013). Flavonoid glycosides are easily soluble in water, methanol, ethanol, acetone, and ethyl acetate, but are insoluble in ethyl ether and chloroform (Chebil et al., 2007). Flavonoid aglycone is generally not soluble in water, but is soluble in methanol, ethanol, acetone, ethyl acetate, ethyl ether, and other organic solvents (Ferreira and Pinho, 2012). Saponins are generally soluble in water, methanol, and dilute ethanol and are easily soluble in hot water, hot methanol, and hot ethanol but are hardly soluble in acetone, ethyl acetate, and ethyl ether (Guclu-Ustundag and Mazza, 2007). Saponin aglycones are generally soluble in ethyl acetate, ethyl ether and benzene, insoluble in water (Podolak et al., 2010). Polysaccharides are easily soluble in water but are insoluble in organic solvents (Tomasik, 2003). Therefore, we speculated that in sequential extraction, the EEE of Am mainly contains flavonoid and saponin aglycones, while the EAE of Am mainly contains saponin and flavonoid aglycones, and flavonoid glycosides. In this study, the EEE and EAE of Am showed the highest cytotoxicity in rat hepatocytes and fish erythrocytes among all of the examined extracts. This is in good agreement with the report that the EAE of *Astragalus* has cytotoxic and apoptosis-inducing effects on HeLa cells (Gül Özcan, 2012). Their cytotoxicity may be ascribed to the aglycones of saponin and flavonoids. Studies have suggested that the flavonoid aglycones induce cytotoxicity in ovarian cancer cells (Wang et al., 2015). The saponin aglycones induce high cytotoxicity in malignant tumour cells (Wojtkielewicz et al., 2007). The AE of Am may primarily contain flavonoid glycosides. In this study, the AE of Am showed the most anti-apoptotic effects among all of the examined extracts. This was consistent with the reports that flavonoids can prevent apoptosis in rat hepatocytes (Blankson et al., 2000). Based on the solubility, the EE of Am may contain a small amount of saponins at room temperature. Studies have indicated that the saponins isolated from Am protect against apoptosis in rat cardiomyocytes (Jia et al., 2014). The saponins in the EE of Am may be the key factors in the anti-apoptotic effects in fish erythrocytes. The AQE of Am may primarily contain polysaccharides and saponins. Studies have demonstrated that the polysaccharides of Am can delay zebra fish cell apoptosis (Xia et al., 2012). The observed anti-apoptotic effects in fish erythrocytes may be due to the saponins and polysaccharides in the AQE of Am. However, other ingredients in the AQE of Am may obstruct the antioxidative and anti-apoptotic effects in fish erythrocytes (Kuhn, 2002). Further research is needed to uncover the detailed mechanism by which this occurs.

It has been reported that the some polysaccharides, saponins and flavonoids can induce cytotoxicity *in vitro* (Engen et al., 2015; Podolak et al., 2010; Thetsrimuang et al., 2011). However, no

previous study has addressed whether EAm could directly enter the blood circulation and induce cytotoxicity *in vivo*. Moreover, sub-chronic toxicity studies demonstrated that the EAm, which consists of *Astragalus* polysaccharide and saponins, was safe without any distinct toxicity and side effects in rats and dogs (Yu et al., 2007). Long-term studies revealed that no toxic side effects were found in flavonoids-treated experimental animals (Nijveldt et al., 2001). The 100 g/kg of raw herbs of Am have been given by lavage to rats with no adverse effects (Monograph, 2003). Thus, it is possible that the EAm can be used as a feed ingredient for inhibiting oxidative stress and the apoptosis of erythrocytes in fish.

5. Conclusion

In summary, our study first showed that EAm is toxic at high concentrations, and at the same concentrations, BHA is more toxic than EAm in rat hepatocytes and fish erythrocytes. Moreover, the toxicity order of BHA and EAm in fish erythrocytes was consistent with that in the rat hepatocytes. Thus, the fish erythrocyte system can be used as an experimental model to evaluate the cytotoxicity of feed ingredients. Furthermore, this study is the first to reveal that EAm inhibits $\cdot\text{OH}$ -induced apoptosis by decreasing the generation of ROS, inhibiting the oxidation of cellular components and restoring the activities of antioxidants in fish erythrocytes. Of all of the examined EAm, the AE of Am showed the strongest effects. The effects of AE on $\text{O}_2^{\cdot-}$, H_2O_2 , Met-Hb, and GSH levels as well as GR activity were equivalent to those of BHA, and the effects on apoptosis were stronger than that of BHA. Therefore, the AE of Am could be used as a potential natural antioxidant and inhibitor of apoptosis in fish erythrocytes.

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