Prevention of Cellular Oxidative Damage by an Aqueous Extract of *Anoectochilus formosanus*

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ABSTRACT: *Anoectochilus formosanus* (AF) is a popular folk medicine in Taiwan whose pharmacological effects have been characterized. In this work we investigated the antioxidant properties of an aqueous extract prepared from AF. The AF extract was capable of scavenging H_2O_2 in a dose-dependent manner. We induced oxidative stress in HL-60 cells, either by the addition of hydrogen peroxide (H_2O_2) or by the xanthine/xanthine oxidase reaction. Apoptosis caused by oxidative damage was displayed by DNA fragmentation on gel electrophoresis, and the apoptotic fraction was quantified with flow cytometry. The cell damage induced by oxidative stress was prevented by the plant extract in a concentration-dependent manner. Furthermore, the proteolytic cleavage of poly(ADP-ribose) polymerase during the apoptotic process was also inhibited by AF extract. Our results provide the basis for determining an AF extract to be an antioxidant.

KEYWORDS: *Anoectochilus formosanus*; reactive oxygen species; apoptosis; PARP

INTRODUCTION

Reactive oxygen species (ROS) play an important role in the development of tissue damage and pathological events in living organisms.[1] Imbalance of oxidation–reduction in a healthy living system may lead to cellular dysfunction and various diseases. Selected natural products from plant sources share antioxidant actions. The genus *Anoectochilus (Orchidaceae)*, comprising more than 35 species, is a perennial herb. Its distribution spread from India, the Himalayas, Southeast Asia, and Indonesia to New Caledonia and Hawaii. Of the 35 species, four were found in Taiwan, including *Anoectochilus formosanus* (AF), *A. inabai, A. koshunensis*, and *A. lanceolatus.* AF and *A. koshunensis* were first used by local people to treat snakebite. AF is still a popular folk medicine used to treat hepatitis, hypertension, and can-

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cer in Taiwan. A crude AF extract, with high antioxidant content, was found to have hepatoprotective action in a rat model of CCl_4-induced liver damage.[2] This finding prompted us to examine the protective effect of an aqueous extract of AF in H_2O_2- or superoxide-induced apoptosis in HL-60 cells.

MATERIALS AND METHODS

Preparation of Plant Extracts

AF whole plant was collected from Puli in central Taiwan. The species of the plant was authenticated, and a voucher specimen (accession no. SP 9703010) was deposited in the herbarium of the Taipei Medical University.[3] One hundred grams of the whole plant was homogenized with 900 mL of cold water and stirred at 4°C for 30 min and then centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant was freeze-dried to powder form. The yield of crude extracts was 1.92 g. The dry extract was maintained at –20°C until used. The extract was dissolved in the appropriate medium immediately prior to the experiments.

Cells and Cell Culture

The promyelocytic cell line HL-60 was obtained from the American Type Culture Collection (Rockville, MD) and maintained as a continuous culture in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (Life Technologies, Merelbeke, Belgium), 100 U/mL penicillin G, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. Medium was refreshed every 3–4 days. Cell cultures were free of mycoplasma.

Hydrogen Peroxide Scavenging Assay

The ability of the extract to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.*[4] Hydrogen peroxide concentration was determined spectrophotometrically with absorption at 230 nm. Because the AF extract has an absorption spectrum covering 230 nm (A_{230}), the scavenging of hydrogen peroxide activity was determined by the decrease of the A_{230} after 10 min of incubation at room temperature in the presence of various concentrations of AF and 10 mM hydrogen peroxide in 1 mL phosphate-buffered saline (PBS, pH 7.4) against a blank solution containing AF extracts in PBS without hydrogen peroxide.

DNA Fragmentation Assay

Cells under various treatments were harvested and lysed with DNA extraction buffer (50 mM Tris–HCl, 10 mM EDTA, 0.5% *N*-lauroylsarcosine, 0.5 mg/mL proteinase K, and 0.5 μg/mL RNase A) overnight at 50°C. The DNA samples were isolated by phenol–chloroform extraction and then analyzed by a 1.8% agarose gel electrophoresis.[5] The DNA fragmentation was visualized by UV illumination after ethidium bromide staining.

Protection of HL-60 Cells from ROS Injury

A total of 2×10^5 HL-60 cells were incubated with various concentrations of AF extracts for 10 min and then treated with 50 μM xanthine and 3.2 U/L xanthine oxidase for 4 h. The resulting cell apoptosis was characterized with a DNA fragmentation assay.

Quantification of Apoptosis by Flow Cytometry

The percentage of cells displaying DNA fragmentation was quantified using propidium iodide. Cells were washed with PBS, fixed in 70% ethanol, and then incubated in extraction buffer (0.1 M Na_2HPO_4/0.05 M citric acid, pH 7.8) at room temperature for 10 min in the dark. The cells were stained with 10 μg/mL propidium iodide containing 0.5% Triton X-100 and 0.1 mg/mL RNase A for 30 min at room temperature. The apoptotic fraction, taken as the sub-G_1 peak, was analyzed on a FACScan (Becton Dickenson, San Jose, CA).[6]

Immunoblot Analysis

Cells under various treatments were lysed in 2× sodium dodecyl sulfate (SDS)/sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% β-mercaptoethanol) and boiled for 5 min at 100°C. Samples containing 10 μg of protein were subjected to electrophoresis in an 10% SDS-PAGE

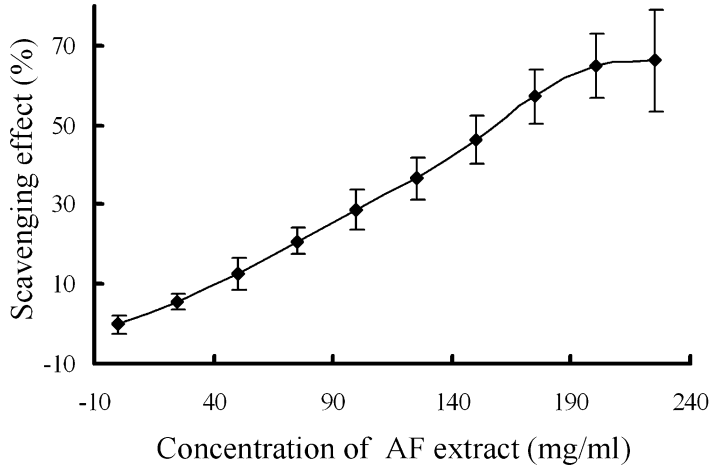

FIGURE 1. The scavenging effect of AF extract on hydrogen peroxide. Each data point represents a triplicate measurement of three preparations and was expressed as mean ± SD. Calculation of scavenging effect as following. (**A**) A_{230} (mixture of various concentration of AF and 10 mM hydrogen peroxide at room temp, 10 min) minus A_{230} (mixture of various concentration of AF at room temp, 10 min). (**B**) A_{230} (mixture of various concentration of AF and 10 mM hydrogen peroxide at room temp, 0 min) minus A_{230} (mixture of various concentration of AF at room temp, 0 min). Scavenging effect (%) = $[(B - A)/B] \times 100$.

and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was blocked in 10 mM Tris–HCl buffer (pH 7.5) containing 100 mM NaCl, 0.05% Tween 20 and 4% skimmed milk for 1 h and then probed with the antibodies raised against PARP (Clontech, Palo Alto, CA) and β-actin (Sigma, St. Louis, MO) for 1 h. The membrane was then incubated with a horseradish peroxidase–conjugated secondary antibody, and the immunoreactive bands were visualized with enhanced chemiluminescent reagents.[7]

RESULTS AND DISCUSSION

Hydrogen Peroxide Scavenging Effect by AF Extract

The aqueous extract of AF was capable of scavenging H_2O_2 in a dose-dependent manner during 10 min of incubation (FIG. 1), suggesting its antioxidant activity.

Reduction of Hydrogen Peroxide–Induced Apoptosis by AF Extract

Oxidative stress has been implicated in several pathological processes. The antioxidant effect provides a mode of action of several selected dietary items in disease prevention. The protection of HL-60 cells from H_2O_2-induced apoptosis by the aqueous extract of AF was compared with a number of known antioxidants. HL-60 cells were exposed to 50 μM H_2O_2 for 4 h. A characteristic DNA laddering pattern suggestive of apoptosis was noted following H_2O_2 exposure. H_2O_2-scavenging en-

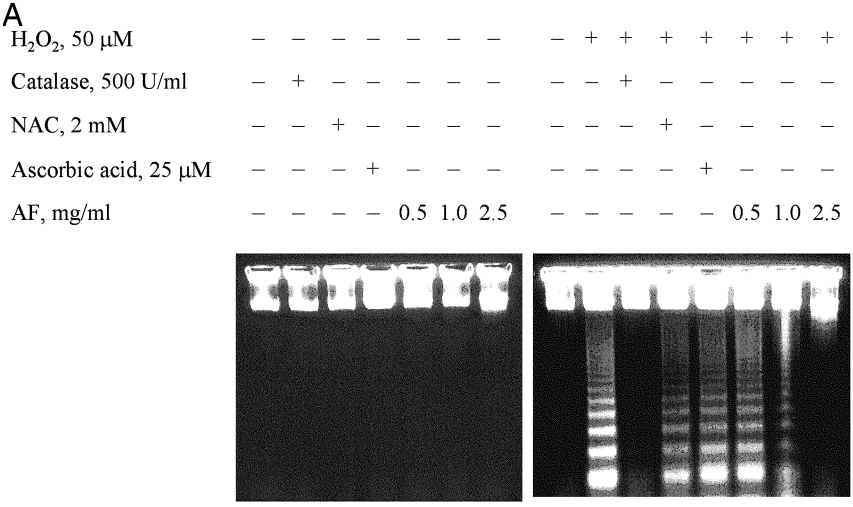

FIGURE 2. Effects of antioxidants on hydrogen peroxide induced cell apoptosis. (**A**) Effects of antioxidants on DNA fragmentation of apoptotic HL-60 cells. Cells were pretreated with catalase, NAC, ascorbic acid, or AF extract for 10 min prior to 50 μM H_2O_2 treatment for 4 h. The DNA ladder was analyzed using 1.8% agarose gel electrophoresis. The results in the left panel were control without hydrogen peroxide treatment, all showing genome integrity.

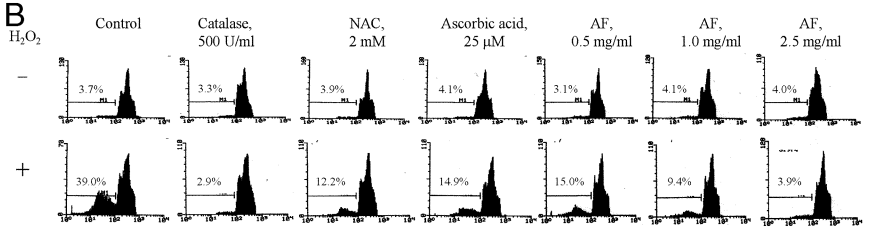

FIGURE 2 (*continued*). (**B**) Quantification of the apoptotic fraction upon various treatments using flow cytometry. Cells were treated as described in panel A. The cells were fixed in 70% ethanol, stained with 10 μg of propidium iodide/mL, and then the sub-G_1 peaks were analyzed on FACS. The percentage of sub-G1 peak fraction is indicated.

zyme catalase (500 U/L), or antioxidants including ascorbic acid (25 μM) and *N*-acetylcysteine (NAC, 2 mM), or the aqueous extract of AF (0–2.5 mg/mL) reduced the extent of H_2O_2-induced DNA laddering (FIG. 2A, right panel). The aqueous extract of AF reduced the extent of DNA laddering in a dose-dependent manner with the potency of 2.5 mg/mL comparable to that of 500 U/L catalase. To further assess the extent of apoptosis under various treatment conditions, the sub-G1 peak area was quantified using flow cytometry (FIG. 2B). Exposure to 50 μM H_2O_2 resulted in a 39% cell fraction in the sub-G1 peak area. The aqueous extract of AF dose-dependently reduced the extent of apoptosis based on flow cytometry, with the cell fraction in the sub-G1 peak area reduced to 3.9% at a concentration of 2.5 mg/mL, consistent with results shown in FIGURE 2A.

Inhibition of Xanthine/Xanthine Oxidase–Induced Cell Apoptosis by AF Extract

Xanthine/xanthine oxidase (X/XO) reaction is an important biological source of ROS contributing to the oxidative stress on the organism and is involved in many pathological processes. AF with ROS scavenging activity might be useful candidates for the prevention or suppression of X/XO reaction–induced damage. The extent of DNA laddering was suppressed when cells were treated with 0–5 mg/mL of AF extract (FIG. 3). AF effectively protected HL-60 cells from ROS-induced apoptosis in the X/XO reaction in a dose-dependent manner.

AF Extract Rescued PARP from Apoptotic Cleavage in Apoptotic Cells

When the apoptotic signaling was triggered by H_2O_2 in the HL-60 cells, the PARP was effectively cleaved from its native 113-kDa form to an 89-kDa fragment. Therefore, the cleavage pattern of PARP is used as an indication of apoptosis progression. HL-60 cells were treated with H_2O_2, and the PARP level was assessed by western blot analysis (FIG. 4). The processed 89-kDa cleavage forms of PARP appeared in samples derived from H_2O_2-treated HL-60 cells.[8] Upon the addition of various antioxidants, the levels of the 89-kDa fragment were decreased with catalase (500 U/mL), NAC (2 mM), ascorbic acid (25 μM), or AF (0.5, 1.0 and 2.5 mg/mL, respectively).

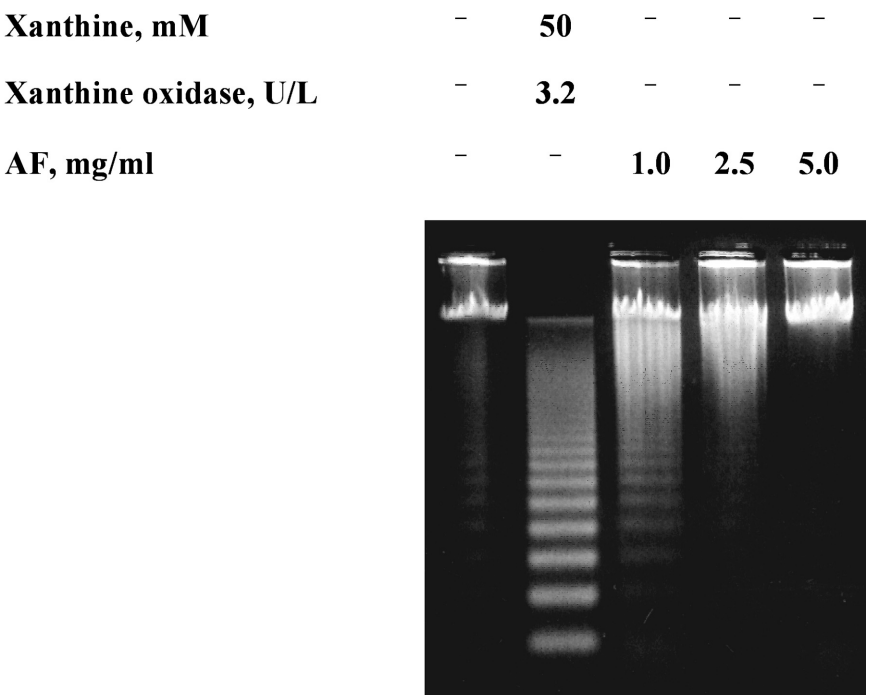

FIGURE 3. AF extract protected HL-60 cells from ROS-induced damage in the xanthine/xanthine oxidase reaction. DNA fragmentation of HL-60 cells treated with X/XO for 4 h in the presence of AF extract (0–5 mg/mL) is shown.

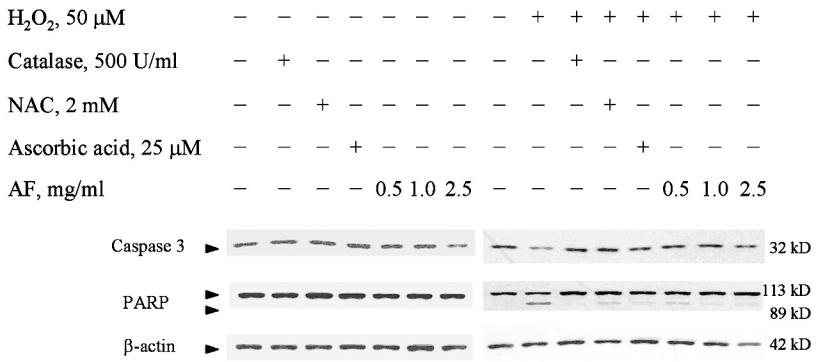

FIGURE 4. Effect of antioxidants on H_2O_2-induced PARP cleavage in HL-60 cells. Catalase, NAC, ascorbic acid, and an aqueous extract of AF were contained in various cell treatments, respectively, as described in FIGURE 2. Protein samples of whole-cell lysates were prepared, and 10 μg of protein for each lane was separated by 10% SDS-PAGE. Western immunoblots were performed using anti-PARP and –β-actin monoclonal antibodies to probe protein levels.

The pharmacological effects of aqueous extract of AF include hepatoprotective activity,[9] hypoglycemic and antioxidant effects in diabetic rats,[10] inhibitory action on the oxidation of low density lipoprotein,[11] and anti-inflammatory actions.[12] Excessive and persistent formation of ROS by inflammatory cells is thought to be a key factor in the ROS genotoxic effect. ROS was also proposed to be the common second messenger for activation of NF-κB, and some have suggested that the modulation of these oxidation–reduction reactions provides therapeutic benefit for controlling diseases involving inflammatory processes.[13] The aqueous AF extract exhibited the effects on protecting cells from hydrogen peroxide– or X/XO reaction–triggered apoptosis. Apoptotic cleavage of PARP in HL-60 was also reduced by the aqueous extract of AF. Results from this study provide pharmacological findings supporting the antioxidant action of this aqueous extract of AF.

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REFERENCES

1. Cuzzocrea, S., D.P. Riley, A.P. Caputi, *et al*. 2001. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol. Rev. **53:** 135–159.
2. Lin, C.C., P.C. Huang & J.M. Lin. 2000. Antioxidant and hepatoprotective effects of *Anoectochilus formosanus* and *Gynostemma pentaphyllum*. Am. J. Chin. Med. **28:** 87–96.
3. Cheng, K.T., L.C. Fu, C.S. Wang, *et al.* 1998. Determination of the components in a Chinese prescription, yu-ping-feng san, by RAPD analysis. Planta Med. **64:** 46–49.
4. Ruch, R.J., S.J. Cheng & J.E. Klaunig. 1989. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis **10:** 1003–1008.
5. Lin, C.M., C.S. Chen, C.T. Chen, *et al.* 2002. Molecular modeling of flavonoids that inhibits xanthine oxidase. Biochem. Biophys. Res. Commun. **294:** 167–172.
6. Lin, C.M., C.T. Chen, H.H. Lee, *et al*. 2002. Prevention of cellular ROS damage by isovitexin and related flavonoids. Planta Med. **68:** 363–365.
7. Wang, I.K., S.Y. Lin-Shiau & J.K. Lin. 1999. Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. Eur. J. Cancer **35:** 1517–1525.
8. DiPietrantonio, A.M., T. Hsieh, & J.M. Wu. 1999. Activation of caspase 3 in HL-60 cells exposed to hydrogen peroxide. Biochem. Biophys. Res. Commun. **255:** 477–482.
9. Du, X.M., N.Y. Sun, J. Hayashi, *et al.* 2003. Hepatoprotective and antihyperliposis activities of *in vitro* cultured *Anoectochilus formosanus*. Phytother. Res. **17:** 30–33.
10. Shih, C.C., Y.W. Wu & W.C. Lin. 2002. Antihyperglycaemic and anti-oxidant properties of *Anoectochilus formosanus* in diabetic rats. Clin. Exp. Pharmacol. Physiol. **29:** 684–688.
11. Shih, C.C., Y.W. Wu & W.C. Lin. 2003. Scavenging of reactive oxygen species and inhibition of the oxidation of low density lipoprotein by the aqueous extraction of *Anoectochilus formosanus*. Am. J. Chin. Med. **31:** 25–36.

12. LIN, J.M., C.C. LIN, H.F. CHIU, *et al*. 1993. Evaluation of the anti-inflammatory and liver-protective effects of *Anoectochilus formosanus, Ganoderma lucidum* and *Gynostemma pentaphyllum* in rats. Am. J. Chin. Med. **21:** 59–69.
13. TSE, H.M., M.J. MILTON & J.D. PIGANELLI. 2004. Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation-reduction reactions in innate immunity. Free Radic. Biol. Med. **36:** 233–247.