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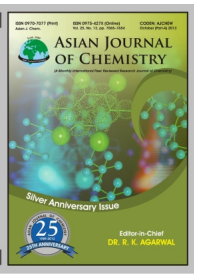

Antioxidant, Antiinflammatory and Anticancer Activities of *Amaranthus viridis* L. Extracts

YING-SHAN JIN[1], YONGHAO XUAN[2], MANLI CHEN[1], JINCHUAN CHEN, YUNZHE JIN[3], JIYU PIAO[3] and JUN TAO[4,*]

[1]College of Bioscience and Biotechnology, Yangzhou University, Yangzhou 225009, Jiangsu Province, P.R. China
[2]Yangzhou University Library, Yangzhou University, Yangzhou 225009, Jiangsu Province, P.R. China
[3]Longjing City Forestry Bureau in Jilin province, Longjing 133400, , Jilin Province, P.R. China
[4]College of Horticulture and Plant Protection, Yangzhou University, Yangzhou 225009, Jiangsu Province, P.R. China

*Corresponding author: Fax: +86 514 87347537; Tel: +86 514 87997219; E-mail: taojun@yzu.edu.cn; ysjin@yzu.edu.cn

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In this study, we investigated antioxidant, antiinflammatory and anticancer activities of ethyl ether extract and ethyl acetate extract of *A. viridis* L. Antioxidant activity were evaluated with DPPH free radical scavenging, total antioxidant activity assay and protein damage protection assay. The ethyl acetate extract exhibited higher antioxidant ability than that of ethyl ether extract. Moreover, the ethyl acetate extract not only showed strong antiinflammatory activity on RAW 264.7 cells, but also had greater anticancer activities against HT-29 and HepG2 cancer cells. These results suggest that *A. viridis* L. may be used as a potential source of antioxidant, antiinflammatory and anticancer materials.

Key Words: *Amaranthus viridis* L., Antioxidant activity, Antiinflammatory activity, Anticancer activity.

INTRODUCTION

Recently, increases in the prevalence rates of chronic diseases along with rapid increases in aging populations have led to great demands for foods with health-improving functionalities. A considerable body of literature supports the role of oxidative stress in the pathogenesis of age-related human diseases such as cancer, diabetes, immune system decline and brain dysfunction[1,2]. There have been various types of phytonutrients with multiple biological effects including anti-inflammatory, anticancer, antiallergic, antiviral and antiaging activities[3-5], therefore much attention has been focused on the biological properties of natural foods and herbs.

Amaranthus plants (Amaranthaceae) are spread throughout the world, growing under a wide range of climatic conditions and they are known as grains and leafy edible vegetables[6]. Traditionally, *Amaranthus viridis* L. (Amaranthaceae) has been used in the treatment of dysentery, enteritis, hemorrhoids and kidney diseases in China. *A. viridis* L. has been reported for its antiinflammatory, antioxidant, antihyperlipedemic and antidiabetic activities[7,8]. At the same time, the young leaves of *A. viridis* are used as a vegetable.

There are almost no relevant reports about the investigation of the bioactivities of *Amaranthus viridis* L. extracts. Thus, in this study, we evaluated the antioxidant, antiinflammatory and anticancer activities of ethyl ether and ethyl acetate extracts from *Amaranthus viridis* L. using *in vitro* assays.

EXPERIMENTAL

Preparation of extracts: Wild type *A. viridis* L. were collected from Yangzhou and identified by Professor H.Y. Huai (College of Bioscience and Biotechnology, Yangzhou University, China). 250 g dried sample of the powder was extracted with methanol. The crude extract was extracted sequentially with petroleum ether, ethyl ether, ethyl acetate and *n*-butanol.

DPPH free radical scavenging activity: The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of the extracts were determined by the method of Kilani *et al.*[9] with some modifications. Aliquots of varying concentration of the extract were mixed with freshly prepared DPPH in methanol (final concentration 100 μM) and the absorbance at 514 nm was measured after incubation for 0.5 h in the dark at room temperature. Methanol was used as control and ascorbic acid as reference compound. Inhibition (IC_{50}) was calculated from the graph of DPPH scavenging activity.

Total antioxidant activity: The effects of ethyl ether and ethyl acetate extracts from *A. viridis* L. on total antioxidant were investigated according to the method of Prieto *et al.*[10] with several modifications. Briefly, 0.2 mL of each extract (at concentration of 1.0 mg/mL) was mixed with 0.6 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95 ºC for 90 min. The absorbance of the cooled mixture was measured at 695 nm against a blank.

The total antioxidant activity was expressed as the absorbance of the sample. The higher absorbance value indicates higher antioxidant activity. BHA was used as standard.

Protein damage protection assay: The effect of ethyl ether and ethyl acetate extracts from *A. viridis* L. on protein oxidation was carried out according to the method of Hu *et al.*[11].

Cell lines and cell culture: RAW264.7 cell, HT-29 cell and HepG2 cell lines were purchased from the Korean Cell Bank (Seoul, Korea) and grown in RPMI 1640 (Roswell Park Memorial Institute medium 1640), supplemented with 10 % fetal bovine serum, 100 µg/mL and streptomycin 100 U/mL penicillin. Cells were cultured in a humidified atmosphere and incubated at 37 ºC in 5 % CO_2.

Determination of nitric oxide (NO) level: The inhibitory effects of *A. viridis* L. extracts on NO production were determined as described by Li *et al.*[12]. *A. viridis* L. extracts solubilized with DMSO were diluted with RPMI 1640. RAW264.7 cells (2×10^6 cells/mL) were incubated with LPS (1 µg/mL) in the presence or absence of *A. viridis* L. extracts for 24 h. Supernatants were assayed for NO.

MTT assay: The cytotoxicity of ethyl ether and ethyl acetate extracts from *A. viridis* L. was determined by a conventional MTT assay, as previously reported[13].

RT-PCR analysis: Total RNA was isolated using a Trizol reagent (Life Technologyies). For cDNA synthesis, 1 µg of RNA was reverse transcribed with moloney murine leukemia virus reverse transcriptase (Invitrogen). The primers used in this experiment were: COX-2 (F-5'-CACTACATCCTGACCCACTT-3' and R-5'-ATGCTCCTGCTTGAGTATGT-3'); iNOS (F-5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' and R-5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3') and GAPDH (F-5'-CACTCACGGCAAATTCAACGGCAC-3' and 5'-GACTCCACGACATACTCAGCAC-3'; β-actin(F-5'-TCACCCTGAAGTACCCCATC-3' and R-5'-CCATCTCTTGCTGCAAGTCC-3'); capspase-3(F-5'-TCACAGCAAAAGGAGCAGTTT-3' and, R-5'-CGTCAAAGGAAAAGGACTCAA-3').

Statistical analyses: All tests were carried out independently in triplicate (n = 3). Data are expressed as the mean ± standard derivation (SD). The results were processed using Excel 2003 (Microsoft, Redmond, WA, USA).

RESULTS AND DISCUSSION

Antioxidant activity: Amaranthus plants have been reported as one of many vegetables which are rich in antioxidant components. The leaves of amaranth constitute an inexpensive and rich source of protein, carotenoids, vitamin C, flavonoids and phenolic acids[14].

Fig. 1 shows the experimental results of antioxidant activity. High absorbance value of a sample indicates high antioxidant activity. Ethyl ether extract (EEA) and ethyl acetate extract (EAA) exhibited varying degrees of antioxidant activity. The order of total antioxidant activity was: BHA > EAA > EEA (Fig. 1A).

It was reported that DPPH is the method of choice for evaluating the free radical scavenging activity of natural compounds[15]. As shown in Fig. 1B, the EAA (IC_{50} = 70.0 µg/mL) had greater DPPH radicals scavenging activities. These results were consistent with the previous observation that Amaranthus

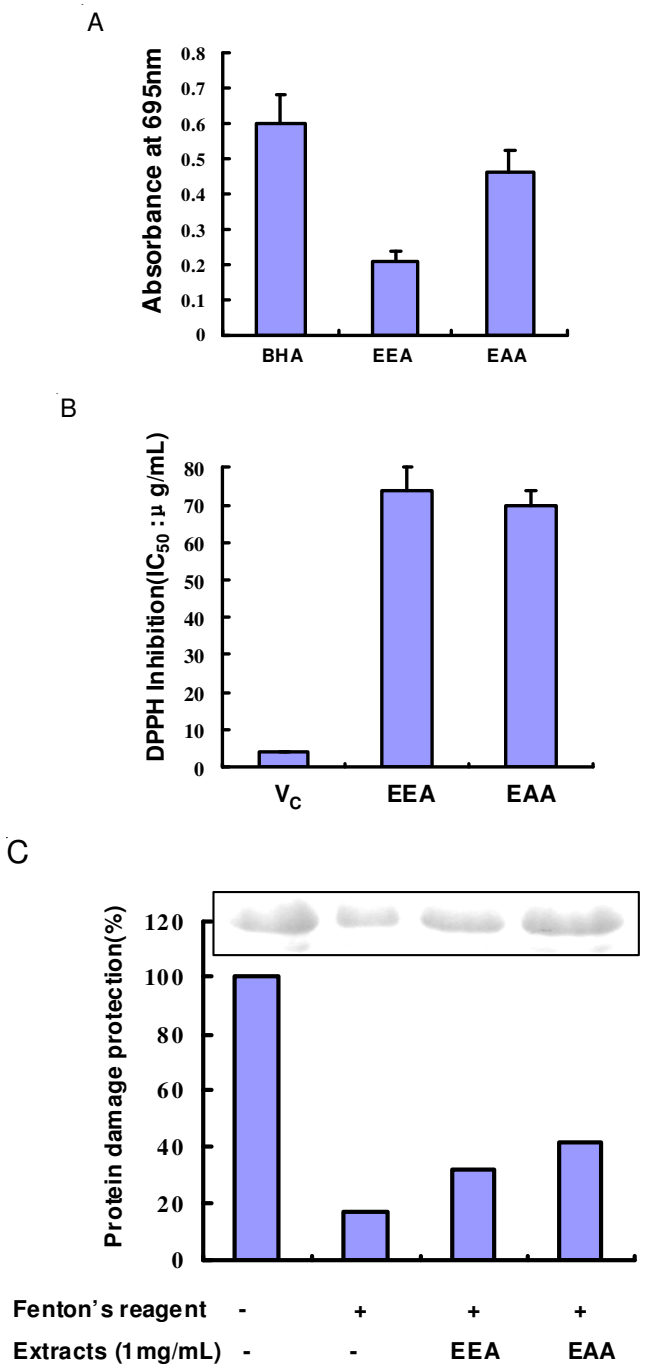


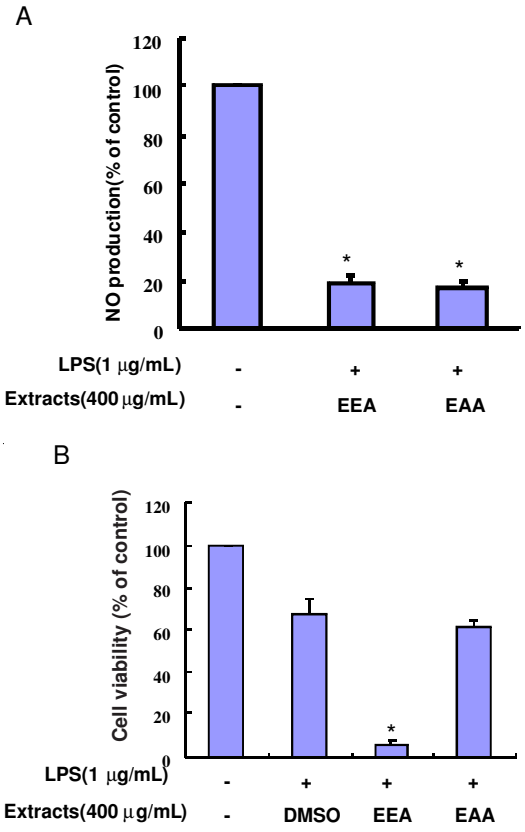
Fig. 1. Antioxidant activity. (A) Total antioxidant activity of extracts of *A. viridis* L. (100 µg/mL). BHA (80 µg/mL) were used as a standard. (B) DPPH free radical scavenging activity (IC_{50}: µg/mL) of EEA and EAA. (C) SDS-PAGE profile of the BSA protein treated with Fe^{3+}/H_2O_2/ascorbic acid system in the presence of the fractions of *A. viridis* L. (1 mg/mL) and histogram showing the protective effect densitometric measurements

varieties[16] contained radical scavenging agents that could directly react with and quench stable DPPH radicals. *A. paniculatus* extract acting as a free radical scavenger or hydrogen donor was reported[14]. Moreover, the antioxidative properties of the ethyl acetate extract of *A. lividis* on scavenging DPPH were found to be superior to those of *A. cruentus*[16].

The oxidative protein damage induced by free radicals has been shown to play a significant role in several pathological events in aging[17]. Hydroxyl radical is recognized as a

protein-damaging agent with physiological significance. The protein damage was induced by the Fe^{3+}/H_2O_2/ascorbic acid system. The ethyl ether and ethyl acetate extracts restored the BSA band intensity and EAA showed slightly greater protein protection than that of EEA (Fig. 1C).

Antiinflammatory activity: Lymphocytes and macrophages play important roles in the host immune defense mechanism, composed of innate and adaptive immunity[18]. However, certain conditions with over-production of inflammatory molecules by these immune cells can give the host severe immunopathological symptoms such as acute and chronic inflammatory diseases[19]. Therapy of inflammatory diseases is usually directed at the inflammatory processes. Many anti-inflammatory drugs have been prepared and marketed[20]; however, these complex drugs are known to provoke gastro-intestinal irritation. Thus, more gentle antiinflammatory natural herbs are being investigated. In this study, the antiinflammatory activity of *A. viridis* L. extracts was measured on LPS (lipopolysaccharide)-induced RAW264.7 cells. Compared to the group with LPS treatment, the EEA and EAA reduced the nitric oxide (NO) production (Fig. 2A). LPS can induce apoptosis in the cells, with a viability of 67.99 % (Fig. 2B). EEA also can induce apoptosis in the cells, with a viability of 5.59 %. The cytotoxicity of EEA is too high, so we stopped using it to do the subsequent experiment. EAA protected the cells against LPS-induced apoptosis. EAA inhibited mRNA levels of COX-2 and iNOS to suppress NO (Fig. 2C).The antiinflammatory effect of EAA is considerable, the results suggest that EAA is suitable to be used as an antiinflammatory agent. The antiinflammatory mechanism of *A. viridis* L. will be investigated in further study.



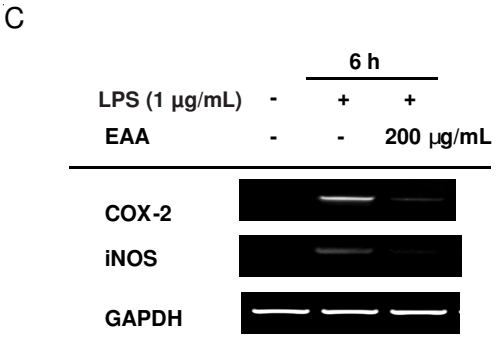


Fig. 2. Antiinflammatory activity. (A) Effect of EEA and EAA on the production of NO in LPS-activated RAW264.7 cells. RAW264.7 cells (1×10^6 cells/mL) were incubated with EEA and EAA in the presence of LPS (1 μg/mL) for 24 h. Culture supernatants were assayed for NO determination by Griess assay. (B) RAW264.7 cells ($1 \times 10_6$ cells/mL) were incubated with EEA and EAA for 24 h. Cell viability was determined by MTT assay. *:$p < 0.05$ compared to control. (C) Effect of EAA on the mRNA expression of iNOS and COX-2 from LPS-activated RAW264.7 cells. The mRNA levels of iNOS, COX-2 and GAPDH were determined by semi-quantitative RT-PCR

Anticancer activity: Cancer is a neoplastic disorder caused by excessive cellular proliferation. Apoptosis is a key regulator of tissue homeostasis and imbalances between cell death and proliferation may result in tumor formation[21]. The objective of using anticancer agents is to induce apoptosis-related signaling, disrupt cell proliferation in cancer cells. Cancer cell line HT-29 and HpeG 2 cells were used to examine the antiproliferation activity and cytotoxicity of the EEA and EAA using the MTT based assay. In HT-29 and HepG2 cells, extract of EEA inhibited cell growth and showed inhibition of growth rate on 24 h basis by 96.9 and 85.9 % at concentrations of 400 μg/mL, respectively (Fig. 3A). However, EAA can't inhibit cancer cells growth.

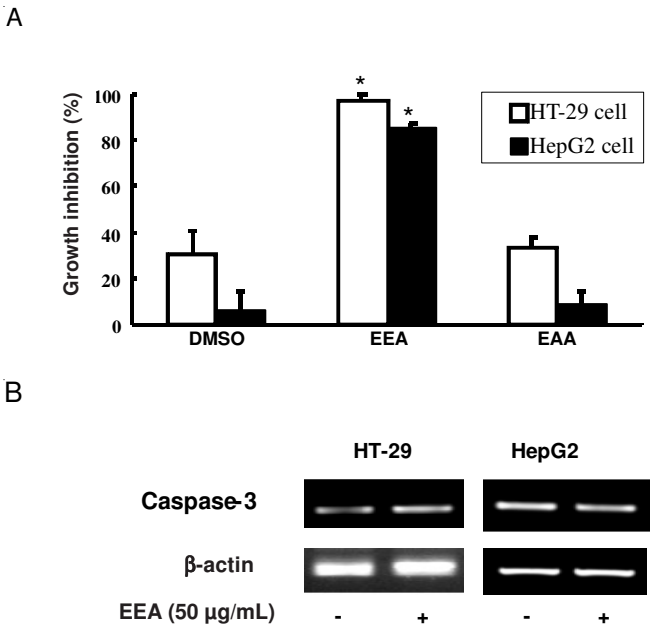


Fig. 3. Anticancer activity. (A) Cell viabilities of HT-29 or HepG2 cancer cells after incubated with 400 μg/mL EEA and EAA for 24h. (B) RT-PCR analysis of caspase-3 mRNA expression on HT-29 and HepG2 cells. Cancer cells were treated with EEA (50 μg/mL) for 24 h

Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Excessive apoptosis causes atrophy, whereas an insufficient amount results in uncontrolled cell proliferation, such as cancer. The activation of caspase-3 has been regarded as a primary mechanism of apoptosis[22,23]. Reverse transcription PCR was used to examine in caspase-3 expression. As shown in Fig. 3B, EEA up-regulated the expression of caspase-3 in HT-29 cells. However, there was no difference in the expression of caspase-3 in HepG2 cells. The anticancer mechanism of EEA will be investigated in further study.

Conclusion

The two extracts from *A. viridis* L. showed different biological activities in this study. The ethyl acetate extract showed higher free radical scavenging and antiinflammatory activities. The ethyl ether extract showed slightly lower antioxidant activity and strong anticancer activities. These results suggest that ethyl acetate extract should be investigated for further research of its antiinflammatory mechanisms. The antioxidant and anticancer effects elicited by ethyl acetate extract will be explored in further studies.

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