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ORIGINAL PAPER

Antioxidant Properties of *Amaranthus hypochondriacus* Seeds and their Effect on the Liver of Alcohol-Treated Rats

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Abstract Amaranth constitutes a valuable pseudocereal, due to its nutritional quality and its nutraceutical properties, which contribute to improve human health. This work evaluated the effect of a diet based on *Amaranthus hypochondriacus* (*Ah*) seed on oxidative stress and antioxidant status in the liver of rats sub-chronically exposed to ethanol. The seed extract was investigated for antioxidant capacity *in vitro,* showing an adequate content of total phenols and antioxidant activity elevated. For *in vivo* assays, four groups of six rats each were fed with an AIN-93 M diet for 28 days. In groups III and IV casein was replaced by *Ah* as the protein source; groups II and IV were received ethanol in the drinking water (20% *v*/*v*). When comparing groups IV and II, the following was observed: significant decrease in the activity of aspartate aminotransferase and content of malondialdehyde ($p<0.001$) in serum; decrease of malondialdehyde and increase in the activity and gene expression of Cu,Zn-superoxide dismutase, also, decrease in the NADPH oxidase transcript levels ($p<0.05$) in liver. Our data suggest that *Ah* is a good source of total phenols and exerts a protective effect in serum and in liver of rats intoxicated with ethanol.

Keywords *Amaranthus hypochondriacus* · Antioxidant enzymes · Ethanol · Oxidative stress · Total phenols

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Abbreviations

ADH alcohol dehydrogense
Ah *Amaranthus hypochondriacus*
ALP alkaline phosphatase
ALT Alanine aminotransferase
AST Aspartate aminotransferase
BHT Buthylated hydroxy toluene
CAT Catalase
CYP2E1 Cytochrome P450-2E1
DNPH 2,4-dinitrophenylhidrazine
DPPH 1,1-diphenyl-2-picrylhydrazyl
GGT Gamma glutamyl transferase
GPx Glutathione peroxidase
MDA Malondialdehyde
M-MLV Moloney Murine Leukemia Virus Reverse Transcriptase
NADPH Nicotinamide adenine dinucleotide phosphate
NO Nitric oxide
NO test Scavenging activity against nitric oxide
NOX NADPH oxidase
PCR Polymerase chain reaction
RNS Reactive nitrogen species
ROS Reactive oxygen species
RSA Radical scavenging activity
RT Reverse transcription
SOD Superoxide dismutase
TBARS Thiobarbituric Acid Reactive Substances
TMP 1,1,3,3-tetramethoxypropane

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism

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that at low/moderate concentrations act as beneficial species [1]. However, ROS/RNS overproduction and/or inadequate antioxidant defense leads to injury due to oxidative stress of various biomolecules, including proteins, lipids, lipoproteins and DNA.

The cell antioxidant defense system includes antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) as well as small molecules, such as glutathione, urate, ubiquinol and plasmatic protein. Also, a wide variety of natural antioxidants of vegetable origin, such as polyphenols, carotenoids and ascorbate, play a crucial role in the prevention of several diseases [2].

Epidemiological studies have examined the potential interaction between dietary habits and oxidative stress. Alcohol is one of the most widely consumed psychoactive substances in the world. The metabolism of ethanol induces ROS generation and depletion of the cell antioxidant activity, which leads to development of alcohol-related pathologies [3]. Otherwise, the consumption of grains has been associated with a lower risk of diseases related with oxidative stress [4]. Amaranth grains have an excellent nutritional quality; they contain approximately 15% protein with an adequate balance of aminoacids, high lysine content, 60% starch and 8% fat, besides minerals and dietary fiber [5]. Polyphenolic compounds, such as phenolic acids and flavonoids, have been characterized in amaranth grains [6]. Furthermore, amaranth grains are considered to be an important source of food for celiac patients, since they are gluten-free [7].

The aims of this work were: (a) to assess the content of total phenols, flavonoids, anthocyanins and antioxidant activity *in vitro* in methanolic extract of *Ah* seeds and (b) to evaluate the effect of the administration of a diet based on *Amaranthus hypochondriacus* (*Ah*) as a source of antioxidants on liver of male Wistar rats sub-chronically (for 28 days) intoxicated with ethanol.

Materials and Methods

Plant Materials

Amaranthus hypochondriacus (Ah) var. Antorcha was provided by the Agronomy Department of National University of Río Cuarto, Argentina, from a 2008 harvest of an experimental cultivation.

In Vitro Assays Determination of Bioactive Compounds and Antioxidant Activity

The extraction of total phenols was done from amaranth flour with 1.2 mol/l HCl in 50% methanol:water. The sample was heated at 90 °C for 3 h, cooled and diluted with methanol [8]. The supernatant was used for determination of phenols, flavonoids, anthocyanins and antioxidant activity. The concentration of the obtained extract was 5 mg/ml.

The determination of total phenols was performed using Folin Ciocalteu reagent with gallic acid as a standard. The absorbance was measured at 750 nm (UV–vis Beckman DK-2ª). The result was expressed as mg/100 g of dry weight of gallic acid equivalent [9].

Total flavonoids content was determined by a colorimetric method using Al_3Cl as complex-forming reagent with known catechin concentrations as a standard. The absorbance was measured at 510 nm. The results were expressed as mg/100 g of dry weight of catechin equivalent [10].

Total anthocyanins contents were estimated by a pH differential method [11]. An aliquot of methanolic extract was adjusted at pH 1.0 and another aliquot at pH 4.5. Absorbance of both mixtures was measured at 510 nm and at 700 nm. The monomeric pigment concentration in the extract was calculated by means of molar extinction coefficient of cyanidin-3-glucoside (26,900) and was expressed as mg/100 g of dry weight.

Scavenging activity against nitric oxide (NO test) uses sodium nitroprusside in 0.02 mol/l phosphate buffer (pH 7.4), to generate nitric oxide (NO), which interacts with oxygen to produce stable nitrite ions. These can be estimated by using Griess reagent at 542 nm [12]. Results were expressed as percentage (%) of radical scavenging activity (RSA) with respect to blank.

The DPPH free radical-scavenging assay relates the sample capacity to inhibit the action of free radicals generated by 0.004% methanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) [13, 14]. The absorbance was measured at 517 nm. Buthylated hydroxy toluene (BHT) was used as a positive control. Results were expressed as percentage (%) of RSA.

The β-carotene-linoleic acid assay involves measuring β-carotene bleaching at 470 nm resulting from β-carotene oxidation by linoleic acid degradation products at 50 °C [15]. The absorbance at 470 nm was taken at zero time (t=0) and was measured at intervals of 15 min until the color of β-carotene disappeared in the control tube (t=60 min). A mixture prepared without β-carotene served as blank. BHT was included in experiments as a positive control. Results were expressed as percentage (%) of RSA.

In Vivo Assays

Animal and Experimental Design

Twenty-four male Wistar rats, weighing 200 g, were used for the experiment. They were divided into four groups of six rats each, housed in individual cages and maintained under a regular light–dark cycle with free access to food

and water. All groups were fed according to recommendations of the American Institute of Nutrition 1993 AIN-93 M for four weeks [16]. In groups III and IV casein was replaced by *Ah* as protein source; groups II and IV received ethanol in the drinking water (20% *v*/*v*) [17]. Group I is standard control, without alcohol administration. An intake between 4 and 6 g/kg/day of alcohol per rat was estimated. All studies involving experimental animals in this work were conducted in accordance with national and institutional guidelines for the protection of animal welfare. The study was approved by the Animal Care Committee of the National University of San Luis, Argentina.

Determinations in Serum

Thiobarbituric acid reactive substances (TBARS) were assayed according to Draper & Hadley [18], and their concentration in serum was expressed as nmol/ml. The activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) were studied spectrophotometrically according to the standard procedures using Clinical Chemical Analyst Metrolab I model 2300 Plus, and were expressed as IU/l.

Determinations in Liver

Livers were homogenized (1/10 p/v) in cold 30 mM phosphate buffer pH 7.4, 120 mM KCl and 1% Triton X-100 (1:20 *v*/*v*). The TBARS and activities of catalase, superoxide dismutase, and glutathione peroxidase were determined in the supernatant.

The level of lipidic peroxidation was assessed spectrophotometrically at 234 nm by means of thiobarbituric acid reactive substances determination (TBARS), using malondialdehyde (MDA) as standard, prepared by hydrolysis of 1,1,3,3-tetramethoxypropane (TMP). The result was expressed as MDA (nmol/g tissue) [18].

The content of protein carbonyls was determined by the 2,4-dinitrophenylhydrazine (DNPH) method, according to Rreznick & Packer [19]. The maximum absorbance was determined at 360–390 nm. The results were calculated as μmol of carbonyls groups/mg of protein.

CAT activity was determined by following the decomposition of H_2O_2, measured by the decrease in absorbance at 240 nm in a medium containing 50 mM phosphate buffer (pH 7) [20]. One catalase unit is defined as the amount of the enzyme required to decompose 1 μM of H_2O_2/min/ml at pH 7 and 25 °C. The CAT activity was expressed as IU/mg protein.

Cu,Zn-SOD activity was evaluated by the method of McCord & Fridovich [21] modified by Flohé & Otting [22]. Activity of SOD was determined on the basis of its inhibitory action on the rate of superoxide-dependent reduction of cytochrome c by xanthine oxidase at 550 nm. One unit of SOD is defined as the amount of enzyme that inhibits cytochrome c reduction by 50%. The enzyme activity was expressed in IU/mg protein.

GPx activity was determined by following reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm in a reaction medium containing glutathione (GSH), glutathione reductase, tert-butyl hydroperoxide, phosphate buffer and EDTA (pH 7.7) [23]. One unit of activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of NADPH/min per mg protein, at 30 °C and pH 7.7. The enzyme activity was expressed as IU/mg protein.

The mRNA expression of Cu,Zn-SOD; CAT; GPx-1 and NADPH oxidase 2 (NOX-2) was estimated using RT-PCR analysis. Total RNA was isolated from liver tissues by means of TRIZOL® Reagent (Life Technologies). Reverse transcription (RT) reaction was performed using 200 IU of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) and random hexamer primers. The RT products (cDNA) were then subjected to polymerase chain reaction (PCR) at 94 °C for 5 min, followed by three cycling steps (35 cycles), each cycle consisting of denaturing at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and finally by the final extension at 72 °C for 5 min. All values for each PCR product were normalized and were expressed as a ratio with respect to the quantity of β-actin used as internal standard. The intensity of each band was measured with the NIH Image software and was reported as values of arbitrary units (AU).

Statistical Analysis

Results were expressed as the mean values ± SD. Statistical differences were tested by One-way Analysis of Variance (ANOVA). A probability of 0.05 or less indicated significant difference [24].

Results and Discussion

Antioxidant supplements or antioxidant containing foods may be used to help the human body to reduce oxidative damage [25]. Table 1 shows the content of total phenols, flavonoids and anthocyanins in *Ah* var. Antorcha. The amount of phenols found in this study was slightly higher than that reported for the seeds of other amaranth ecotypes, while the levels of flavonoids were found in adequate amounts and were not significantly different from values reported by other authors [26]. The content of anthocyanins was lower than values reported by Czerwinski et al. [26]. A number of factors can influence in the concentration of anthocyanins, for example, their synthesis is controlled by

Table 1 Total phenols, flavonoids and anthocyanins content and antioxidant activity in *Amaranthus hypochondriacus* seeds extract

Antioxidant compounds	(mg/100 g of dry weight)	Antioxidant activity	% RSA
Phenols	57.07±1.70	NO	35.20±1.60
Flavonoids	18.66±2.10	DPPH	86.93±1.40
Anthocyanins	35.33±1.70	scavenging β-carotene	69.51±1.50

NO Scavenging activity against nitric oxide; *DPPH* DPPH free radical-scavenging assay; *β-carotene* β-carotene-linoleic acid assay. *RSA* radical scavenging activity. Values are means ± SD (n=3)

various regulatory genes, can be induced by light [27] and also is inhibited by ionic stress and/or other environmental factors [28]. In general, the small variations observed in the content of these components can be explained by differences in environmental conditions or in the genetic background of the different vegetal species.

The extract showed the following order of antioxidant activity expressed as percentage (%) of RSA: DPPH: 86.93± 1.40; ß-carotene: 69.51±1.50; NO: 35.20±1.60 (Table 1). These data are similar to reports by Nsimba et al. [29] and Czerwinski et al. [26]; however, the values for ß-carotene were higher, which could be indicative of very good protection against the lipoperoxidation of membranes. It must be taken into account that the total antioxidant activity involves not only phenols but also non-phenolic compounds, such as ascorbic acid, tocopherols, sterols and carotenoids, among others.

During ethanol metabolism free radicals are generated, which contribute to cellular damage. It has been demonstrated that pretreatment with antioxidants such as vitamin E, vitamin C, and agents that enhance antioxidant capacity, can attenuate alcohol-induced injury [30]. In order to assess the potential protective role of the bioactive compounds with antioxidant properties present in *Ah,* an *in vivo* study was performed.

In serum, MDA (nmol/ml) and AST (IU/l) of group IV (Alcohol + *Ah*) decreased as compared to group II (Alcohol + Casein). MDA (nmol/ml) IV: 1.15±0.10, II: 1.49±0.11, $p<0.001$ and AST (IU/l) IV: 103.50±15.93, II: 157.17± 18.30, $p<0.001$ (Table 2). These results show that ethanol caused a significant increase in the lipid oxidation and levels of AST and that administration of *Ah* in the diet restored these values. The serum enzymes did not suffer modifications with alcohol intake when the diet included *Ah*, this may be indicative of the protective effect of *Ah*. When the diet included casein, ALP (IU/l) increased in group II (Alcohol + Casein) as compared to group I (Control Casein) ($p<0.05$).

In liver tissue, the MDA values (nmol/g tissue) exhibited a significant decrease in group IV: 0.11±0.03, as compared to group II: 0.20±0.03, $p<0.05$ (Table 3). This might suggest that alcohol provokes oxidative stress and that *Ah* plays an important role in the protection against lipid peroxidation in the liver. As can be seen in Table 3, SOD activity (IU/mg prot) increased in group IV: 26.66±1.65 as compared to group II: 21.78±2.44, $p<0.05$ and an increasing tendency was observed in the activities of CAT (15.41%), which might be due to a better response of the antioxidant mechanisms of the organism under a vegetable diet. Both the increased SOD and CAT activities are correlated with a significant decrease in the MDA content. This may reflect a compensatory response to increased oxidative stress. Glutathione peroxidase activity was not modified. No alteration in GPx suggests enhanced tolerance or low susceptibility in liver in response to the chronic ethanol stress. The carbonyl groups were also studied, which constitute the best markers of oxidation protein [31]. Evaluation of the presence of carbonyl groups showed no differences between rats under different diets, which suggests that in the studied period, lipid oxidation is the predominant damage, as shown by MDA values (Table 3). Likewise, the difference in duration of ethanol exposure may have contributed to the disparate results. Although a number of studies have shown an increase in lipid peroxidation or the formation of carbonyl groups caused by alcohol, it is not always clear whether this is a cause or an effect of the tissue injury induced by alcohol.

The gene expression of the enzymes involved in the antioxidant system was measured by RT-PCR using gene-specific primers. Up-regulation of Cu,Zn-SOD expressed as

Table 2 Levels of lipids peroxidation and hepatic function enzymes in serum

	I: Control Casein	II: Alcohol + Casein	III: Control *Ah*	IV: Alcohol + *Ah*
MDA (nmol/ml)	1.22±0.10	1.49±0.11**	1.16±0.12	1.15±0.10[a]
AST (IU/l)	99.30±19.25	157.17±18.30**	101.00±11.2	103.50±15.93[a]
ALT (IU/l)	51.80±7.98	44.50±3.45	43.80±6.10	48.80±6.14
ALP (IU/l)	214.50±34.15	300.75±32.94*	269.40±49.88	227.40±30.01
GGT (IU/l)	2.83±0.98	2.50±1.05	3.99±1.87	3.80±1.30

Ah Amaranthus hypochondriacus; *MDA* malondialdehyde; *AST* aspartate aminotransferase; *ALT* alanine aminotransferase; *ALP* alkaline phosphatase; *GGT* gamma glutamyl transferase

[a] $p<0.001$ as compared to group II; *$p<0.05$ as compared to group I; **$p<0.01$ as compared to group I. Values are means ± SD (n=6)

Table 3 Levels of lipids peroxidation and activity of antioxidant enzymes in liver

	I: Control Casein	II: Alcohol + Casein	III: Control *Ah*	IV: Alcohol + *Ah*
MDA (nmol/g tissue)	0.11±0.01	0.20±0.03*	0.12±0.03	0.11±0.03[a]
Cu,Zn-SOD (IU/mg prot)	17.31±2.39	21.78±2.44*	19.58±1.79	26.66±1.65[a ##]
CAT (IU/mg prot)	159.01±42.51	158.46±47.45	171.31±11.01	182.88±49.10
GPx (IU/mg prot)	0.65±0.07	0.53±0.12	0.62±0.11	0.52±0.08
Carbonyls (μmol/mg prot)	4.75±0.57	4.65±0.56	4.89±0.70	5.09±0.55

Ah Amaranthus hypochondriacus; *MDA* malondialdehyde; *Cu,Zn-SOD* Cu,Zn-superoxide dismutase; *CAT* catalase; *GPx* glutathione peroxidase

[a] $p<0.05$ as compared to group II; *$p<0.05$ as compared to group I; ## $p<0.01$ as compared to group III. Values are means ± SD ($n=6$)

arbitrary units (AU) was observed in group IV (Alcohol + *Ah*) as compared to group II (Alcohol + Casein) (IV: 0.51± 0.05; II: 0.45±0.04; $p<0.05$) The transcript levels of CAT and GPx-1 showed no significant differences (Fig. 1). The apparent discordance among transcripts and biological activities of these enzymes suggests regulation by multiple factors at different levels. The expression of the SOD and GPx mRNA exhibited the same behavior as enzymatic activity (Table 3); this parallelism between mRNA levels and enzymatic activity suggests that the expression of these enzymes may be regulated by transcriptional control [32]. The expression of CAT mRNA did not exhibit variations; however, the activity of this enzyme showed an increasing tendency, which may indicate that one or more mechanisms of post-transcriptional control might be involved.

During ethanol metabolism, cytochrome P450-2E1 (CYP2E1), alcohol dehydrogenase (ADH) and NADPH oxidase (NOX) generate H_2O_2, acetaldehyde and superoxide radicals, respectively [33]. The present study demonstrates that NOX-2 transcript level (AU) decrease in group IV as compared to group II (IV: 1.89±0.23; II: 2.72±0.17; $p<0.05$) (Fig. 1), which may suggest low generation of superoxide radicals and, as a consequence, a decrease in lipid peroxidation, in agreement with the MDA values previously obtained (Tables 2 and 3).

The correlation between the increase in mRNA expression and SOD activity, along with the reduced MDA values in

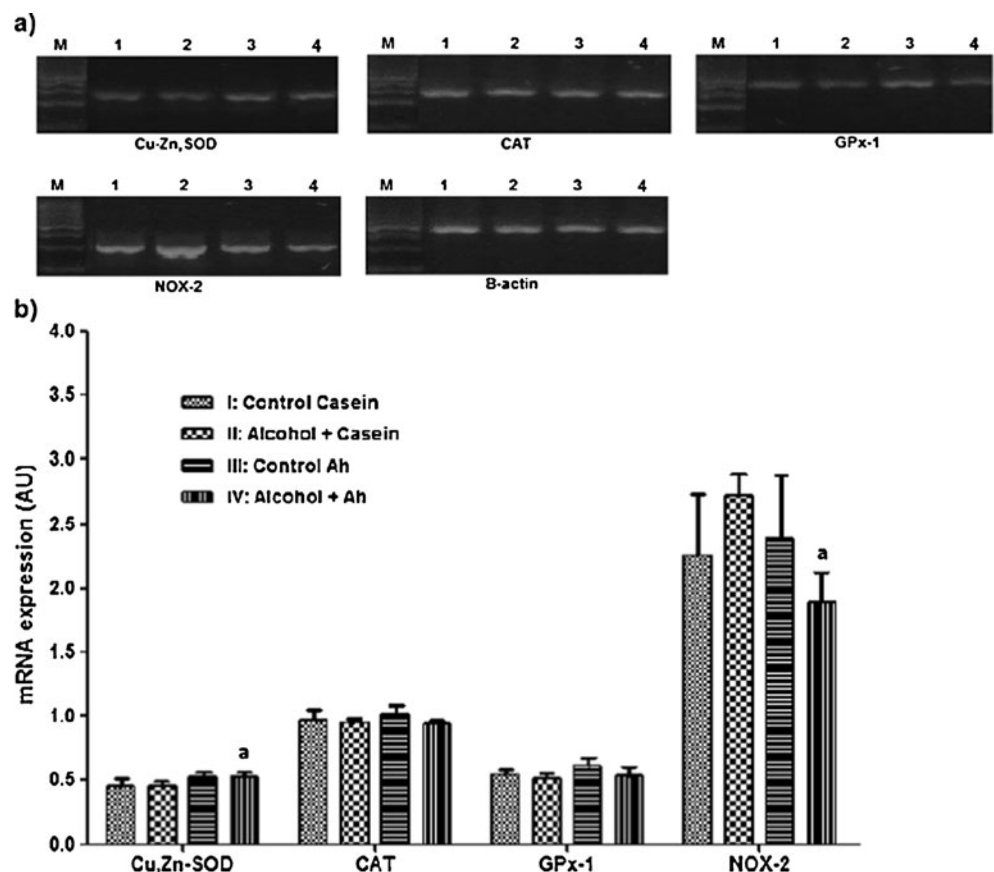

Fig. 1 RT-PCR of liver antioxidant enzymes against β-actin as internal standard. **a** Agarose gel electrophoresis of RT-PCR products from total RNA of Cu,Zn-superoxide dismutase *(Cu,Zn-SOD)*, catalase *(CAT)*, glutathione peroxidase *(GPx-1)*, NADPH oxidase-2 *(NOX-2)* and β-actin. *Lane 1* (Group I: Control Casein), *lane 2* (Group II: Alcohol + Casein), *lane 3* (Group III: Control *Ah*), *lane 4* (Alcohol + *Ah*). **b** Densitometric analyses as arbitrary units (*AU*). [a]$p<0.05$ as compared to group II. Values are means ± SD ($n=6$)

group IV as compared to II, indicates that the presence of antioxidants in *Ah* may have a beneficial effect. On the other hand, the reduction of the enzymatic antioxidant defense and the MDA increase in group II might be a contributing factor in the pathogenesis of alcoholic hepatic disease.

In conclusion, this work has shown that *Ah* extract has adequate contents of total phenols, flavonoids and anthocyanins, which, along with tocopherols, peptides and other bioactive components present in the seed, act as a source of dietary antioxidants. Also, the extracts were able to inhibit lipid oxidation and scavenge several free radicals in the *in vitro* experiments. *In vivo*, the diet based on *Ah* has a beneficial effect on the liver of alcohol-intoxicated rats. Taking into account that the metabolism of ethanol is very similar in rats and humans, the results obtained suggest that *Ah* may protect the human liver against oxidative injury caused by ethanol. It may also prevent oxidative stress produced by other agents which affect other systems and that are also involved in numerous pathological situations. Therefore, the present study expects to contribute to the promotion of the use of these pseudocereals in human nutrition as source of antioxidant compounds, proteins and minerals.

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