Determination and Toxicity of Saponins from *Amaranthus cruentus* Seeds

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The concentrations of four triterpene saponins present in amaranth seeds were determined with high-performance liquid chromatography. It was shown that the total concentration of saponins in seeds was 0.09−0.1% of dry matter. In germinating seeds an increase in concentration to 0.18% was observed after 4 days of germination, which remained stable for the next 3 days and later dropped to 0.09%. Highly purified extracts from the seeds were tested for their toxicity against hamsters. The hydrophobic fraction obtained by the extraction of seeds with methylene chloride showed no toxicity; the behavior of tested animals was similar to that of the group given an equivalent dose of rapeseed oil. A crude saponin fraction, containing ∼70% of pure saponins in the matrix, showed some toxicity; the approximate lethal dose was calculated as 1100 mg/kg of body weight. It is concluded that low contents of saponins in amaranth seeds and their relatively low toxicity guarantee that amaranth-derived products create no significant hazard for the consumer.

Keywords: *Amaranth; Amaranthus cruentus; saponins; concentration; toxicity*

INTRODUCTION

Amaranth is a nongrass plant producing significant amounts of edible "cereal" grain and is by some authorities called the "grain of the 21st century". Its protein content is as high as 16%, which is somewhat higher than that found among commercial varieties of common cereals. This protein is unusual because its balance of amino acids is very close to the optimum required in human nutrition. In comparison to other cereals it has high lysine and tryptophan contents. Low gluten concentrations make products based on amaranth very valuable for the diet of persons afflicted with diabetes (*Amaranth*, 1984). Amaranth contains ∼8% oil, of which fatty acids comprise ∼70% oleic and linoleic, ∼20% steraric, and ∼1% linolenic acids. The oil also contains a high level of squalene.

The limited literature data indicate, however, the presence of some antinutritional factors such as saponins, trypsin inhibitors, and tannins. Despite some recent progress in the identification of saponins in amaranth species (Rastelli et al., 1995; Junkuszew et al., 1998), nothing is known about their concentrations in seeds and their toxicity against animals. Thus, in our present work saponins in amaranth seeds were determined with high-performance liquid chromatography (HPLC), and purified saponin extracts were fed to hamsters to determine their toxicity. Moreover, because some procedures for preparing amaranth-derived food require soaking the seeds (*Amaranth*, 1984), we deemed it of interest to study how saponin concentrations may change during 0−10 days of the germination process.

MATERIALS AND METHODS

Plant Material. Seeds of amaranth (*Amaranthus cruentus* L.) were purchased at Szarlat Co., Lomza, Poland. These seeds were germinated on a filter paper in glass Petri dishes (0.5 g per dish) at room temperature (21−22 °C, 80% relative humidity). The papers were kept moist by subirrigated paper wicks. Three plates were terminated every day for 10 days. The germinated seeds were freeze-dried and powdered.

HPLC Analysis. *Extraction.* For extraction, 200 mg of powdered seeds was refluxed for 2 h with 100 mL of methanol. The extract was filtered and evaporated to dryness, and the resulting solid was redissolved in 10 mL of distilled water.

Purification. Five milliliters of the aqueous solution was passed through a C_{18} Sep-Pak cartridge (Waters Associates) preconditioned with 5 mL of water. The cartridge was washed with 10 mL of 30% methanol, and saponins were then removed with 5 mL of 70% methanol (Oleszek, 1988).

Derivatization. After evaporation of solvent, saponins were derivatized with 4-bromophenacyl bromide (Oleszek et al., 1990).

Identification and Quantitation. The saponins derivatized with 4-bromophenacyl bromide were analyzed by an HPLC system (Knauer, Germany) with an ultraviolet (UV) detector operating at 260 nm. Separations were performed on a 5 μm (250 × 4.6 mm i.d.) Eurospher 80 C_{18} column (Säulentechnik, Germany). Chromatographic runs were carried out using a mobile phase (AcN/H_2O) gradient as previously described (Nowacka and Oleszek, 1992). Three independent runs were performed for each sample, and saponins were identified by comparing their retention times with those of authentic standards (Junkuszew, 1998; Junkuszew et al., 1998). Quantification was based on external standardization by employing calibration curves over the range 0.25−1.0 mg/mL of the individual reference compounds (Figure 1).

Statistical Analysis. Each sample was submitted to three replicate analyses, and results were subjected to the ANOVA test. The relative standard deviation for saponins was 8−10%.

Toxicity Evaluation. *Extraction and Purification of Samples.* Amaranth seeds (5 kg) were extracted in Soxhlet with methylene chloride. Evaporation of solvent resulted in oil (*Hdp*-hydrophobic, 800 g). Defatted plant material was then extracted with methanol. Evaporation of the solvent yielded a solid, which was redissolved in water and loaded on the column (C_{18}, 3 × 10 cm). The column was washed with 30% methanol, and crude saponins were removed with 70% methanol. Evaporation of solvent provided dry crude saponins *Cs* (4

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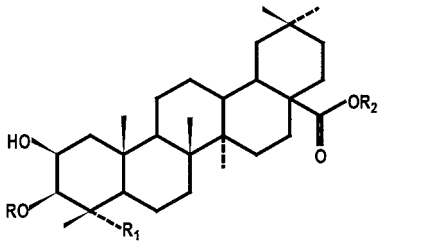
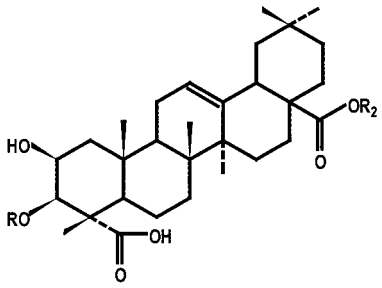


1: R = Rha (1→3) GlcA R_1= CH_3 R_2 = Glc
2: R = Rha (1→3) GlcA R_1= CH_2OH R_2 = Glc
3: R = Rha (1→3) GlcA R_1= CHO R_2 = Glc



4: R = GlcA R_2 = Glc

Figure 1. Chemical formulas of saponins from *A. cruentus* seeds used as standards.

g). Concentration of pure saponins in *Cs* was determined with the HPLC, as above.

Animal Experiments. Experiments with hamsters were performed in the Pharmacology and Toxicology Department, State Veterinary Institute, Pulawy. Four-month-old males weighing 80 g (±10%) were randomly distributed into six groups of four animals each. For several hours prior to the experiment animals were not given any food. The *Hdp* and *Cs* samples were administered in one dose with a tube straight to the stomach.

For the *Hdp* fraction, the following preparations were fed to animals (doses calculated in relation to 100 g of animal body weight): group I, 0.5 mL of *Hdp* + 0.5 mL of rapeseed oil; group II, 1 mL of *Hdp*; group III, 2 mL of *Hdp*; and group IV, 1 mL of rapeseed oil (as a control).

The *Cs* samples were dissolved in the smallest possible amount of ethanol, and water was added in such an amount that one animal was obtaining 1 mL of solution of an appropriate saponin concentration. Animals were first given 500 mg/kg, and then, due to the lack of any toxicity symptoms, after 2 weeks they were given 1500 and 1750 mg/kg. The performance of experimental animals was observed continuously.

RESULTS AND DISCUSSION

The four triterpene saponins from *A. cruentus* seeds, which were isolated and identified previously (Junkuszew et al., 1998), were used as standards for calibration of the HPLC procedure for their determination in plant materials. Because the compounds did not possess any chromophores allowing registration of chromatograms with a UV detector, precolumn derivatization with 4-bromophenacyl bromide was performed. This derivatization was possible for all four saponins because they had at least one free COOH group in the molecule (Figure 2), which is a perequisite for derivatization (Oleszek et al., 1990). The calibration curves prepared for the concentrations in the range 0.25−1 mg/mL showed very good linearity with a correlation coefficient of 0.99.

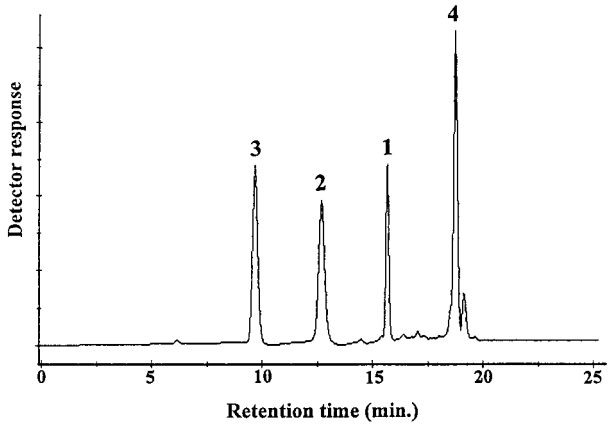


Figure 2. HPLC separation of 4-bromophenacyl bromide derivatives of amaranth saponin standards.

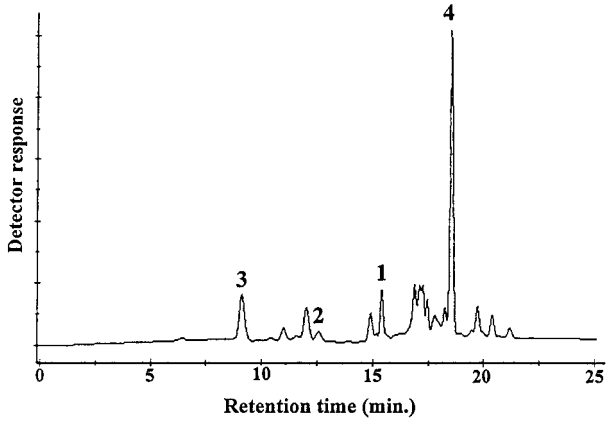


Figure 3. HPLC profile of saponins from amaranth extracts.

Table 1. Concentration of Saponins in Germinating Amaranth Seeds

germination time (h)	concn (mg/g)	germination time (h)	concn (mg/g)
24	0.79 ± 0.08	168	1.69 ± 0.12
48	0.94 ± 0.07	192	0.85 ± 0.07
96	1.86 ± 0.16	216	0.92 ± 0.08
120	1.78 ± 0.14	240	0.95 ± 0.07
144	1.39 ± 0.14		

The concentrations of individual saponins established with this procedure were 0.017, 0.020, and 0.060% (±15%) in dry matter for compounds **1**, **3**, and **4**, respectively (Table 1). The concentration of compound **2** was below the detection limit of the method and was not determined (Figure 3). Thus, the total concentration of saponins in *A. cruentus* seeds was ∼0.1% of dry matter and can be recognized as low if compared to those found in legumes and in some other grains (Price et al., 1987).

To check if soaking and germination processes change the saponin content, seeds were germinated for 10 successive days. It was evident (Table 1) that after 48 h the concentration of saponins did not change. Increase in their concentration was registered after 96 h of germination, but this reached the level of 0.18% in dry matter, which despite being double the concentration in ungerminated seeds was still relatively low. This concentration persisted through the next 4 days and after that dropped again to the level of 0.088% of dry matter. The relative concentration of individual saponins did not differ statistically during the testing period. This experiment undoubtedly shows that during

the germination of amaranth seeds there is an increased synthesis of saponins, but this process is not as intensive as might be expected.

For evaluation of toxicity, gram amounts of saponin preparations were needed. Because the concentration of saponins in amaranth seeds is low and separation of individual compounds was time-consuming, highly purified fractions were used in experiments. The hydrophobic fraction (*Hdp*) was obtained in the form of oil, containing besides fatty acids high amounts of squalene, chondrillasterol, and its 3-*O*-glucoside (Junkuszew et al., 1998). As a control, rapeseed oil fed in similar concentration was used. These tests show that even 2 mL of *Hdp* had no influence on animal performance. No symptoms of disturbance in the nervous system were observed, and the behavior of test group animals did not differ from that of the group getting rapeseed oil.

To obtain crude triterpene saponins, the extract was purified on a C_{18} short column. This procedure allowed us to obtain crude saponins in which, as determined by HPLC, saponins made up 70% of the matrix. When *Cs* was introduced by tube to the stomach at a concentration of 1500 mg/kg, no toxicity symptoms were observed in hamsters and the behavior of the animals did not differ from that of the control group. In the group of animals in which the saponin dose was increased to 1750 mg/kg, during the first few hours after administration of saponins, breathing problems were observed. After 10−15 h, animals suffered nervous system perturbation followed by death after 36 h. No further tests were performed to establish the exact lethal dose, but on the basis of the experiments, the approximate lethal dose value can be estimated and is thought to be somewhere between 1500 and 1750 mg/kg of body weight. Considering that the purity of *Cs* was ∼70%, the approximate lethal dose for pure saponins can be calculated as 1100 mg/kg.

According to the scale of toxicity, amaranth saponins can be regarded as moderately toxic (Zbinden and Flury-Roversi, 1981). Also, in relation to other saponins, amaranth saponins show low toxicity. The LD_{50} for rats evaluated for six orally administered structurally divergent saponins ranged between 50 and 160 mg/kg (Vogel and Marek, 1962). The dose for ginseng saponins evaluated with mice was 765 mg/kg (Chandel and Rastogi, 1980), for hederagenin glycosides from *Sapindus mukorosi* was 1625 mg/kg (Agrawal and Rastogi, 1974), and for zanhic acid tridesmoside measured for hamsters was 562 mg/kg (Oleszek, 1996). Gaunt et al. (1974), studying *Quillaya saponaria* saponins fed to rats, concluded that 400 mg/kg is a safe daily rate. Extracts of the same plant were studied by Drake et al. (1982), who showed that a dose of 1500 mg/kg resulted in weight depression at the beginning of experiment, but after some period animals recovered and performed very well during two years of study. The *Quillaya* extracts are recognized as generally regarded as safe (GRAS) products and are accepted for being used as food additives. Similarly, low concentrations of saponins in amaranth grain as well as their relatively low toxicity in relation to other saponins seem to indicate that the presence of these compounds does not create any hazard for consumer health. From a saponin point of view, amaranth-derived products can be recognized as safe.

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