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Identification of novel saponins in vegetable amaranth and characterization of their hemolytic activity

Jenny Zehring [a,b,1], Valeria Reim [a,1], David Schröter [b], Susanne Neugart [b], Monika Schreiner [b], Sascha Rohn [a,*], Ronald Maul [a,b]

[a] University of Hamburg, Hamburg School Of Food Science, Institute of Food Chemistry, Grindelallee 117, D-20146 Hamburg, Germany
[b] Leibniz-Institute of Vegetable and Ornamental Crops, Großbeeren/Erfurt e.V., Department of Quality, Theodor-Echtermeyer-Weg 1, D-14979 Großbeeren, Germany

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ABSTRACT

Amaranth is a plant genus of global importance comprising more than 60 species that are dually used for human consumption. While the grains are used as pseudo-cereals mainly in America and Asia, leaves are also consumed as leafy vegetable in African countries. Besides further secondary plant metabolites, saponins are described as major bioactive constituents in amaranth species. These triterpenoid saponins belonging to the oleanane-type are assumed to be part of the plant defense system and are often also associated with potential health risks for the consumer, mainly due to hemolytic properties. However, data concerning amaranth saponins are limited to the grains of single cultivars of only a few species investigated.

The aim of the present work was to determine the saponin profile in leaves of various amaranth cultivars grown under identical conditions. Out of 15 cultivars, six did not show any indications for the presence of saponins in HPLC–MS analysis. Two saponin-rich cultivars (one of *Amaraanthus hybridus* and one of *Amaranthus hypochondriacus*) as well as commercially available amaranth grains were selected for an in-depth analysis using a combined approach of high resolution and multi stage mass spectrometry. Three previously undescribed monodesmosidic and four bidesmosidic saponins could be assigned according to the MS data. Four novel saponins were also found in commercial grain amaranth analyzed for comparison. The investigation of the hemolytic effects revealed that only one saponin exerts significant activity whilst the further saponins did not lyse erythrocytes in vitro.

The results show that the saponin profile of amaranth cultivars is more diverse than reported so far. However, the biological activity seems to be different for the single structures. Thus, a more comprehensive case-by-case investigation of amaranth saponins is required to evaluate the impact of these secondary metabolites on humans and plants.

1. Introduction

Amaranth is a plant genus consisting of more than 60 species distributed worldwide. Unlike most other crop plants, it is used versatile, e.g. as vegetable, grain, or ornamental plant. Amaranth belongs to the family Amaranthaceae, not being a member of the grasses as the most common staple foods (wheat, rice, corn). Therefore, the seeds are considered a pseudo-grain or pseudo-cereals (National Research Council, 1984). Due to its socio-economic importance and its nutritional value, amaranth is claimed as the new millennium crop (Rastogi & Shukla, 2013).

Depending on the size, taste and quantity of the leaves and grains, some amaranth species are cultivated solely as vegetable or grain crop, although irrespective of usage, leaves and grains of all amaranth species are edible. The leaves are prepared similar as spinach (*Spinacia oleracea*): cooked or stewed. Thus, *Amaranthus dubius* is also known as Chinese spinach. In Asia and Africa, leafy amaranth represents one the most commonly eaten vegetables and fulfills strong importance in the supply with essential proteins and minerals (Rastogi & Shukla, 2013). The consumption of amaranth as vegetable is mainly exclusive in Africa and Asia, whereas the grain is popular all over the world.

Amaranth grains and leaves provide a wide profile of secondary plant metabolites, e.g. flavonoids, tannins, coumarins, betalains, alkaloids, carotenoids, and saponins (Nana, Hilou, Millogo, & Nacoulma, 2012). In this context, saponins represent one of the most important classes of secondary plant metabolites having various, partly controversially discussed, biological properties. Their occurrence extends to all plant parts including the roots, steams, bulbs, leaves, flowers, and fruits (Manik, Subrata, & Pranabesh, 2010). The quantitative amount and qualitative composition of saponins is affected by ecophysiological factors such as irradiation, temperature, water, and nutrition supply (Szakiel, Pączkowski, & Henry, 2011).

* Corresponding author.
E-mail address: rohn@chemie-uni-hamburg.de (S. Rohn).
[1] These authors contributed equally to this work.

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The chemical structure of saponins is characterized by an aglycone (non-sugar part) and a corresponding sugar moiety, with the aglycone backbone being referred to as sapogenin. Saponins are classified into two groups based on the chemical structure of the sapogenin: steroidal and triterpenoid saponins (Vincken, Heng, de Groot, & Gruppen, 2007). It is further possible to align saponins with regard to the number of sugar chains bound to the sapogenin. If there is only one sugar chain (monodesmosidic), it is usually linked to the sapogenin at the carbon atom C-3. The second chain (bidesmosidic) is then preferably bound at C-28. Tridesmosidic structures have been hardly ever encountered. Sugar chains can be linear or branched and in most cases consist of 1–3 sugar units. Glucuronic acid and glucose are the most frequent monomers directly attached to the sapogenin in Amaranthaceae (Mroczek, 2015).

To date, in amaranth triterpenoid saponins have been described exclusively. One characteristic structural feature for all amaranth saponins is a carboxyl group at C-17 leading to ester bound sugar moieties attached to this part of the molecule by condensation. The first NMR-verified data concerning saponins in amaranth were provided by Kohda, Tanaka, Yamaoka, and Ohhara (1991). The four identified saponins in *Amaranthus hypochondriacus* seeds have been named amaranthus saponins I to IV, being bidesmosidic and having identical sugar moieties consisting of rhamnose, glucose, and glucuronic acid with only minor differences in the sapogenin (Kohda et al., 1991). Further investigations on seeds and leaves of *A. caudatus* led to the identification of seven new saponins (Rastrelli, Pizza, Saturnino, Schettino, & Dini, 1995; Rastrelli et al., 1998).

Several biological activities have been assigned to saponins including anti-inflammatory, anti-microbial, anti-parasitic, anti-tumor, and anti-viral, but also adverse cytotoxic and hemolytic effects (Mroczek, 2015; Sparg, Light, & van Staden, 2004). Most of these effects result from the structural peculiarity of the non-polar sapogenin in combination with the high polar sugar moieties. Due to these structural features, they act as surfactants and are able to form complexes with molecules such as cholesterol (Story et al., 1984) or even lipid bilayers (Champ, 2002). Saponins can enhance the permeability of membranes, easing the passageway of macromolecules such as proteins to the target (Gauthier, Legault, Girard-Lalancette, Mshvildadze, & Pichette, 2009). One of the possible adverse health effect described for some of the saponins is their hemolytic activity. They emulsify lipids of the erythrocyte membrane, causing the disruption of the Na^+/K^+ balance. The cells swell till the erythrocyte membrane is raptured and hemoglobin is shed into the plasma. Studies determined no linkage between the hemolytic activity and the ability to form cholesterol complexes or the reduction of the surface activity (Hostettmann & Marston, 1995). However, valid information on the structure-dependent functional effects of single saponins, which are in charge of the hemolysis, is rare and partly conflictive.

As there is little information on the saponin profile of vegetable amaranth, amaranth leaves require detailed investigations, also with regard that it is consumed by roughly more than one billion of people worldwide.

Thus, the aims of this work were (I) to identify saponins in amaranth matrices, particularly in the leaves, using diverse mass spectrometric approaches. In addition and due to the lack of information on the structure-related hemolytic effects of saponins, (II) the saponins of vegetable amaranth were tested with regard to their hemolytic activity using an in vitro blood-gelatin assay.

2. Material and methods

2.1. Chemicals and plant material

Acetonitrile (ACN; HPLC grade for the preparative HPLC; LC–MS grade for mass spectrometry) and methanol (HPLC grade) were purchased from Merck KGaA (Darmstadt, Germany). Formic acid (purity >96%) was purchased from Carl Roth GmbH & Co.KG (Karlsruhe, Germany). Water was double distilled by a Purelab flex system (Veolia Water Solutions & Technologies). Digitonin (isolated from *Digitalis purpurea*) was purchased from AppliChem GmbH (Darmstadt, Germany) and oleanic acid (≥97%) was from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). PBS buffer tablets (pH 7.2) used for blood gelatin assay were purchased from Merck KGaA (Darmstadt, Germany). Chromabond C18 polypropylene columns (500 mg/6 mL) for solid phase extraction (SPE) were purchased from Macherey-Nagel GmbH & Co. KG (Düren, Germany).

Seeds of 15 amaranth cultivars were grown at Leibniz Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V. (IGZ), Germany greenhouses. The set of seeds was originally provided by The Asian Vegetable Research and Development Center — World Vegetable Center, Tanzania (AVRDC) and consisted of five cultivars of *Amaranthus hypochondriacus*, four cultivars of *A. cruentus*, two cultivars of *A. tricolor*, by one cultivar of *A. dubius*, *A. hybridus*, and *A. graecizans* and one further non-characterized amaranth species. Seeds were seeded in standard potting soil (Nitrogen: 340 mg/L; P_2O_5: 380 mg/L; K_2O: 420 mg/L) purchased from Einheitserdewerke Werkverband e.V. (Sinntal-Altengronau, Germany). After 2–3 weeks, the seedlings were pricked out using the same soil and further cultivated in the greenhouse for 9 weeks (June/July 2014, average temperature 23.3 °C and 25.3 °C respectively) until harvest without fertilization. The fully developed leaves were harvested, freeze-dried and ground to a fine powder.

For a comparative analysis of saponins from amaranth grains, a package of amaranth grains (500 g; Indian origin, non-defined species) was purchased at a local supermarket. When growing these seeds, it became obvious that based on the phenotype, this package of grains contained at least two different amaranth species and was therefore considered as “grain-mix“.

2.2. Mass spectrometric characterization of amaranth saponins using LC–TOF–MS

1 mL of a methanol/water mixture (60:40; *v*/*v*) was added to 50 mg of the milled material (grains and dried leaves) and extracted for half an hour under continuous shaking at 1000 rpm (20 °C). The extract was filtered through Spin-X® centrifuge tube filters (cellulose acetate membrane, pore size 0.22 μm; Sigma-Aldrich, Germany) and subsequently diluted by a factor of 100 in the extraction solvent mixture. For the chromatographic separation 1260 Agilent Series LC system was used, consisting of a binary pump, an online-degasser, an autosampler, and a thermostatically controlled column compartment. Separation was

Table 1
Overview of amaranth cultivars applied and results of the saponin-screening.

Cultivars	Origin	Species	Semi-quantitative estimation of total saponins
Arkasugna	India	*Amaranthus tricolor*	+
Mombo-2	Tanzania	*Amaranthus dubius*	−
Kongei	Tanzania	Unknown	+
IP-7	Unknown	*Amaranthus hybridus*	++
IP-11	Unknown	*Amaranthus graecizans*	+
TZ SMN 102	Tanzania	*Amaranthus hypochondriacus*	++
DB 2006 306	USA	*Amaranthus hypochondriacus*	−
AH-NL	Tanzania	*Amaranthus hypochondriacus*	++
AH-TL	Tanzania	*Amaranthus hypochondriacus*	++
TZ SMN 82	Tanzania	*Amaranthus hypochondriacus*	+
Red Sudan	Sudan	*Amaranthus cruentus*	−
Ex Zim	Zimbabwe	*Amaranthus cruentus*	−
AC 25	Tanzania	*Amaranthus cruentus*	++
AC-NL	Tanzania	*Amaranthus cruentus*	−
DB 2003 889	USA	*Amaranthus tricolor*	−

− = no or low signal intensity found for potential saponins.
+ = decent amount of known and novel potential saponins.
++ = high amount of known and novel potential saponins.