Food Research International 78 (2015) 361–368

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

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

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ARTICLE INFO

Article history:
Received 5 August 2015
Received in revised form 7 September 2015
Accepted 10 September 2015
Available online 13 September 2015

Keywords:
Vegetable amaranth
Saponins
Hemolysis

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).

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http://dx.doi.org/10.1016/j.foodres.2015.09.010
0963-9969/

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.

Table 2
Matching of high resolution masses of potential saponins of *A. hybridus* (L1), *A. hypochrondriacus* (L2) and the "grain-mix" (G) in positive and negative ESI-mode.

Peak no.	t_R [min]	Molecular ion adduct $[M + NH_4]^+$	Quasi molecular ion $[M\text{-}H]^-$	Monoisotopic mass	NUMBER of peaks (structuralisomers)	Occurrence
1	10.7	1002.418	983.380	984.386	2	L2 + G
2	11.5	974.425	955.386	956.392	1	L1 + L2
3	11.8	842.416	823.376	824.383	1	L1 + L2 + G
4	12.3	828.435	809.399	810.404	1	L2
5	12.6	1164.506	1145.465	1146.472	2–3	L1
6	12.9	972.476	953.440	954.445	1	G
7	13.0–14.3	1018.449	999.410	1000.416	2	L1 + L2
8	13.9	964.419	962.410	963.415	1	G
9	14.2	1020.464	1001.428	1002.433	1	L1
10	14.7	990.454	971.417	972.422	1	L1 + L2
11	15.2	858.450	839.410	840.417	1	L2 + G
12	15.9	844.468	825.433	826.437	1	L2
13	16.6	988.511	969.475	970.479	1	L1 + G
14	17.3 + 20.5	974.529	955.494	956.498	2	L1 + G
15	18.8	840.368	821.329	822.335	1	L2
16	20.2	812.371	793.334	794.339	1	L2
17	21.0	1104.594	1085.558	1086.562	1	L1
18	21.7	680.367	661.325	662.333	1–2	L2 + G
19	22.5–24.0	972.481	953.443	954.449	2	L1
20	23.4	958.537	939.502	940.506	1	L1
21	24.1	856.397	837.358	838.364	1	L1 + L2
22	24.5	944.486	925.449	926.454	1	L1
23	25.5	828.403	809.363	810.370	1	L1 + L2
24	27.4	696.396	677.358	678.363	1–2	L1 + L2
25	27.9	682.415	663.366	664.377	1	L2
26	29.7	826.459	807.420	808.426	1–2	L1

*as quasi-molecular ion $[M + H]^+$.

performed on an Ascentis Express F5 column 15 cm × 4.6 mm, 5 μm (Supelco, Sigma-Aldrich Co. LLC, USA) at 30 °C and a flow rate of 0.400 mL/min. The mobile phase consisted of 0.05% formic acid water (A) and ACN (B) using a gradient elution of 12% B at 0–1 min, 22% at 6 min, 32% at 29 min, 90% at 32–33 min and 5% at 34–39 min. The injection volume was 2 μL.

High-resolution mass spectrometric analysis was performed using an Agilent 6230 TOF mass spectrometer (Agilent Technologies, USA) equipped with an ESI interface. The operating parameters of the ESI-TOF-MS were: gas flow rate, 10 L/min (N_2); drying gas temperature, 350 °C; nebulizer, 35 psi; sheath gas flow, 11 L/min; sheath gas temperature, 330 °C capillary, 3500 V; skimmer, 130 V, and Octopole RF voltage, 750 V. Each sample was analyzed in both positive (fragmentor, 275 V) and negative mode (fragmentor, 100 V). The system operated with Masshunter workstation software version B.02.00 (Agilent Technologies) that was also used for data handling.

The analysis of a crude extract demands the evaluation of certain criteria to distinguish between potential saponins and non-saponin compounds. These criteria were derived from hrMS analysis of NMR-proven amaranth saponins I and II already described in the literature (Kohda et al., 1991). Considering the composition of the known saponins of amaranth, the target compounds consist only of carbon, oxygen, and hydrogen and are free of nitrogen atoms. The molar mass should reside between *m/z* 600 and 1200 considering other triterpenoid saponins. The elemental composition only based on C, H, and O forming a triterpenoid aglycone and sugar moieties leads to a limited range of possible decimals for the exact mass of 0.38 at least and 0.53 at most (refers to monoisotopic mass in negative ionization mode). The second criterion was the abundant parallel presence of the compound in positive and negative ionization mode. Every compound applying to these two criteria and featured an intensive mass signal was therefore considered being a potential saponin.

2.3. Mass spectrometric characterization of amaranth saponins using ion trap-MS following purification with preparative HPLC

To further characterize the saponins, they were purified and analyzed in an ion trap-MS to enable detailed investigations at hand of specific fragmentation patterns.

The chromatographic fractionation and purification was performed on an Agilent 1200 Infinity Series LC system (Agilent Technologies, Germany) equipped with a binary pump, an autosampler, and a fraction collector. The stationary phase was a Nucleodur PFP column 250 × 21 mm; 5 μm (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

Table 3
Summary of results including structural suggestions concerning new saponins and those already described in literature.

Peak no.	t_R [min]	Molecular ion adduct $[M + NH_4]^+$	Quasi-molecular ion $[M\text{-}H]^-$	Calc. molecular mass ESI (+)	Calc. molecular mass ESI (−)	Formula	Theoretical monoisotopic mass	Error (+) [ppm]	Error (−) [ppm]
1	11.8	842.417	823.377	824.383	824.385	$C_{41}H_{60}O_{17}$	824.383	0.0	1.6
2	12.3	828.435	809.399	810.400	810.407	$C_{41}H_{62}O_{16}$	810.404	4.9	2.8
3	14.7	990.457	971.417	972.422	972.426	$C_{46}H_{68}O_{22}$	972.420	2.1	4.4
4	15.2	858.450	839.410	840.416	840.418	$C_{42}H_{64}O_{17}$	840.414	2.4	3.9
5	15.9	844.468	825.432	826.433	826.439	$C_{42}H_{66}O_{16}$	826.435	2.4	5.2
6	16.6	988.509	969.473	970.475	970.481	$C_{48}H_{71}O_{20}$	970.477	2.1	3.4
7	20.5	974.530	955.492	956.496	956.500	$C_{48}H_{76}O_{19}$	956.498	2.1	1.4
8	21.7	680.367	661.325	662.333	662.333	$C_{35}H_{50}O_{12}$	662.330	4.5	3.5
9	24.0	972.480	953.442	954.446	954.449	$C_{46}H_{68}O_{22}$	954.446	0.0	3.5
10	23.4	958.536	939.496	940.502	940.504	$C_{48}H_{76}O_{18}$	940.503	1.1	0.3
11	27.4	696.397	677.356	678.363	678.364	$C_{36}H_{54}O_{12}$	678.362	1.5	1.9

(+) = positive ion mode; (−) = negative ion mode.

The mobile phase consisted of double distilled water (A) and ACN (B) using a gradient elution of 20% B at 0–15 min, 90% 17–20 min and an equilibration period of 15 min before the start of a next run. The flow rate was kept at 7.5 mL/min und the sample volume injected was 400 μL. Fractions were collected in time intervals of 1.0 min.

For the structural investigation, a Bruker amaZon SL ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) was used. The sample admission was conducted via an automated syringe pump with a flow rate of 1 mL/h. The operating parameters of the ESI-ion trap-MS were: positive polarity; drying gas flow rate, 8.5 L/min (N_2); drying gas temperature, 300 °C; nebulizer, 35 psi; capillary, 4500 V. The scan range was set from 100 to 2200 m/z. The first fragmentation (MS^2) of the isolated target compound already showed the characteristic fragmentation pattern, which was further refined by selecting fragments resulting from characteristic neutral mass losses for further sequential MS^n fragmentation. This procedure was continued until only the decarboxylated sapogenin remained, which was depending on the number of sugar moieties and favored leaving groups at MS^4 to MS^6. Fragmentation further than the decarboxylated sapogenin was manageable but resulted in the elimination of water or unspecific fragments.

2.4. Characterization of the hemolytic activity

Among the bandwidth of biological properties, selected saponins possess the ability to lyse cell membranes of erythrocytes. For this purpose, the crude extract, chromatographic saponin fractions as well as an isolated compound have been tested for their hemolytic activity.

10 mL of a methanol/water mixture (60:40; *v*/*v*) were added to 1.0 g of dried *A. hybridus* powder which was extracted and purified using preparative HPLC as described above. In eight chromatographic runs 3.2 mL of the supernatant extract were injected into the HPLC system and the fractions from 15 to 20 min were collected. The combined fractions were concentrated using a rotary evaporator and dissolved in 0.1 mL methanol/water (60:40; *v*/*v*). For a comparison, a concentrated crude extract was obtained from the remaining 6.8 mL crude extract by complete evaporation and dissolving in 0.1 ml methanol/water (60:40; *v*/*v*).

For the hemolysis assay, a gelatin solution was prepared by dissolving commercially gelatin in PBS buffer (pH 7.2) at 37 °C (2%, *w*/*v*). After cooling down to room temperature human blood from an expired blood preservation provided by the Universitätsklinikum Hamburg-Eppendorf (UKE), Hamburg, Germany, was added to obtain a final concentration of 3% blood (*v*/*v*) in 2% gelatin (*w*/*v*). The solution was poured in a petri dish and put in a refrigerator until complete solidification. Cavities with an approximate diameter of 3 mm were stamped in the blood gelatin and filled with different volumes (2.5 μL; 5.0 μL; 10 μL) of the saponin solutions or 10 μL of standard solutions (digitonin, c = 0.2–1.2 mg/mL in ethanol), respectively. In addition, a blank (ethanol) was applied to exclude a positive effect caused by the solvent. Incubation was performed for 48 h at 4 °C. Zones of decolourisation illustrated a hemolytic activity which was documented with a TLC visualizer (CAMAG AG, Muttenz, Switzerland) at white light.

3. Results and discussion

3.1. Analysis of amaranth saponins using LC–TOF–MS

The initial LC–TOF analysis of the 15 amaranth cultivars of six different species revealed a heterogeneous profile of potential saponins. Based on the diversity of the peak profile and the intensity of the signals, two cultivars were selected containing high amounts of potential saponins and covered the range of all target compounds found in all 15 cultivars. Of the further cultivars, six did not show any pronounced signals for potential saponins in the LC–TOF-analysis (Table 1). As no amaranth saponin standards are commercially available, potential compounds were preselected based on mass spectrometric features resulting in 16 potential candidate substances in the *A. hybridus* (IP-7) extract, 15 substances in the *A. hypochondriacus* (AH-NL) extract, and 8 substances in the commercial "grain mix"(Table 2). As no intense mass spectrometric signals for additional saponin candidates were detectable in any of the

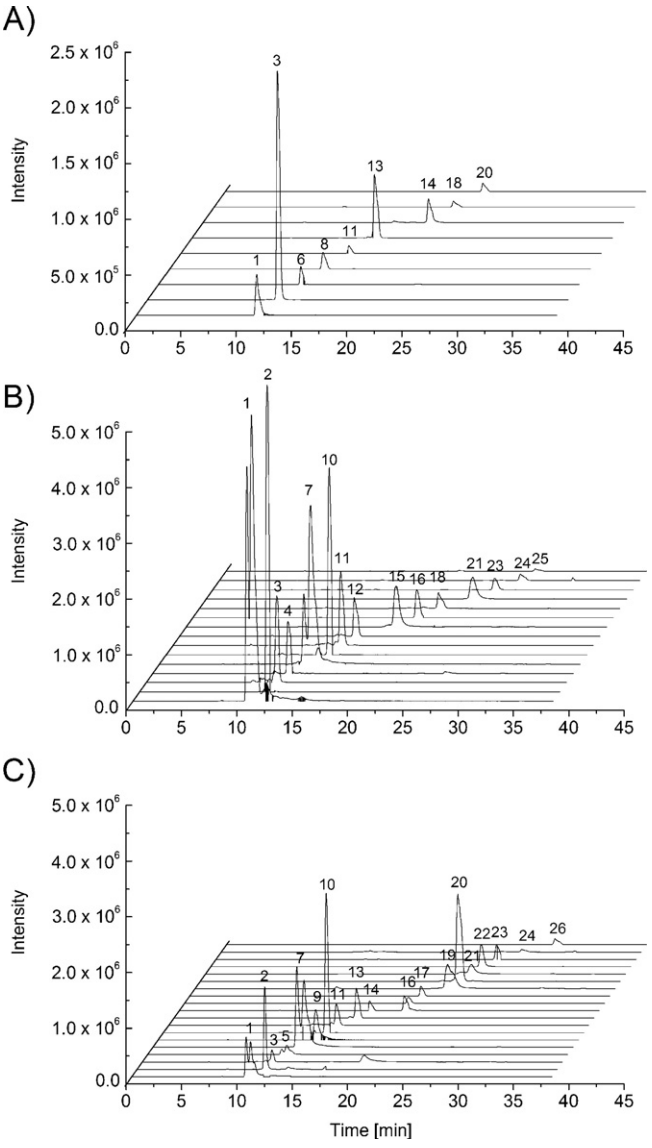


Fig. 1. A–C. Extracted ion chromatograms of potential saponins in *A. hybridus* (A), *A. hypochondrias* (B) and "grain-mix" (C). For each saponin structure, the extracted ion chromatogramm of the $[M–H]^-$ ion mass as given for every peak in Table 1.

Table 4
Saponin properties related to descriptions in the literature.

Peak no.	Sugar units	Sample origin	Occurrence in grain-mix	NMR data available in literature
1	2	L1 + 2	y	–
2	2	L2	n	Rastrelli et al., 1998
3	3	L1 + L2	n	–
4	2	L1 + L2	y	–
5	2	L2	n	–
6	3	L1	y	Kohda et al., 1991
7	3	L1	y	Kohda et al., 1991
8	1	L1 + L2	y	–
9	3	L1	y	Kohda et al., 1991
10	3	L1	y	–
11	1	L1 + L2	n	–

L1 = *Amaranthus hybridus*; L2 = *Amaranthus hypochondriacus*; y = yes; n = no.

other amaranth cultivars, further analyses were carried out for the extracts of *A. hybridus*, *A. hypochondriacus*, and the "grain-mix".

For all potential saponins, ammonia adducts $[M + NH_4]^+$ represented the dominant ion in ESI positive ionization mode with one exception. Only compound no. 8 formed the $[M + H]^+$ ion as the most abundant one (Table 2). For all saponins, the sensitivity was higher in ESI negative ionization mode, leading to $[M\text{-}H]^-$ ions without any adduct formation. However, both polarities led to abundant signals of the main saponin candidates. Only compounds detectable in both ionization polarities at identical retention times were considered for further evaluation. Additional to the (quasi) molecular signals, it was feasible to detect the sapogenin signal for the investigated saponins enabling the tentative assignment of the compounds to an amaranthus sapogenin sum formula. In all cases the detected mass difference from the experimental data to the theoretical monoisotopic mass was less than 5 ppm (Table 3). For several *m/z* ratios, signals were detected at two or more retention times (Fig. 1). This indicated the presence of several isomers of a corresponding saponin.

3.2. Purification with preparative HPLC and fragmentation using ion trap-MS

The saponin fractions collected by preparative HPLC were analyzed by direct infusion mass spectrometry in order to gain first structural

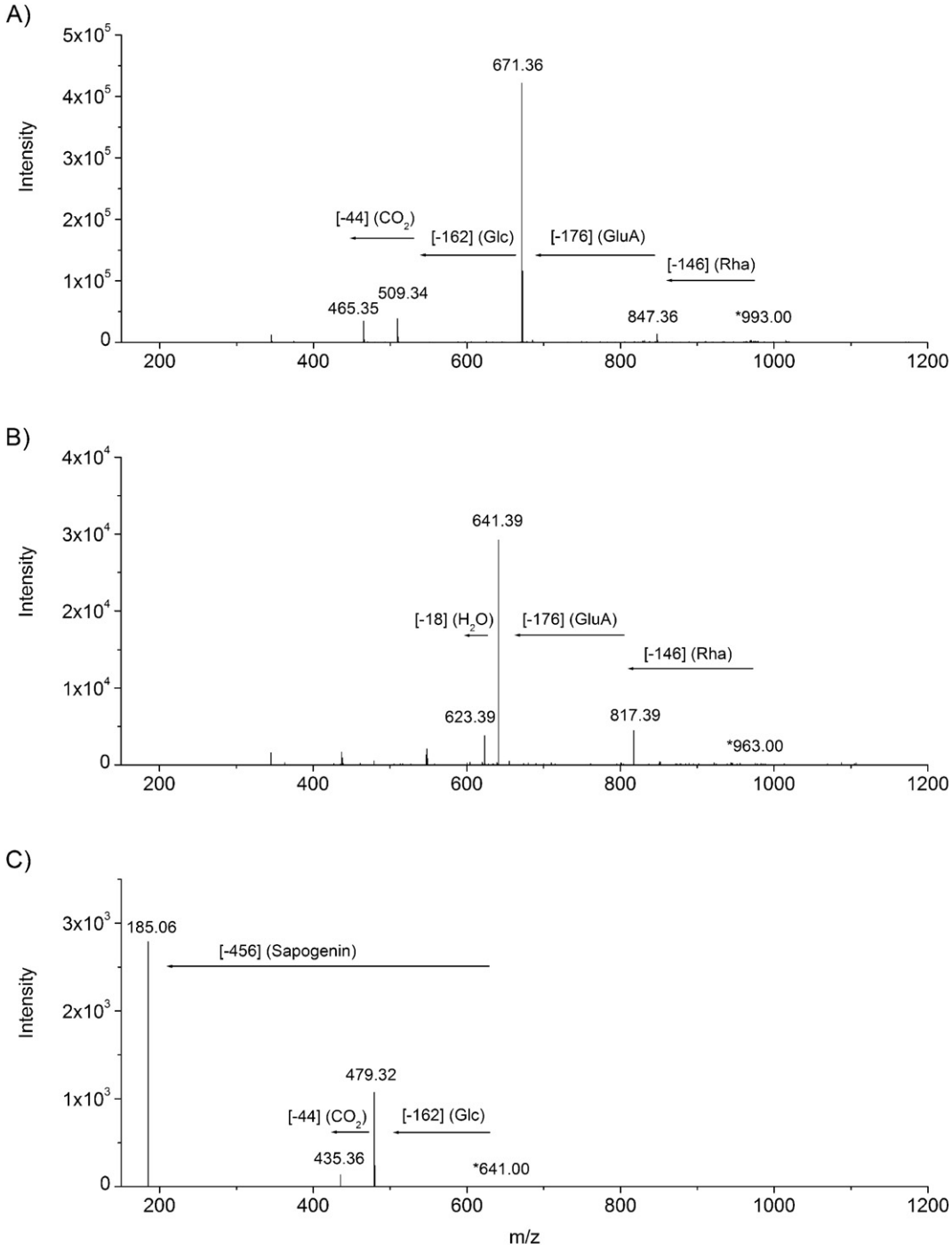


Fig. 2. A–C. MS^2-fragmentation of (A) amaranthus saponin II ($m_i = 970.477$) and (B) potential saponin (compound 10; $m_i = 940.503$) in positive ionization mode as Na^+ adduct. (C) MS^3-experiment on m/z 641 as the main MS^2-fragment of compound 10.

information on novel amaranth saponins as well as to confirm the structures identified earlier in *A. hypochondriacus* by Kohda et al. (1991). Stepwise fragmentation using ion trap-MS was carried out by manually selecting characteristic fragments resulting from subsequent cleavage of the sugar moieties and carboxyl groups until only the sapogenin remained. Thus, the sugar moieties of the saponins were characterized by their specific neutral loss masses. These fragmentation patterns were compared to available literature data describing four amaranth saponins already identified (Kohda et al., 1991; Rastrelli et al., 1998). For 11 out of the 26 candidate substances, the signal intensities allowed for a sequential fragmentation of the intact molecular ion up to the level of the sapogenin in positive ionization mode. However, negative ionization did not enable a stepwise fragmentation of neutral loss transitions. Additional to previous work carried out on other saponins using Q-TOF-MS, the analysis using syringe pump infusion and ion trap-MS enabled monitoring one particular branch of the collision induced fragmentation exclusively, whilst in Q-TOF-MS analysis all fragments are present in parallel (Li et al., 2010).

For amaranth, only sapogenins of the olean-5-en-type, carboxylated in C-17-position had been identified so far (Mroczek, 2015). Based on the similarity of the fragmentation patterns of the compounds known from literature and the uncharacterized signals in the present analysis, it is likely that they possess the same sapogenin backbone. For compounds nos. 1 to 11 (Table 3), this assumption is supported by the sum formula derived from exact masses measured during LC–TOF analysis. Furthermore, literature data indicates glucose (Glc) and rhamnose (Rha) as the dominating sugars for triterpeniod saponins in Amaranthaceae. A peculiarity of many saponins in amaranth is the presence of glucuronic acid (GluA), which is bound to the sapogenin in C-3-position (Mroczek, 2015).

Only for compounds nos. 2, 6, 7, and 9, NMR-data are available, while for compounds nos. 1, 3, 4, 5, 8, 10, and 11, an assignment was based on MS data (Table 4). For all analyzed compounds the Na^+ adduct led to the most abundant signals in ESI-positive mode when using syringe pump direct infusion. Fig. 2 exemplarily depicts the fragmentation of compound no. 6, a saponin with NMR-data available, and compound no. 10, a potential novel amaranth saponin. Table 5 shows the resulting fragmentation patterns of all 11 compounds.

3.2.1. Fragmentation of amaranth saponins with available NMR-data.

As already described by Kohda et al. (1991), compounds nos. 6, 7, and 9 differ only in the position of the substituents at C-4 and C-20 of the aglycone, while the sugar derivatives are identical (Fig. 3). As expected for all three compounds, rhamnose was eliminated primarily leading to the fragment with *m/z* [(M + Na)-146], followed by a loss of glucuronic acid (*m/z* [(M + Na-Rha)-176]), glucose (*m/z* [(M + Na-Rha-GluA)-162]), and finally the carboxyl group (*m/z* [(M + Na-Rha-GluA-Glc)-44]). These data indicate that these saponins tend to split the C-3 bound substituent completely off, before losing the C-28 bound substituent. In all cases, *m/z* resulting from [M + Na-C3-moiety] showed the most intense signal and led therefore to the conclusion that the fragment molecule is stabilized for this ion. After cleavage of the ester-bond at C-28, a glucose unit was split off and a free carboxyl group remains. This group represents a further good leaving group which was eliminated in the following step. At this point only the decarboxylated sapogenin remained.

Compound no. 2, previously described by Rastrelli et al. (1998), showed a different fragmentation (Table 5), which can be explained by the different substitution pattern of the sapogenin including a free carboxyl group at position C-4 and the different sugar moiety composition compared to compounds nos. 6, 7, and 9 (Rastrelli et al., 1998). Initially, a glucose unit, preferably from the less stable C-3 position, was eliminated. Afterwards the loss of a carboxyl group at C-4 led to the fragment *m/z* 627, followed by the loss of the second glucose unit (*m/z* 465) and the carboxyl group (*m/z* 421) bound to the glucose at C-17.

3.2.2. Fragmentation of novel amaranth saponins.

Compounds nos. 1 and 4 showed an identical fragmentation pattern, which was characterized by the initial loss of a glucuronic acid unit at C-3, followed by loss of a glucose unit at C-28, and a carboxyl group at C-17 (Table 5). Compounds nos. 8 and 11 showed a fragmentation behavior analogous to compounds nos. 1 and 4 (Table 5). This similarity was due to the same chemical structure except for the lack of one additional glucose unit in C-28-position compared to compounds nos. 1 and 4, respectively.

Compound **no. 3** initially also split off the sugar chain at C-3 consisting of one rhamnose bound to a glucuronic acid (Table 5). The side chain at C-28 was cleaved starting by a glucose unit followed by the carboxyl group. The exact mass for the sapogenin fragment (466.306 amu) indicated the presence of a hydroxyl group instead of a methyl group, most likely in position C-4.

In the first ESI-MS step, a water-molecule was eliminated from compound no. 5 (Table 5). This finding led to the conclusion that the hydroxyl group in position C-3 of the sapogenin is non-conjugated allowing for a β-elimination of water with the adjacent hydroxyl group in C-2.

Also other saponins occasionally showed the cleavage of a water-molecule with low abundance at later stages of the fragmentation cascade. The elimination of the oxygen at C-3 with a hydrogen atom in β-position is possible, but not characteristic for any structure and therefore negligible. In the following steps, a glucose unit and a glucuronic acid unit were cleaved. The fact that glucose was eliminated prior to the glucuronic acid indicated that in this case the glucuronic acid was bound to the carboxyl group at C-28, connected to the glucose unit.

Analysis of compound no. 10 led to the subsequent cleavage of the following units (Table 5): rhamnose, glucuronic acid, glucose, and a carboxyl group. The exact mass obtained for the aglycone (434.352 amu)

Table 5
Fragmentation of saponins in positive ESI-mode.

No.	Monoisotopic mass m_i	Precursor ion $[M + Na]^+$	Fragmentation (m/z)	Fragmentation
1	824.4	847	671	$[M + Na\text{-GluA}]^+$
			509	$[M + Na\text{-GluA-Glc}]^+$
			465	$[M + Na\text{-GluA-Glc-COO}]^+$
2	810.4	833	671	$[M + Na\text{-Glc}]^+$
			627	$[M + Na\text{-Glc-COO}]^+$
			465	$[M + Na\text{-Glc-COO-Glc}]^+$
			421	$[M + Na\text{-Glc-COO-Glc-COO}]^+$
3	972.4	995	849	$[M + Na\text{-Rha}]^+$
			673	$[M + Na\text{-Rha-GluA}]^+$
			511	$[M + Na\text{-Rha-GluA-Glc}]^+$
			467	$[M + Na\text{-Rha-GluA-Glc-COO}]^+$
4	840.4	863	687	$[M + Na\text{-GluA}]^+$
			525	$[M + Na\text{-GluA-Glc}]^+$
			481	$[M + Na\text{-GluA-Glc-COO}]^+$
5	826.4	849	831	$[M + Na\text{-}H_2O]^+$
			511	$[M + Na\text{-GluA-Glc}]^+$
6	970.5	993	847	$[M + Na\text{-Rha}]^+$
			671	$[M + Na\text{-Rha-GluA}]^+$
			509	$[M + Na\text{-Rha-GluA-Glc}]^+$
			465	$[M + Na\text{-Rha-GluA-Glc-COO}]^+$
7	956.5	979	833	$[M + Na\text{-Rha}]^+$
			657	$[M + Na\text{-Rha-GluA}]^+$
			495	$[M + Na\text{-Rha-GluA-Glc}]^+$
			451	$[M + Na\text{-Rha-GluA-Glc-COO}]^+$
8	662.3	685	509	$[M + Na\text{-GluA}]^+$
			465	$[M + Na\text{-GluA-COO}]^+$
9	954.5	977	831	$[M + Na\text{-Rha}]^+$
			655	$[M + Na\text{-Rha-GluA}]^+$
			493	$[M + Na\text{-Rha-GluA-Glc}]^+$
			449	$[M + Na\text{-Rha-GluA-Glc-COO}]^+$
10	940.5	963	817	$[M + Na\text{-Rha}]^+$
			641	$[M + Na\text{-Rha-GluA}]^+$
			479	$[M + Na\text{-Rha-GluA-Glc}]^+$
			435	$[M + Na\text{-Rha-GluA-Glc-COO}]^+$
11	678.4	701	525	$[M + Na\text{-GluA}]^+$
			481	$[M + Na\text{-GluA-COO}]^+$

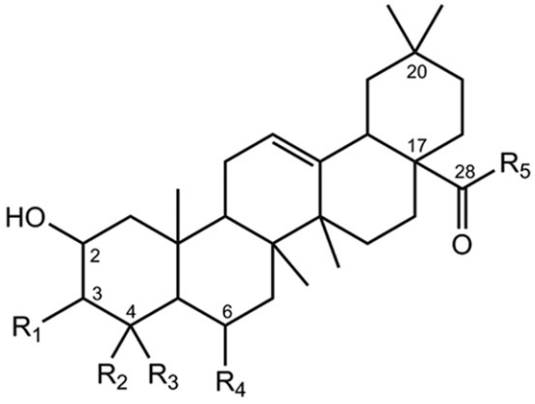
suggested that there is no oxygen containing group bound to the aglycone at C-2. Of note, this lack of a hydroxyl group at C-2 marks a major difference to all other saponin compounds in the amaranth varieties investigated in the present study. Table 3 sums up the results of the structural analysis. However, final structural identification requires NMR-analysis of chromatographically isolated compounds, which is hardly feasible in many cases due to the highly complex saponin profile hampering the isolation of pure substance.

3.3. Characterization of the hemolytic activity of amaranth saponins isolated

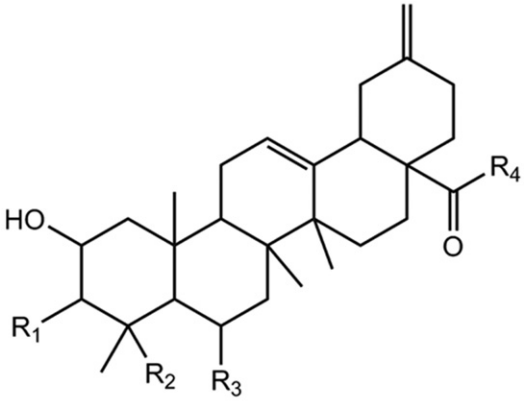
In order to test the potential hemolytic activity of amaranth saponin extracts of *A. hybridus*, *A. hypochondriacus* and the amaranth "grain-mix" were fractioned using RP-SPE, spotted on a HPTLC matrix and overlain with blood gelatin for a first screening experiment crude. Hemolytic effects were limited to *A. hybridus* extracts and restricted to only one fraction (data not shown). As only compound no. 10 was highly abundant exclusively in the respective *A. hybridus* fraction this substance was investigated more in detail. The crude extract of *A. hybridus* and a fraction predominantly containing compound no. 10 were investigated for their hemolytic activity comparatively in a modified blood-gelatin diffusion assay. The target compound was preparatively isolated as described in section 2.4. The purity of this extract was checked using LC–MS. It contained at least 80% of compound no. 10. Crude extract and isolated compound caused an approximately equal extent of hemolysis estimated by the diameter of the decolorized zone of the blood-gelatin (Fig. 4, e and c).

The application of digitonin standard solutions enabled for a semi-quantitative estimation of the hemolytic target compound no. 10. The decolourisation zone of 2.5 μL crude extract (Fig. 4, e-1) is equivalent to the 10 μL application of the 0.6 mg/mL digitonin standard. When assuming that digitonin and compound no. 10 exhibited the same hemolytic activity, the content of saponin no. 10 in *A. hybridus* amounts to 0.1% dry weight.

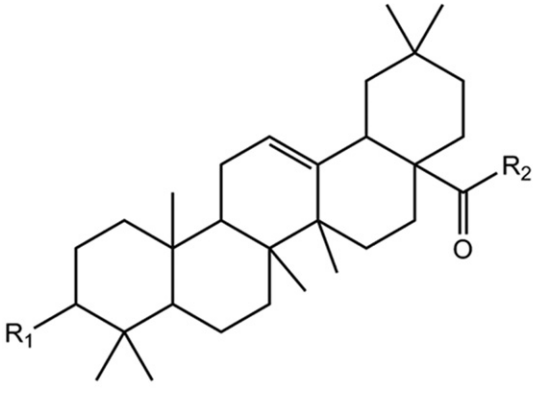
As expected, oleanolic acid, which represents the most common sapogenin, showed no hemolytic activity (Voutquenne, Lavaud, Massiot, & Le Men-Olivier, 2002). The potato sprout extract comprising the steroidal glycoalkaloids α-chaconine and α-solanine (Fig. 4, p) indicates that the plant matrix does not hinder the hemolytic effect of the contained saponins. It rather appears that the matrix might facilitate the diffusion of the saponins through the blood-gelatin. Thus, the decolourised zone of the crude extract is slightly wider than the zone of the single saponin. However, the fraction of compound no.10 is only of about 85% purity according to LC–TOF–MS analysis and contains minor amounts of similar almost co-eluting substances (data not shown). Hence, besides the target compound no. 10 other saponins in the crude extract might also possess weak hemolytic activity resulting in an additive response. Gauthier et al. (2009) suggested that many oleanane-type saponins are



1 R_1=GluA; R_2=CH_3; R_3=CHO; R_4=OH; R_5=Glc
2 R_1=Glc; R_2=CH_3; R_3=COOH; R_4=H; R_5=Glc
3 R_1=GluA→Glc; R_2=H; R_3=CHO; R_4=OH; R_5=Glc
8 R_1=GluA; R_2=CH_3; R_3=CHO; R_4=OH; R_5=H
9 R_1=GluA→Rha; R_2=CH_3; R_3=CHO; R_4=H; R_5=Glc



4 R_1=GluA; R_2=CHO; R_3=OH; R_4=Glc
5 R_1=OH; R_2=CH_3; R_3=OH; R_4=Glc→GluA
6 R_1=GluA→Rha; R_2=CHO; R_3=H; R_4=Glc
7 R_1= GluA→Rha; R_2=CH_3; R_3=H; R_4=Glc
11 R_1=GluA; R_2=CHO; R_3=OH; R_4=H



10 R_1=GluA→Rha; R_2=Glc

Fig. 3. Suggested structures of saponins considering literature data, high resolution mass analysis, and fragmentation patterns.

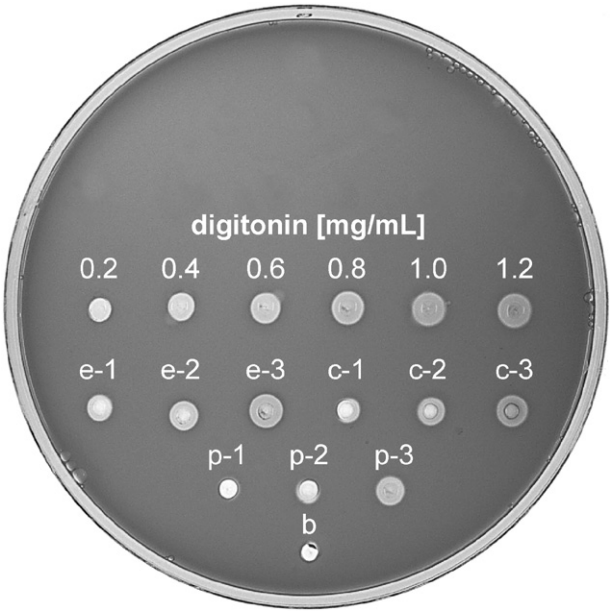


Fig. 4. Hemolytic activity determined via diffusion test. Digitonin standards (0.2–1.2 mg/mL) in ethanol: 10 μL each; crude extract of *A. hybridus*: 2.5 μL (e-1), 5.0 μL (e-2), 10.0 μL (e-3); purified compound 10: 2.5 μL (c-1), 5.0 μL (c-2), 10.0 μL (c-3); potato sprout extract with α-chaconine and –solanine: 2.5 μL (p-1), 5.0 μL (p-2), 10.0 μL (p-3); ethanol blank: 10.0 μL (b).

hemolytic while lupane-type saponins are not. However, results of the present study indicated that only one out of more than ten amaranth saponins of the oleanane-type is hemolytic.

Studies indicated that the ability to lyse erythrocytes depends on a multitude of structural features. Hemolysis cannot be pinpointed to one certain sapogenin or sugar, but rather to the combination of both (Francis, Kerem, Makkar, & Becker, 2002). Among all of the previously identified substances, compound no. 10 differs by the lack of free hydroxyl groups at the sapogenin. The combination of a particularly nonpolar sapogenin and highly polar sugar moieties might facilitate the linkage to the erythrocyte membrane.

Esterified saponins are considered to be more hemolytic than nonester compounds (Hostettmann & Marston, 1995; Voutquenne et al., 2002). However, all saponins in the present study except compounds 8 and 11 are esters of the carboxy function in position C-28 with only one compound (no. 10) appearing to have major hemolytic activity.

4. Conclusion

The investigations of the present work revealed that the saponin profile of amaranth leaves is highly diverse as demonstrated for *A. hybridus* and *A. hypochondriacus* as well as for amaranth grains marketed as cereals for human consumption. This diversity applies to the overall abundance as well as to the profile of the saponins. Especially, two leafy amaranth cultivars rich in saponins were investigated in detail in this study. However, a pre-screening revealed that other cultivars of the same amaranth species either have a very similar saponin profile or contain only minor amounts of saponins at all. This leads to the assumption that saponins are not essential for the survival of the plant. However, the selectivity of bioactivities indicates that saponins may play a considerable role in the plant defense system.

By determining the hemolytic activity, one relevant toxicological endpoint for the bioactivity of amaranth saponins was investigated. Nevertheless, due to the large variety of saponins multifaceted biological effects have to be expected, likewise. Moreover, bioactivity cannot be correlated to the overall saponin content. Rather a case-by-case identification and evaluation is required to estimate health impact or plant physiological properties of a distinct saponin. In general, a hemolytic activity is an undesirable property, possibly affecting the human health adversely. On the other hand, saponins are poorly absorbed by the human intestine, as already proven for soy saponins fed to mice (Koratkar & Rao, 1997). Nonetheless, the fate of saponins in the gastrointestinal tract still needs further investigation.

Considering all the stated facts – particularly the differences in hemolytic activity – exhaustive studies concerning the contribution of isolated amaranth saponins to the plant defense system as well as evaluating their health and nutritional impact are urgently required.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The present work was financially supported as part of the project "Horticultural Innovation and Learning for Improved Nutrition and Livelihood in East Africa" (FKZ 031A248J and FKZ 031A248K), funded within the global food security (GlobE) initiative of the German Federal Ministry of Education and Research and the German Federal Ministry for Economic Cooperation and Development. The authors would also like to thank Dr. Benard Ngwene for his advice and expertise in amaranth cultivation.

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