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Protein content and glucosinolates from *Moringa oleifera* Lam. – New insights into an auspicious commodity

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Summary

Moringa oleifera is considered to be one of the most valuable and beneficial crop tree species. The great nutritiousness is assigned to its high leaf protein content, and its health-promoting effect to the anti-carcinogenic properties of its genuine glucosinolates and their degradation products.

From a plant physiological perception protein contents of 30% seem to be quite high. Accordingly, a reconsideration of these findings became necessary. The related inquiry unveils that also in the leaves of many other plant species such high protein contents are reported, provided that they are quantified by Kjeldahl nitrogen determinations. But, it is rather likely that the nitrogen accounting for the putative high protein contents is due to insoluble cell wall bound hydroxyproline-rich glycoproteins. Due to their extreme insolubility, these compounds cannot be digested easily, and thus, they do not contribute to the nutritiousness of *M. oleifera* leaves.

In contrast to classical glucosinolates, those occurring in *M. oleifera* are characterized by an attachment of a rhamnose to the aglycone. In consequence, the products generated during the myrosinase-catalysed hydrolysis correspond to non-volatile rhamnosides of isothiocyanates. Since over time, olfactorily active substances emerge, the rhamnose moiety has to be cleaved off, putatively by a corresponding rhamnosidase.

Key words: Glucosinolates; *Moringa oleifera*; protein contents.

Introduction

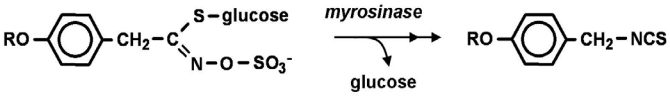
Moringa oleifera Lam. is considered as a valuable and beneficial crop plant, and especially the leaves of this tree species are consumed and employed all over the world for nutrition and health care (ANWAR et al., 2006; FALOWO et al., 2018). Whereas their high nutritional value is particularly attributed to the tremendously high protein content (MAKKAR and BECKER 1997; MOYO et al., 2011), the reports on the health beneficial effects (PATEL et al., 2010; JUNG, 2014) are primarily based on the exceptional glucosinolates (RAZIS et al., 2014), which are present in high concentrations in *M. oleifera* leaves as well as on various antioxidants (DA SILVA ALVES et al., 2017), vitamins and minerals (PATEL et al., 2010; MOYO et al., 2011; RAZIS et al., 2014). This case study focuses on the high protein content and on the unusual glucosinolates.

The "*extremely high protein content*" of *M. oleifera* leaves

In many popular scientific papers *M. oleifera* is denoted as the "*most nutritious plant of the world*" because of the high protein contents of its leaves, ranging from 25% to over 30% (d.w.) (e.g. DHAKAR et al., 2011; MBAILAO et al., 2014; TEIXEIRA et al., 2014, BIEL et al., 2017). However, from a plant physiological perception such putative tremendous high protein concentrations seem to be weird. In contrast to seeds, which represent storage organs exhibiting a retarded metabolism, in photosynthetically active organs an extremely high protein concentration is not reasonable. Accordingly, related statements have to be challenged. A thorough review of the relevant literature unveils that the estimation of the putative protein content of *M. oleifera* leaves is always based on the quantification of the total nitrogen, either determined by basic Kjeldahl analyses (MAKKAR and BECKER, 1996; MBAILAO et al., 2014; BIEL et al., 2017) or by applying the Dumas method (OLSON et al., 2016). Provided that the reported tremendously high protein concentration for *M. oleifera* leaves would be due to authentic genuine proteins, their quantity should also be evaluated on the basis of the amount of amino acids, which are liberated in the course of a complete hydrolysis of the proteins. Alternatively, the proteins should easily be detected in corresponding electrophoretic analyses. In this approach, in addition to Kjeldahl-based protein quantifications, we estimated the *M. oleifera* leaf proteins on the basis of the amino acids liberated in the course of an acidic hydrolysis as well as by various electrophoretic analyses.

The unusual glucosinolates of *M. oleifera* leaves

Leaves of *M. oleifera* exhibit very unique glucosinolates (AMAGLO et al., 2010), which in particular, correspond to rhamnosyl derivatives of sinalbin (Fig. 1).



R = rhamnose: glucomoringin
R = *O*-acetyl-rhamnose (3 isomers): acetyl-glucomoringin

R = rhamnose: "moringin"
R = *O*-acetyl-rhamnose: acetyl-moringin

Fig. 1: Structure of the *M. oleifera* glucosinolates and the related isothiocyanates.

The particular isothiocyanate (4-(*a*-L-rhamnopyranosyloxy)-benzyl-isothiocyanate, Fig. 1) released in the course of myrosinase-catalysed cleavage of glucomoringin is frequently denoted as moringin (e.g. MÜLLER et al., 2015; KARIM et al., 2016). However, the name moringin is fallacious and misleading, since the hitherto described glucomoringin does not represent the glucose-derivative of the isothiocyanate moringin, but the genuine glucosinolate. In case of the acetyl-rhamnosyl-hydroxybenzyl-glucosinolates (= acetyl-glucomoringin isomers I-III), the rhamnose moiety of glucomoringin is *O*-acetylated (AMAGLO et al., 2010; PATEL et al., 2010) which can occur at three different positions (FAHEY et al., 2018) (Fig. 1).

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In addition to the various glucosinolates, also their putative degradation products, i.e. isothiocyanates, nitriles or carbamates, are reported to occur in *M. oleifera* leaves (RAZIS et al., 2014; WADHWA et al., 2013). However, AMAGLO et al. (2010) did not detect corresponding degradation products and deduced that these substances had been generated in the course of tissue damage (AMAGLO et al., 2010). Moreover, these authors reported the occurrence of glucotropaeolin in stems of *M. oleifera*.

With respect to their health-promoting effects (PATEL et al., 2010; RAZIS et al., 2014; KARIM et al., 2016), apart from an anti-inflammatory (WATERMAN et al., 2014) and an antibacterial activity (GALUPPO et al., 2013), the anti-carcinogenic action of the unusual glucosinolates and their various degradation products is in the centre of interest (JUNG, 2014; RAJAN et al., 2016). In this context, the corresponding isothiocyanates are thought to be of high relevance (MIYACHI et al., 2004; BRUNELLI et al., 2010; RAJAN et al., 2016; GALUPPO et al., 2013). Based on their studies, these authors assume that the relevant isothiocyanates are mostly generated – just as in the case of "classical" glucosinolates – by their hydrolysis catalyzed by myrosinases. However, in contrast to the typical glucosinolates present in plants of the Brassicaceae, in injured *M. oleifera* leaves, the liberated isothiocyanates are still attached to a rhamnose moiety, and thus are not volatile. Since with time volatile, olfactory active compounds are liberated, the rhamnose moiety must be cleaved off, presumably by a rhamnosidase. In contrast to the numerous studies dealing with the health-promoting effects of these compounds, only very few reports had been published concerning their ecological significance for the plants (MÜLLER et al., 2015). It is assumed that – just in the same manner as for "classical" glucosinolates (HALKIER & GERSHENZON, 2006) – also the glucosinolates in *M. oleifera* leaves represent phytoanticipins by protecting the plant against herbivores and pathogens. As the repellent and protective effect is due to the generation of degradation products (MÜLLER et al., 2015), also the *M. oleifera* glucosinolates can be classified as typical inactive precursors, which – after tissue disruption – generate active agents (BRUNELLI et al., 2010), exhibiting their repellent effect on potential herbivores by their pungent taste (e.g. CHODUR et al., 2018). Yet, the manifestation of the pungent taste of *M. oleifera* leaves is reported to be quite different (DOERR, 2009; CHODUR et al., 2018) and may strongly vary between different varieties, origins and proveniences, respectively. CHODUR et al. (2018) attributed these differences in the markedness of the pungency primarily to variations in the overall amount of glucosinolates. In contrast, the studies of DOERR et al. (2009) did not establish any correlation between "pungency" or "peppery taste" on the one hand, and content and spectrum of glucosinolates on the other hand. As consequence, these authors deduced that the variations in the differences of pungency are caused by different activities of myrosinases (DOERR et al., 2009). This assumption seems to be – at least in part – plausible, since the pungent, peppery taste and the characteristic flavour are not generated by the intact glucosinolates, but are determined by their degradation products. In order to provide further information on this topic and to disclose the basic processes involved, the hydrolysis of the *M. oleifera* glucosinolatess has been studied.

Material and methods

Sample preparation for Kjeldahl and quantification of amino acids

For protein quantifications, 5 g of commercial *M. oleifera* powder (*africrops!*, Berlin) were extracted two times with 50 mL McIlvaine buffer (0.1 M citric acid / 0.2 M Na_2PO_4; pH 5.6) (1:5 diluted). The extract was centrifuged (4.000 × g, 10 min) and the supernatant was filtered. The combined aqueous supernatants (**S**) were divided equally and freeze-dried. Accordingly, the soluble protein fraction **S** comprises all proteins that are soluble in plain buffers. The related pellet (**P**) was re-extracted for 30 min at 95 °C with 40 mL SDS-buffer (McIlvaine buffer containing 2% SDS and 4% β-mercaptoethanol) and centrifuged (4.000 × g, 10 min). The residual supernatant (**R**) was separated from the pellet (**P**). Both fractions were divided equally and freeze-dried (Fig. 2). The freeze-dried samples **S**, **R**, and **P** as well as the dried leaves (total leaf extract = **T**) were either used for the Kjeldahl analysis or for the quantification of amino acids.

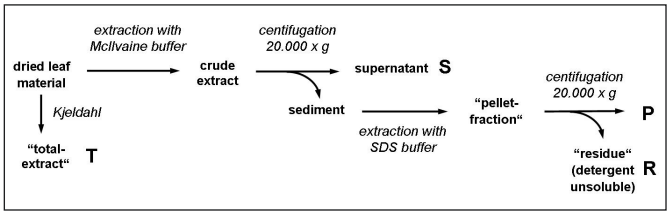

Fig. 2: Scheme of protein sample preparation.

Protein determination - Kjeldahl

Protein determination was conducted via Kjeldahl nitrogen quantification according to MATISSEK and STEINER (2006).

Digestion: Each sample was mixed with 20 mL concentrated sulfuric acid, one Kjeldahl tablet (Thompson & Capper, Runcorn, UK) and some glass beads in a 250 mL Tecator Kjeldahl tube. After gentle shaking, the Tecator Kjeldahl tubes were placed in the Tecator block digester (FoodAlyt IR 1200) and heated at 375 °C until the digestion solutions were clear and only slightly bluish in colour. Subsequently the tubes were removed from the Tecator block and cooled down to room temperature (RT). Then the solutions were carefully diluted with distilled water, transferred to a 100 mL volumetric flask with rinsing distilled water and filled up to the mark after cooling.

Distillation: 10 mL of the digestion solutions were alkalized with 40 mL NaOH before a steam distillation was carried out for 5 min. For titration, the distillates were transferred into a 20 mL boric acid-indicator mixture and a Tashiro indicator was added, exhibiting colour changes from violet-red through grey to light green.

Titration: The steam distillates were titrated dropwise with HCl (0.1 M) until the first colour change (green-grey) occurred.

Calculation: The protein content in percent (P) was calculated according to the formula:

$$P = \frac{(a - b) \times 1.4008 \times F}{E}$$

P: percentage of protein content (%); a: consumption of HCl in the titration (mL) of the related samples; b: consumption of HCl in the titration (mL) of the related blanks (water); F: 6.25 (general conversion factor for calculating the protein content); E: sample weight (g).

Protein determination via quantification of amino acids liberated by acidic hydrolysis

Alternatively to the Kjeldahl approach, the protein content was estimated on the basis of amino acids liberated in the course of acidic proteolysis. For this, the contents of amino acids present in the hydrolysed as well as in the non-hydrolysed samples (commercial *M. oleifera* powder, *africrops!*, Berlin) were determined. Quantification was performed by HPLC according to BYTOF et al. (2005).

Hydrolysis: Of each sample 200 mg (P, R, S, T; Fig. 2) were mixed with 2 mL 6 M HCl in small glass ampullae. To minimize the amount of oxygen, ampullae had been sealed by melting the ampulla neck. Hydrolysis was performed at 100 °C for 6 h in three independent

replicates. Subsequently, the samples were neutralized with NaOH, diluted 1:5 before norvaline was added as internal standard to yield a final concentration of 160 nmol. Prior to HPLC analysis, samples were centrifuged (4.000 × g, 10 min).

Extraction: To determine the content of free amino acids, 200 mg of each sample were extracted with 3 mL sulphosalicylic acid (4% w/v) containing exactly 160 nmol norvaline as internal standard according to Bytof et al. (2005). For each assay, three independent replicates had been conducted.

HPLC: Prior to HPLC analysis, the extracted amino acids were derivatised with *o*-phthaldialdehyde (OPA) using a Spark Holland Midas autosampler for derivatisation and sample injection (Bytof et al., 2005); injection volume was 30 µL. Separation was performed on a C18 column (Nucleosil 120, 5 µm, Macherey & Nagel, 250 × 4.0 mm) employing a binary gradient (A: 5% MeOH, 5% acetonitrile (ACN), 2% tetrahydrofuran (THF), 88% 50 mM sodium acetate buffer, pH 6.2; B: 40% MeOH, 40% ACN, 20 % sodium acetate buffer). Flow rate: 1.0 mL/min, oven temperature: 35 °C. The amino acid derivatives were detected employing a RF-551 Shimadzu fluorescence detector (λ_{ex} = 334 nm; λ_{em} = 425 nm) and quantified using external calibration.
The protein amounts were quantified as difference of the amounts for free amino acids and those released by acidic hydrolysis.

Analyses of glucosinolates and their degradation products

Extraction and degradation of glucosinolates

Plant material was harvested from plants grown in the greenhouse of the Institute of Botany, Leibniz University Hannover, Germany. After freezing in liquid nitrogen, the material was ground and freeze-dried. Of the lyophilized powder 25 mg were incubated with 500 µL ultrapure water at two different temperatures (4 °C and 20 °C) for a time period of 60 s. At various times (15, 30, 45 and 60 s), degradation of glucosinolates was stopped by adding 500 µL MeOH and heating the samples at 95 °C for 10 min. Extracts were centrifuged (4.000 g; 10 min) and filtered. Samples were analysed in triplicates.

LC-MS analysis

For identification of the glucosinolates in *M. oleifera* samples were analysed by liquid chromatography-mass spectrometry (LC-MS). A volume of 10 µL was injected into the HPLC system (Shimadzu, Darmstadt, Germany) and separated on a Vertex Plus column (250 × 4 mm, 5 µm particle size, packing material ProntoSIL 120-5 C18-H) (Knauer, Berlin, Germany) equipped with a pre-column (Knauer). Solvent A: water (18.2 µS cm^{-1}, obtained by Elga Purelab Ultra GE MK2, Veolia Water Technologies Deutschland GmbH, Celle, Germany; solvent B: MeOH (VWR, Darmstadt, Germany; LCMS grade, 99.9% purity); both containing 2 mM ammonium acetate (Merck, Darmstadt, Deutschland; LC MS grade, 90.0% purity). Flow rate: 0.8 ml min^{-1} at 30 °C, gradient: 10-90% B for 35 min, 90% for 2 min, 90-10% B for 1 min and 10% B for 2 min. HPLC was coupled to a mass spectrometer (AB Sciex TripleTOF 4600, Canby, USA). At a temperature of 600 °C and an ion spray voltage floating of -4500 V the negative electrospray ionization (ESI) was performed. For the ion source gas one and two 50 psi were used and for the curtain gas 35 psi. In the range of 100-1500 Da in the TOF range the mass spectra as well as the MS/MS spectra from 100-800 Da at a collision energy of -10 eV were recorded (total ion chromatogram, TIC). Peaks were identified by analysing the characteristic masses (according to Förster et al., 2015; Rambulana, et al., 2017; Fig. 3). The acetylation site of the three isomers of acetyl-4-α-L-rhamnopyranosyloxybenzyl glucosinolates could not be certainly identified because of lacking NMR data. In order to be able to discuss obtained results, the isomers were named according to their elution time from I to III. Quantification was performed by using peak areas obtained by monitoring samples at 229 nm. Due to lack of authentic standards, contents of glucosinolates were calculated by using the standard curve of sinigrin (Phytolab, Vestenbergsgreuth, Germany, 99.8% purity).

Degradation of glucosinolates in **M. oleifera** *leaf extracts*

One g of commercially available *M. oleifera* powder (*africrops!*, Berlin) was incubated with 20 mL tap water at two different temperatures (4 °C and 20 °C) for a time period of 5 d. At various time points (6, 18, 30, 42, 54, 66, 78 and 90 h) the extracts were centrifuged (4.000 g; 10 min) and filtered before being injected into the HPLC. For each assay, two independent replicates had been conducted.

HPLC: Estimation of glucosinolate degradation products was performed according to Kleinwächter (2007) employing a C18 column (Nucleosil 120, 5 µm, Macherey & Nagel, 250 × 4.0 mm) and a binary gradient (A: MeOH, B: H_2O dest.; 10 min 40% A and 60% B, 10 min 80% A and 20% B, 5 min 40% A and 60% B). Flow rate: 0.8 mL/min. For detection a photodiode array detector (220 nm) was used. 4-Hydroxybenzyl alcohol (Alfa Aesar, Kandel) was employed as authentic standard.

Results and discussion

The dissent of high protein contents

Protein determination based on the quantification of total nitrogen (Kjeldahl)

In order to clarify the inconsistencies on the alleged high protein contents, extracts of *M. oleifera* leaves had been fractionated and analysed. In accordance with the literature for the dried leaf material (**T**) - based on the total nitrogen quantification – a protein content of 26.5 % was estimated (Tab. 1). This value corresponds to the amount of 24.3%, which results as sum of the particular factions, i.e. **S** + **P** + **R** (Fig. 2). However, the determination of the protein content in the "water soluble" and "SDS-extractable" faction (sum **S** + **P**) reveals only 14.3%. This value is in the same range as reported for other plant species, e.g. 7% - 11% for barley (Dai et al., 2009) and 9% - 16% for spinach (Jacobi et al., 1975). Obviously, in *M. oleifera* leaves, a very high share of nitrogen remains in the insoluble fraction **R**. Unfortunately, only few related data on this issue are available.

Tab. 1: Kjeldahl based nitrogen and protein content of the various fractions from *M. oleifera* leaves.

Fraction	Nitrogen content [%]	Putative protein content [%]	Share of total protein [%]
S (supernatant "buffer extract")	0.96	**6.0**	**22.6**
P (supernatant of the SDS-solubilised pellet)	1.33	**8.3**	**31.3**
R (SDS-insoluble)	1.60	**10.0**	**37.7**
sum: **S + P**	2.29	**14.3**	**53.9**
sum: **S + P + R**	3.89	**24.3**	**91.7**
T ("total extract")	4.25	**26.5**	**100**

Data represent mean values from three independent replicates.

Protein determination via quantification of amino acids liberated by acidic hydrolysis

To scrutinise whether or not the putative protein contents of *M. oleifera* leaves estimated via the Kjeldahl nitrogen quantification could be attributed to authentic, genuine proteins, the related samples had been hydrolysed employing hydrochloric acid. Subsequently, the amount of amino acids was quantified. The difference in the con-

tent of amino acids in the hydrolysed and the non-hydrolysed fractions corresponds to the amount of proteins (Tab. 2). When comparing these results with the putative protein contents quantified on the basis of Kjeldahl nitrogen determinations (Tab. 1), it turned out that the total protein content in the dried leaves (**T**) is only 13.8% (Tab. 2) instead of 26.5% (Tab. 1). These results are in accordance with protein contents of other plant species, estimated by standard protein quantifications. Thus, the protein determinations based on their acidic hydrolysis fully confirm the assumption that a major share of nitrogen (corresponding to a calculated, putative protein content of about 10%) is present in the SDS-insoluble fraction (**R**) and is not due to authentic proteins.

In addition to protein quantification, various electrophoretic studies had been conducted. After staining the gels with Coomassie, no major differences in the amount of proteins derived from the three different plant species were visible (Fig. 1, Supplementary material), underlining the lacking of major differences in protein contents between *M. oleifera* and other plant species.

Protein contents in M. oleifera leaves are as high as in other species

The combined data on protein analyses suggest that the key to understand the inconsistencies in the protein content of *M. oleifera* leaves is the elucidation of the source of the nitrogen present in the insoluble sediment. When thoroughly evaluating the literature dealing with *M. oleifera*, some hints on the not yet identified insoluble, nitrogen-containing compounds can be found. KAKENGI et al. (2005) studied the feasibility of employing *M. oleifera* leaves as protein supplement for ruminants. In addition to the determination of the putative total protein content, the authors quantified the share of protein, which is degradable in the rumen and which part is not. The latter one was identified as putative proteins which are insoluble in detergent-containing acids. The authors ascertained a total protein content of 26.5% and outlined that about 40% of this amount (corresponding to about 10% of the dry mass as putative protein) is not degradable in the rumen (KAKENGI et al., 2005). Accordingly, they concluded that about two fifths of the entire protein could not be solubilised with detergents. Hence, these data are fully in accordance with the data presented here. However, it has to be questioned, whether or not the nitrogen present in the compounds, which are not rumen-degradable, are authentic proteins.

Based on further literature screening, it became obvious that the putative high protein concentration in *M. oleifera* leaves is not exceptional. For example, in cassava leaves – when estimated on the basis of Kjeldahl nitrogen determinations – it is reported to be in the range between 29% and 38% (YEOH and CHEW, 1976). In a comprehensive study employing many tropical plants, BYERS (1961) determined the putative protein content of the leaves of 70 different plant species growing in Ghana. Based on Kjeldahl determinations of crude extracts, the author reported that 10 of the studied plant species revealed a leaf protein content higher than 28%, and 28 of them exhibited a percentage even higher than 24%. Of course, also for this study, it has to be doubted that the Kjeldahl-based protein determination indeed outlines the correct amount of authentic proteins. Nonetheless, these data state that the very high putative protein content reported for *M. oleifera* is not exceptional.

When contemplating the nature of the SDS-insoluble nitrogen containing substances present in the leaves, the focus inevitably shifts to cell wall components. It is well known that – in addition to cellulose, various other polymeric carbohydrates, and lignin – also proteins are part of plant cell walls (HOUSTON et al., 2016). Already more than half a century ago, LAMPORT and NORTHCOTE (1960) reported that the main share of the proteins bound to the cell walls of higher plants is due to hydroxyproline-rich glycoproteins (HRGP), which may account for about 10% of the dry weight of the cell wall (TALMADGE et al., 1973; ROBERTS et al., 1985). According to LAMPORT (1966) these highly insoluble cell wall proteins are denoted as extensins. Until now, no clear picture on the complex nature of extensins could be drawn. Yet, the explanation for their highly insoluble character is given by tyrosine-based bindings resulting in isodityrosine moieties (LAMPORT, 1980; FRY, 1982), which results in a highly cross-linked network of extensins (LAMPORT, 1986).

Whereas at the end of the last century many efforts had been made to elucidate the structure of the HRGP, to date the evolutionary and genetic aspects of these proteins are in the focus of interest (JOHNSON et al., 2017). So far no studies are available that combine basic plant biochemical insights on HRGP or extensins, respectively, and the data elaborated for the relevance of leaf proteins with respect to human or animal nutrition as outlined above. Accordingly, we do not know whether or not the insoluble proteins, putatively non-degradable in the rumen (KAKENGI et al., 2005), correspond to the HRGP.

These coherences outline that there is a massive need for interdisciplinary as well as for applied research to further elucidate the marvel of the putative high protein contents. This especially accounts when considering that - without any doubt – *M. oleifera* leaves exhibit many benefits for human nutrition and a significant growth promoting effect on livestock (NOUMAN et al., 2014). As reported by

Tab. 2: Protein content in *M. oleifera* leaves estimated on the basis of acidic protein hydrolysis.

Fraction	Content of amino acids [mg/g]	Amino acids liberated by hydrolysis = protein [%]	[mg/g]	Protein content (Kjeldahl) [%]
T (total extract = free amino acids)	12	138	13.8	26.5
T*h* (total extract, hydrolysed)	150			
S (aqueous supernatant = free amino acids)	7.1	33.6	3.36	6.0
S*h* (aqueous supernatant, hydrolysed)	40.7			
P (SDS supernatant = free amino acid)	1.05	22.1	2.21	8.3
P*h* (SDS supernatant = hydrolysed)	23.1			
R (SDS insoluble = free amino acids)	1.4*	46.7	4.67	10.0
R*h* (SDS insoluble = hydrolysed)	48.1			
sum "free amino acids" (S + P + R)	9.5	102.4	10.24	24.3
sum "hydrolysed" (Sh + Ph + Rh)	111.9			

The protein content of each sample was calculated as difference of the amino acid contents in the original and the hydrolysed fractions. *In principle, in this fraction no free amino acids could be present. However, their occurrence points to the fact that proteins might be hydrolysed by proteases present in this fraction.

ANHWANGE et al. (2004), the amino acids spectrum of *M. oleifera* leaf protein is not really exceptional: apart from glutamic and aspartic acid, the semi-essential amino acid arginine and the essential leucine represent the most abundant ones in the related hydrolysates. In consequence, all tremendously high number of positive nutritional effects described in the literature must have another origin, which has to be elucidated in the future.

Composition and degradation of the *M. oleifera* glucosinolates

General state of affairs

As outlined above, the well-known health-promoting effects of *M. oleifera* glucosinolates are attributed to their various degradation products, in particular to the corresponding isothiocyanates. Accordingly, the extent and velocity of glucosinolate degradation are of special interest. A plain finding in daily life may open the door for a better understanding of the entire process: when an extract of *M. oleifera* leaves is kept in the cold, e.g. in the fridge, for several days, there is a strong retardation in the generation of the characteristic pungent taste and flavour, whereas the sensory properties develop much quicker at RT (H. HEINRICHS, pers. comm.). However, the appendent intensity and pungency as well as the overall sensory quality are quite different. Obviously, the relevant degradation products of the glucosinolates differ and are generated at different times. The basis for the initiation of the various reactions – also in *M. oleifera* – is the hydrolysis of the genuine glucosinolates catalysed by myrosinases (DOERR et al., 2009; CHODUR et al., 2018). However, due to the presence of rhamnose attached to the isothiocyanates in *M. oleifera* leaves, the primary hydrolysis products might not be volatile, and then would be aroma-neutral. Yet, a thorough investigation of the related degradation processes requires a sound determination of the genuine glucosinolates. According to WATERMAN et al. (2014) and FAHEY et al. (2018) apart from glucomoringin (4-α-L-rhamnopyranosyloxy-benzyl glucosinolate), three different acetyl-derivatives are described (acetyl-4-α-L-rhamnopyranosyloxy-benzyl glucosinolate = acetyl-glucomoringin isomers I-III; Fig. 3A). By employing LC-MS chromatography, 4-α-L-rhamnopyranosyloxy-benzyl glucosinolate could be assigned reliably. Identification of the isomers of acetyl-4-α-L-rhamnopyranosyloxy-benzyl glucosinolates was also performed but with lack of NMR data, position of the acetylation site could not be determined (Fig. 3B). No other glucosinolates were identified in the analysed plant material.

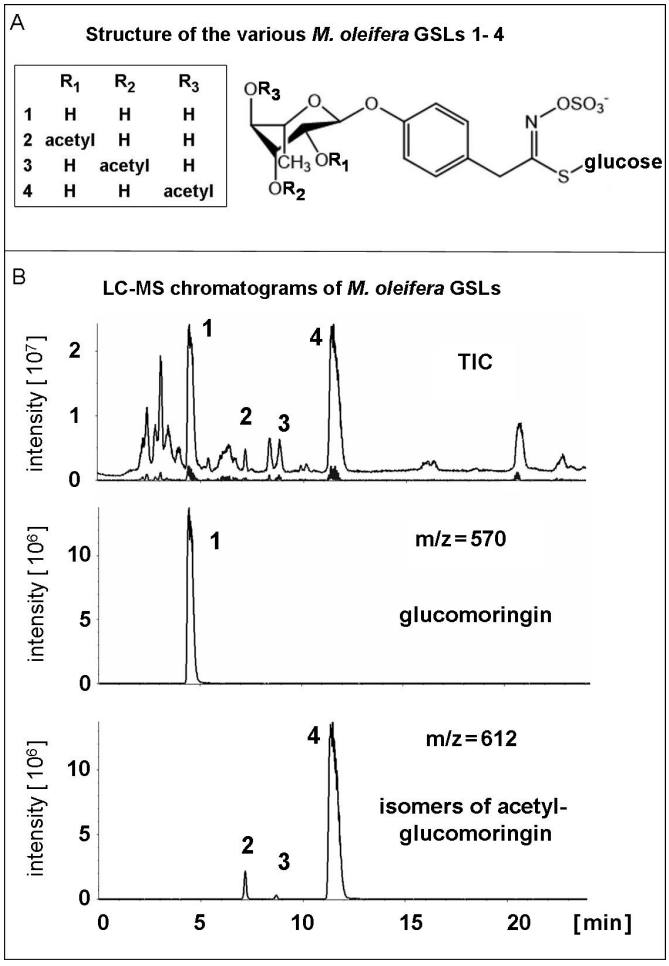

Fig. 3: Glucosinolates present in *M. oleifera* leaves.

A: Structure of the common *M. oleifera* glucosinolates. Apart from glucomoringin (4-α-L-rhamnopyranosyloxy-benzyl glucosinolate; **1; $R_{1,2,3}$, = H**), three isomers of its acetylated form (acetyl-4-α-L-rhamnopyranosyloxy-benzyl glucosinolate = acetyl glucomoringin; isomers I-III R_1 or R_2 or R_3 = acetyl) are present in *M. oleifera* leaves.

B: Identification of the *M. oleifera* glucosinolates by LC-MS. Mass chromatograms of a *M. oleifera* leaf extract containing all glucosinolates. At the top: total ion chromatogram (TIC; mass range: 100-800 Da). In the middle: extracted ion chromatogram of m/z = (570) $[M]^-$ representing glucomoringin (4-α-L-rhamnopyranosyloxy-benzyl glucosinolate; **1**). At the bottom: extracted ion chromatogram of m/z = (612) $[M]^-$ representing the three isomers of acetyl-glucomoringin (acetyl-4-α-L-rhamnopyranosyloxy-benzyl glucosinolate; isomers I-III). The MS/MS spectra of the four different *M. oleifera* glucosinolates are displayed in Fig. S2 (Supplementary material).

Time-dependent glucosinolate degradation

In order to elucidate whether the known differences in the generation of degradation products might be related to differences in the myrosinase activity, leaf extracts have been incubated at 4 °C and 20 °C, respectively. The concentration of the four glucosinolates was determined by LC-MS. The results proved that all glucosinolates are hydrolysed efficiently (Fig. 4, 1-4).

Degradation of all glucosinolates was significantly lower at 20 °C compared to samples incubated at 4 °C. This unexpected observation was especially evident in samples incubated for 15 s. In several cases, the counteracting effects of elevated temperatures on either enzyme activity or on its stability could result in a higher substrate turn-over at lower temperatures. However, myrosinase exhibits a high stability (VAN EYLEN et al., 2005; GHAWI et al., 2012). Accordingly, this explanation can be excluded. Currently, no physical explanation for this phenomenon, which putatively leads to a solubilisation and thus a faster hydrolysis of glucosinolates, is available. Wetting of samples was carefully observed and samples that were not homogenized within the first second of shaking were discarded because of the very short incubation time. Since experiments are conducted with finely ground leaf powder in an artificial experimental setup, a high degradation of glucosinolates probably happens during the first few seconds in samples incubated at 20 °C, when it is assumed that the myrosinases present in *M. oleifera* perform better at 20 °C compared to 4 °C, similarly to other myrosinases (LI and KUSHAD, 2005). This could lead to an inhibition of myrosinases by breakdown-products of glucosinolates in samples incubated at 20 °C leading to lower degradation rates and therefore higher glucosinolate contents. Lower contents in samples incubated at 4 °C for 15 s could be explained by a slower, but steadier degradation of glucosinolates by myrosinases at 4 °C, which is also reflected in the contents of glucosinolates observed after 30 s, which were higher in samples incubated at 4 °C compared to samples incubated at 20 °C.

Nonetheless, it has to be stated that the glucosinolates are entirely hydrolysed within the first minute of incubation. Accordingly, the

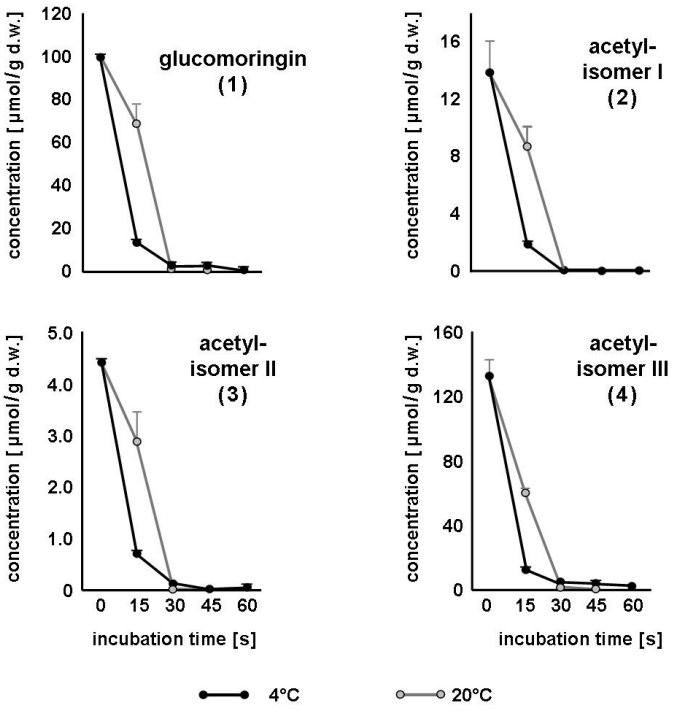

Fig. 4: LC-MS analysis of glucosinolates in *M. oleifera* extracts. Lyophilized plant material from the greenhouse was incubated for 15 to 60 s either at 4 °C (black line) or at 20 °C (grey line), respectively. Data points represent the mean of three technical replicates for each glucosinolate. Contents were calculated with the standard curve of sinigrin.

myrosinase-catalysed hydrolysis cannot be responsible for the observed sensory differences in extracts, which are kept at different temperatures for many hours or several days, respectively.

The generation of olfactorily active substances in the further course of incubation signifies that the rhamnose moiety is cleaved off from the assumed non-volatile primary degradation products, i.e., the rhamosylated isothiocyanates, as time goes on. No detailed information is available with respect to the reaction mechanism and the enzymes putatively involved, such as rhamnosidases. The different degradation rates at the two temperatures could also have an effect on these putative rhamnosidases degrading the rhamnosylated degradation products. In general, activity of enzymes could also be inhibited by an oversupply of substrates (breakdown-products). The steadier generation of breakdown-products at 4 °C could lead to subsequent steadier generation of olfactorily active substances, whose volatility is additionally diminished at lower temperatures. The retarded generation of the olfactorily active substances as well as the related differences at 4 °C and 20 °C also seem to be related to experimental *in vitro* conditions, since all glucosinolates are hydrolysed by myrosinase already within one minute of incubation (Fig. 4).

Due to the differences in stability of potential hydrolysis products (BUSKOV et al., 2000), the situation becomes more complex. The products of the myrosinase reaction, i.e., the various rhamnosylated isothiocyanates (Fig. 1) are quite stable. However, the elimination of the rhamnose moiety results in 4-hydroxybenzyl isothiocyanate (Fig. 5), a very instable compound, which is also generated in the course of the hydrolysis of sinalbin in white mustard (*Sinapis alba*). Analyses of the corresponding degradation products in crushed seeds of *S. alba* revealed that 4-hydroxybenzyl isothiocyanate is converted quickly to 4-hydroxybenzyl alcohol, and other reaction products; e.g. in the presence of ascorbic acid 4 hydroxybenzyl-ascorbigen is generated (Fig. 6) (KAWAKISHI and MURAMATSU, 1966; BUSKOV et al., 2000; BOREK and MORRA, 2005; PAUNOVIĆ et al., 2012). In contrast, in black mustard (*Brassica nigra*) the related degradation products, allyl isothiocyanate and benzyl isothiocyanate, which are derived from sinigrin and glucotropaeolin, respectively, are far more stable (FENWICK et al.,1983). Moreover, the various isothiocyanates reveal different affinities to the transient receptor potential ankyrin, which is responsible for the perception of pungent taste (TERADA et al., 2015).

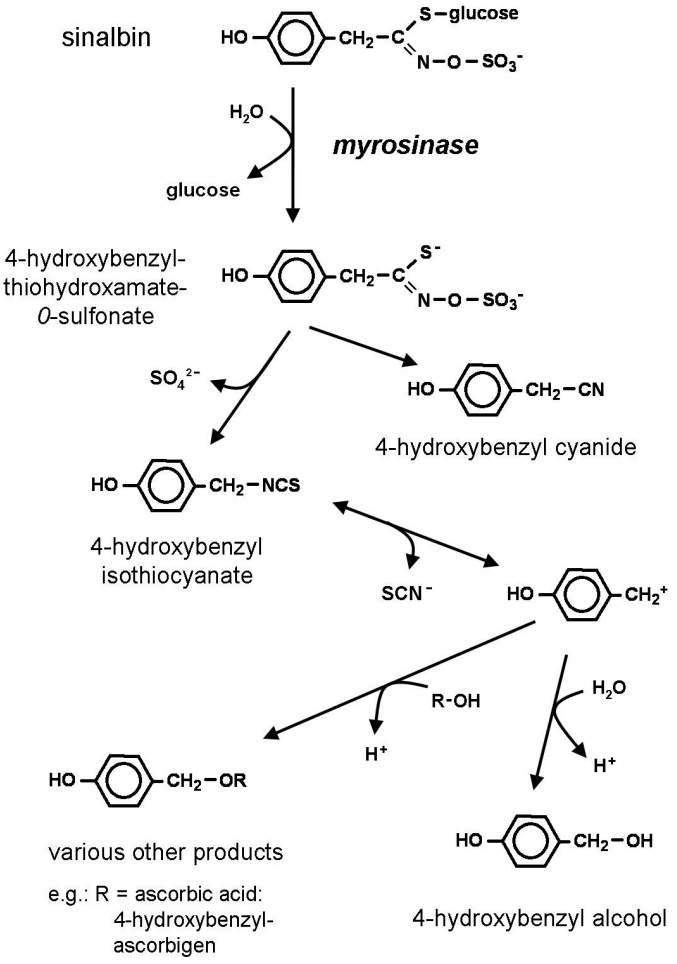

Fig. 5: Potential degradation products of 4-hydroxybenzyl isothiocyanate produced by myrosinase-catalysed hydrolysis of sinalbin according to BUSKOV et al. (2000).

In order to get a survey on the related degradation products, which are generated in disintegrated *M. oleifera* leaves, HPLC analyses of aqueous extracts of fresh *M. oleifera* leaves as well as of commercially available dried *M. oleifera* leaf material had been conducted. The material was homogenized with tap water at RT and stored subsequently at 4 °C. Every day, samples had been taken and analysed by HPLC (Fig. 6). To facilitate the identification of putative degradation products of the glucosinolates, authentic reference substances need to be employed. Only 4-hydroxybenzyl alcohol (Fig. 6) could be used as reference substance, since neither the rhamnosylated nor the non-substituted 4-hydroxybenzyl isothiocyanates are commercially available.

The HPLC analyses exhibited massive differences in the spectrum of glucosinolate degradation products in the extracts of fresh leaves in comparison to those derived from dried leaf material (Fig. 6). The only compound identified reliably is 4-hydroxybenzyl alcohol. Accordingly, it could be deduced that in analogy to the degradation

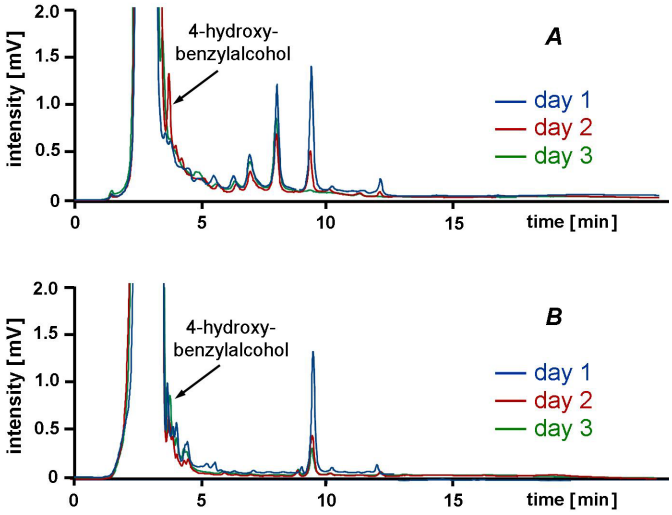

Fig. 6: Variation of degradation products pattern in *M. oleifera* cold extracts. **A:** Dried leaf powder; **B:** fresh crushed leaves. Directly after homogenization with tap water, the samples had been stored at 4 °C. For detection, a diode array detector was employed (ʎ = 220 nm). Due to the HPLC conditions, in contrast to the LC-MS analyses mentioned above, glycosylated compounds, e.g. all glucosinolates, elute in the void volume.

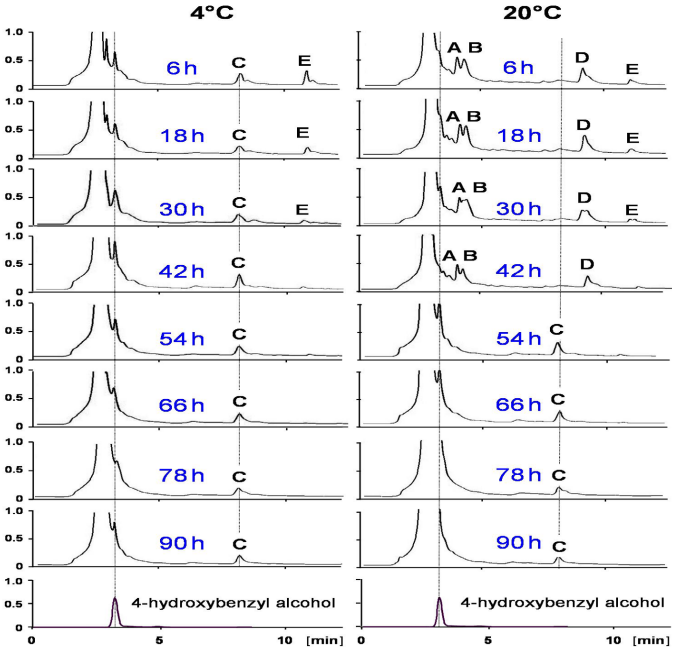

Fig. 7: HPLC analyses of *M. oleifera* extracts stored at 4 °C or 20 °C. Samples had been taken every 12 h. 4-Hydroxybenzyl alcohol was employed as authentic reference substance. For detection, a diode array detector was employed (ʎ = 220 nm). Due to the HPLC conditions, in contrast to the LC-MS analyses mentioned above, glycosylated compounds, e.g. all glucosinolates, elute in the void volume.

of sinalbin (Fig. 5) 4-hydroxybenzyl alcohol was produced as final degradation product of *M. oleifera* glucosinolates. Other substance peaks cannot be unequivocally assigned to putative degradation products of glucosinolates. Their absorbance spectra point to glucosinolate-related compounds. Hence, all glucosinolates are already cleaved within the first minute of incubation (Fig. 5) the observed differences must be related to reactions following the myrosinase-catalysed hydrolysis of glucosinolates. Obviously, these processes, i.e. rearrangements and further degradations, are quite different in extracts of fresh and dried plant material. In general, such differences could be a consequence of differential inactivation of enzymes. This underlines the assumption that – in addition to myrosinases – further enzymes, such as rhamnosidases, could be involved in the degradation of glucosinolates and their breakdown-products. Accordingly, the observed substantial differences could result from varying activities of these putative enzymes, caused by their different stabilities in fresh and dried leaves.

From these coherences it could be deduced that the characteristic differences in the release of olfactorily active substances in *M. oleifera* extracts which are kept at either 4 °C or at 20 °C (H. Heinrich, pers. comm.) might be related to variations in enzyme activities. To get more information on this phenomenon, a further row of experiments was conducted. Commercially available *M. oleifera* leaf material was homogenized with tap water and stored either at 4 °C or at 20 °C, respectively. Every 12 h, a corresponding sample had been taken and analysed by HPLC (Fig. 7).

The HPLC analyses reveal that in the *M. oleifera* extracts various substances are released as time goes on (Fig. 7). Obviously, also in the case of the various *M. oleifera* glucosinolates 4-hydroxybenzyl alcohol represents the final degradation product (Fig. 5). This verifies that the rhamnose moiety of the *M. oleifera* glucosinolates has to be cleaved off in the course of their further degradation. Although the various intermediates of the complex degradation process are not yet identified, the comparison of the related products and their time-dependent generation at 4 °C and 20 °C, respectively, indicates that various additional reactions are involved in the complex degradation processes and suggest the involvement of a putative rhamnosidase. Although up to now no definite information on this enzyme is available. Apart from the basic scientific point of view, especially with regard to the health-protective effects of *M. oleifera* preparations, the elucidation of these complex processes is of special interest. Due to their anti-carcinogenic effect, the stability of the rhamnosylated isothiocyanates (moringin and analogues), are of particular importance, since the elimination of the rhamnose moiety induces the putative further degradation. Accordingly, the elucidation of these complex processes, i.e. the overall degradation of the *M. oleifera* glucosinolates and the subsequent rearrangements of their degradation products, need to be further investigated.

Conclusion

Moringa oleifera is thought to be one of the most valuable and beneficial tree crop plant species. Apart from great benefits for human nutrition and a significant growth improvement of livestock, outlined in numerous studies, especially the health-promoting properties are of special interest. Up to now, the nutritional properties had been attributed primarily to the putatively high protein content of *M. oleifera* leaves. Yet, this study unveiled that the protein content of *M. oleifera* leaves is not exceptional and is in the same range as found for leaves of other plant species. Moreover, the amino acids composition of *M. oleifera* leaves is not that exceptional as commonly mentioned (ANHWANGE et al., 2004). Thus, the high number of positive effects described in the literature must have another origin, which needs to be elucidated.

The health-promoting effects is frequently assigned to the anti-carcinogenic properties of the *M. oleifera* glucosinolates, in particular, to their degradation products. By analysing the degradation of glucosinolates at various conditions, the complexity of these processes is illustrated. Due to their anti-carcinogenic effect, the degradation of the rhamnosylated isothiocyanates is of special interest, since the elimination of the rhamnose moiety induces the further decay, and thus the loss of the desired health-protective properties. Thus, further elucidation of these complex processes is of particular relevance.

Conflict of interest

On behalf of all authors, the corresponding author declares that there is no financial and personal relationships with other people or organizations that could inappropriately have influenced our work.

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Supplementary material

In addition to protein quantification, various electrophoretic studies on *M. oleifera* proteins had been conducted. For these analyses, the soluble protein fraction as well as the sediment had been treated with SDS and employed for the electrophoretic studies. For comparison, extracts from spinach (*Spinacia oleracea*) and the rubber tree (*Hevea brasiliensis*) had been analysed analogously. After staining the gels with Coomassie, overall, no major differences in the amount of proteins derived from the three plant species were visible, underlining the lacking of major differences in protein contents between *M. oleifera* and ordinary plants.

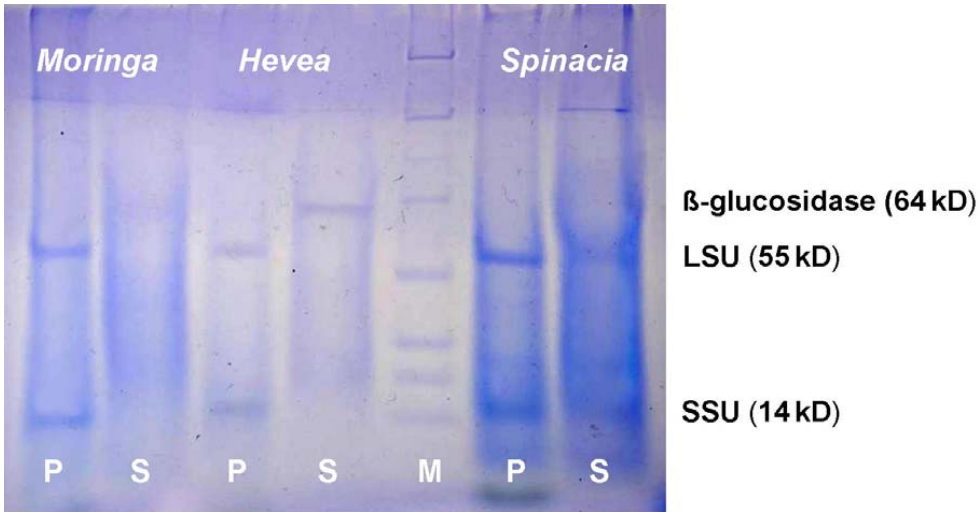

Fig. S1: SDS gel electrophoresis of various protein fractions. Protein was extracted from dried leaf material (commercial *M. oleifera* powder, *africrops!*, Berlin, and freeze-dried leaves from spinach and *Hevea*) applying McIlvaine buffer (pH 5,6). The related supernatant (30,000 g) is denoted as S. Before electrophoresis, samples had been treated with SDS and mercaptoethanol. Analogously, also the sediment was re-suspended in SDS-mercaptoethanol containing buffer. After centrifugation, the supernatant was also employed for electrophoresis (S). M = protein marker. Electrophoresis was performed according to http://www.genstrom.net/public/biology/botany/plant_physiology_module/protocols/protokoll_3.html

Indeed, in all three species, the most prominent protein bands are due to the large (LSU) and small subunit (SSU) of the ribulose-bisphosphate carboxylase (RuBisCo). Whereas this enzyme from spinach is quite soluble in McIlvaine buffer (pH 5,6), in *M. oleifera* and in *Hevea* this enzyme has to be solubilised with SDS. In *Hevea*, the most prominent band in the soluble fraction is due to the ß-glucosidase.

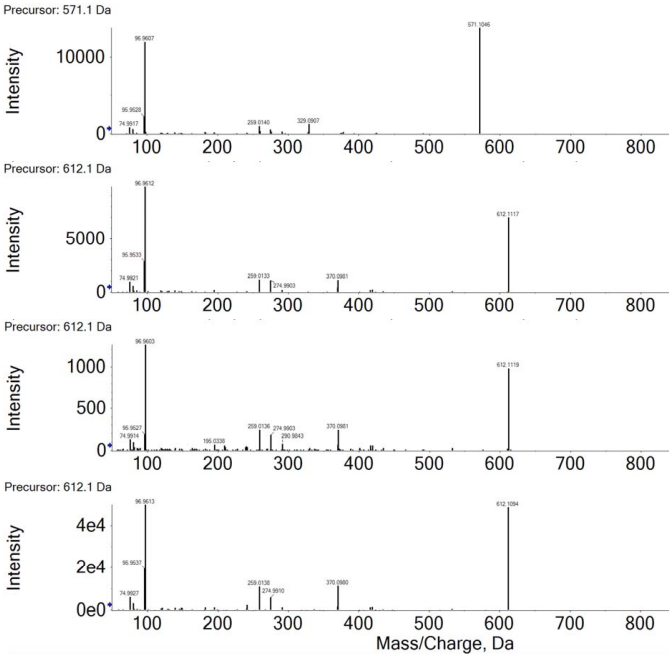

Fig. S2: MS/MS spectra for glucosinolates found in *M. oleifera*. Mass fragments at -10 eV for the precursor ions 571.1 (4-α-L-rhamnopyranosyloxy-benzyl glucosinolates) and 612 (acetyl-4-α-Lrhamnopyranosyloxy-benzyl glucosinolates isomers 1-3).