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Original Article

Gamma radiation treatment activates glucomoringin synthesis in *Moringa oleifera*

Tsifhiwa Ramabulana[a], Risimati D. Mavunda[b,c], Paul A. Steenkamp[a,d], Lizelle A. Piater[a], Ian A. Dubery[a], Ashwell R. Ndhlala[e], Ntakadzeni E. Madala[a,*]

[a] *Department of Biochemistry, University of Johannesburg, Auckland Park, South Africa*
[b] *Department of Physics, University of Johannesburg, Auckland Park, South Africa*
[c] *South African Nuclear Energy Corporation, Pretoria, South Africa*
[d] *Council of Scientific and Industrial Research, Biosciences, Natural Products and Agro-processing Group, Pretoria, South Africa*
[e] *Agricultural Research Council, Vegetable and Ornamental Plants, Pretoria, South Africa*

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ABSTRACT

Plants are a very rich source of pharmacologically relevant metabolites. However, the relative concentrations of these compounds are subject to the genetic make-up, the physiological state of the plant as well as environmental effects. Recently, metabolic perturbations through the use of abiotic stressors have proven to be a valuable strategy for increasing the levels of these compounds. Oxidative stress-associated stressors, including ionizing radiation, have also been reported to induce metabolites with various biological activities in plants. Hence, the aim of the current study was to investigate the effect of gamma radiation on the induction of purported anti-cancerous metabolites, glucomoringin and its derivatives, in *Moringa oleifera* Lam., Moringaceae. Here, an UHPLC-qTOF-MS-based targeted metabolic fingerprinting approach was used to evaluate the effect of gamma radiation treatment on the afore-mentioned health-beneficial secondary metabolites of *M. oleifera.* Following radiation, an increase in glucomoringin and three acylated derivatives was noted. As such, these molecules can be regarded as components of the inducible defense mechanism of *M. oleifera* as opposed to being constitutive components as it has previously been assumed. This might be an indication of a possible, yet unexplored role of moringin against the effects of oxidative stress in *M. oleifera* plants. The results also suggest that plants undergoing photo-oxidative stress could accumulate higher amounts of glucomoringin and related molecules.

Introduction

Glucosinolates (GS) are secondary metabolites found in almost all plants of the order Brassicales (Fahey et al., 2001; Mithen, 2001). These compounds are diverse in origin, side chain modification, degradation and final biological functions (Grubb and Abel, 2006), and comprise short- and long-chain aliphatic glucosinolates (Ile, Leu, Val, Ala and Met), indolic glucosinolates (Trp) and aromatic glucosinolates (Tyr and Phe) (Brown et al., 2003; Clarke, 2010; Agerbirk and Olsen, 2012; Leone et al., 2015). Under normal conditions, GS are chemically stable, however, during plant wound responses these compounds are hydrolyzed by the enzyme myrosinase to produce isothiocyanates, nitriles, thiocyanates, epithionitriles and oxazolidines which are responsible for the reported biological activities thereof (Bones and Rossiter, 2006; Zandalinas et al., 2012). GS-derived molecules are highly water-soluble due to the hydroxyl-amino sulfate group and a β-thioglucosyl residue attached to the variable R-group on the GS skeletal structure (Clarke, 2010; Vo et al., 2013; Förster et al., 2015a; Leone et al., 2015), thereby contributing to a high bioavailability following human consumption. In plants, GS are known to be responsive to both biotic and abiotic stresses, and have been shown to be induced by various environmental factors such as solar radiation, temperature variation and climate changes (Bones and Rossiter, 2006; Zandalinas et al., 2012). Almost all the afore-mentioned stressors of plants are associated with oxidative stress (Bajguz and Hayat, 2009; Demidchik, 2015), thereby suggesting a possible role of these compounds in mitigating the damages imposed as a result of such stress, a phenomenon which has also been extended to human-related diseases (Tumer et al., 2015; Williamson et al., 1998).

* Corresponding author.
E-mail: emadala@uj.ac.za (N.E. Madala).

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Recently, the GS components (4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate followed by three isomeric acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate (Ac-isomer-GS I, II, III) of *Moringa oleifera* Lam. have been reported to possess indirect anti-oxidant activity due to the ability to regulate an anti-oxidant enzymatic processes in mammalian systems (Tumer et al., 2015). GS have also been reported to control the damages of other physiological conditions associated with oxidative stress such as reducing the risks of several cancers (colon, bladder and breast cancer) (Björkman et al., 2011). It has been shown that GS have the ability to inactivate phase I enzymes (cytochrome P-450) or to stimulate phase II enzymes (glutathione-*S*-transferase), thereby eliminating carcinogenic metabolites (Zhang and Talalay, 1994). More recently, consumption of GS derived compounds such as isothiocyanates (ITC) has been shown to be beneficial for mammals, since they lead to the up-regulation of xenobiotic metabolism (phase II metabolic enzymes), associated with an increases in the antioxidant capacity, thus leading to improved protection against various chronic physiological conditions (Traka and Mithen, 2009).

As previously mentioned, the levels of GS in plants are subject to several environmental factors and, as such, create conditions favoring the production of these compounds by plants, as has been investigated (Förster et al., 2015a). An increase in a GS, glucotropaeolin, due to UV-B radiation treatment of nasturtium (*Tropaeolum majus* L.) plants has been reported (Schreiner et al., 2009). Different forms of radiation are known to induce oxidative stress in plants (Esnault et al., 2010; Hollósy, 2002; Kovács and Keresztes, 2002), and thus the involvement of GS could be to control the damages of radiation-induced oxidative stress.

Moringa oleifera is a versatile and widely cultivated species in the monogeneric family of Moringaceae, and is known to contain GS molecules (Fahey, 2005; Förster et al., 2015a; Moyo et al., 2011; Popoola and Obembe, 2013). Almost all parts of the *M. oleifera* plant contain varying amount of aromatic GSs, with the leaves containing the highest levels (Clarke, 2010; Moyo et al., 2011). Some of the reported pharmacological potency of the plant have been directly correlated to the presence of these GSs (Clarke, 2010).

Recently, we have shown that *M. oleifera* does not produce other highly sought after pharmacologically relevant metabolites (rutin for an example) in comparison to other related species, *M. ovalifolia* (Makita et al., 2016). Moreover, we further speculated that production of such metabolites could be influenced by various factors such as environmental conditions and the genetic make-up of the plants (Makita et al., 2016). Elsewhere, the levels of health-promoting metabolites have been shown to be affected by ionizing radiation (Ramabulana et al., 2015, 2016). Radiation is a potent inducer of oxidative stress, and it has been used to identify metabolites with anti-oxidative properties in various plants (Mittler, 2002). With increasing evidence on the anti-oxidative properties of GS molecules (Guerrero-Beltrán et al., 2012) and as potential agents for ameliorating oxidative stress-associated diseases (Dinkova-Kostova and Kostov, 2012), it is important to study biotic and abiotic factors with potential of enhancing the levels of these compounds. As such, in the current study, a potent form of radiation, namely gamma radiation was used to trigger oxidative stress in *M. oleifera* leaves. Subsequent perturbations in the levels of GSs were monitored using UHPLC-ESI-qTOF-MS-based fingerprinting.

Materials and methods

Plant material

Two month old *Moringa oleifera* Lam., Moringaceae, plants were obtained from the Patience Wellness Centre farm in Lebowakgomo, South Africa. The plant species was authenticated, and a voucher specimen (with voucher number NEM001) was prepared and deposited at the Department of Botany, University of Johannesburg, South Africa.

Gamma radiation procedure

Plants were irradiated as previously described (Ramabulana et al., 2015, 2016). Irradiation was performed at Nuclear Energy Cooperation of South Africa (NECSA) (Phelindaba, Pretoria, South Africa). Briefly, fifteen plants were irradiated with a Cobalt-60 source (at a dose rate of 22 kGy/h) inside a well-protected chamber, along with fifteen non-irradiated control plants. Various radiation doses (0.1–8 kGy) were tested and 2 kGy dose was found to be more potent as shown previously (Ramabulana et al., 2015). Total radiation dose absorbed by plants was further confirmed by Harwell Perspex Poly Methyl Methacrylate Amber (PMMA) 3042 dosimeters (Harwell Co, United Kingdom).

Metabolite extraction

From the optimization results achieved with our preceding studies, plant leaf material was harvested a day (24 h) post-radiation and dried at 50 °C for 72 h (Ramabulana et al., 2015, 2016). The dried plant material was ground and extracted with 80% aqueous methanol as described by Ramabulana et al. (2015, 2016). The extracts were concentrated, reconstituted in 50% aqueous methanol and stored at −20 °C until analyzed.

Chromatography and mass spectrometry analyses

Three technical repeats of the hydromethanolic extracts (5 μl) were analyzed using an Acquity UHPLC equipped with an Acquity BEH C18 reverse phase column (150 mm × 2.1 mm, 1.7 μm) (Waters Corporation, MA, USA). The mobile phase A consisted of 0.1% formic acid in deionized water, while the mobile phase B consisted of 0.1% formic acid in acetonitrile (Romil Pure Chemistry, Cambridge, UK). The elution gradient started at 98% A until 5% at 26 min for 2 min, and then returned to initial conditions of 98% A at 28 min for 2 min with a run time of 30 min at a constant flow rate of 0.4 ml/min. Chromatographic separation/elution was monitored using a photodiode-array detector (PDA) collecting 20 spectra/s between the 200 and 500 nm range. In a second detection, a Synapt G1 high-definition mass spectrometer (MS) was used operating in both positive and negative electrospray ionization (ESI) modes. Briefly, the following MS conditions were used as optimal experimental conditions: the capillary voltage of 2.5 kV, multichannel plate detector potential of 1600 V, sample cone potential of 30 V, desolvation temperature of 450 °C, source temperature of 120 °C, cone gas flow of 50 l/h and desolvation gas flow of 550 l/h. For MS fragmentation experiments, the MS acquisition method with low collision energy ramp of 10–30 eV and a high collision energy ramp of 15–60 eV was used to generate typical MS^E fragmentation patterns. MassLynx™ and MarkerLynx™ software (Waters Corporation, MA, USA) were used to visualize and analyze the UHPLC-qTOF-MS raw data so as to generate data matrix for further statistical modeling.

Metabolite identification and statistical analyses

The UHPLC-ESI-MS data collected in negative ionization mode was analyzed using MarkerLynx™ XS software for peak alignment, peak finding, peak integration and retention time (Rt) correction with the following parameters: Rt range of 1–27 min, mass range of 100–1000 Da, mass tolerance of 0.05 Da, Rt window of 0.2 min. Data was normalized to total intensity (area). The acquired data matrix was exported to SIMCA-P software (Umetrics, Umea,

Sweden) for Principal component analysis (PCA) and Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) computation (Ramabulana et al., 2015) and, using these models, possible bio-markers showing differential accumulation across different treatments were identified (Madala et al., 2012; Ramabulana et al., 2015). The data matrix was also exported to Microsoft Excel and, using the area under the peak corresponding to the respective masses (*m/z*) of known GS molecules from *M. oleifera* (Förster et al., 2015a,b), were searched for and further used to create box-and-whiskers plots using SPSS version 22 software (IBM, United States of America, www.ibm.com/SPSS_Statistics). Furthermore, GS molecules with statistical significance were computed using the student t-test in Microsoft Excel. Here, a *p*-value of <0.01 indicates that the fold increases of the identified metabolites are statistically significant.

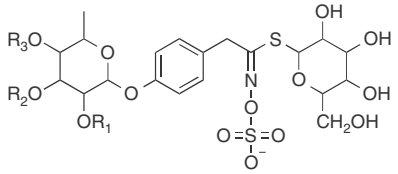
To further confirm the identification of metabolites, the fragmentation patterns generated with the use of different collision energies were compared with the already existing knowledge. Briefly, the molecular formulae of all the peaks corresponding to GS molecules were computed and selected based on the criterion that these are within 5 mDa mass accuracy when compared to the calculated mass of the corresponding molecules. Metabolites were thus annotated according to the Metabolomic Standards Initiatives, level 2 identification (Sumner et al., 2007).

Results and discussion

Gamma radiation is an inducer of oxidative stress that subsequently activates complicated defense mechanisms in plants (Ahuja et al., 2014; Esnault et al., 2010). *M. oleifera* is able to synthesize GS as part of its secondary metabolites. The predominant GS molecule in this plant species is 4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate (**1**), known as glucomoringin (Clarke, 2010; de Graaf et al., 2015; Tumer et al., 2015). The structural uniqueness of these GS derives from the presence of a second glycosyl residue in addition to the already glycosylated side chain (Amaglo et al., 2010). Other structural derivatives of glucomoringin such as the acylated forms thereof have also been reported in this plant (Fig. 1) (Förster et al., 2015a), making these molecules interesting to study. More remarkably, glucomoringin has always been thought to exist only in *M. oleifera*. However, it has also been recently reported in *Noccaea caerulescens* but the authors could not identify the acetylated forms (de Graaf et al., 2015). This suggests the acylation of glucomoringin to be an exclusive phenomenon of *M. oleifera*.

In the current study, gamma radiation-induced oxidative stress resulted in changes to the metabolome in *M. oleifera* plants (Fig. 2, Table 1). Using an UHPLC-ESI-qTOF-MS-based targeted metabolite fingerprinting approach, increased levels of GS molecules were found in plants irradiated with a 2 kGy dose of gamma radiation as compared to the control plants (Fig. 2; Table 1). Here, the box-and-whiskers plots display an increase in the concentrations of glucomoringin and related GS molecules in *M. oleifera* following gamma radiation treatment (Fig. 2). The above results provide a semi-quantitative overview of the amount of GS and its derivatives since there are no commercially available standards of these molecules to achieve absolute quantification. Moreover, the results indicate that the fold increase in the identified GS were statistically significant, with almost all having *p*-values of less than 0.01 as shown in Table 1. Interestingly, it should be re-emphasized that a dose of 2 kGy was found to be more potent and non-lethal, thus inducing the highest levels of GS and its derivatives. Preliminary optimization showed lower doses (0.1, 0.5 and 1.0 kGy) to minimally affect the levels of GS and its derivatives but the levels above 2 kGy such as 4 kG and 8 kGy were found to be lethal, thus killing the plants immediately after radiation. The above phenomenon was also highlighted in studies conducted with another plant, *Phaseolus vulgaris* (Ramabulana et al., 2015).

Furthermore, the characterization of these metabolites was achieved by means of accurate mass MS results (as shown in Fig. 3) with the use of fragmentation patterns and comparison to already published data. Briefly molecule **1** with precursor ion ($[M-H]^-$) at *m/z* 570.0927 ($C_{20}H_{29}NO_{14}S_2$) and Rt of 3.17 min was identified as 4-(α-L-rhamnosyloxy)-benzyl glucosinolate (glucomoringin). The acylated forms of glucomoringin (**2–4**) produced isobaric precursor ions at *m/z* 612.102 ($C_{22}H_{31}NO_{15}S_2$). Interestingly, these molecules eluted at different Rt and, in accordance with already published results (Förster et al., 2015a; Tumer et al., 2015), these three isomers were identified as acetyl 4-(α-L-rhamnopyranosyloxy)-benzyl GS isomer I (**2**), II (**3**) and III (**4**) eluting at Rt of 5.60 min, 6.46 min and 9.63 min respectivey (Bennett et al., 2003; Förster et al., 2015a,b) (Table 1).



1 $R_1=R_2=R_3=H$
2 $R_1=Ac$; $R_2=R_3=H$
3 $R_1=R_3=H$; $R_2=Ac$
4 $R_1=R_2=H$; $R_3=Ac$

The presence of these acetylated isomers pose another interesting but challenging dimension to our results since the function of

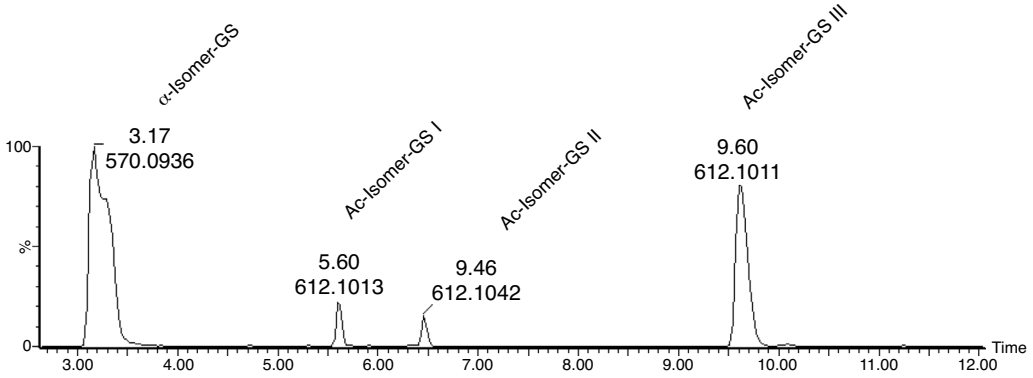


Fig. 1. UHPLC-ESI-qTOF-MS analyses in negative ionization mode of hydromethanolic extracts from 2 kGy gamma irradiated *Moringa oleifera* showing base peak intensity (BPI) chromatograms of 4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate (α-rhamno GS), acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate isomer I (Ac-isomer-GS I), II (Ac-isomer-GS II) and III (Ac-isomer-GS III).

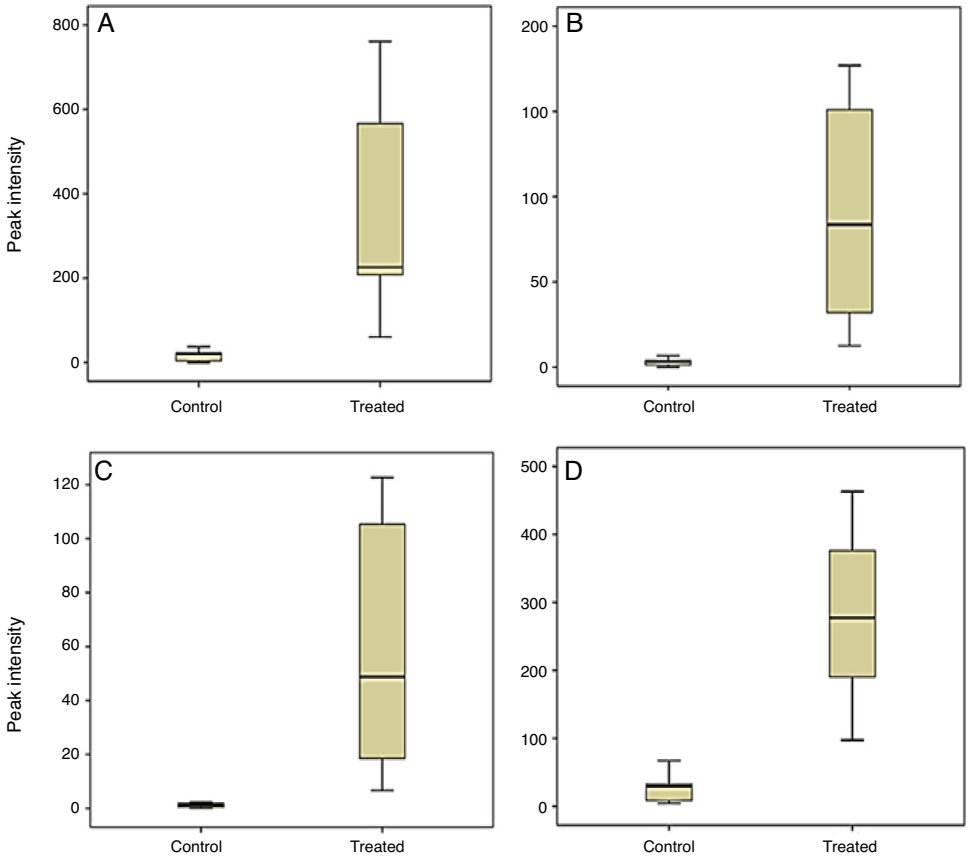


Fig. 2. Box-and-whiskers plots showing relative composition of four identified glucosinolates (moringin derivatives), increased due to 2 kGy gamma radiation treatment of *M. oleifera* with statistical significance of $p < 0.01$. (A) 4-(α-L-Rhamnopyranosyloxy)-benzyl glucosinolate; (B) acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate isomer I; (C) acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate isomer II; and (D) acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate, isomer III.

Table 1
Gamma radiation-induced glucomoringin molecules in *Moringa oleifera*.

Peak	Rt (min)	Compound name	Mass (*m/z*)	Elemental composition	*p*-values	Fold change
1	3.09	4-(α-L-Rhamnopyranosyloxy)-benzyl glucosinolate (**1**)	570.0922	$C_{20}H_{29}NO_{14}S_2$	1.7×10^{-5}	22
2	5.60	Acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate isomer I (**2**)	612.1029	$C_{22}H_{31}NO_{15}S_2$	1.4×10^{-5}	30
3	6.46	Acetyl-4-(α-L-rhamnopyranosyloxy)- benzyl glucosinolate isomer II (**3**)	612.1004	$C_{22}H_{31}NO_{15}S_2$	3.7×10^{-5}	51
4	9.54	Acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate isomer III (**4**)	612.1044	$C_{22}H_{31}NO_{15}S_2$	1.5×10^{-8}	10

this modification and the effect on the biological activity of glucomoringin are not known. The presence of structurally related (isomeric) metabolites in plants is a known phenomenon with a classical example being positional isomers of chlorogenic acids (Ncube et al., 2014, 2016). However, the presence of positional isomers of chlorogenic acids in plants is also not fully understood; but recently it has been speculated to be a strategy deployed by plants to increase the concentration of these molecules through diversification, so as to create a rich reserve to be utilized when needed (Karaköse et al., 2015). As such, the same phenomenon could be true for the case of *M. oleifera* but more research is needed to validate this hypothesis. Although all these isomers increased concomitantly, the relative abundance levels in irradiated plants differed (Fig. 1), suggesting varying stability amongst these compounds. However, elsewhere these acetyl isomers were found to be affected by the type of extraction method and significant rearrangements were noted, with a standard of acetyl-isomer-GS III being converted to acetyl-isomers-GS I and II in a buffered system due to an apparent acetyl migration (Förster et al., 2015a). Thus, it can be postulated that the diversity of GS molecules in *M. oleifera* could be the result of both enzymatic and non-enzymatic reactions in a biological system responding to an oxidative stress environment.

Even though the MS data was acquired using both positive and negative ESI modes, only the ESI negative data was found to be suitable for identification of the GS molecules and this could be due to the fact that these molecules are inherently negatively charged). The accurate MS spectra of these molecules collected at elevated collison energy (30 eV) are shown in Fig. 3.

Using a combination of multivariate and univariate statistical models (data not shown), underlying differences in peak intensities of the extracts obtained from both control and irradiated plants were noted. These differences in the levels of GS molecules is an indication of induction of the glucomoringin biosynthesis pathway in reponse to the oxidative stress triggered by the radiation treatment. As previously stated, GS molecules have been shown to accumulate in plants irradiated with UV-radiation (Schreiner et al., 2009), while other research has reported these molecules to be constitutively present in *M. oleifera* leaf extracts (Fahey, 2005; Förster et al., 2015b; Jansen et al., 2008; Vo et al., 2013). In this regard, our results suggest that the GS compounds are

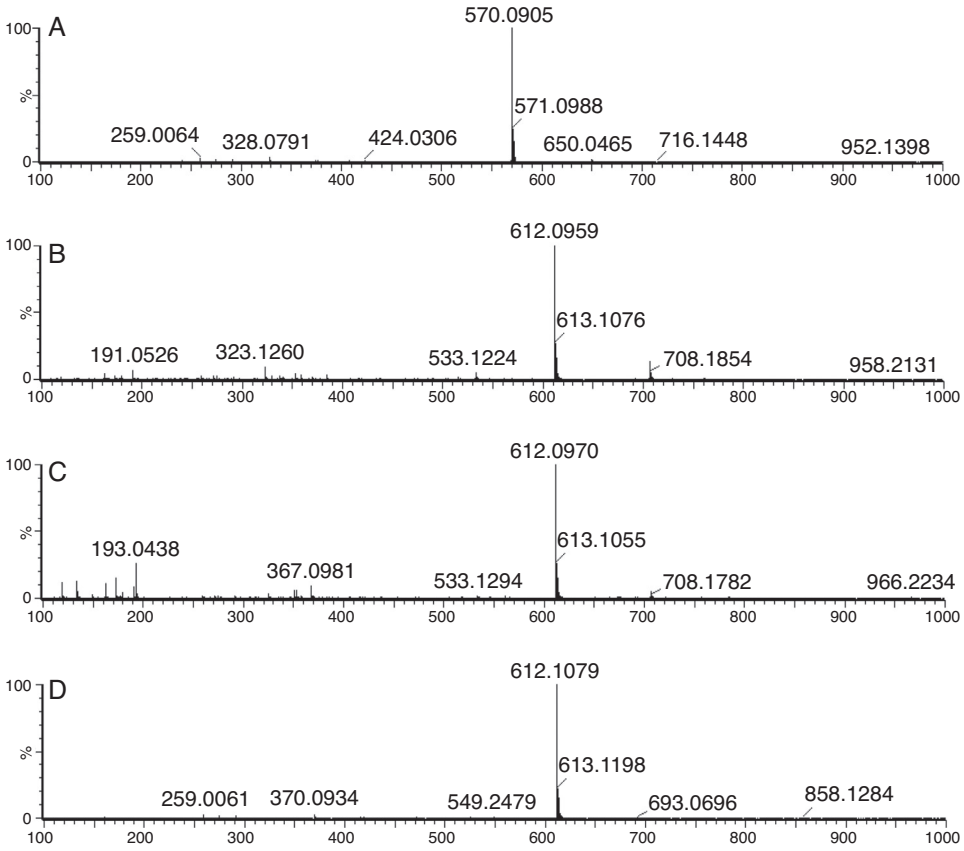


Fig. 3. Spectra of identified GSs in *M. oleifera* leaf extracts of plants irradiated with 2 kGy dose of gamma radiation. (A) 4-(α-L-Rhamnopyranosyloxy)-benzyl glucosinolate; (B) acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate isomer I; (C) acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate isomer II; and (D) acetyl-4-(α-L-rhamnopyranosyloxy)-benzyl glucosinolate isomer III.

inducible components of this plant species as these were found to increase upon radiation treatment. Generally, GS molecules are known to respond against plant wounding (Bodnaryk, 1992), a phenomenon which is inevitable during leaf harvesting and could further explain why these compounds are reported in non-induced leaves elsewhere (Rodríguez-Pérez et al., 2015). Previously, the distribution and presence of these molecules in *M. oleifera* has also been reported with mixed outcomes. For instance, only one glucomoringin molecule was identified in the current study but three distinct glucomoringin molecules were identified in *M. oleifera* from Madagascar (Rodríguez-Pérez et al., 2015). As previously stated, glucomoringin was also recently identified in *N. caerulescens* plants, but the distribution was only limited to a few samples analyzed and absent in other accessions/cultivars (de Graaf et al., 2015). The same authors concluded that these differences are due to regional genetic variation rather than the initially thought environmental factors such as metal toxicity (de Graaf et al., 2015). Genetic variation was further used to justify why glucomoringin was never detected in some species related to *N. caerulescens* (Tolrà et al., 2000; Asad et al., 2013). Recently, acetyl-(4-α-L-rhamnopyranosyloxy)-benzyl GS isomers were found to only accumulate in some, but not all *M. oleifera* plants of the same ecotype (Förster et al., 2015b). Taken together, all the above results are an indication that the presence and relative concentration of these compounds are subject to underlying cellular conditions or genetic makeup of plants. As such, not all GS-containing *M. oleifera* plants will have similar GS-mediated bio-activities. Therefore, studies of conditions with the ability to increase the levels of GS molecules in plants capable of GS synthesis are important. In this regard, the distribution of GS molecules in *M. oleifera* has been studied by varying the cultivation conditions such as sulfur fertilization and water availability, and it has been shown that the GS content increased under a water-deficient regiment, with the effect more pronounced in selected ecotypes (Förster et al., 2015b). This again highlights the importance of genetic variation and abiotic stress conditions.

In the current study, an increase in GS content due to gamma radiation was noted and, more importantly, all the irradiated plants exhibited a consistent response. In general, the involvement of GSs against oxidative stress caused by biotic and abiotic stresses has been reported elsewhere (Björkman et al., 2011; Sardans et al., 2011; Zhang et al., 2011; Zandalinas et al., 2012). Accumulation of the GS content in plants treated with radiation (*i.e.* UV light) has been reported in *T. majus* (Schreiner et al., 2009), *Arabidopsis thaliana* (Wang et al., 2011) and broccoli (Pérez-Balibrea et al., 2008; Mewis et al., 2012). Therefore, taken together, the increase in GS molecules in response to a more potent stimulator of oxidative stress in the form of gamma radiation is an indication of possible anti-oxidative properties of these molecules in plants. Hitherto, there are very limited reports on the direct anti-oxidant activity of GS molecules and whether these compounds function as independent entities or synergistically (Förster et al., 2015a,b). Though the current results has indicated gamma radiation as a potent inducer of medicinally important metabolites, care needs to be taken since this type of radiation is known to cause irreversible damages to food vitamins such as vitamin C (Dionísio et al., 2009). As such, prolonged exposure to milder forms of radiation can be used instead (Zhang and Björn, 2009).

Conclusion

The study represents a proof on concept manipulation of health-beneficial neutraceuticals in a medicinal plant where the inducer leaves no chemical residue. Here, the targeted metabolite profiling confirms the presence of structurally diverse glucomoringin molecules in *M. oleifera* and demonstrates the relative accumulation post-gamma radiation treatment. The current results also show the GS molecules of *M. oleifera* to be part of the inducible defense mechanism of plants rather than constitutive components as previously perceived. Our results supports an *in planta* anti-oxidative role for glucomoringin and acylated derivatives from *M. oleifera*, and by extension in the human body when consumed as herbal supplement. As such, consumption of non-induced *M. oleifera* leaf material does not necessarily guarantee the reported activities associated with these molecules. However, the use of radiation may provide an attractive way to enhance GS content and, as such, *Moringa* plants grown under light intensive environments contributing to photo-oxidative stress, are expected to contain a higher content thereof. Moreover, irradiated plants are also expected to exhibit enhanced pharmacological properties and, as such, future studies should focus on evaluation and biological testing of extracts prepared from irradiated plants.

Authors' contributions

NEM, RDM and IAD conceived of the study, TR conducted the experiments. TR, ARN, NEM and PAS analyzed the MS data. NEM, RDM, LAP and IAD supervised the project and LAP participated in critical reading of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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