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Research note

# Variation in pharmacologically potent rutinoside-bearing flavonoids amongst twelve *Moringa oleifera* Lam. cultivars

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ABSTRACT

Recent evidence has shown that consumption of plants rich in phenolic compounds has health-promoting benefits. Amongst these plants, include *Moringa oleifera* Lam. (Moringaceae) which has gained significant attention due to its high contingency of secondary metabolites. It has also been shown that the quantity of nutritional and health promoting secondary metabolites in *M. oleifera* is cultivar specific. As such, in the current study, the distribution of three pharmacologically potent flavonoids [quercetin rutinoside (rutin), kaempferol rutinoside and isorhamnetin rutinoside] was investigated using untargeted metabolomic approach with the aid of UPLC-qTOF-MS. The results of this study, suggest only three out of the twelve cultivars investigated were found to contain these flavonoid molecules in significant amounts. The current results, coupled with other techniques therefore, suggest rutinoside-bearing flavonoids could be used to group different cultivars of *M. oleifera* which can definitely help with selection of pharmacologically potent cultivars.



## 1. Introduction

*Moringa oleifera* Lam., belonging to the Moringaceae family is native to India and is commonly cultivated in Asia and in many African countries. In Africa, countries such as Malawi, Ghana and Senegal are said to have the highest cultivation rate of *M. oleifera* trees (Pakade et al., 2013). The trees are cultivated mainly for their leaf material because they exhibit high contents of metabolites such as phenolic acids, especially flavonoids associated with various pharmacological activities (Förster et al., 2015). Besides this, *M. oleifera* has been labelled a functional food as a number of studies to determine the nutritional composition have confirmed the usage of *M. oleifera* as a dietary supplement (Habtemariam and Varghese, 2015) its potential as a heath supplement because of its medicinal properties such as antimicrobial, anti-inflammatory, detoxifying and anti-cancerous properties (Förster et al., 2015).

The presence of rutin in plant cells could be responsible for the nutritional and therapeutic potential of many medicinal and traditional food plants (Habtemariam and Varghese, 2015). Some studies have attributed the pharmacological activities of certain plants to the derivatives of common aglycones such as quercetin, kaempferol and isorhamnetin (Chen et al., 2013). However, very few plants have the ability to store rutin in large amounts. Plants such as *Eucalyptus macrorhyncha* F. Muell. ex. Benth. and *Nicotiana tabucum* L. (tobacco plant) leaf was found to contain less than 1.5% in dry weight of rutin while *M. stenopetala* leaves were found to contain 2.34%. *Moringa oleifera* could be a suitable alternative for large scale production of rutin for medicinal and nutritional purposes (Habtemariam and Varghese, 2015).

It has been shown that *M. oleifera* ecotypes/cultivars differ from each other and show diverse differences in growth performance, leaf-mass production and secondary plant metabolite content (Ndhlala et al., 2014; Förster et al., 2015). Apart from the genetic makeup, cultivation practises were also shown to have the most significant influence on these important factors (Förster et al., 2015). Only recently has the differences in the composition of secondary plant metabolites amongst cultivars been the basis for cultivation of ecotypes, which this present study addresses (Fahey, 2005; Ferreira et al., 2008; Amaglo et al., 2010), Moreover, the presence/absence of some of these molecules can further serve as pharmacological markers which can suffice better understanding of the bioactivity variation of *M. oleifera* cultivars which can further aid in quality control of commercial herbal products thereof. Therefore, in the current study, an investigation into the distribution patterns of rutinoside-bearing flavonoids in twelve cultivars of *M. oleifera* based on UPLC-qTOF-MS results was carried out.

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## 2. Materials and methods

### 2.1. Plant collection

*Moringa oleifera* Lam. cultivars were obtained from different geographical locations around the world [through the World Vegetable Centre (AVRDC)] and grown at the Agricultural Research Council (ARC) (Roodeplaat, South Africa). Cultivars TOT4893, TOT4951, TOT4977, TOT5028, TOT5077, TOT5169, TOT5330 and TOT7266 originated from Thailand; TOT4100 originated from Taiwan; TOT4800 originated from USA; SH originated from Silver Hill, South Africa; CHM originated from Limpopo province, South Africa. Voucher specimens were prepared for each cultivar and were submitted at the University of Johannesburg for authentication.

### 2.2. Metabolite extraction

Briefly, ground leaf powder (2 g) for each cultivar was extracted with 20 mL of 80% aqueous methanol (MeOH) with sonication for 30 min. The mixture was centrifuged for 15 min at 25 °C at a high speed of 5000 ×*g* with a swinging–bucket to remove any excess tissue debris. Thereafter, the liquid supernatant was transferred to a round bottom flask, filtered through a Whatman No. 1 filter paper and reduced to approximately 1 mL with the aid of a Büchi rotary evaporator at 40 °C. The resulted extract was then transferred to a 2 mL Eppendorf tube and further dried to completeness under vacuum at 45 °C. The dried extracts were then stored in a freezer at −20 °C so as to avoid degradation until they were analysed on the UPLC-qTOF-MS.

Metabolite separation and detection was achieved using an Acquity UPLC system hyphenated to SYNAPT G1 HDMS mass spectrometer (Waters, MA, USA), of which, three repeats of the extracts from each cultivar was analysed. The chromatographic separation of the *M. oleifera* extracts was accomplished on a Waters BEH $C_{18}$ reverse phase column (150 mm × 2.1 mm, 1.7 μm) (Waters, MA, USA) and the column temperature controlled at 60 °C. The mobile phase consisted of 0.1% formic acid in deionized water (Eluent A) and 0.1% formic acid in acetonitrile (Eluent B). The gradient was programmed as follows: the initial conditions were 98% eluent A followed by multiple gradients to 5% At 26 min. The conditions were kept constant for 1 min and then changed to the initial conditions. The injected volume of each extract was 1 μL at a constant flow rate of 0.4 mL/min which was maintained for 1 min. The chromatographic method was a total run time of 30 min. Chromatographic separation was monitored using a photodiode-array detector (PDA), which was set to scan 200–500 nm and collecting 20 spectra per second, was coupled in tandem to an electrospray ionization mass spectrometer (ESI–MS) detector. The PDA detector was set to scan.

MS detection parameters were applied with the following parameters: an electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-qTOF-MS) detector was used and Leucine enkephalin (556.227/554.2615 Da) was used as reference calibrant at a constant flow rate of flow rate of 0.1 mL/min to obtain typical mass accuracies of at least 3 ppm. The mass spectrometer was operated in both negative and positive ionization modes with a capillary voltage of 2.5 kV, the sampling cone at 30 V, and the extraction cone at 4 V, multichannel plate detector potential at 1600 V, source temperature at 120 °C, desolvation temperature at 450 °C, cone gas flow at 50 L/h, and desolvation gas flow at 550 L/h. The scan time was 0.2 s and an interscan delay of 0.02 s covering the 100 to 1000 Da mass range. For identification purposes, different fragmentation experiments were achieved by alteration of the trap collision energy levels to mimic $MS^E$ experiments. MassLynx™ software (Waters Corporation, MA, USA) was used to visualise the raw data from data matrix generated from UHPLC-qTOF-MS for accurate statistical modelling.

### 2.3. Statistical analysis and metabolite identification

The UHPLC-ESI-MS data was analysed using MarkerLynx ™ software to obtain peak alignment, peak finding, peak integration and retention time (RT) with the following parameters: Rt range of 1–27 min, mass range of 100–1000 Da, mass tolerance of 0.05 D, Rt window of 0.2 min. This highly dimensional data was exported to a universal excel data matrix was transferred to Microsoft Excel. The peak area under the curve with respect to the masses (*m/z*) of known kaempferol, quercetin and isorhamnetin from the twelve *M. oleifera* cultivars was used to create Box-and-Whiskers plots with the aid of SPSS version 22 software (IBM, United States).

## 3. Results and discussion

### 3.1. HPLC-MS identification of compounds

Through the use of metabolite fingerprinting approach with the aid of UPLC-qTOF-MS and multivariate data models, chromatographic peaks which showed significant differences across the different cultivars were identified (Results not shown). Amongst these potential bio-markers, three were of importance since they exhibited an interesting distribution/trend across different cultivars. Upon further investigation through accurate mass, fragmentation pattern and comparison to authentic standards, these three peaks were identified as kaempferol rutinoside, rutin and isorhamnetin rutinoside (Fig. 1A). Rutin eluted at retention time (RT) 14.07 min with precursor ion $[M\text{-}H]^-$···at *m/z* 609.1441 and produced fragmentation product ions at *m/z* 300.0217 representing quercetin aglycone (Hvattum, 2002; Martucci et al., 2014). Another peak at retention time 16.48 min and precursor ion at *m/z* 593.1526 was identified as kaempferol rutinoside. This molecule produced a product ion at *m/z* 285.0364 representing the kaempferol aglycone (Vagiri et al., 2015). The third peak was identified as isorhamnetin rutinoside eluting at retention time 16.98 min and precursor ion $[M\text{-}H]^-$···at *m/z* 623.1693 (Gutzeit et al., 2007). Similar to the other former two metabolites, the identity of this molecule was further confirmed by the presence of an isorahamnetin aglycone ion at *m/z* 315.0472. Ironically, even though the ESI negative data was sufficient to carry out positive annotation of these metabolites, ESI positive data was found to be more efficient to deduce the sugar sequence and for strengthening of our metabolite identities.

### 3.2. Distribution of the compounds in cultivars using Box-and-Whiskers plots

The distribution of these three metabolites across the studied cultivars was found to vary significantly (Fig. 1B–D). These results suggest that rutinoside bearing flavonoids are unique for certain cultivars and are not produced by other cultivars. The presence of these three molecules was shown to be remarkably higher in cultivars TOT4977, CHM and TOT5330 as opposed to the other cultivars where significant variation occurs. Recently, other pharmacologically relevant metabolites were also found to be ecotype specific in *Citrus grandis* L. Osbeck (Zhang et al., 2014). This could be due to a possible difference in genetic make-up across the different cultivars currently cultivated around the world.

Rutin appeared as the most abundant flavonoid compound in cultivars TOT4977, CHM and TOT5330 with relative peak intensity varying between 100 and 130% (Fig. 1B–D), when compared to the other two rutinoside-bearing flavonoids. Isolation of flavonoids, rutin in particular, has also been shown to present challenging exercise owing to the fact that it is easily transformed and it is also sensitive to certain extraction methods (Dawidowicz et al., 2016). This could be one reason why these metabolites are rarely reported in plants (Khoza et al., 2014; Rodriguez-Perez et al., 2015). Rutin accumulation was, however, reported to be significantly different between matured leaves and other