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Comparative analyses of flavonoid content in *Moringa oleifera* and *Moringa ovalifolia* with the aid of UHPLC-qTOF-MS fingerprinting

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ABSTRACT

Moringa species are multipurpose plants with nutritional, medicinal and industrial benefits. The flavonoids content of *Moringa oleifera* and *Moringa ovalifolia* was studied using an Ultra high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UHPLC-ESI-qTOF-MS). The results revealed that the two species contain at least 17 flavonoids compounds between them. However, *M. oleifera* was found to contain the most flavonoids than *M. ovalifolia* which contained only three of the total flavonoids. Furthermore, all flavonoids in *M. ovalifolia* were shown to be glycosylated with only rutinoside. Based on the current findings, the two species seem to have a different composition of flavonoids, therefore suggesting an underlying variation at the genetic level for flavonoid biosynthesis. The difference in the flavonoids composition of the two species as seen from the results is mainly due to glycosylation capabilities, with *M. oleifera* being more superior in this case. Prior to this study, there has been no comprehensive investigation into the flavonoid content (or any phytochemical studies) of *M. ovalifolia* and its comparison to other *Moringa* species. Furthermore, vicenin-2 a molecule that has recently been linked to various medicinal properties has been identified in *M. oleifera*. Overall, *M. oleifera* (as compared to *M. ovalifolia*) is expected to exhibit wider pharmacological activities owing to its glycosylation complexity.

1. Introduction

The study of the flavonoid composition in natural plants has stimulated considerable interest because of the health benefits (Cook and Samman, 1996). New natural flavonoids have been isolated and characterized with many biological activities (Robak and Gryglewski, 1996). Scientific evidence has confirmed that the regular intake of dietary flavonoids (specifically flavanols, proanthocyanidins and anthocyanins) from natural plants reduces the effects of oxidative damage such as cardiovascular diseases, diabetes and other diseases associated with aging (Nijveldt et al., 2001). Amongst such, *Moringa oleifera*, a plant native to India has been proven to be a multipurpose plant containing medicinal and nutritional benefits (Pakade et al., 2012). Several researchers have also reported treatment of severe medical conditions such as malaria, bronchitis, fever and even symptoms associated with HIV/AIDS (Rodriguez-Perez et al., 2015). Such benefits are correlated to the large contingency of phenolic acids and flavonoid molecules of these plants (Rodriguez-Perez et al., 2015).

Flavonoids are dispersed secondary plant metabolites with various metabolic functions. These compounds differ from one another and exist as either free forms (aglycones), unsaturated or commonly linked to a sugar moiety (glycosides). The sugars can be attached as monosaccharides, disaccharides or oligosaccharides through *C*- or *O*-glycosylation (Ferreyra et al., 2012). Flavonoids such as kaempferol, quercetin and isorhamnetin are the most common and exist in abundance in plant tissue as glycosides (Ono et al., 2010).

The current development in plant metabolomics using mass spectrometry (MS) has made it possible to characterize a number of natural compounds including flavonoids (Geng et al., 2009; Iswaldi et al., 2011; Rodriguez-Perez et al., 2015). This technique has given researchers the ability to compare samples based on their similarities and differences in a semi-automated and untargeted manner (Bedair and Sumner, 2008; Schripsema, 2010; Sumner and Hall, 2013). It also provides an accurate analysis of a wide-range of metabolites of different polarities compared to standard LC methods (Madala et al., 2014). In this current study, ultra high-performance liquid chromatography (UHPLC) coupled with high resolution quadrupole time-of-flight mass spectrometer (q-TOF-MS) was utilized. This technique is advantageous compared to other LC methods because of its high sensitivity and selectivity. To the best of our knowledge, this is the first report on the flavonoid composition of *M. ovalifolia*. Therefore, the objective of this research was to evaluate the differences in flavonoids composition of *M. ovalifolia* and *M. oleifera* using ultra high-performance liquid chromatography

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coupled with high resolution quadrupole time-of-flight mass spectrometer (UPLC-ESI-qTOF-MS).

2. Materials and methods

2.1. Plant collection

The leaves of *M. oleifera* were sampled from the *Moringa* farm in Lebowakgomo, Limpopo, South Africa. Additionally, *M. oleifera* samples were also collected from three different locations in Namibia (Windhoek, Rundu and Katima Mulilo). The *M. ovalifolia* samples were sampled from the following locations in Namibia: Rocky hills located in the *Moringa* Safari Farm in the Otjozondjuba Region (site 1), Sprokieswoud "enchanted forest" located in Etosha National Park (site 2), Dolomite Hills located near Halalo (site 3) and Tsumeb located along B1 road side (site 4). All *M. ovalifolia* leaves were pooled together.

For experimentation, all the leaves were plucked by hand and laid on shelves, 30 cm apart, in a dry cool place with good ventilation and in shade. Prior to extraction, leaves were ground to a fine powder with a pestle and mortar.

2.2. Metabolite extraction

The ground leaf powder (2 g) was extracted with 20 mL of 80% aqueous methanol (MeOH) by sonicating for 30 min using an ultrasonic cleaning bath (SB-120DT, Loyal Key Group, Hong Kong). Following, the samples were centrifuged at 5000 rpm for 10 min at room temperatures (25 °C) to remove the tissue debris from the homogenate. The supernatant liquid was decanted into a round bottom flask and the solvent evaporated to approximately 1.5 mL using a rotary evaporator under reduced pressure at 60 °C. The extract was then transferred to a 2 mL Eppendorf tube and further dried using a speed vacuum concentrator at 45 °C under negative vacuum (Eppendorf, Merck, South Africa). The dried extracts were reconstituted in 1 mL 50% MeOH and finally filtered through 0.22 μm nylon filters. The resulting extracts were then stored in a freezer at −20 °C to avoid degradation until they were analyzed on the UHPLC-qTOF-MS.

2.3. UHPLC conditions

For the evaluation of metabolites in *M. oleifera* and *M. ovalifolia*, UHPLC coupled to a SYNAPT G1 HDMS mass spectrometer was used to generate accurate mass data (Waters, MA, USA). The chromatographic separation of the *Moringa* extracts (1 μL) was accomplished using a 30 min long gradient chromatographic method on a Waters BEH C_{18} reverse phase column (150 mm × 2.1 mm, 1.7 μm). For chromatographic elution, a binary solvent mixture was used consisting of 0.1% formic acid in deionized water (Eluent A) and 0.1% formic acid in acetonitrile (Eluent B). The chromatographic gradient was as follows: the initial conditions were 98% eluent A followed by multiple gradients to 5% A at 26 min. The conditions were kept constant for 1 min and then changed to the initial conditions. The flow rate was set at 0.4 mL/min and maintained for 1 min after which the column was finally re-equilibrated for 3 min prior to the next run. Chromatographic separation was monitored initially using a photodiode-array (PDA) detector coupled in tandem to an electrospray ionization mass spectrometer (ESI-MS) detector. The PDA detector was set to scan 200–500 nm and collecting 20 spectra per second.

2.4. MS-conditions

For MS detection, an electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-qTOF-MS) detector was used. Leucine enkephalin (556.227/554.2615 Da) was used as reference calibrant to obtain typical mass accuracy of at least 5 MDa at a constant flow rate of 0.1 mL/min. The mass spectrometer was operated in negative and positive ionization mode 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 inter-scan delay of 0.02 s covering the mass range of 100 to 1000 Da. The fragmentation patterns of the compounds were obtained in a MS file with three functions. The settings were as follows: Function 1 unfragmented using tune page settings; Function 2 a Trap Collision Energy Ramp (TCER) of 10–30 eV; Function 3 a TCER of 15–50 eV.

3. Results and discussion

Metabolites were extracted from dried leaves and subsequent targeted flavonoid profiling of extracted material was carried out for both species. From the crude UHPLC–MS chromatograms (Fig. 1), a total of 17 chromatographic peaks showing typical flavonoid fragmentation patterns were detected (Table 1). From the structures of the identified flavonoid (Fig. 2), it can be seen that almost all harbor kaempferol, quercetin, apigenin or isorhamnetin aglycone core moieties (Table 1). The results further reveal the presence of a wide spectrum of sugar moieties attached to these flavonoid core structures, showing interesting glycosylation capabilities of the two species.

3.1. Kaempferol-O-glycosides

In *M. oleifera* leaves from South Africa, six kaempferol-*O*-glycoside molecules (**2**, **8**, **9**, **13**, **14**, and **15**) were tentatively identified. In *M. oleifera* leaves from Namibia, three out of the six kaempferol-*O*-glycoside molecules were identified (**2**, **9** and **15**). Molecule **2** showed a precursor ion at *m/z* 651.1537 $[M-H]^-$ was identified as kaempferol acetyl dihexose. The product ions shown in the MS spectrum were indicative of the elimination of hexosyl and acetyl-hexosyl-hexose moieties, respectively (Table 1). Similarly, the same molecule with similar fragmentation patterns was identified in *M. oleifera* sampled from Namibia. To the best of our knowledge, this is the first time kaempferol acetyl dihexose has been detected in *M. oleifera* species, which is noteworthy because of its beneficial effects on human kind. Molecule **9** was identified as kaempferol hexose with a precursor ion at *m/z* 447.0917 $[M-H]^-$ in *M. oleifera* samples from both locations. The product ion shown in the MS spectrum was due to a loss of a hexosyl moiety. The fragmentation pattern from Molecule **9**, shown in Table 1, was also reported from a herb extract of a Cress plant (*Lepidium sativum*) (Justesen, 2000). Molecule **13** was identified as kaempferol hydroxy-methylglutarate-hexose with a precursor ion at *m/z* 591.1358 $[M-H]^-$ The MS spectrum showed product ions indicating the elimination of a hydroxy-methylglutarate and hydroxy-methylglutarate hexosyl moieties, respectively. The fragmentation pattern of Molecule **13**, shown in Table 1, is similar to previously published data on *M. oleifera* by Rodriguez-Perez et al. (2015). Compound **14** was identified as kaempferol acetyl hexose with a precursor ion at *m/z* 489.0955 $[M-H]^-$ based on the precursor and fragmented ions shown in Table 1, indicating a loss of an acetyl moiety and cleavage of a hexosyl moiety. The fragmentation pattern of Molecule **14** was similar to that identified in strawberry fruit (Kajdžanoska et al., 2010). Molecule **15** was tentatively identified, in the *M. oleifera* samples from both locations, as kaempferol malonyl hexose with a precursor ion at *m/z* 533.0917 $[M-H]^-$ due to the decarboxylation and elimination of the malonyl-hexosyl moiety, respectively (Table 1). The fragmentation profile of Molecule **15** was tentatively identified and found to be similar to one found in strawberry fruits (Aaby et al., 2007).

In *M. ovalifolia*, only one kaempferol-*O*-glycoside molecule was observed. Molecule **8** was identified as kaempferol rutinoside with a precursor ion at *m/z* 593.1460 $[M-H]^-$ with a fragmentation ion indicating the elimination of a rutinoside moiety (Table 1). The fragmentation pattern of Molecule **8** was reported by Vagiri et al. (2015) in the