

Tissue-Specific Metabolic Profile Study of *Moringa oleifera* L. Using Nuclear Magnetic Resonance Spectroscopy

Iqbal Mahmud¹, Kamal Chowdhury¹ and Arezue Boroujerdi^{2*}

Department of ¹Biology and ²Chemistry, Claflin University, Orangeburg SC-29115, USA

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Abstract

Moringa oleifera L. an important multipurpose crop, is rich in various phytochemicals: flavonoids, antioxidants, vitamins, minerals and carotenes. The purpose of this study was to profile the groups of metabolites in leaf and stem tissues of *M. oleifera*. Various sugars, amino acids, and organic acid derivatives were found in all of the *M. oleifera* tissues with different profiles and/or peak intensities depending on the tissue. 1D proton nuclear magnetic resonance (NMR) was applied for collecting metabolite spectra. Approximately 30 metabolites with 2 unknown peaks were identified with Chenomx and verified with MMCD databases. Among these metabolites, 22 were identified as common in both leaf and stem tissues. Of the remaining 8 metabolites, 4-aminobutyrate, adenosine, guanosine, tyrosine, and p-cresol were found only in leaf tissues; however, glutamate, glutamine, and tryptophan were found only in stem tissues. Biochemical pathway analysis revealed that 28 identified metabolites were interconnected with 36 different pathways as well as related to different fatty acids and secondary metabolite-synthesis biochemical pathways. It is well known that different tissues of *M. oleifera* have nutritional, medicinal, and therapeutic values; therefore, our main objective is to provide a publicly available *M. oleifera* tissue specific metabolite database.

Introduction

Moringa oleifera L. has an impressive range of medicinal uses with high nutritional value. It has long been valued as both a food and medicinal tree. The therapeutic benefits, for which different tree parts such as root, bark, gum,

*Author for correspondence.

leaf, stem, fruit (pods), flowers, seed and seed oil are used, include analgesic (Sutar et al. 2008), antipyretic (Oliveira et al. 1999), hypocholesterolemic (Mehta et al. 2003), hypoglycemic (Makonnen et al. 1997), anti-inflammatory and hepatoprotective (Kurma and Mishra 1998), anti-hypertensive (Faizi et al. 1995), anti-spasmodic (Caceres et al. 1992), anti-ulcer (Pal et al. 1995), antioxidant (Wangcharoen and Gomolmanee 2011), anticonvulsant (Amrutia et al. 2011), antimicrobial (Caceres et al. 1991), and antitumor (Murakami et al. 1998) activities.

Moringa leaves have been reported to be a rich source of β -carotene, protein, vitamin C, calcium and potassium and act as a good source of natural antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotenoids (Dillard and German 2000, Siddhuraju and Becker 2003). The high concentrations of ascorbic acid, oestrogenic substances and β -sitosterol, iron, calcium, phosphorus, copper, vitamins A, B and C, α -tocopherol, riboflavin, nicotinic acid, folic acid, pyridoxine, β -carotene, protein, and in particular essential amino acids such as methionine, cystine, tryptophan and lysine present in Moringa leaves and pods make it an ideal dietary supplement (Makkar and Becker 1996). The stem bark has been reported to contain two alkaloids, namely, moringine and moringinine (Kerharo 1969). Vanillin, β -sitosterol, β -sitostenone, 4-hydroxymellin and octacosanoic acid have been isolated from the stem of *M. oleifera* (Faizi et al. 1994). *M. oleifera* leaves and stem are the key sources of those secondary metabolites.

Although, Moringa has versatile utility for medicine and nutrition sources, various active ingredients in the extract of leaves and stem are still unknown (Bose 1980). Tissue culture technology has been widely used to isolate cell lines from callus cultures that produce typical levels of secondary metabolites in several plant species (Mansell and McIntosh 1981). Protocols for the production of secondary metabolites from callus cultures of Moringa have not yet been developed.

Analysis of tissue specific metabolic profiles could shed some light on this gap. With the use of NMR research may contribute to bridge the information gap in this area. In principle ^1H NMR provides a reliable profile of each sample, and quantities are reflected in the integrals of the individual signals of the spectrum. This makes ^1H NMR a unique technique, which enables the quantification of compounds in relation to any other compound in the spectrum (Dagnino and Schripsema 2005). Proton (^1H) NMR spectroscopy is particularly a good choice in plant metabolomics studies given the universal occurrence of protons in organic metabolites (Kim et al. 2007, Mahmud et al. 2014a, Mahmud et al. 2014b, Palama et al. 2010). ^{13}C NMR can be used in combination with proton MMR to verify the

triacylglycerols of *M. oleifera* seed oil to identify oleic-vaccenic acid (Vlahov et al. 2002) and the same technique is also used for profiling of glucosinolates and phenolics in vegetative and reproductive tissues of *M. oleifera* L. and *M. stenopetala* L. (Bennett et al. 2003).

The present study deals with the extraction and identification of metabolites, then builds a metabolite list of various plant parts and ultimately to provide a publicly available *M. oleifera* tissue specific metabolite database which can be utilized to interpret the broad range of issues related with plant metabolomics.

Materials and Methods

About 300 mg of fresh leaves and stems of *Moringa oleifera* L. were collected in separate tubes from Claflin University's greenhouse, in June, 2013. For NMR data verification respective tissue samples were collected in six replications. Samples were collected and immediately submerged into liquid nitrogen to quench all metabolic processes and to prevent any stress responses from the cutting process.

Metabolite extraction: Methanol-chloroform-water extractions of leaf and stem tissues were performed as described by Kim et al. (2010). Briefly, 20 mg of dried sample was used for each replicate and the solvent volumes were calculated using the dry mass to achieve a constant ratio of 2 : 2: 1.8 of chloroform : methanol: water. The dried and homogenized sample material was rehydrated with the appropriate ice-cold methanol : water solution. After vortex-mixing, the mixture was transferred into the ice-cold chloroform : water solution in glass tubes. The sample was incubated on ice for 10 min and centrifuged for 10 min at $2000 \times g$ at 4°C in order to form two liquid layers separated by a solid protein layer. The top liquid layer, i.e. the hydrophilic extract, and the bottom liquid layer, i.e. the hydrophobic extract, were separated. The hydrophilic extracts were dried using a Centrivap centrifuge for ~15 hrs and stored at -20°C until further preparation for NMR analysis. The hydrophobic extracts were stored at -80°C for future analysis.

NMR sample preparation: The dried hydrophilic tissue extracts were resuspended in 620 μl of NMR buffer containing 0.1 M sodium phosphate (pH 7.3), 1 mM 3-(Trimethylsilyl)-2,2,3,3- d_4 acid sodium salt (TMSP), 0.1% NaN_3 in 100% D_2O . Following resuspension, 600 μl of each sample was transferred into standard 5 mm NMR tubes (Norell).

NMR spectroscopy: ^1H NOESY (1-D): All NMR spectra were recorded on a Bruker spectrometer operating at 700 MHz. The experiments were conducted at 298 K using 5 mm NMR tubes. Standard ^1H 1-D pre-saturation (zgpr) and first increment of 1-D NOESY (noesygppr1d) experiments were recorded and

processed for all samples. All data were collected using a spectral width of 16 ppm and 64 K points resulting in an acquisition time of 2.9 s; on-resonance pre-saturation was used for solvent suppression during a 2.0 s recycle delay. The zgpr experiment was used to screen samples and check shimming. For all of the samples, the first increment of 1-D NOESY spectra were collected with 120 scans, 4 dummy scans, 50 ms mixing time, and pre-saturation at the residual 20 water frequency. The 90° pulse widths, measured for each sample using the automatic pulse calculation experiment (pulsecal) in TopSpin2.1 (BrukerBioSpin), were between 9.62 and 10.54 μ s for the callus tissue extract.

¹H-¹³C-HSQC (2-D): Two dimensional edited heteronuclear single quantum correlation (HSQC) spectra with adiabatic ¹³C decoupling (hsqcedetgpsisp2.2) were collected for each tissue type to aid in metabolite identification. A relaxation delay equal to 1.5 s was used between acquisitions and a refocusing delay of 3.60 ms was implemented. The 2048 data points with 256 scans per increment were acquired with spectral widths of 11 ppm in F₂ and 180 ppm in F₁ (¹³C). The FIDs were weighted using a shifted sine squared function in both dimensions. Manual two-dimensional phasing was applied.

Identification of metabolites: The spectral peaks from a spectrum were analyzed using the ChenomX NMR software Suite package (ChenomX Inc.). ChenomX allows users to generate a list of tentatively assigned metabolites via targeted analysis, thus providing us with a spectral metabolic profile. Upon assigning metabolites, these compounds were cross-examined in a 2D¹H-¹³C-HSQC experiment for further validation.

Pathway analysis: Metabolic pathway analysis was performed on hydrophilic metabolite extracts results with MetPA (MetaboAnalyst 2.0) which combines pathway enrichment analysis with pathway topology analysis to help identify the most relevant pathways involved in the conditions under study. Lists of identified metabolites were input and the following parameters were selected for analysis: *Arabidopsis thaliana* as the reference metabolome, hypogeometric tests for the over representation analysis and, relative-betweenness centrality in the pathway topology analysis. This served as a measure of the number of shortest paths running through the nodes within a metabolic network. Of the resulting pathways, significance was determined based on an impact factor threshold value greater than zero and a p - value < 0.05.

Results and Discussion

This research presents the use of NMR for the identification of tissue specific metabolic profile followed by the use of this spectral information for interpretation of biological significance using the biochemical pathway analysis.

1D Proton(¹H) NMR spectral comparison: Stacked 1D ¹H-NMR spectra (0.85 - 9.20 ppm) of leaf and stem tissues of *M. oleifera* were analyzed with expansions of the lower intensity aliphatic region from 0.85 - 3.19 ppm (Fig. 1a, b), sugar region 3.34 - 5.40 ppm (Fig. 1b, c), and aromatic region from 5.9 - 9.2 ppm (Fig. 1d). Using chemomx and online based MMCD metabolite database, 30 different metabolites with 2 unknown peaks were identified from 1D ¹H-NMR spectra. Among these, 22 different metabolites including amino acid such as valine, leucine, isoleucine, threonine, alanine, aspartate, and asparagine, sugar compounds such as glucose, fructose, sucrose, and maltose; and organic acids derivatives such as lactate, acetate, malate, succinate, citrate, ethanolamine, choline, formate, fumarate, and trigonelline were identified as common metabolites in both leaf and stem tissues. Qualitatively different metabolites such as 4-aminobutyrate, adenosine, guanosine, tyrosine, and p-cresol were identified only in leaf tissues, and glutamate, glutamine, and tryptophan were identified only in stem tissues of *M. oleifera*.

1D ¹H-NMR-based metabolomics profile analysis was conducted to build the tissue-specific metabolite database of *M. oleifera*. Currently, NMR-based 1D ¹H spectral comparisons are widely used in the plant science and biotechnology field to decipher the clue related with economically and commercially significant crops. 1D ¹H-NMR spectroscopy was used to compare the inner and outer cells of *Catharanthus roseus* calli (Yang et al. 2009), healthy and infected *C. roseus* leaves (Choi et al. 2004), to distinguish the metabolic profile differences between embryogenic and non-embryogenic callus tissues of sugarcane (Mahmud et al. 2014b), to identify the broad range of compounds such as amino acids, carbohydrates, organic acids and phenolic compounds from callus tissue (Palama et al. 2010) and to distinguish between metabolic profiles of powdery mildew resistant and susceptible watermelon (Mahmud et al. 2014a). Information in this section suggests that the results presented here using 1D ¹H-NMR for identifying metabolites from both tissues of *M. oleifera* can be useful for building the NMR-based metabolite database.

Using 1D ¹H-NMR spectroscopy, we identified various essential amino acids, sugar compounds, organic acid compounds, and aromatic compounds (Table 1). The high concentrations of various organic acids such as ascorbic acid, riboflavin, nicotinic acid, and folic acid, protein content in particular essential amino acids such as methionine, cystine, tryptophan and lysine from *Moringa* leaves and pods were identified (Makkar and Becker 1996). Different simple sugars and their derivatives such as rhamnose (Fahey et al. 2001), L-arabinose, L-galactose, L-glucuronic acid, L-rhamnose, L-mannose, and L-xylose (Bhattacharya et al. 1982) were identified from *Moringa oleifera*. Trigonelline one of the important

metabolically active pyridine alkaloids was identified from both leaf and stem tissues of *M. oleifera*. Trigonelline has been isolated from various plant parts and callus cultures of *M. oleifera* L., Moringaceae, and was identified using TLC, GLC, GC-MS, which was compared to that of the standard trigonelline. The trigonelline recovery was found to be maximum in the pods and minimum in the pods and minimum in flowers (Mathur and Kamal 2012).

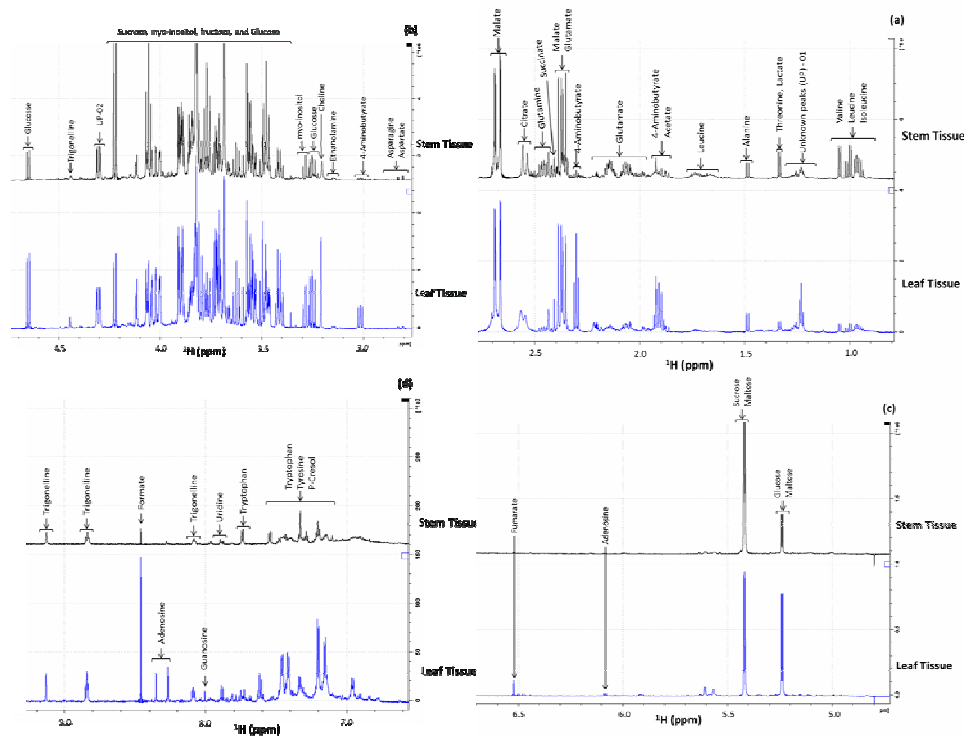


Fig. 1. Stacked 1D ^1H -NMR spectra of both leaf and stem tissues, with enlargements of the aliphatic (0.85 - 3.19 ppm), sugar (3.20 - 5.40 ppm) and aromatic (5.5 - 9.2 ppm) regions. (a) Spectral profile showing aliphatic region, (b) aliphatic and sugar region, (c) sugar region, and (d) aromatic region. UP 01-02 = unknown peak regions.

Metabolic pathway analysis was performed using the 30 different identified metabolites. The pathway analysis suggested that these metabolites have involvement with 36 different metabolic pathways based on p -values < 0.05 and an impact factor threshold greater than zero. Metabolic profile analysis of leaf and stem tissues revealed that biochemical relationships between these two types of tissues regulate an array of metabolites involved in the pathways (Fig. 2).

Based on the biochemical pathway analysis, different aromatic amino acids such as tyrosine, tryptophan, and phenylalanine could be precursors of synthesizing various secondary metabolites as well as could be regulators of

citric acid cycle for producing biological currency. Different organic acid derivatives such as succinate, malate, fumarate, and citrate are involved in the citric acid cycle and can enter into glycolysis or gluconeogenesis to synthesize the

Table 1. The List of identified metabolites and unidentified peaks. Metabolites serial 1 - 17 were identified from the aliphatic region, 18 - 22 were from sugar regions, and 23 - 30 were from aromatic region of 1D ¹H NMR spectra of both leaf and stem tissues. Unknown peaks (UP) - 01 were identified from the aliphatic region and UP - 02 were identified from the sugar region of the same spectral comparisons.

Sl.No.	List of identified metabolites	Leaf tissues	Stem tissues	¹ H chemical shifts (ppm)
1	Valine	√	√	0.98, 1.03
2	Leucine	√	√	0.94, 0.95
3	Isoleucine	√	√	1.00
4	Threonine	√	√	1.32
5	Lactate	√	√	1.32
6	Alanine	√	√	1.47
7	4-Aminobutyrate	√	-	1.89, 2.28, 3.00
8	Acetate	√	√	1.90
9	Glutamate	-	√	2.05
10	Malate	√	√	2.35, 2.66
11	Succinate	√	√	2.40
12	Glutamine	-	√	2.42
13	Citrate	√	√	2.53
14	Aspartate	√	√	2.80
15	Asparagine	√	√	2.85
16	Ethanolamine	√	√	3.13
17	Choline	√	√	3.19
18	Myo-inositol	√	√	3.27, 3.61
19	Glucose	√	√	5.22, 4.63, 3.24
20	Fructose	√	√	4.01, 3.69, 3.58
21	Sucrose	√	√	5.40, 4.20, 3.46
22	Maltose	√	√	5.22, 5.40
23	Adenosine	√	-	6.07, 8.25
24	Trigonelline	√	√	4.43, 8.07, 8.82, 9.12
25	Fumarate	√	√	6.51
26	Guanosine	√	-	7.99
27	Tryptophan	-	√	7.27, 7.31, 7.53
28	Tyrosine	√	-	7.18
29	p-Cresol	√	-	7.14
30	Formate	√	√	8.44
34	Unknown peaks (UP) - 01	√	√	1.16 - 1.27
35	UP - 02	-	√	4.27 - 4.32

different sugar compounds as well as may regulate the various amino acid metabolism/biosynthesis/degradation pathways. Acetyl-CoA, an important metabolite of TCA cycle, and the central compound of metabolism, could be entering into fatty acid biosynthesis/ metabolism process (Fig. 2). The results

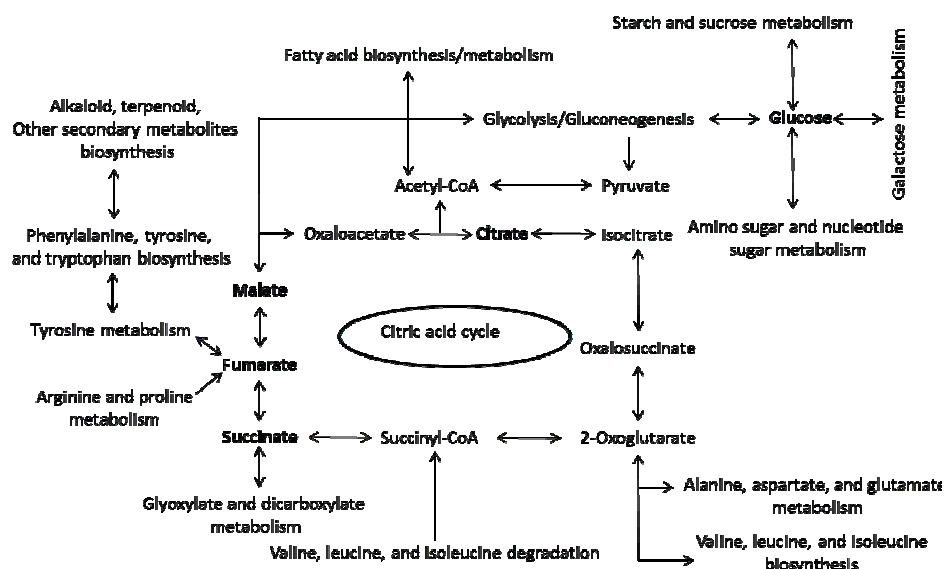


Fig. 2. Metabolic pathways leading to synthesis of metabolites. KEGG database and MetaboAnalyst 2.0 were used to elucidate metabolic networks. The metabolites that accumulated, and identified by NMR, are shown in bold.

explained in this section are in agreement with the relationship among various biochemical pathways in *M. oleifera* leaf and stem tissues that can be applied to identify the bioactive compounds such as various secondary metabolites.

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