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Stable, water extractable isothiocyanates from *Moringa oleifera* leaves attenuate inflammation *in vitro*

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ABSTRACT

Moringa (*Moringa oleifera* Lam.) is an edible plant used as both a food and medicine throughout the tropics. A moringa concentrate (MC), made by extracting fresh leaves with water, utilized naturally occurring myrosinase to convert four moringa glucosinolates into moringa isothiocyanates. Optimum conditions maximizing MC yield, 4-[(α-L-rhamnosyloxy)benzyl]isothiocyanate, and 4-[(4′-O-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate content were established (1:5 fresh leaf weight to water ratio at room temperature). The optimized MC contained 1.66% isothiocyanates and 3.82% total polyphenols. 4-[(4′-O-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate exhibited 80% stability at 37 °C for 30 days. MC, and both of the isothiocyanates described above significantly decreased gene expression and production of inflammatory markers in RAW macrophages. Specifically, both attenuated expression of *iNOS* and *IL-1β* and production of nitric oxide and TNFα at 1 and 5 μM. These results suggest a potential for stable and concentrated moringa isothiocyanates, delivered in MC as a food-grade product, to alleviate low-grade inflammation associated with chronic diseases.

Introduction

Moringa (*Moringa oleifera* Lam.) is a fast growing tropical tree known as the "drumstick" or "horseradish tree." It is one of 13 species in the monogenic family, Moringaceae, within the order Brassicales, to which broccoli and the other cruciferous vegetables belong. Moringa leaves are historically used as nutritious foods and traditional medicine in Asia and Africa. Elevated nutrient content in their leaves can partly be attributed to the relatively low moisture content (*ca.* 76%) of fresh leaves compared with ca. 90% moisture content of most vegetables. Moringa leaves contain approximately 27% protein by dry weight, and all essential amino acids. In addition, they contain high levels of vitamins and beneficial phytoactives (Pandey et al., 2012). The latter include polyphenols and, most interestingly, four unique sugar-modified aromatic glucosinolates (**1**–**4**) (Bennett et al., 2003). In both the Brassicaceae and Moringaceae, isothiocyanates (ITCs) are formed from their glycosylated precursors, glucosinolates (GLSs), via a reaction carried out by myrosinase (thioglucoside glucohydrolase), an enzyme activated during plant tissue wounding or digestion. Myrosinase cleaves the thio-linked glucose in the GLS, leaving the aglycone which rearranges quickly to form the active ITC.

Despite well documented health benefits of ITCs from crucifers, such as sulforaphane (SF) from broccoli and phenethyl isothiocyanate from winter cress on inflammation and cancer, their clinical and dietary use is somewhat restricted because of their inherent chemical instability. For example SF, formed from broccoli glucoraphanin, its GLS precursor, is rapidly converted to several degradation products, mainly dimethyl disulfide and *S*-methyl methylthiosulfinate, making it difficult to formulate and deliver by means other than eating fresh vegetables (Franklin et al., 2013). Consuming ITCs from crucifers in their non-active, but more stable, precursor form as GLSs remains an option. However, GLSs undergo an uncertain and variable degree of enzymatic conversion to ITCs by host gut microbiota (Traka and Mithen, 2009).

Abbreviations: ARE, antioxidant response elements; GLSs, glucosinolates; IL-1β, interleukin-1 beta; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; ITCs, isothiocyanates; Keap1, Kelch-like ECH-associated protein 1; MC, moringa concentrate; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nfr2, nuclear factor (erythroid-derived 2)-like 2; NO, nitric oxide; SF, sulforaphane; TE, trolox equivalents; TNFα, tumor necrosis factor alpha.

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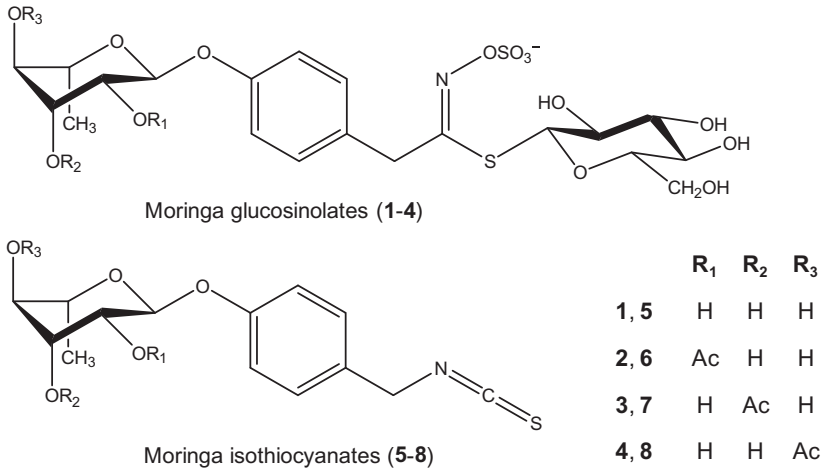

Fig. 1. Moringa glucosinolates (GLSs) **1**–**4** and isothiocyanates (ITCs) **5**–**8**. Molecular masses: **1** = 570, monoacetylated **2**–**4** = 612, **5** = 353, monoacetylated **6**–**8** = 311.

In contrast to crucifers, moringa GLSs (**1–4**) (Fig. 1) contain an additional sugar moiety in the aglycone/ITC portion of the molecule. They can be converted *in situ* to four bioactive and relatively stable moringa ITCs (**5–8**) (Fig. 1). Of these, compound **5** (4-[(α-L-rhamnosyloxy)benzyl]isothiocyanate) and compound **8** (4-[(4′-O-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate) are the most abundant isothiocyanates formed from GLS **1** and **4**, usually making up over 95% of the total ITCs present. In contrast, compound **6** (4-[(2′-O-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate) and **7** (4-[(3′-O-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate) are only formed in small quantities from trace amounts of their respective GLSs precursors, compounds **2** and **3** (Amaglo et al., 2010; Bennett et al., 2003). Our optimized moringa concentrate extract (MC) contained 1.15% of **5**, 0.51% of **8** and approximately 0.06% of **6** and **7** combined. The moringa ITCs are solid and relatively stable compounds at room temperature, in contrast to volatile ITCs from crucifers that are mostly viscous liquids. The retained rhamnose sugar moiety found in moringa ITCs is extremely unique in nature and likely responsible for their high stability and solid appearance (Brunelli et al., 2010). Previous research with moringa extracts has predominantly utilized commercially available dried leaf powder for experimentation. This powder usually contains much lower levels of ITCs (**5–8**) due to the destruction of myrosinase in the drying process. Preparing a MC extract with high ITCs (**5–8**) content takes advantage of endogenous myrosinase in fresh moringa leaves to convert GLSs (**1–4**) to ITCs (**5–8**). There by making MC a useful vehicle for delivering these compounds in the human diet.

Moringa has been used medicinally throughout the centuries to treat a multitude of acute and chronic conditions. *In vitro* and *in vivo* studies with the plant have suggested its effectiveness in treating conditions including inflammation, hyperglycemia, and hyperlipidemia (Bennett et al., 2003; Mbikay, 2012; Fahey, 2005). Moringa's therapeutic effects were linked to the anti-inflammatory, antibacterial and antioxidant properties of its phytochemicals, such as flavonols and phenolic acids (Mbikay, 2012). However, there has been minimal effort focused on the therapeutic activity of GLSs and ITCs present in moringa, even though ITCs from crucifers are some of the most well researched phytoactive therapeutics in human health.

We are particularly interested in the activity of MC and ITCs related to type 2 diabetes mellitus (T2DM) and other chronic conditions associated with metabolic syndrome (MetS). These conditions pose serious and growing health concerns worldwide (Alberti et al., 2009). Effective approaches to combat MetS/T2DM are to maintain a healthy diet and exercise regime. Unfortunately, rates of obesity and T2DM, especially among children, continue to grow. Genetic, social, and economic factors have certainly influenced the epidemic (Wang and Beydoun, 2007). Identifying foods which can help prevent and mitigate manifestations of these conditions is of great interest. Recently, the search for food components that regulate the inflammatory response has attracted particular attention due to evidence linking chronic low-grade inflammation to insulin resistance and obesity. Several key biomarkers of inflammation have been identified as hallmark signs of the pro-inflammatory response found in obesity-induced diabetes. These include cytokines: tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), as well as inducible nitric oxide synthase (iNOS), and nitric oxide (NO), an important cellular signaling molecule in insulin signaling catalyzed by iNOS (Bhargava and Lee, 2012; Ferrante, 2007; Xu et al., 2003). *iNOS* expression and NO overproduction have been implicated in the pathogenesis of disease states, particularly associated with chronic inflammation (Hobbs et al., 1999). TNFα has been shown to directly interfere with insulin signaling (Hotamisligil et al., 1994). Interestingly, deregulated overexpression of TNFα (Mocellin and Nitti, 2008) and other inflammatory regulators (IL-1β, IL-6, C-reactive protein (CRP), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), peroxisome proliferator-activated receptor (PPAR), and cyclooxygenase-2 (COX-2)) have been connected to both metabolic syndrome and cancer development (Bao et al., 2011; Mirza et al., 2012). For example, SF inhibits NF-κB transcription in many cancer models (Srivastava and Singh, 2004; Xu et al., 2005). Activation of NF-κB, an upstream regulator of many inflammatory cytokines, has also been well linked to insulin resistance and diabetes (Mariappan et al., 2010).

Studies of moringa ITCs (**5–8**) show their pharmacological similarity to well-studied crucifer ITCs. For example, compound **5** was a stronger inhibitor of NF-κB expression and myeloma growth in nude mice than SF (Brunelli et al., 2010). Compounds **5–8** also reduced NO formation at low micromolar concentrations in macrophages (Cheenpracha et al., 2010). Isothiocyanate **6** specifically attenuated NO formation more effectively than SF and benzyl isothiocyanate (Park et al., 2011). However, these publications only examined the activity of purified moringa ITCs. This study describes a simple reagent-free method for bioconversion of moringa GLSs (**1–4**) into ITCs (**5–8**) and preparation of a stable isothiocyanate-enriched, food-grade extract from moringa leaves (MC). Additionally evidence of *in vitro* anti-inflammatory activity of MC, **5**, and **8** is presented.