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Characterization of the Glucosinolates and Isothiocyanates in Mustard (*Brassica juncea* L.) Extracts and Determination of Its Myrosinase Activity and Antioxidant Capacity

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Abstract: The research investigated the properties of glucosinolates in mustard (*Brassica juncea* L.) samples including a chemical analysis of the major components in the edible parts, and the activity of their hydrolytic products. HPLC studies showed that raw seeds contain the highest levels of glucosinolates among the samples analyzed. Sinigrin was identified by LC-MS analysis of desulfated sample as the major glucosinolate in the mustard leaves, while sinigrin and gluconapin was found in mustard seeds, confirming previously published work on mustard. Further evidence for the identity of the major compound was seen in the hydrolysis products which were obtained from an optimized procedure. Allyl isothiocyanate was found as the major breakdown product in the reaction catalysed by exogenous myrosinase. Significant amounts of isothiocyanates were detected in seeds and leaves. These may be inherent in the samples or may have been formed during a sample preparation step. The values however markedly increased when samples were made to undergo hydrolysis in the presence of an active hydrolytic enzyme myrosinase, as evidenced by the ability of its extract to catalyze the hydrolysis of sinigrin. The enzyme has a K_M of 0.0436 mM and Vmax of 0.296 g^{-1} min^{-1} for sinigrin. The ability of mustard extracts to scavenge free radicals was measured using the DPPH assay. In this study, the scavenging potential was seen to increase with increased amounts of extract.

Key Words: mustard; glucosinolates; isothiocyanates; myrosinase; DPPH assay

1. INTRODUCTION

Glucosinolate – rich vegetables are fast gaining popularity because several studies have reported their ability to fight certain types of cancers.

A lot of work has been done on glucosinolates in several crucifers including mustard (*Brassica juncea* L) and there is enough evidence to substantiate the health benefits attributed to them.

Mustard is widely cultivated as a vegetable but its seeds are also used in a variety of applications as food condiment and as ingredient in health and medicinal products.

The current investigation aimed to probe the properties of glucosinolates in locally grown mustard samples including a quantitative and qualitative analysis of the major components in the edible parts, their hydrolytic products, the activity of the hydrolytic enzyme, and the antioxidant properties of extracts derived from the plant. The effects of conventional processing – boiling of leaves and roasting of seeds on glucosinolate and isothiocyanate content were also determined.

2. METHODOLOGY

2.1 Chemicals and Reagents

All chemicals used in this study were analytical grade reagents. Sinigrin (allyl glucosinolate) was used as standard glucosinolate, sulfatase (*Helix pomatia*, type H1) (E. C. No. 3.1.6.3), was used for the desulfation of glucosinolates, DEAE Sephadex A25, the ion-exchange resin for desulfation and myrosinase (E.C. No. 3.2.1.147) was used for hydrolysis of glucosinolates and was purchased from Sigma – Aldrich. Other reagents included 1,2 - benzenedithiol, DPPH (2,2-diphenyl-1-picrylhydrazyl) and various solvents *e.g.* methanol, dichloromethane, acetonitrile (HPLC grade).

2.2 Materials

The materials studied consisted of mustard (*Brassica juncea* L.) leaves and seeds obtained from a local supermarket.

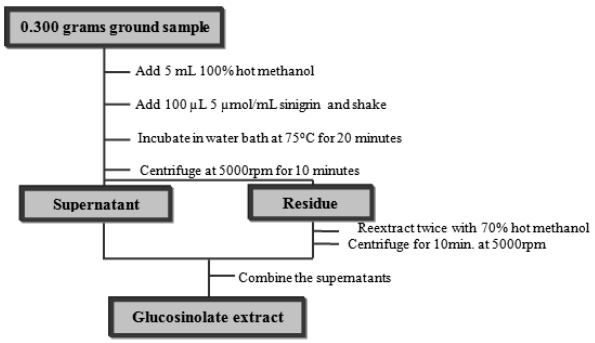
2.3 Sample Preparation

Raw and processed samples were used in the experiments. Leaves were processed by boiling and seeds were subjected to roasting. All the samples were freeze-dried, ground and stored in plastic containers prior to analysis.

2.4 Glucosinolate Analysis

2.4.1 Extraction

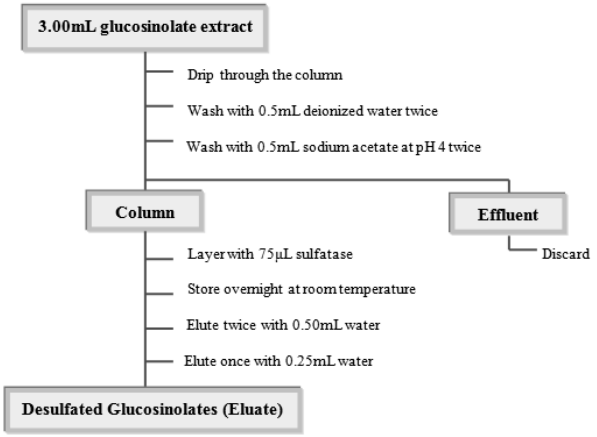
Glucosinolates were extracted from dried, ground mustard samples following a modified method given in Scheme 1 which was patterned from Oerlemans, *et al.*



Scheme 1. Extraction of Glucosinolates

2.4.2 Desulfation

Glucosinolate extracts were desulfated by passing through an ion exchange column (DEAE Sephadex A-25) in the presence of sulfatase (Helix pomatia, type H1) as shown in Scheme 2.



Scheme 2. Desulfation of Glucosinolate Extracts

2.4.3 Analysis by HPLC

Desulfo-glucosinolates were separated on a reverse phase HPLC column (250 x 4.6 mm C18, 5um) with a UV detector set at 230 nm. Elution was carried out using a gradient system consisting of acetonitrile and water at a flow rate of 1.5 mL/min. Glucosinolate concentrations were calculated as sinigrin equivalents.

2.4.4 Characterization by LC-MS

Liquid chromatography – mass spectrometry (LC-MS) was employed in the analysis of glucosinolates. The sample was injected into the LC column (Supelco C18 25 cm x 4.6 mm x 12 µm) and analytes were detected by MS – QTOF. The software allowed the calculation of the molecular masses of specific compounds. The identification of glucosinolates was aided by analysis of mass spectral fragments. Mass comparisons were also done using available literature.

2.5 Assay for Myrosinase Activity

Myrosinase activity was determined in extracts obtained from mustard leaves and seeds using a modified assay procedure (Bellostas et al., 2008; Palmieri et al., 1982). Crude aqueous extracts were added with cold acetone and the residue obtained after centrifugation (7000 rpm, 4°C, 5 min) was dissolved in 33 mM phosphate buffer at pH 7. The assay mixture consisted of measured amounts of enzyme extract, 0.2 mM sinigrin, 0.25 mM ascorbic acid and 50 mM phosphate buffer at pH 6.5. Analysis was done by UV-Vis spectrophotometry. Enzyme activity was expressed as the change in absorbance (ΔAbs_{227}) due to sinigrin per second per gram of sample.

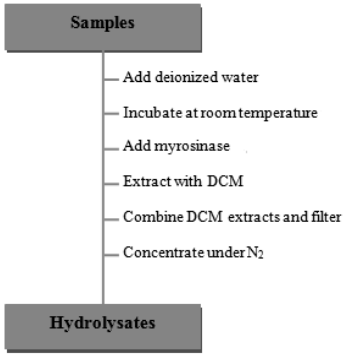
The kinetic properties of malunggay myrosinase were also determined by analyzing assay mixtures containing varying amounts of sinigrin. Samples were incubated for an optimum period prior to spectrophotometric analysis.

2.6 Analysis of Hydrolysis Products

2.6.1 Extraction

Samples were added with measured amounts of deionized water and the mixture was left to hydrolyze for a specific period. Hydrolysis products were then extracted by addition of dichloromethane following the procedure in Scheme 3.

Dichloromethane extracts were also prepared from leaves and seeds without any prior hydrolysis to determine the amounts of isothiocyanates which may be found intact in the sample or formed during the sample preparation steps.



Scheme 3. Extraction of Isothiocyanates

2.6.2 Cyclocondensation and HPLC Analysis

The total isothiocyanate content of the mustard hydrolysate was determined by quantification of the cyclocondensation product between the isothiocyanates and 1,2-benzenedithiol. First, 100µL of allyl isothiocyanate or hydrolysate were made to react with 600µL of 10mM 1,2-benzenedithiol in 500µL of 0.10M potassium phosphate buffer at pH 8.5. The mixture was shaken for 1 minute and was then incubated at 65°C for 2 hours.

The cyclocondensation product(s) formed due to isothiocyanate(s) were determined by HPLC. The chromatographic system consisted of a reverse phase column operated isocratically with 80% methanol in water at a flow rate of 2.0 mL/min for 10 minutes. The eluates were monitored with a UV detector at 365 nm and the area of the 1,3 - benzenedithiole - 2 – thione peak was integrated. A standard curve was generated from measurements using varying amounts of allyl isothiocyanate to estimate the amount of cyclocondensation products (isothiocyanates) in test samples.

2.6.3 Characterization by GC-MS

Dichloromethane extracts of hydrolysates were analyzed by gas chromatography - mass spectrometry (GC-MS) using a Perkin - Elmer gas chromatograph equipped with an Elite 5MS GC column (30 m x 0.25 m x 0.5 µm). A temperature program was determined for the GC analysis. Mass spectra were scanned from 35 to 250 m/z. Hydrolysis products were identified by matching of mass spectra with the GC-MS library and by analysis of mass spectral fragments.

2.7 DPPH Activity

The scavenging activity of extracts of mustard (fresh samples) was determined by monitoring the 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity. Varying amounts of methanolic seed extracts were placed in separate tubes. DPPH were then added to each tube and were placed in the dark for 30 minutes prior to analysis. After the incubation, the samples were subjected to UV-Vis spectrophotometric analysis and the absorbances were read at 517 nm. The analyses were done in triplicate. The scavenging activity of the extract was calculated using the equation:

% DPPH Scavenging = $[(A_{blank} - A_{sample}) / A_{blank}]$

3. RESULTS AND DISCUSSION

3.1 Glucosinolates in Mustard

Total glucosinolates concentrations were found to be higher in seeds (86.18 µmol/g) than in the leaves (54.31 µmol/g). These findings are consistent with published literature citing that glucosinolates concentrations in vegetables are generally higher in seeds compared to the leaves (Shroff et al., 2008; Noel & Malabed, 2012). The glucosinolate concentration and composition varies among plant organs, but all studies report that the seeds contain the highest amount of glucosinolates.

Desulfated glucosinolates from mustard samples were subjected to LC-MS analysis. A predominant peak at 6.2 minutes was observed for mustard leaves (Figure 1). The identity of this peak was characterized using the instrument software which calculates the formula of a compound based on the isotopic patterns. The compound has a m/z of 302 and the formula $[C_{10}H_{17}NO_6S + Na]^+$ is consistent with the theoretical isotopic pattern calculated by the software. The formula corresponds to that of desulfated sinigrin *(1)* which is predominant in mustard according to studies previously done on the plant.

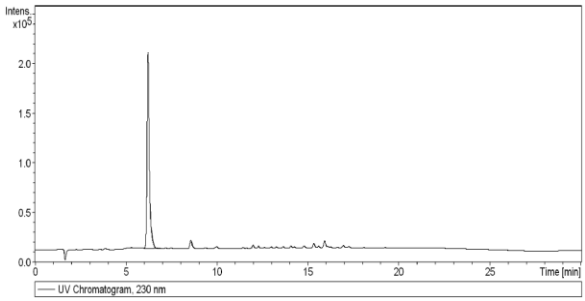
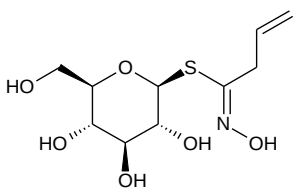


Fig. 1. Chromatogram of mustard leaves



(1) desulfated sinigrin

In mustard seeds, two peaks were observed (Figure 2): the compound with retention time of 6.2 min had mass spectral characteristics corresponding to desulfated sinigrin while the more predominant peak observed at 8.4 min had m/z of 316 and a formula of $[C_{11}H_{19}NO_6S+Na^+]$ based on the instrument's prediction. The formula corresponds to that of desulfated gluconapin *(2)*.

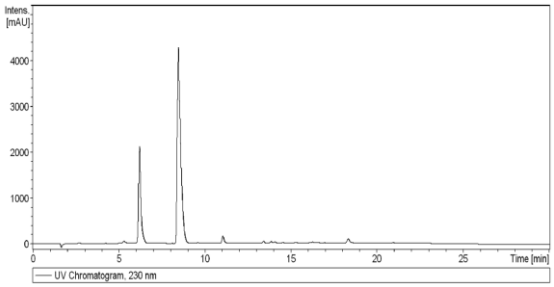
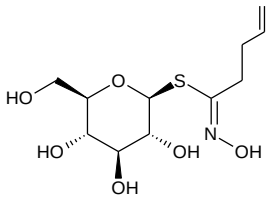


Fig. 2. Chromatogram of mustard seeds



(2) desulfated gluconapin

3.2 Effects of Processing on Mustard

The effect of the length of boiling on glucosinolate content of mustard leaves was investigated. Leaf samples were subjected to boiling for 1, 5, 10, and 15 minutes immediately after samples were obtained. The effect of boiling was studied by comparing the concentration of total glucosinolates with that of the raw samples.

Table 1. Glucosinolate content of mustard leaves

Glucosinolate Content of Mustard Leaves (µmol/g sample)					
	Raw	**1**	**5**	**10**	**15**
Glucosinolate Content	54.31	38.59	37.01	13.63	10.94

The results in Table 1 show that glucosinolate levels decline progressively with boiling. This could be attributed to leaching out of glucosinolates to the boiling medium or to thermodegradative reactions since glucosinolates have been found to be allocated to peripheral tissues in the leaves (Brown et al., 2003).

The effect of roasting on total glucosinolate content of mustard seeds was also investigated. The seed samples were subjected to roasting for 5 minutes. This resulted in a marked reduction in glucosinolate content to 33.70 µmol/g (compared with that of raw seed samples at 86.18 µmol/g). Roasting mustard seeds is a practice in some countries as it could enhance the flavor and increase oxidative stability (Shresthra, 2012). However, there have been no reports citing the effects of roasting on mustard seed glucosinolate. The decrease in the level of glucosinolate in mustard seeds upon roasting could be attributed to thermodegradative reactions when heat is directly applied on the seeds (Brown et al., 2003).

3.3 Isothiocyanates in Mustard

Dichloromethane extracts were obtained directly from leaf and seed samples and from hydrolysates. The isothiocyanate contents were measured from their respective condensation products after the reaction with 1,2-benzenedithiol.

The data given in Table 2 show that in samples analyzed without prior hydrolysis, the isothiocyanate contents of leaves and seeds of mustard do not vary significantly. However, the addition of myrosinase increased isothiocyanate levels reflecting hydrolysis of glucosinolates under the conditions applied. Boiling resulted in decreased isothiocyanate content in the leaves. However, while roasting considerably decreased glucosinolate content in seeds, the changes in isothiocyanate levels were not notable.

Table 2. Isothiocyanate content of mustard

Cyclocondensation Products (µM/g)		
	Unhydrolyzed	**Hydrolyzed**
Raw leaves	0.189	0.198
Boiled leaves	0.174	0.177
Raw seeds	0.182	0.235
Roasted seeds	0.180	0.196

GC-MS analysis of the hydrolysate obtained from mustard samples yielded the chromatogram in Figure 3. The peak which eluted at 8.26 min. had m/z of 99 and gave a strong match with allyl isothiocyanate *(4)*, the hydrolysis product of sinigrin.

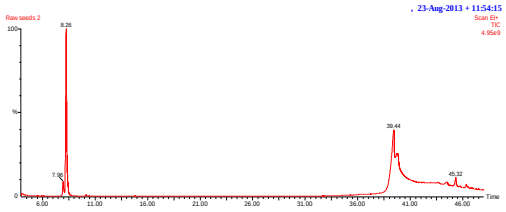
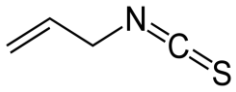


Fig. 3. Gas chromatogram of mustard hydrolysate



(3) allyl isothiocyanate

3.4 Myrosinase Activity of Mustard Seeds

The maximum velocity (V_{max}) and Michaelis' constant (K_M) for sinigrin were found to be 0.296 g^{-1} sample min^{-1} and 0.0436 mM, respectively. These values indicate that the enzyme in *Brassica juncea* L. seeds has high affinity for the substrate and is an effective catalyst towards the conversion of glucosinolates to their corresponding hydrolytic products.

3.5 DPPH Activity of Mustard Leaves

The data given in Table 3 shows a direct linear relationship between the concentration and the scavenging potential of *Brassica juncea* L. extract.

Table 3. Scavenging Activity of Mustard Leaves

%Scavenging Activity	
Amount of Sample (µL)	**Scavenging Activity (%)**
Blank	0
25	35.54 ± 3.977
50	38.74 ± 3.517
75	42.95 ± 2.058
100	44.48 ± 2. 438
125	46.83 ± 0.7629

Studies reporting medicinal properties of mustard leaves claim that individual parts of the plant possess antioxidant activity even if not much scientific evidence is available to support it. Antioxidant activity has been attributed to specific compounds and shown to be directly correlated to phenolic content (Luqman et al., 2012). On the other hand, the antioxidant properties of isothiocyanates are, at best, still debatable. The isothiocyanates may not be directly involved in the scavenging activity of the plant but there are evidences citing that isothiocyanates can indirectly increase the antioxidative capacity of animal cells and their ability to prevent or cope with oxidative stress (Mutanan & Pataji).

4. CONCLUSIONS

This research focused on the characterization of glucosinolates and isothiocyanates derived from *Brassica juncea* L. leaves and seeds. The study revealed that raw mustard seeds showed the highest levels of total glucosinolates among all the samples analyzed by HPLC. The major glucosinolate in leaf extracts was sinigrin (allyl glucosinolate) which was also found in the mustard seeds together with gluconapin (2-butenyl glucosinolate). Significant reductions in glucosinolate and isothiocyanate levels were brought about by boiling of leaves. On the other hand, while roasting resulted in a marked decline in glucosinolate content, no notable changes in isothiocyanate levels were observed.

5. ACKNOWLEDGMENTS

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