Cooking Affects Glucosinolate Concentration in Mustard Leaves and Seeds

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ABSTRACT

Glucosinolates in mustard leaves (*Brassica juncea* L.) and seeds (*Brassica nigra* L.) were investigated via chemical analysis of their major components and the activity of their hydrolysis products. High-performance liquid chromatography (HPLC) revealed that raw seeds have the highest amount of glucosinolates among the samples tested. Boiling of mustard leaves for 15 minutes significantly reduced the total glucosinolate content (10.94 μmol/g) to up to ~80% in comparison with the raw sample (54.31 μmol/g) which is attributed to thermal degradation of glucosinolates or leaching out to the boiling medium. Similarly, roasting of mustard seeds resulted in a marked decline in total glucosinolate content to 33.70 μmol/g compared with that of raw seed samples (86.18 μmol/g) due to thermal degradation. Liquid chromatography-mass spectrometry (LC-MS) revealed that sinigrin (allyl glucosinolate) is the major glucosinolate in mustard leaves, while the seeds contain sinigrin and gluconapin. Allyl isothiocyanate was identified as the major hydrolytic product using gas chromatography-mass spectrometry (GC-MS). The amount of isothiocyanates were quantified via cyclocondensation reaction and HPLC analysis. Boiling of leaves and roasting of seeds have no significant effect on the total isothiocyanate in comparison with the raw samples. However, the amount of isothiocyanates increases upon hydrolysis in the presence of exogenous myrosinase. Lastly, mustard leaf extracts were found to possess antioxidant properties due to its ability to scavenge free radicals as evidenced by the DPPH assay, which could be attributed to bioactive isothiocyanates inherent in the plant samples.

Keywords: mustard; sinigrin, gluconapin; allyl isothiocyanate; scavenging activity

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INTRODUCTION

Glucosinolates are sulfur-rich secondary metabolite exclusively found in cruciferous vegetables. These compounds were first observed when the mustard flavor was investigated (Fahey *et al.*, 2011). Glucosinolates were found to be responsible for the pungent aroma and the sharp and biting taste of cruciferous vegetables (Ortner and Granvogl, 2017). The structure of glucosinolates contains an aglycone group linked to a central carbon atom via a sulfur atom. The carbon is also bound to an oxime group linked by a nitrogen atom. The central carbon atom is also attached to a side chain (R-group) which differs for every kind of glucosinolate (Clarke, 2010). Glucosinolates can be converted to isothiocyanates, thiocyanates or nitriles via the enzyme myrosinase, which is inherent to the plant. Thiocyanates have been found to have goiter-promoting properties in humans. On the other hand, isothiocyanates have exhibited potential anticancer properties via modulation of phase I and phase II enzymes (Das *et al.*, 2000). Among the isothiocyanates isolated in cruciferous vegetables, sulforaphane, a hydrolysis product of glucoraphanin, gained popularity due to its ability to inhibit various kinds of cancer. Each vegetable contains specific or predominant glucosinolate and isothiocyanate. Processing and storage of vegetables can generally affect their glucosinolate and isothiocyanate content (de Vos and Blijleven, 1988). Glucosinolate and the enzyme myrosinase are found in different plant organelles (Andersson *et al.*, 2009). If the plant is cut or wounded during processing, such as chopping or crushing, glucosinolate will come together with myrosinase and will be converted to the hydrolysis product including isothiocyanates (Verkerk *et al.*, 2001). During cooking or heating, myrosinase can be heat-inactivated and the conversion to the hydrolysis product will be affected. Most glucosinolates are heat-stable although some can undergo thermal degradation. Although glucosinolates are higher than the amount of hydrolysis product during heat-inactivation, the gut microflora can also hydrolyze glucosinolates due to the presence of myrosinase-like activity (Conaway *et al.*, 2000). Also, prolonged storage can lead to chemical degradation and conversion to nitrile and epithionitrile.

Several studies have been conducted on glucosinolates in several crucifers including mustard (*Brassica juncea* L) and enough evidence has been published regarding the health benefits attributed to them. Mustard is widely used as a vegetable but the seeds are also used as food condiments and medicine. This study probes the glucosinolates in locally grown mustard samples via qualitative and quantitative analysis of the major glucosinolate component of mustard leaves and seeds and its corresponding hydrolytic products, the activity of myrosinase enzyme and the antioxidant properties of bioactive isothiocyanates. The effect of boiling of leaves and roasting of seeds on glucosinolate and isothiocyanate contents were also determined in comparison with the raw samples. Techniques employed to quantify and characterize glucosinolates and isothiocyanates include high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and ultraviolet-visible (UV-Vis) spectrophotometry.

EXPERIMENTAL

Materials and Chemicals. Mustard leaves (*Brassica juncea* L.) and seeds (*Brassica nigra* L.) were obtained from a local supermarket. All chemicals used in this study were analytical grade reagents. Sinigrin (allyl glucosinolate) was used as standard glucosinolate, allyl isothiocyanate was used as standard isothiocyanate sulfatase (*Helix pomatia*, type H1) (E. C. No. 3.1.6.3), was used for the desulfation of glucosinolates, DEAE Sephadex A25 ion-exchange resin used for desulfation and myrosinase (E.C. No. 3.2.1.147) used for hydrolysis of

glucosinolates and were all purchased from Sigma-Aldrich. Other reagents included were 1,2-benzenedithiol, DPPH (2,2-diphenyl-1-picrylhydrazyl) and various solvents *e.g.* methanol, dichloromethane, acetonitrile (HPLC grade). All buffers used are freshly prepared.

Sample Preparation. Raw and samples subjected to cooking (boiling and roasting) were used in the experiments. Leaves were subjected to boiling in water at medium heat for 1, 5, 10 and 15 minutes, while seeds were subjected to roasting for 5 minutes under direct heat. All the samples were cooled down, freeze-dried, ground and stored in plastic containers at 4 °C before analysis.

Glucosinolate Extraction. Glucosinolates were extracted from dried, ground mustard samples using a modified method patterned from Oerlemans, *et al*. (2006). To a tube containing 0.300-gram powdered sample, 5.00 mL of hot methanol (75 °C) was added and the mixture was shaken. One hundred (100) µL of the internal standard (5.0 µmol/mL sinigrin) was added into the sample and was shaken. Samples were incubated in a water bath at 75 °C for 20 minutes. The solution was mixed several times during this period. The samples were centrifuged at 5000rpm for 10 minutes. The residue was re-extracted twice with 70% hot methanol (3.00 mL and 2.00 mL) and then centrifuged as before. The combined supernatants contained the intact glucosinolate.

Desulfation of Glucosinolates. Glucosinolate extracts were desulfated by passing through an ion-exchange column in the presence of sulfatase (*Helix pomatia,* type H1). The resin was prepared by adding DEAE Sephadex (A-25 anion exchange resin) to an excess of 20 mM sodium acetate (CH_3COONa) at pH 4.0. The mixture was gently stirred and then allowed to settle for 30 minutes. The resin was allowed to expand. The resulting resin was washed with deionized water, followed by resuspension in deionized water and storage

at 4 °C until use. In preparing the column, 5 mL pipette tips were plugged with cotton wool, which was wetted with deionized water. The resin was added (1 mL bed volume) to each column and allowed to settle. This was then followed by washing twice with 0.50 mL deionized water. Three (3.00) mL of glucosinolate extract was dripped through the column, which was washed twice with 0.5 mL deionized water and twice with 0.5 mL 20 mM acetate buffer at pH 4.0. The effluent was discarded. A layer of 75 µL sulfatase (25 mg/mL) was added to the column which was left to stand for 12-16 hours at room temperature. The column was eluted twice with 0.50 mL deionized water and once with 0.25 mL deionized water. The eluate was collected in 1 mL aliquots and was stored at -20 °C.

HPLC Analysis of Desulfated Glucosinolates. Desulfated glucosinolates were separated by Agilent Technologies 1200 Series HPLC using a reverse-phase column (250 mm x 4.6 mm C18, 5µm) 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.

Characterization of Major Glucosinolates by LC-MS Analysis. Liquid chromatography-mass spectrometry (LC-MS) was used to characterize the major desulfated glucosinolates in mustard samples. LC-MS analysis was carried out using Dionex Ultimate 3000 HPLC & Bruker microTOF-Q II MS. 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 identification of glucosinolates was carried out by analysis of mass spectral fragments. Mass comparisons were done using available literature.

Assay for Myrosinase Activity. The activity of the enzyme myrosinase inherent in mustard seeds using a modified assay procedure

(Bellostas et al., 2008; Palmieri et al., 1982). Approximately 5 grams of mustard seed was mixed with 10 mL of cold, deionized water and were ground in a pre-cooled ceramic mortar and pestle placed over an ice bath. The resulting mixture was filtered by vacuum filtration and the filtrate was collected in a volumetric flask. The residue was re-extracted twice with cold, deionized water and then filtered as before. The filtrates were combined and the resulting extracts were diluted to 50 mL with deionized water. This made up the crude aqueous extract. A 25 mL aliquot portion of the combined filtrates was centrifuged at 7000 rpm at 4 °C for 5 minutes. Ten (10) milliliters of the supernatant were kept and were used as a crude extract for the optimization of the assay method. The remaining supernatant was measured and subsequently mixed with 40 mL of cold acetone. This made up the acetone extracts. The mixture was incubated at 12 °C for 90 minutes and then centrifuged as before. The precipitate was collected and then dissolved in 1.5 mL of 33 mM potassium phosphate buffer at pH 7.0 before analysis. The prepared enzyme solution was used in the determination of myrosinase activity. The analysis was done by UV-Vis spectrophotometry (Hitachi U-2000). In a quartz cuvette, 40 µL of 18.75 mM ascorbic acid, 60 µL of 5 mM sinigrin and 40 µL of crude extract were mixed and then diluted with phosphate buffer at pH 6.5 to bring the mixture to a total volume of 3 mL. The cuvettes were then inserted into the instrument and the absorbance was read at 227 nm after the addition of crude extract to determine the optimum incubation conditions. Another assay mixture was prepared but this time, 40 µL of acetone extract was added instead of the crude extract to make a 3 mL solution. The absorbance was then read and recorded after a specified incubation period. The activity of the enzyme was calculated based on the decrease in the absorbance of the mixture which was due to a decrease in the amount of sinigrin. Enzyme activity was expressed as the change in absorbance (ΔAbs_{227}) due to sinigrin per second per gram of sample. The kinetic properties of mustard myrosinase were also determined by analysis of the assay mixtures containing varying amounts of sinigrin. The samples were incubated for an optimum period before analysis.

Analysis of Hydrolysis Products. The freeze-dried mustard sample was either autolyzed (reaction without added myrosinase) or hydrolyzed in the presence of exogenous myrosinase added at various concentrations. Approximately 1-gram of mustard leaves and seed samples were obtained and was mixed with 5-mL deionized water and was allowed to undergo autolysis or hydrolysis at 1 hour incubation period. The reaction was stopped by the addition of 5-mL of dichloromethane (DCM) and the mixture was centrifuged for 5 minutes at 5000 rpm. The DCM extracts were transferred to another container and the aqueous extract was re-extracted with 5-mL DCM and centrifuged as before. All the DCM extracts were combined and dried over sodium sulfate. The extracts were then concentrated under N_2 and were stored at room temperature in a container with desiccant to prevent contamination with moisture. DCM 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. 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 used 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.

HPLC Analysis of Cyclocondensation Products. The isothiocyanate content of the unhydrolyzed, hydrolyzed, and autolyzed samples were determined by subjecting the extracts to cyclocondensation reaction with
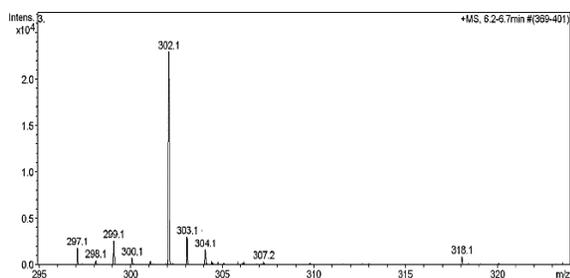
1,2-benzenedithiol (Scheme 1). A 100-μL of allyl isothiocyanate or dichloromethane extracts were made to react with 600 μL of 10 mM 1,2-benzenedithiol in 500 μL of 0.10 M potassium phosphate buffer at pH 8.5. The mixture was shaken for 1 minute and was incubated at 65 °C for 2 hours. The cyclocondensation product(s) formed due to isothiocyanate(s) were determined by HPLC-UV analysis. HPLC analysis was carried out using Agilent Technologies 1200 Series HPLC using a reverse-phase column (250 mm x 4.6 mm C18, 5μm) with a UV detector set at 365 nm. The chromatographic system was operated isocratically with 80% methanol in water at a flow rate of 2.0 mL/min for 10 minutes. The area of the 1,3-benzenedithiole-2-thione peak was integrated and was quantified using a standard curve generated from measurements using varying amounts of allyl isothiocyanate.

1,2-benzenedithiol   isothiocyanate   1,3-benzodithiole-2-thione

Scheme 1. Cyclocondensation of isothiocyanates by 1,2-benzenedithiol (Zhang *et al.*, 1992)

DPPH Activity. The ability of mustard extracts to scavenge free radicals was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity. A 250-mL solution of 60 μM DPPH was prepared and was kept in the dark before the assay. About 1 gram of freeze-dried leaves was extracted twice with 100% hot methanol and the mixture was centrifuged at 5000 rpm. The supernatants were collected and combined. Various amounts of leaf extracts were placed into different tubes. Ten (10) mL of DPPH were subsequently added to each tube. The tubes were shaken in a vortex mixer and placed in the dark for 30 minutes before analysis. After the incubation period, the samples were subjected to UV-Vis analysis and the absorbance of the different samples was read at 517 nm. The analyses were done in

triplicate. The scavenging activity (%SA) of the extract was calculated using the equation:

$$\%SA = \left[\frac{A_{blank} - A_{sample}}{A_{blank}}\right] * 100$$

A_{blank} = Absorbance of blank at 517 nm

A_{sample} = Absorbance of sample at 517 nm

RESULTS AND DISCUSSION

Glucosinolates in Mustard. Desulfated glucosinolates in mustard leaves and seeds were quantified by HPLC-UV analysis. Raw mustard seeds contained higher levels of total glucosinolates (86.18 μmol/g) than the raw leaves (54.31 μmol/g). Published data suggest that glucosinolates are distributed in different plant organs but are mostly concentrated in the seeds (Shroff et al., 2008). In a separate study conducted by our group (Noel & Malabed, 2012) on malunggay *(Moringa oleifera)* samples, significant amounts were found in the seeds or pods of malunggay (722.12 μmol/g) as compared to the leaves (375.79 μmol/g). The identities of major or predominant glucosinolates in mustard leaves and seeds were characterized by LC-MS analysis. A predominant peak at 6.2 minutes was observed in mustard leaves (Fig. 1). Mass spectral analysis of the peak (Fig. 2) revealed a compound with m/z of 302.1 with a formula of $[C_{10}H_{17}NO_6S+Na]^+$ corresponding to a sodium adduct of desulfated sinigrin (Fig. 3). This result is consistent with published literature citing the presence of sinigrin in mustard (Mazumder *et al.*, 2016).

Figure 1. HPLC-UV chromatogram of mustard leaves monitored at 230 nm.

Figure 2. Mass spectrum of predominant desulfated glucosinolate in mustard leaves eluted at 6.2 minutes.

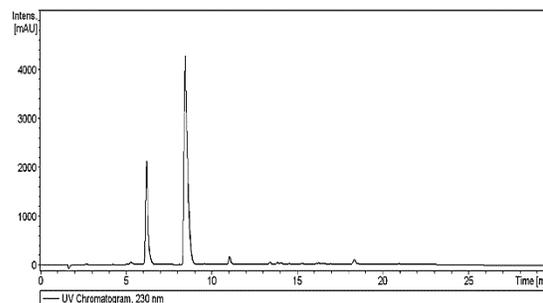**Figure 4.** HPLC-UV chromatogram of mustard seeds monitored at 230 nm

Figure 3. Chemical structure of desulfated sinigrin.

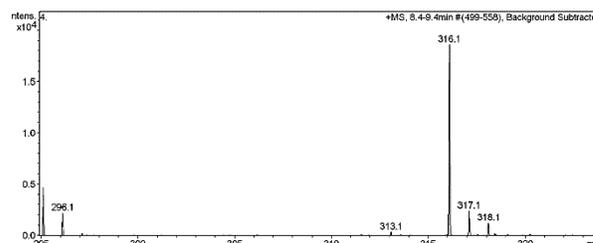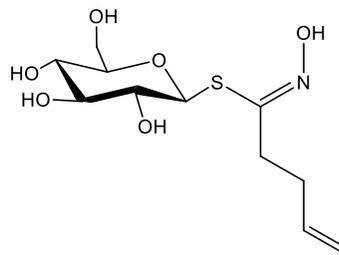**Figure 5.** Mass spectrum of predominant desulfated glucosinolate in mustard seeds eluted at 8.4 minutes.

The desulfated sinigrin peak was likewise detected in mustard seeds (Fig. 4; eluted at 6.2 minutes) together with a more predominant desulfated glucosinolate peak eluted at 8.4 minutes. Mass spectral analysis of the latter peak (Fig. 5) showed a fragment having m/z 316.1 with a formula of $[C_{11}H_{19}NO_6S+Na^+]$ corresponding to a sodium adduct of gluconapin (Fig. 6). According to literature, gluconapin is a major glucosinolate found in mustard seed and oil (Wang *et al*., 2014).

Figure 6. Chemical structure of desulfated gluconapin

Table 1. Effect of boiling on glucosinolate content of mustard leaves.

Time	Total Glucosinates (μmol/g)
Raw	54.31
1 minute	38.59
5 minutes	37.01
10 minutes	13.63
15 minutes	10.94

Effects of Processing on Mustard. The boiling of mustard leaves revealed a reduction of total glucosinolate (sinigrin) content as the boiling time increases (Table 1). Boiling for 15 minutes loses 80% of the glucosinolates in the leaf sample.

The decrease in glucosinolates upon boiling is attributed to the thermal degradation of glucosinolates and/or leaching out into the boiling medium. The exposure of glucosinolates in the peripheral tissues in the leaves made it more susceptible to degradation (Brown et al., 2003). Similarly, applying heat on mustard seeds via the roasting process significantly decreased the level of glucosinolates (33.70 μmol/g) compared to the raw seed samples (86.18 μmol/g). Roasting of mustard seeds is common in some countries to enhance its flavor and increase oxidative stability (Shresthra, 2012). A cursory survey of the literature showed that this is the first report on the effect of roasting on mustard seed glucosinolates. The reduction in the level of glucosinolate in mustard seeds upon roasting is attributed to the thermal or chemical degradation when heat is directly applied to the seeds (Brown et al., 2003).

Isothiocyanates in Mustard*.* The hydrolysis products, including isothiocyanates derived from a precursor glucosinolate inherent in the plant sample, were analyzed using dichloromethane (DCM) extracts of mustard leaves and seed. Quantitative determination of isothiocyanate concentration from unhydrolyzed and hydrolyzed samples was carried out by analysis of cyclocondensation products after the reaction of DCM extracts with 1,2-benzenedithiol. Results show (Table 2) that boiling of leaves and roasting of seeds do not significantly alter the isothiocyanate content in the unhydrolyzed sample although a minimal decrease in the isothiocyanate amount was observed. The results observed are in stark contrast with the effect of heat on glucosinolates. This suggests that the properties of isothiocyanate are considerably different from glucosinolates and that the isothiocyanate present in mustard might be heat stable and may not be affected by either boiling or roasting process. On the other hand, hydrolysis in the addition of exogenous myrosinase showed a minimal increase in the isothiocyanate content in raw and boiled leaves, as well as in the roasted seeds. A more significant increase in isothiocyanate content is observed in raw seeds from 0.182 μM/g for an unhydrolyzed samples to 0.235 μM/g after hydrolysis.

Furthermore, GC-MS analysis of hydrolysates showed the presence of a peak eluted at 8.26 minutes (Fig. 7). Mass spectral analysis showed the presence of a compound with m/z of 99 (Fig. 8), which gave a strong match with allyl isothiocyanate (Fig. 9), the hydrolysis product of sinigrin.

Myrosinase Activity of Mustard Seeds. Assay for myrosinase enzyme isolated from mustard seeds was done to assess the activity of the enzyme in hydrolyzing glucosinolates and converting to isothiocyanates and other hydrolysis products. Sinigrin was used as representative glucosinolate. Measurements were carried out by monitoring the UV

Table 2. Isothiocyanate content of mustard.

Sample	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

absorbance, which is usually in a decreasing manner due to the disappearance of sinigrin and formation of hydrolysis products.
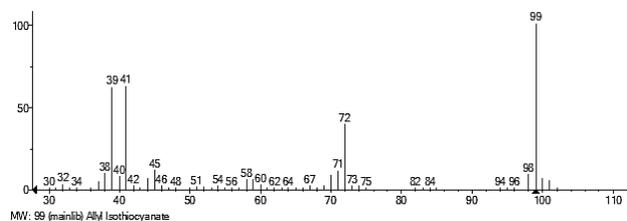

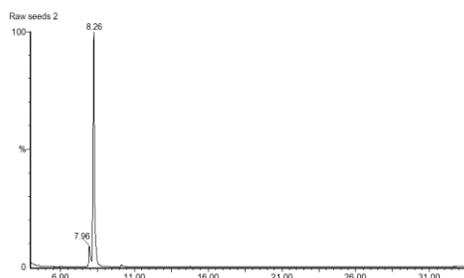**Figure 7**. Gas chromatogram of seed hydrolysate

Figure 8. Mass spectrum of hydrolysis product eluted at 8.3 minutes
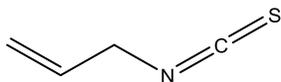

Figure 9. Chemical structure of allyl isothiocyanate

The maximum velocity (V_{max}) and Michaelis' constant (K_m) for sinigrin were reported at 0.296 g^{-1} sample min^{-1} and 0.0436 mM, respectively. These values imply that the enzyme inherent in mustard seeds shows a high affinity for the sinigrin substrate and is an effective catalyst towards its conversion to its corresponding hydrolysis product/s.

DPPH Activity of Mustard Leaves. Investigation on the antioxidant activity of Mustard leaf samples by DPPH assay showed a direct linear relationship between the concentration of bioactive compounds present in the leaf extracts and % scavenging activity. Although the highest concentration of extracts

used did not exceed 50% scavenging activity (Fig. 10), a higher concentration of leaf extracts contributes to an increase in the
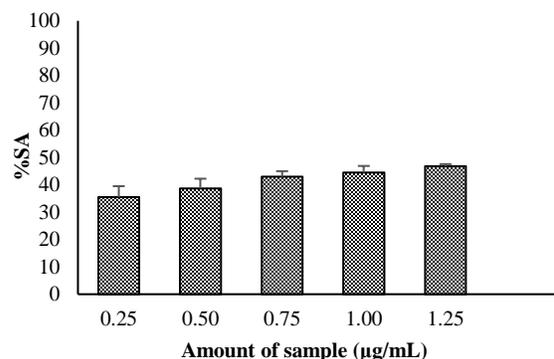
activity.

Figure 10. Scavenging activity of mustard leaves

The antioxidant activity could be attributed to specific compounds and is directly correlated to phenolic content (Luqman et al., 2012). However, isothiocyanates in mustard may not be directly involved in the scavenging activity of the plant but evidence in the literature pointed out that isothiocyanates could indirectly increase the antioxidant capacity of animal cells and their ability to prevent or cope with oxidative stress (Mutanen and Pajari, 2011).

CONCLUSIONS

This study was able to quantify and characterize the major glucosinolates and isothiocyanates present in mustard leaves (*Brassica juncea* L.) and seeds (*Brassica nigra* L.) The study revealed that higher glucosinolate content is found in raw seeds and that the application of heat by boiling of leaves and roasting of seeds considerably reduced the amount of glucosinolates present in plants. Desulfated sinigrin was found to be the major glucosinolate in mustard leaves, while the seeds contained both desulfated derivatives of sinigrin and gluconapin. Although we were able to achieve a substantial amount of results, further testing on the biological activity of biologically active

compounds including glucosinolates and isothiocyanates should be made. Antioxidant assay on mustard seed should also be carried out. In the future of this research, we are planning to isolate the major glucosinolate and isothiocyanate in mustard and assess its direct antioxidant activity and anticancer properties.

ACKNOWLEDGEMENT

Studies conducted on mustard suggests that individual part of the plant may possess different antioxidant activities. In this study, the antioxidant properties were only tested on leaf extracts, the seed might have been a good source of higher levels of antioxidants although we do not have evidence to support our claim.

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