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IDENTIFICATION OF MAJOR GLUCOSINOLATES IN BROCCOLI (*Brassica oleracea* var. *italica*) BY LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY (LC-MS) AND DETERMINATION OF ANTICANCER PROPERTIES OF BROCCOLI EXTRACTS

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Abstract: The health benefits attributed to broccoli *(Brassica oleracea* var *italica)* and vegetables belonging to the family *Brassicaceae* have largely been attributed to the presence of glucosinolates, some of which are precursors of compounds proven to have anticancer properties. This study done on broccoli florets identified specific glucosinolates and isothiocyanates by liquid chromatography – mass spectrometry (LC-MS) and investigated potential anticancer properties of extracts. Better signal intensities, mass spectral data and mass and isotopic matches were obtained when desulfoglucosinolates were analyzed by ESI LC–MS in the negative mode. The major glucosinolates, identified as their desulfated derivatives were glucoraphanin, glucobrassicin and neoglucobrassicin. These findings were consistent with those obtained from the LC-MS analysis in the positive ion mode. For the identification of isothiocyanates, positive ion ESI enabled molecular weight determination of hydrolysis products expected from the glucosinolates identified. The hydrolysates contained detectable levels of sulforaphane and indole -3 - carbinol. The total isothiocyanate content of DCM extracts of the hydrolysates were quantified by HPLC anaysis of the cyclocondensation product and results showed higher isothiocyanate concentrations in boiled broccoli compared to the raw samples. Potential anticancer properties of aqueous extracts and the broccoli hydrolysate were assessed using *in vitro* tests for cytotoxicity against normal (HDFn), colorectal (HT-29) and breast (MCF-7) cell lines as well as for cytoprotective and anti - Fenton properties. The extracts were found to be non-cytotoxic to normal cells as well as to the cancer cell lines at the concentrations tested. The dose - response curves for cancer cell lines however showed increasing cell death with increasing concentrations and indicate that IC_{50} could have been obtained at higher doses. Evidence is given to suggest that broccoli extracts, more specifically the hydrolysate, exhibit cytoprotective properties to normal cells subjected to oxidative stress. Reaction of extracts with Fe^{3+} resulted in the formation of complexes illustrating their ability to act as indirect antioxidants. LC-MS analysis of the products revealed the formation of iron complexes with glucoraphanin and glucobrassicin.

Key Words: Brassica oleraceae**,** glucosinolate, liquid chromatography – mass spectrometry,

1. INTRODUCTION

Studies have shown that inclusion of broccoli (*Brassica oleraceae* var *italica*) in a regular diet the potential of impeding the growth of cancerous neoplasm. The chemical constituents of this vegetable together with the vegetables in the family Brassicaceae, include significant amounts of glucosinolates, which when hydrolyzed yield a variety of compounds such as isothiocyanates, some of which have been shown to be responsible for the inhibition of tumors.

The investigations carried out in our laboratory on broccoli glucosinolates confirmed many literature reports on the presence of high concentrations of these compounds in various parts of the plant and the effects of processing and storage on specific glucosinolates, their hydrolysis products and the activity of the enzyme myrosinase (Malabed and Sandoval, 2008; Salmasan, 2010).

The present study explored the use of liquid chromatography – mass spectrometry in the identification of specific glucosinolates and their hydroysis products in broccoli florets. In addition, extracts of the plant were used in *in vitro* assays for the determination of cytotoxic, chemopreventive and anti – Fenton properties.

2. METHODOLOGY

Preparation of Desulfated Glucosinolate Extracts from Broccoli Florets

Broccoli heads used in the analyses were purchased from a local supermarket. The florets were carefully separated from the stalks, frozen and then lyophilized. The samples were extracted with methanol and desulfated by passing through an anion exchanger in the presence of the desulfating enzyme sulfatase (Helix pomatia type H1) (Oerlemans et al., 2006).

LC-MS Analysis of Desulfated Glucosinolates

A gradient program involving water and acetonitrile was used as the mobile phase. For analysis in the positive ion mode, the solvent was acidified with 0.1% formic acid. The UV detector was set at 230 nm, temperature at 25^{O}C, flow rate at 1 mL/min, and injection volume was 20 µL. Spectra were recorded between 0 to 30 min, in the mass range of m/z 50 - 3000. Elution was done on a 150 mm x 2.1 mm C18, 3 µm, 120 Å reverse phase HPLC column. The effluent from the liquid chromatograph was directly introduced into a mass spectrometer equipped with APCI and ESI probes operated in positive or negative ion mode.

Hydrolysis of Broccoli Glucosinolates and Analysis of Hydrolysis Products

Freeze-dried samples were added with measured amounts of deionized water, ascorbic acid and myrosinase and the mixture was left to hydrolyze for one hour. Hydrolysis products were extracted with dichloromethane.

Total isothiocyanate content of the hydrolysate was determined by HPLC analysis of the cyclocondensation product formed following the reaction with 1,2 – benzenedithiol (Shapiro et al., 2001). Hydrolysis products were identified by LCMS.

Determination of Anticancer Properties

The effect of broccoli extracts on cell viability was evaluated using the PrestoBlue™ assay. Cells used in the determination of cell viability were HDFn (human dermal fibroblasts), HT-29 (human colon adenocarcinoma grade II) and MCF-7 (human breast adenocarcinoma). The cytotoxic and cytoprotective properties of the extracts were compared against a positive control, colchicine.

Anti-Fenton Test

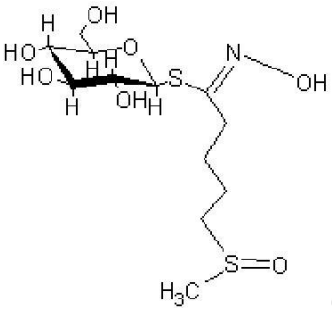
Solutions with varying concentration of Fe^{3+} solution in 0.1 M HCl and 20 mM of phosphate buffer at pH 7.2 were prepared and added to a fixed volume of the broccoli extract in a quartz cuvette for analysis by UV/Vis spectrophotometry at 440 nm.

3. RESULTS AND DISCUSSION

LC-MS Analysis of Desulfated Glucosinolates

Using default settings for both APCI and ESI probes, better signal intensities and peak separation were obtained with electrospray ionization (ESI). Analysis in both the negative and positive ion mode generated spectra which offered structural information for identifying the compounds.

The major glucosinolates in broccoli extracts were identified from the negative ion ESI mass spectra. The formulas derived were consistent with the isotopic patterns and best mass fit generated by the software and corresponded to the deprotonated molecular $[M - H]^-$ ions of the specific glucosinolates. The tests confirmed the presence of glucoraphanin *(1)*,m/z 356; glucobrassicin *(2)* m/z 367; and neoglucobrassicin *(3)* m/z 397 in broccoli florets.



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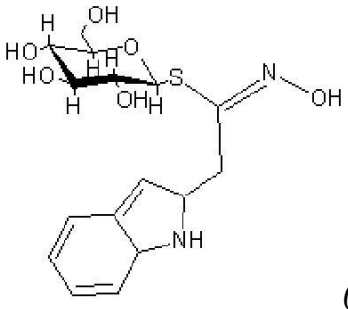

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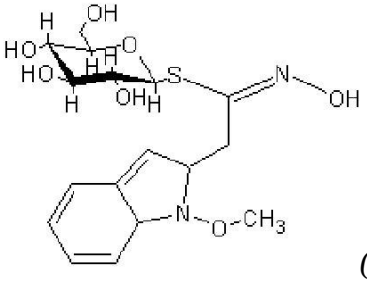

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Analysis using the positive ion mode of compounds which eluted at the same retention times as the three major glucosinolates yielded mass spectra showing ions which could support the results obtained from negative ion ESI mass spectroscopy. All compounds yielded ions which corresponded to the protonated molecular ions of the respective glucosinolates $[M+H]^+$ as well as fragments which may have resulted from the loss of the glucose moiety.

Analysis of Hydrolysis Products

Enzymatic hydrolysis of broccoli extracts yielded isothiocyanates which were quantified by measurement of the cyclocondensation product formed with 1.2- benzenedithiol. The boiled samples had higher isothiocyanate content than the raw samples. LC-MS analysis revealed that the hydrolysates contained detectable levels of sulforaphane and indole -3 – carbinol. These compounds are hydrolysis products expected from the glucosinolates identified in the broccoli samples.

Anti-cancer Properties of Broccoli Extracts

The hydrolysate and aqueous extracts of broccoli were assessed for their cytotoxic and cytoprotective properties. The extracts were not proven to be cytotoxic when tested against normal (HDFn), colorectal cancer (HT-29) and breast cancer (MCF-7) cell lines. The dose - response curves for cancer cell lines however showed increasing cell death with increasing concentrations and indicate that IC_{50} could have been obtained at higher doses. The results of this study also reveal that broccoli extracts, more specifically the hydrolysate, exhibit cytoprotective properties to normal cells subjected to oxidative stress.

Anti-Fenton Test

Anti-Fenton reactions are the result of metal chelation. Chelation involves the use of a chemical substance to bind molecules and occurs when two or more separate coordinate bonds form between a multi-bonded ligand and a single central atom. Through this process, a certain compound is able to grasp tightly onto a molecule or a toxic mineral or metal. Once this happens, the metal can be excreted or removed from the body as a metal complex. This experiment determined whether methanol extracts of broccoli would be able to form a complex with $Fe3^{+}$ since iron overload could lead to the formation of free radicals.

The anti – Fenton test was done on broccoli methanol extracts and results indicated the possible formation of a complex. LC-MS analysis verified the presence of reaction products between $Fe3^{+}$ and the glucosinolates glucoraphanin and glucobrassicin. Confirmation was done by comparison of masses and HRMS isotopic patterns. The results are summarized in Table 1.

Table 1. LC-MS Analysis of Fe^{3+} - broccoli extract reaction products

Observed molar mass	Molecular formula	Glucosinolate in Product
491.29	$C_{12}H_{21}N_1O_{10}S_3Fe$	glucoraphanin
501.99	$C_{16}H_{18}N_2O_9S_2Fe$	glucobrassicin

4. ACKNOWLEDGEMENT

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