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Consumption of Glucosinolate-Rich *Nasturtium officinale* and *Brassica oleracea* L. var. sabellica May Reduce the Risk of Cancer Occurrence in Humans

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ABSTRACT

Vegetables containing glucosinolates, commonly referred to as crucifers, are gaining popularity due to their anticancer benefits in human. Glucosinolates are secondary metabolites that are usually found in cruciferous vegetables belonging to the family Brassicaceae. Several studies pointed out that glucosinolates could undergo hydrolysis and produce bioactive isothiocyanates, via the action of enzyme myrosinase. In this study, the total glucosinolates in watercress (*Nasturtium officinale*) and kale (*Brassica oleracea* L. var. *sabellica*) were quantified using high-performance liquid chromatography (HPLC). HPLC analysis revealed that watercress contains 228.93 ± 26.39 μmol/g, while kale contains 87.60 ± 80.12 μmol/g of total glucosinolates. Analysis of cyclocondensation products via HPLC directly correlates the amount of isothiocyanates present in the extracts, which were found to be 0.398 ± 0.018 and 0.378 ± 0.014 for unhydrolyzed watercress and kale, respectively. An increase in isothiocyanate content was observed for both watercress and kale after subjecting them to hydrolysis in the presence of myrosinase enzyme. Moreover, the biological activity of the extracts derived from the plants was determined using standard assay methods. Free radical DPPH assay showed that methanolic extracts of both watercress and kale exhibit antioxidant properties with the IC_{50} values of 13.04 ± 1.11 μg/mL and 33.14 ± 1.44 μg/mL, respectively. On the other hand, the hydrolysates showed higher scavenging activity values reported at the IC_{50} values of 7.69 ± 0.93 μg/mL and 20.40 ± 1.26 μg/mL, for watercress and kale, respectively. PrestoBlue™ assay also revealed the ability of the aqueous extracts to selectively target cancer cells. Watercress showed anticancer potential towards breast adenocarcinoma (MCF-7, IC_{50} = 17.67 ± 1.64 μg/mL). On the other hand, kale showed anticancer potential towards small lung cancer cell (H69PR, with IC_{50} = 19.35 ± 1.54 μg/mL) and colon cancer cell (HT-29, IC_{50} = 18.32 ± 2.41 μg/mL). Moreover, the extracts were found to be non-cytotoxic to

normal cells (HDFn, human dermal fibroblast neonatal, IC_{50} >100 μg/mL), which indicates that the extracts are selective towards targeting cancer cells.

Keywords: *watercress; kale; glucosinolates; isothiocyanates; antioxidant; anticancer*

INTRODUCTION

Cancer is becoming one of the major diseases that humans need to deal with because of its agonizing effects to our body. This disease has been found to be the second leading cause of death in the world and is one of the leading causes of mortality in the Philippines (Tacio, 2018). Consumption of fruits and vegetables could prevent the occurrence of cancer and could also lead to its elimination or cure in our system. Cruciferous vegetables including mustard, radish, broccoli, cauliflower, cabbage, watercress, kale, etc. were found to contain high amounts of phytochemicals including glucosinolates and isothiocyanates (Larson, 2018, Mazumder et al., 2016). Glucosinolates and isothiocyanates are sulfur-rich compounds, which have the ability to prevent and cure various types of cancer. Among the two compounds, greater attention is given to isothiocyanates, the breakdown products of glucosinolates, which is likely more responsible for the exhibited biological activity of the vegetables (Higdon et al., 2007; Fahey et al., 2001). The conversion of glucosinolates to their corresponding isothiocyanates is due to the presence of the enzyme called myrosinase, which upon action to glucosinolates, hydrolyzes it to produce the breakdown products including thiocyanates, isothiocyanates, and nitriles (Clarke, 2010 and Higdon et al., 2019). Thiocyanates are compounds which have goiter-promoting properties in humans while isothiocyanates have exhibited potential anticancer properties via modulation of phase I and phase II enzymes (Das et al., 2000), as well as the inhibition of NF-kB induced inflammation and cell cycle arrest via the apoptotic pathway (Navarro et al., 2011).

Studies on the glucosinolates and isothiocyanates in various cruciferous vegetables have been done to assess their benefits in humans. Watercress (*Nasturtium officinale*), is a Brassica vegetable known to have small, round leaves and edible stems and have a slight spicy and peppery flavor. The intake of watercress daily may significantly reduce DNA damage of blood cells, which is considered to be a major cause of the development of cancer (Smith, 2007). Watercress contains glucosinolates called gluconasturtiin (2-phenetyl glucosinolate), which when hydrolyzed through the action of myrosinase, produces 2-phenetyl isothiocyanate. Isothiocyanates such as sulforaphane and phenethyl isothiocyanate have been found to protect the body against cancer by safeguarding the healthy cells from damage, inactivating the carcinogenic chemicals, and hindering the growth and spread of tumors (Groves, 2018). According to Barrington (2015), the bitter taste of watercress is due to the presence of glucosinolates, which gives watercress few medicinal properties. In addition, an experiment conducted by Zeb (2015) revealed that watercress supplementation in diet shows that the increase in the blood antioxidant is related to the significant antioxidant activity of watercress. Kale (*Brassica oleracea* L. var. sabellica), are green or purple leafy cruciferous vegetable, which can have either curly or smooth shape (Gunnars, 2018). Recent studies have shown that kale is a rich source of organosulfur compounds, which have the ability to reduce the risk of colon cancer. One of the major glucosinolates found in kale is glucobrassicin, which when hydrolyzed forms indole-3-carbinol known to prevent the development of tumors in mice (Gander, 2018).

Watercress and kale are incorporated in salads and are also used as powdered condiment, food garnish, and medicine. In this research, the presence of glucosinolates and isothiocyanates were determined using qualitative and quantitative approaches. HPLC (high performance liquid chromatography) analysis of the extracts was carried out to determine the concentration of the major compounds. Furthermore, the biological activities of the extracts were determined via

DPPH and PrestoBlue™ assays to establish their antioxidant and anticancer properties, respectively.

METHODS

Materials and Equipment. Locally grown watercress and kale were purchased from supermarkets (Cartimar and Dizon Farms). Telstar LyoQuest Freeze Dryer was used for lyophilization. Agilent Technologies 1200 HPLC with a UV detector was used for the quantification of glucosinolates. Biological assays were performed using available kits and analyses were carried out using FLUOstar Omega Microplate Reader and BioTek ELx800 Absorbance Microplate Readers. 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. Other reagents included 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. Healthy leaves were separated from the stem/stalk. The leaves were then washed with tap water and distilled water consecutively, drained in a colander, freeze-dried, powdered, and stored in plastic containers at 4 °C prior to analysis.

Methanolic Extraction of Glucosinolates. Glucosinolates from watercress and kale samples were extracted using methanol following a modified method by Oerlemans et al. (2006) To each tube containing ~0.3 g of powdered samples, 5 mL of 100% hot methanol was added. The samples were vortex-mixed and incubated in a heating block for 20 minutes at 70 °C. The tubes were cooled-down to room temperature and centrifuged for 10 minutes at 5,000 rpm. The supernatant was collected in a separate tube and the pellet was re-extracted twice sequentially with 3 mL and 2 mL portions of 70% methanol. The supernatants were combined and diluted to 10 mL with methanol. A volume of 100 µL of sinigrin was added to each tube containing the extracts. Another tube was also prepared containing only 100 µL sinigrin in methanol/water, which was used as the standard for the analysis.

Desulfation of Glucosinolates. In preparing the column, 5 mL pipette tips were fitted with cotton wool. A 1 mL bed of DEAE Sephadex A-25 anion exchange resin was allowed to settle in each column. The column was washed twice with 0.50 mL deionized water. A 3.0 mL of glucosinolate extract was then allowed to drip through the column followed by washing with 0.5 mL deionized water and 0.5 mL sodium acetate twice. The column was treated with 75 µL sulfatase (25 mg/mL) and was allowed to undergo enzymatic reaction at room temperature for about 16 hours. The column was eluted using 0.50 mL deionized water twice and once using 0.25 mL deionized water and the eluates were collected in an HPLC vial prior to analysis.

Analysis of Desulfated Glucosinolates via HPLC. Glucosinolate analysis was carried out based on a standard HPLC method (ISO 10633-1:1995). Glucosinolates were separated using a 250 x 4.6 mm C18, 5 µm reverse phase HPLC column. A 20 µL sample was injected and eluted using a gradient system that consists of acetonitrile and water with a flow rate of 1.5 mL/min. Glucosinolate concentrations were calculated as sinigrin equivalents.

Extraction of Hydrolysates. For the extraction of hydrolysates, four sets of approximately 0.1 g of samples were obtained and subjected to different treatments. In the unhydrolyzed treatment, 5 mL of dichloromethane (DCM) was added to the tubes containing watercress and kale samples. The samples were soaked in DCM for 30 minutes with cover to avoid evaporation of the solvent. The mixture was filtered and the filtrate was collected in a separate glass vial. The residue was washed with DCM to maximize the extraction of target compounds. For the autolyzed sample, 5

mL H_2O was added to the tube containing the sample and is incubated for 30 minutes. For the hydrolyzed treatment, 5 mL H_2O, 5 µL of exogenous myrosinase and 40 µL 18.75 mM of ascorbic acid (present or absent) were added to each separate tube containing the sample. After which, the samples were mixed and incubated for 30 minutes. Five (5) mL of DCM was added to the autolyzed and hydrolyzed samples to arrest further hydrolysis. The samples were vortexed and the organic and aqueous layer was allowed to separate. The organic layer was separated and filtered into a separate vial. The aqueous layer was re-extracted with 5 mL DCM and the organic layers were combined. The organic layers were dried over anhydrous sodium sulfate to get rid of moisture and was transferred to a new vial. The solvent was completely evaporated and the crude product was placed in a covered cool dry container with desiccant. The mass of the hydrolysates was determined after the solvent has completely dried.

HPLC Analysis of Cyclocondensation Products. The isothiocyanate content of the unhydrolyzed, autolyzed, and hydrolyzed samples were determined by allowing the hydrolysates to undergo cyclocondensation reaction with 1,2-benzenedithiol (Zhang et al., 1992). A 100-µL of allyl isothiocyanate or hydrolysates were made to react with 600 µL of 10 mM 1,2-benzenedithiol and 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 in a heating block. 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 with a reverse phase column (250 mm x 4.6 mm C18, 5µm) monitored using UV detector set at 365 nm. The mobile phase was set isocratically using 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 at ~3.2 min was integrated and was quantified using a standard curve generated from measurements using varying concentrations of allyl isothiocyanate (1 to 5 µM).

Determination of Antioxidant Activity of Extracts via DPPH Assay. The free-radical scavenging activity of extracts was determined by monitoring the 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity. A 1.0 mg/mL sample of the methanolic extracts and hydrolysates were prepared. From the stock solution, a 150 µL volume of extracts with various concentration (0 to 100 µg/mL) were pipetted in a 96-well microplate. Fifty (50) µL DPPH was added to the extracts making the final concentration of DPPH 60 µM per well. The plate was incubated in the dark for 30 minutes. The samples were subjected to UV-Vis analysis at 517 nm using FLUOstar Omega Microplate Reader. The analyses were done in triplicate. Methanol and distilled water were used as blank, while ascorbic acid was used as positive control. The scavenging activity (%SA) of the extract was calculated using the equation:

$$\%SA = [(A_B - A_S) / A_B] \times 100$$

$\mathbf{A_B}$ is the absorbance of blank at 517 nm

$\mathbf{A_S}$ is the absorbance of sample at 517 nm

Determination of Anticancer Activity of Extracts via PrestoBlue™ Assay. A 1 mg/mL of juice extracts was prepared from watercress and kale samples. The extracts were filtered in 45-micron filter to remove particulates, which may interfere with the assay. The samples were brought to Molecular Biology Unit of CENSER, and cell culture and assay were conducted using various cell lines. Human dermal fibroblast (HDFn), human colon adenocarcinoma grade II (HT-29), human breast adenocarcinoma (MCF-7), human small lung cancer (H69PR), and monocytic leukemia (THP-1) cells were cultured using standard protocol. The cells were placed in a 75-cm^2 Falcon flask with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1x antibiotic/antimycotic. The cells were placed in a controlled environment at 37° C in a humidified atmosphere of 5% CO_2 and subcultured after

trypsinization. Cells were transferred in a 96-well plate and were grown in DMEM with 10% FBS and 1x antibiotic/antimycotic. The culture medium was replaced every 24 hours of incubation.

The cells were washed with phosphate-buffered saline (PBS) at pH 7.2 followed by addition of 100 µL culture media. Serial dilution of extracts was done in the 96-well plate. Zeocin was used as the positive control and the untreated wells as the negative control. The cells were incubated for 3 hours at 37 °C in a humidified atmosphere of 5% CO_2. The treated cells were analyzed using the PrestoBlue™ assay. To each well containing the treated cells, 10 µL of PrestoBlue™ dye was pipetted followed by incubation for 30 minutes. The absorbance was read at 570 nm using a BioTek ELx800 Absorbance Microplate Reader.

$$\% \text{ CI} = \{100 - [(A_U - A_M) / A_M]\} \times 100$$

% CI is the number of dead cells

$\mathbf{A_U}$ is the absorbance of untreated sample and

$\mathbf{A_M}$ is the absorbance measured per well

RESULTS AND DISCUSSION

Glucosinolates in Watercress and Kale Leaves. Watercress and kale leaves were investigated via HPLC analysis of desulfated glucosinolate derivatives to determine the total glucosinolate (GSL) content in the sample. The watercress sample contains a significant amount of glucosinolates amounting to 228.93 ± 26.39 µmol/g. The HPLC chromatogram (Figure 1) showed the presence of a glucosinolate eluted at 15.871 min, which could be assigned as gluconasturtiin ***(1)***, a predominant glucosinolate in this plant. The identity of the major peak was previously reported using mass spectrometry (Barrington, 2015).

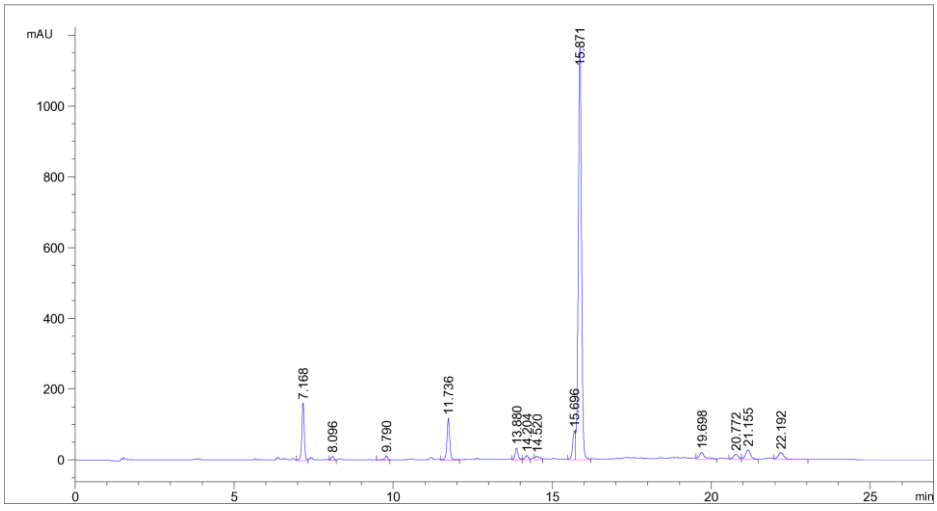
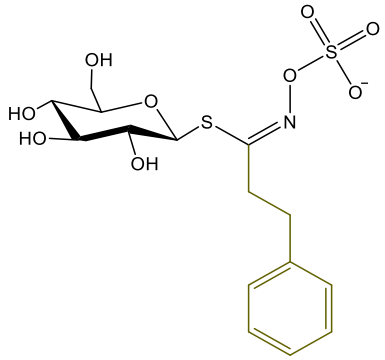


Figure 1. HPLC-UV chromatogram of watercress leaves monitored at 230 nm



(1) Structure of gluconasturtiin

On the other hand, kale contains 87.60 ± 80.12 µmol/g of total glucosinolates. The chromatogram (Figure 2) revealed the presence of two predominant glucosinolates, which eluted at 5.893 and 7.174 mins. Although kale is found to be rich in glucobrassicin ***(2, right)***, further analysis should be done to fully characterize the identity of the two predominant glucosinolates present in kale.

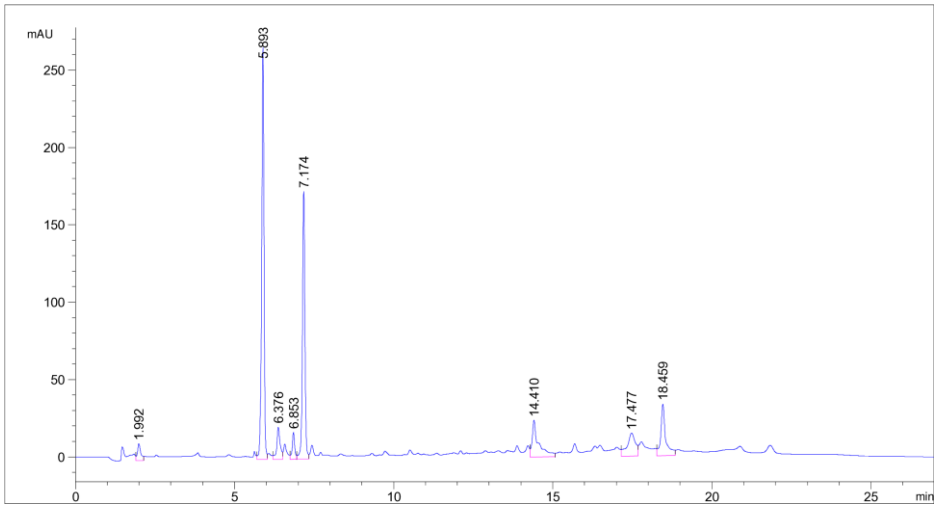
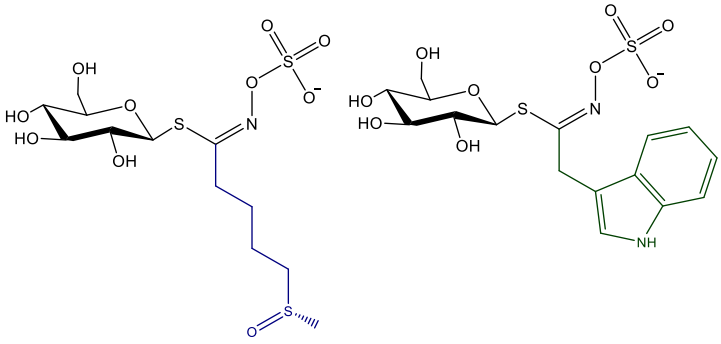


Figure 2. HPLC-UV chromatogram of kale leaves



(2) Structures of glucoraphanin (left) and glucobrassicin (right)

Previous studies also suggested that kale and other *Brassica* spp. including broccoli, cauliflower and cabbage, contain glucoraphanin ***(2, left)***, which could be the other predominant glucosinolate in the sample analyzed (Boddupalli et al., 2012). The analysis of major glucosinolates observed in watercress and kale should be further verified using LC-MS/MS and/or NMR analysis.

Isothiocyanates in Watercress and Kale Leaves. The hydrolysates were used to analyze the presence of hydrolysis products, including isothiocyanates. Quantification of isothiocyanate concentration from unhydrolyzed, autolyzed, and hydrolyzed samples were carried out by analysis of cyclocondensation products from the reaction of isothiocyanates with 1,2-benzenedithiol. Table 1 shows that isothiocyanates are inherent in watercress and kale with the values of 0.398 ± 0.018 and 0.378 ± 0.014, respectively. Figure 3 shows a slight increase in the isothiocyanates when the samples were autolyzed in the presence of water. Water might have an effect in the activation of myrosinase enzyme inherent in the sample (Clarke, 2010 and Higdon et al., 2019). Surprisingly, a significant increase in the isothiocyanate content was observed upon the addition of exogenous myrosinase in comparison to the unhydrolyzed samples. Moreover, the presence of ascorbic acid does not have a huge effect in the concentration of isothiocyanate in comparison to samples without ascorbic acid. Previous studies suggest that ascorbic acid activates the myrosinase enzyme via conformation changes (Ohtsuru and Hata, 1979). The similarities between the concentration of isothiocyanates in the hydrolyzed samples, in the absence or presence of ascorbic acid could be due to the presence of ascorbic acid in the powdered samples even before the samples were subjected to hydrolysis, however, the differences in ascorbic acid concentration were not accounted in the study. The increase in the total amount of isothiocyanates is brought about by the action of exogenous myrosinase, which is responsible for further hydrolysis of glucosinolates present in watercress and kale samples (Higdon et al., 2019; Fahey *et al*., 2001).

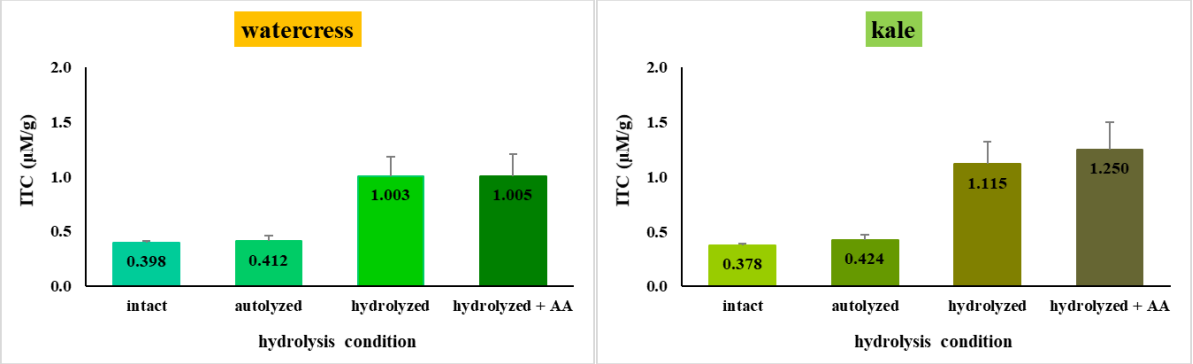


Figure 3. Concentration of hydrolysis products in watercress (*Left*) and kale (*Right*) samples under various conditions.

Table 1. Isothiocyanate content of watercress and kale

Concentration of cyclocondensation products (µM/g)		
	watercress	**kale**
unhydrolyzed	0.398 ± 0.018	0.378 ± 0.014
autolyzed	0.412 ± 0.047	0.424 ± 0.049
hydrolyzed	1.003 ± 0.180	1.115 ± 0.206
hydrolyzed with ascorbic acid (AA)	1.005 ± 0.199	1.250 ± 0.247

± refers to standard deviation measured from 3 replicates

Antioxidant Activity of Watercress and Kale Leaves. The antioxidant properties of watercress and kale leaves were assessed by their free radical scavenging ability. Kiers et al. (1976) showed how DPPH free radical undergoes neutralization by an effective scavenging compound and convert radicals into their corresponding products. Methanolic extracts and hydrolysates of watercress and kale showed free radical scavenging activity as described by the IC_{50} values (Tables 2 and 3).

Table 2. Antioxidant activity of methanolic extracts from watercress and kale leaves

sample	IC_{50} (μg/mL)
watercress	13.04 ± 1.11
kale	33.14 ± 1.44
ascorbic acid	2.51 ± 0.03

± refers to standard deviation measured from 3 replicates

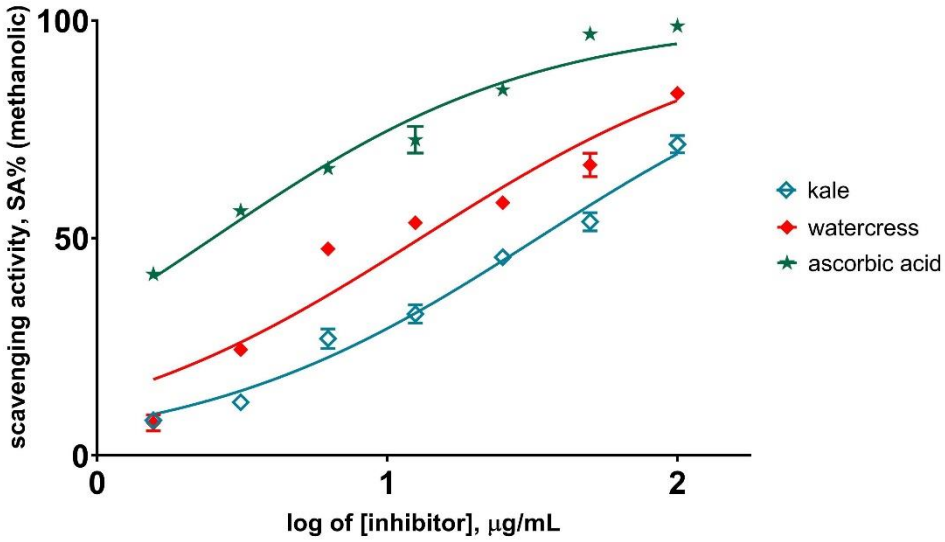


Figure 4. Non-linear response curve for methanolic extracts towards DPPH. Ascorbic acid was used as positive control, while methanol was used as blank.

Table 3. Antioxidant activity of hydrolysates from watercress and kale leaves.

sample	IC_{50} (μg/mL)
watercress	7.69 ± 0.93
kale	20.40 ± 1.26
ascorbic acid	2.77 ± 0.03

± refers to standard deviation measured from 3 replicates

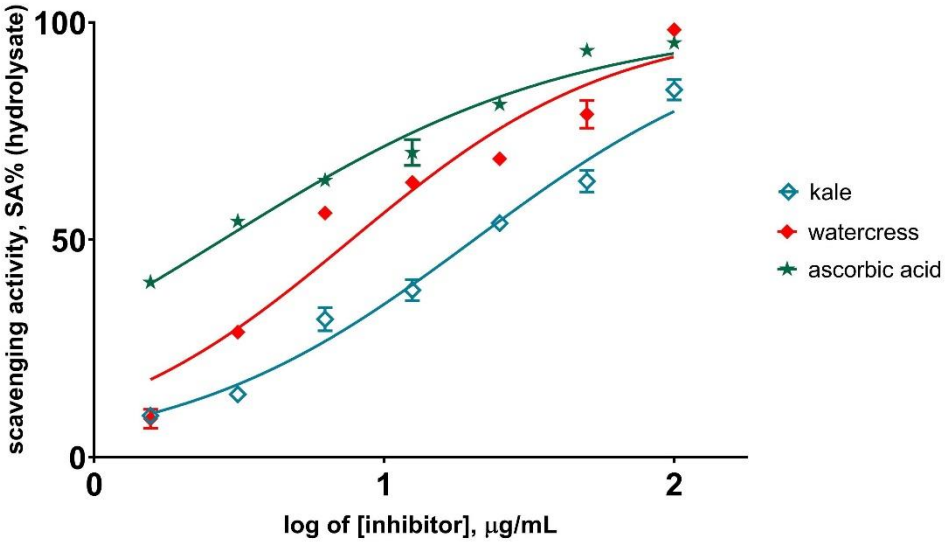


Figure 5. Non-linear response curve hydrolysates towards DPPH. Ascorbic acid was used as positive control, while methanol was used as blank.

Lower IC_{50} values usually indicate higher efficiency towards scavenging of DPPH radicals by the leaf extract. The ability of a plant or vegetable sample to exhibit an effective antioxidant activity is due to the bioactive compounds present in the extracts (Ahmed et al., 2015, Luqman et al., 2012). Compounds which might be responsible for the observed antioxidant activity could be found in the methanolic extracts and hydrolysates, which may include glucosinolates and isothiocyanates (Figures 4 and 5). Moreover, ascorbic acid is known to have significant antioxidant activities, which was also proven by the analysis as evidenced by the data generated. The presence of glucosinolates and isothiocyanates in the leaf extracts could be accountable for the observed antioxidant properties, but further evidence should be presented to support the claim. Previous reports have cited the importance of glucosinolates and isothiocyanates towards inhibition of oxidation and reduction in the formation of free radicals, which could support the results of this assay (Boddupalli et al., 2012).

Anticancer Activity of Watercress and Kale Leaves. The effect of the extracts towards cancer cells and normal cells was evaluated using PrestoBlue™ cell viability assay. The ability of cells to convert resazurin to resorufin (Shenoy et al., 2017) was carried out using absorbance measurements. The IC_{50} values, the concentration of extracts, which causes 50% cell death are summarized in Table 4.

Table 4. Summary of PrestoBlue™ assay. Zeocin was used as the standard. The highlighted values correspond to samples which exhibit high activity within the IC_{50} value limits of 20 µg/mL for crude extracts. Lower IC_{50} values indicate higher cytotoxic properties.

	IC_{50} (µg/mL)		
	watercress	**kale**	**zeocin**
H69PR	65.85 ± 3.50	19.35 ± 1.54	9.81 ± 1.06
MCF-7	17.67 ± 1.64	61.95 ± 2.35	3.82 ± 0.89
HT-29	22.74 ± 1.74	18.32 ± 2.41	4.89 ± 1.10
THP-1	91.37 ± 4.50	90.11 ± 4.66	4.73 ± 0.94
HDFn	> 100	> 100	13.25 ± 1.12

± refers to standard deviation measured from 3 replicates

Watercress was found to be effective towards MCF-7 (IC_{50} = 17.67 ± 1.64 µg/mL), while kale is effective towards H69PR (IC_{50} = 19.35 ± 1.54 µg/mL) and HT-29 (IC_{50} = 18.32 ± 2.41 µg/mL). Most importantly, both extracts were found to be non-cytotoxic towards HDFn (IC_{50} > 100 µg/mL) suggesting selectivity in targeting cancer cells.

The individual non-linear response curves for watercress and kale leaf samples towards specific cancer cell lines and normal cell line are shown in the succeeding figures (Figures 6-10). The data generated showed that higher concentrations of extract could exhibit cytotoxic activities for each cell lines except HDFn, where the highest concentration of extracts did not kill 50% of the cells.

The result of our study suggests that cruciferous vegetables, including watercress and kale, contain bioactive compounds such as isothiocyanates, which could highly be responsible for the observed cytotoxic properties (Sarikamis, 2009; Conaway et al., 2009; Molina-Vargas, 2013). Although the mechanism of action has not been fully characterized, it is worth noting that eating the vegetable alone may have the ability to reduce the risks of various cancer types in humans (Conaway et al., 2009; Mutanen and Pajari, 2011; Beevi et al., 2010; Seyed et al., 2016).

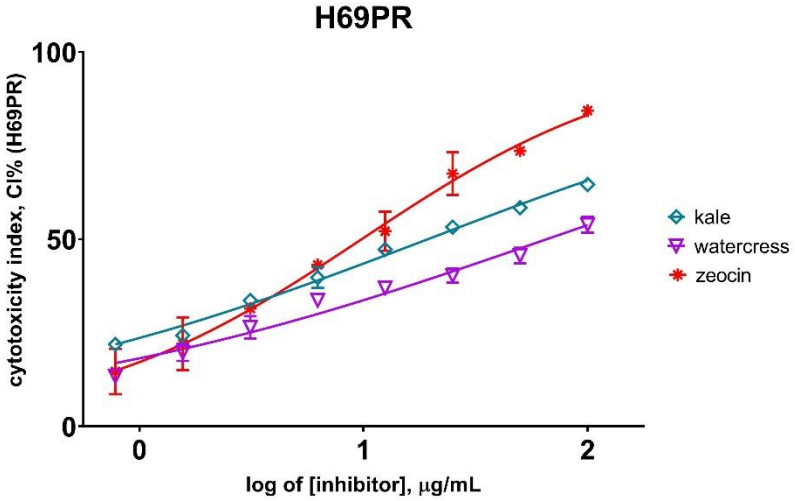


Figure 6. Non-linear response curve for juice extracts towards H69PR. Zeocin was used as the standard.

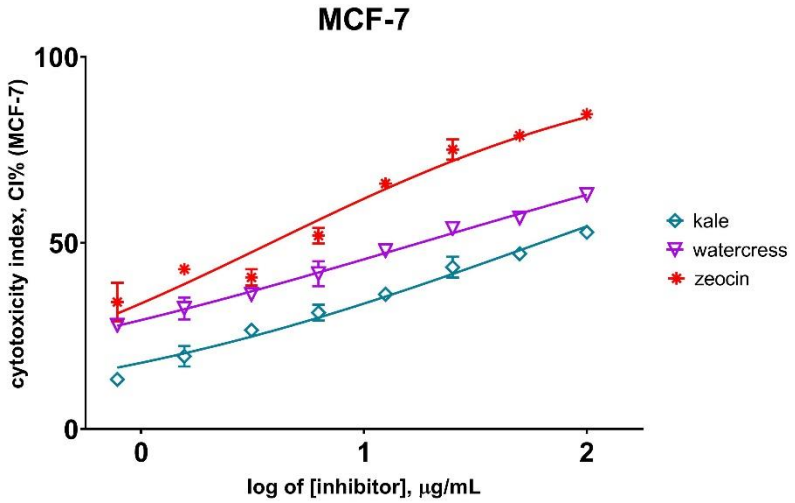


Figure 7. Non-linear response curve for juice extracts towards MCF-7. Zeocin was used as the standard.

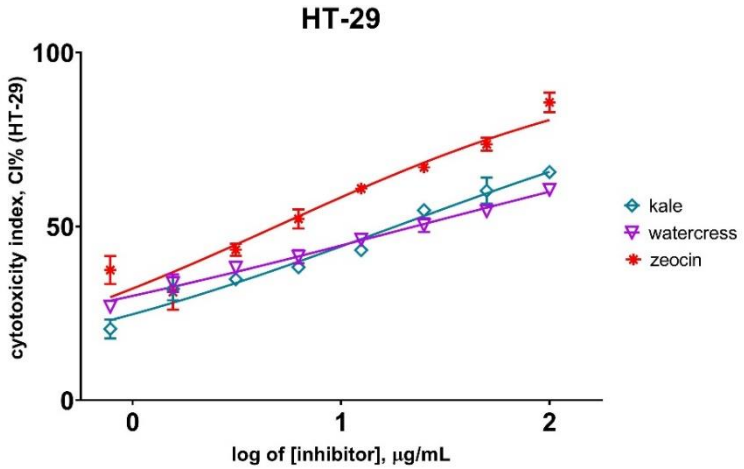


Figure 8. Non-linear response curve for juice extracts towards HT-29. Zeocin was used as the standard.

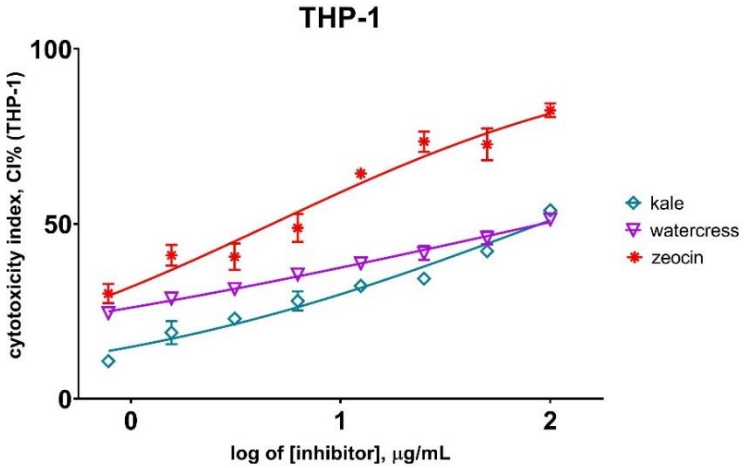


Figure 9. Non-linear response curve for juice extracts towards THP-1. Zeocin was used as the standard.

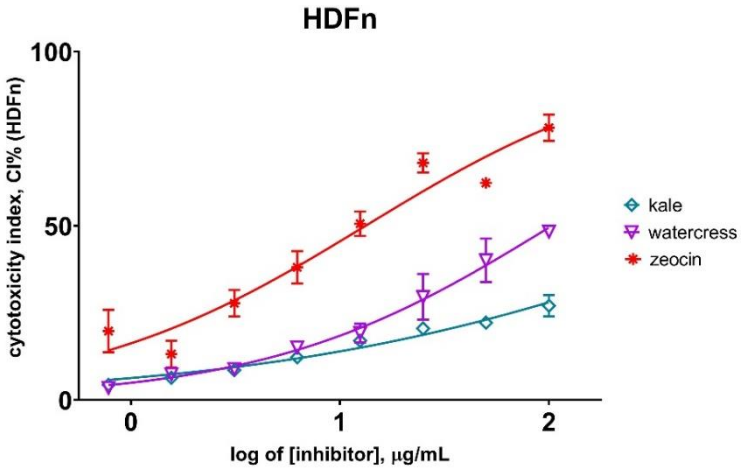


Figure 10. Non-linear response curve for juice extracts towards HDFn. Zeocin was used as the standard.

CONCLUSIONS

This research focused on the analysis of glucosinolates found in watercress and kale leaves. HPLC analysis revealed that the glucosinolate content of watercress is significantly higher than that of kale. The methanolic extracts and hydrolysates were found to have potential antioxidant activity measured using the DPPH assay. Moreover, the vegetables were found to have anticancer potential towards specific cancer cell lines used in the study without exhibiting cytotoxicity to normal cells. Further analysis should be conducted to fully characterize the glucosinolates and isothiocyanates in the plant. Additional assays can be done in order to further determine the mechanism of action of the extracts towards free radicals and cancer cells. The action of the extracts towards specific cell lines can be assessed using reverse transcription polymerase reaction (RT-PCR) while monitoring the gene expression, i.e., the down-regulation and up-regulation. The anticancer properties of both watercress and kale should be further studied in animal models and humans in order to determine its medical properties.

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