DISSERTATION

# ISOTHIOCYANATES FROM *CHORISPORA TENELLA* AND THEIR TOXICITY TO LIVER AND THYROID CELL LINES

Submitted by

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# ABSTRACT

## ISOTHIOCYANATES FROM *CHORISPORA TENELLA* AND THEIR TOXICITY TO LIVER AND THYROID CELL LINES

Consumption of *Brassica* plants like *Chorispora tenella* that contain isothiocyanates can be detrimental to the health of livestock, specifically horses. Late term gestational mares who ingest *Brassica* plants, in particular *Chorispora tenella,* have had foals born with Congenital Hypothyroid Dysmaturity Syndrome. Symptoms include underbite, contracted tendons, uncharacteristic silky coat, and incompletely formed hock and carpal joints. Unfortunately, most of the foals do not survive. Additionally, isothiocyanates can cause renal dysfunction, liver and thyroid toxicity. In particular, isothiocyanates have a profound antithyroid effect by interfering with the synthesis of thyroid hormones. In this study, *Chorispora tenella* in the Fort Collins area was quantified for isothiocyanate content in 2010, 2016 and 2017. In 2010, there were three isothiocyanates present in this *Brassica* plant: allyl isothiocyanate, propyl isothiocyanate and butyl isothiocyanate. However, in the 2016 and 2017 plant samples there was only allyl isothiocyanate present. Lastly, HepG2 human hepatocarcinoma and K1 human thyroid carcinoma cells were used as cytotoxicity models for the liver and thyroid pathology seen after ingestion of plants from the Brassica family. Results show that HepG2 cells were more sensitive to the various isothiocyanates and to *Chorispora tenella* plant extracts than the K1 cell line. These findings show that exposure to isothiocyanates and *Chorispora tenella* may impact livestock health even at a subclinical level and provide baseline information for further studies investigating the molecular mechanism of cytotoxicity by isothiocyanates.

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# 1. INTRODUCTION

*Chorispora tenella* is a nonnative plant that is widespread in North America, including northern Colorado. These plants are part of the Brassicaceae family and as part of this family they have glucosinolates. Glucosinolates are natural compounds in the plants that aid the plant in preventing herbivory. When glucosinolates come in contact with the enzyme myrosinase in the plant upon tissue disruption, these compounds undergo hydrolysis to form isothiocyanates. Isothiocyanates have a range of potential effects on livestock that consume these plants fresh or through silage. For example, late term gestational mares who consume plants that have particular isothiocyanates have had foals born with Congenital Hypothyroid Dysmaturity Syndrome. Symptoms in foals include underbite, contracted tendons, uncharacteristic silky coat, and incompletely formed hock and carpal joints. Additionally, isothiocyanates can cause renal dysfunction, liver and thyroid toxicity. In particular, isothiocyanates have a profound antithyroid effect by interfering with the synthesis of thyroid hormones.

The overall goal of this study was to determine if *Chorispora tenella*, poses a potential threat to livestock consuming it. In the past, it had been believed that sulfur was the cause for the toxicity upon consumption of some the *Brassica* species, however, the effects seen are not indicative of sulfur exposure. I hypothesize that the health effects seen in livestock are due to the isothiocyanate content present in the plant. In particular, I hypothesize that the liver will be a target tissue for isothiocyanate effects resulting in decreased liver function. Additionally, I hypothesize that the thyroid will also be affected by isothiocyanate and result in altered thyroglobulin production.

The first aim was to determine if *Chorispora tenella* had toxic isothiocyanates present. Plants were collected at six different locations in the Fort Collins area in 2010, 2016 and 2017. *Chorispora tenella* plant autolysis extracts were analyzed for isothiocyanate content, thiocyanate anion content and for total sulfur content.

The second aim had two equal parts. The first was to determine toxicity of the known pure isothiocyanates and *Chorispora tenella* plant autolysis extract in HepG2 human hepatocarcinoma cells. The second part was to determine toxicity of the known pure isothiocyanates and *Chorispora tenella* plant autolysis extract in K1 human thyroid carcinoma cells. Cytotoxicity of the known pure isothiocyanates and *Chorispora tenella* plant autolysis extract was measured through cell metabolism, DNA content, ATP content and for the K1 thyroid cells, thyroglobulin synthesis.

These questions have been addressed and are described in this dissertation. The literature review will give a thorough background of the plant chemistry and health effects followed by the materials and methods used throughout the research. Each aim will have a results and discussion sections. Lastly, there will be a final conclusion to summarize the research.

# 2. LITERATURE REVIEW

*Chorispora tenella*, *Descurainia sophia* and *Sisymbrium altissimum* are three plants non-native to North America, originating in Eurasia. These three plants are currently found throughout the United States and are part of the Brassicaceae family (Figures 2-1, 2-2, 2-3, 2-4, 2-5, 2-6; plants.usda.gov).

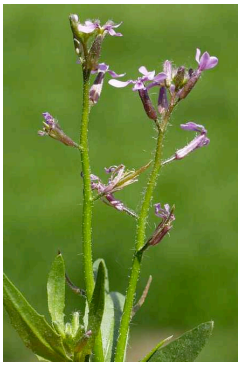

**Figure 2-1**. *Chorispora tenella*.

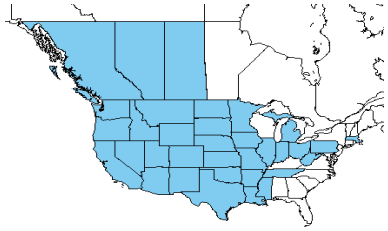

**Figure 2-2**. Occurrence of *Chorispora tenella*.

**Figure 2-3.** *Descurainia sophia*.

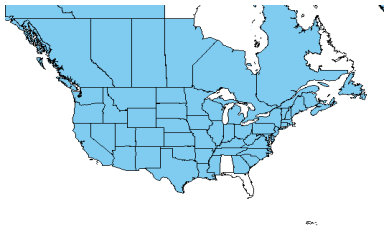

**Figure 2-4.** Occurrence of *Descurainia sophia*.

**Figure 2-5.** *Sisymbrium altissimum*.

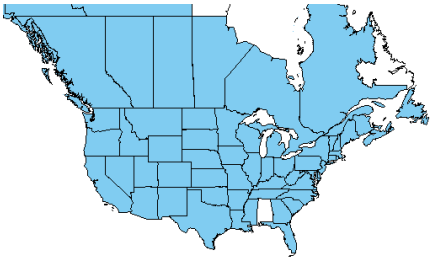

**Figure 2-6.** Occurrence of *Sisymbrium altissimum*.

These annual plants which are predominantly found in early spring, are ingested fresh or dry through feeding of hay or silage to horses and various domestic livestock. The ingestion of these plants may cause Congenital Hypothyroid Dysmaturity Syndrome (a syndrome of thyroid gland hyperplasia and musculoskeletal deformity) in foals, if consumed by the mare in late gestation (Hines 2006). It is believed that the syndrome is related to 'mustard weed' consumption, in particular the species *Chorispora tenella*, *Descurainia sophia* and *Sisymbrium altissimum* (Gay 2004; Hines 2006; Assayed and El-Aty 2009). This syndrome was first reported in 1981 in western Canada, but more recently has occurred in the USA (Doige and McLaughlin 1981; Kreplin and Allen 1991). Symptoms of Congenital Hypothyroid Dysmaturity Syndrome include prolonged gestation, underbite, contracted tendons, uncharacteristic silky coat, and incompletely formed hock and carpal joints (Hines 2006; Koikkalainen et al. 2014; Gay 2004; Kreplin and Allen 1991). The thyroid hormones, T3 and T4, are low however the thyroid's response to thyroid stimulating hormone is decreased (Koikkalainen et al. 2014; McLaughlin et al. 1986). Affected foals are either stillborn or weak at birth and survive only for a short time (Doige and McLaughlin 1981).

Plants in the Brassicaceae family are known for having a pungent flavor upon consumption due to the presence of glucosinolates. These are part of the plant's natural herbivory defense. The general glucosinolate structure (Figure 2-7) contains a glucose molecule, sulfur, carbon nitrogen double bond, sulfate ester and an amino acid derived side chain. The side chain (R) is highly variable and determines the specific glucosinolate structure.

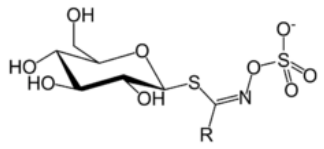


**Figure 2-7.** Generic Glucosinolate Structure

The glucosinolate is normally kept separate from the enzyme myrosinase, however, upon disruption of the plant tissue it comes in contact with myrosinase and the glucosinolate is metabolized to different products depending upon the hydrolysis conditions (Rask et al. 2000; Fenwick et al. 1983; McGregor et al. 1983; Fahey et al. 2001; Brown et al. 2003; Wang et al. 2012;Molina-Vargas 2013). Upon mastication or tissue disruption, there is hydrolysis of the thioglucosidic bond in the glucosinolate which yields a glucose and an unstable aglycone, the thiohydroxamate-O-sulfonate (Holst and Williamson 2004). The thiohydroxamate-O-sulfonate can undergo spontaneous rearrangement into different possible products: isothiocyanates, nitriles and elemental sulfur, thiocyanates, epithionitriles, oxazolidine-2-thiones or indolyl compounds depending upon the structure of the side chain and the pH environment (Holst and Williamson 2004). At more neutral pH of 6-7, the major hydrolysis products are stable isothiocyanates. Under acidic conditions, nitriles are the main degradation product. However, at basic pH limited information is available as to the nature of the products (Figure 2-8).

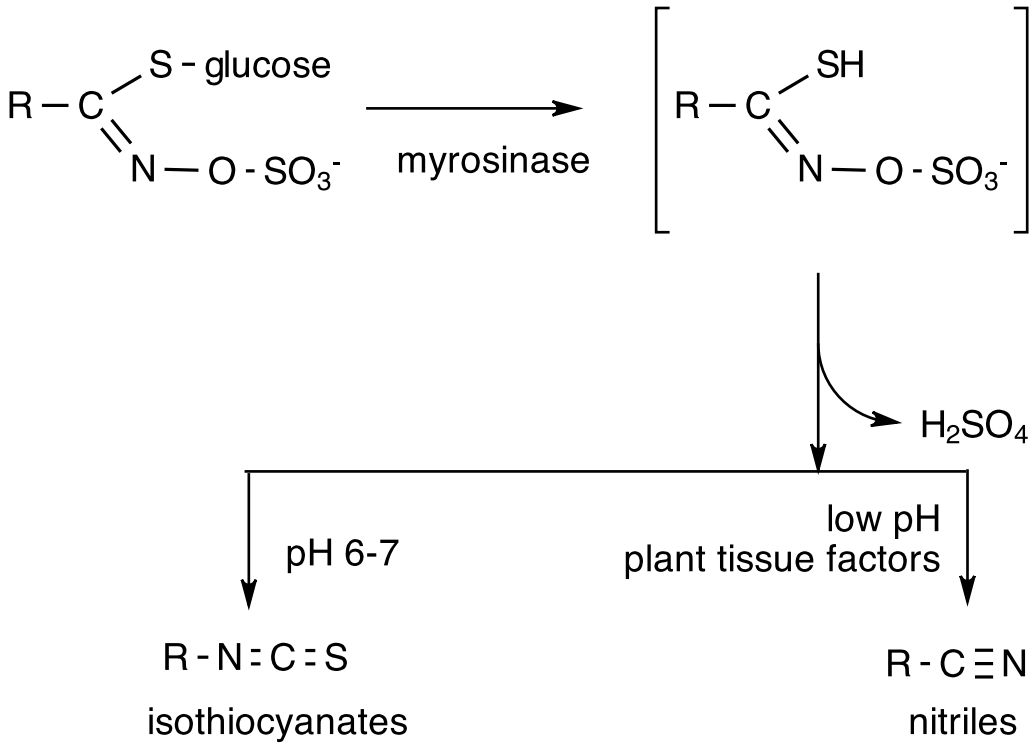


**Figure 2-8.** Glucosinolate Hydrolysis

Glucosinolates and isothiocyanates resulting from myrosinase-induced hydrolysis occur in all parts of the plant, however in differing concentrations and profiles. Occurrence and concentration vary according to species and cultivar, tissue type, physiological age, plant health, environmental factors (agronomic practice, climatic conditions), insect attack and microorganism intrusion (Fahey et al. 2001; Brown et al. 2003; Holst and Williamson 2004). The content of glucosinolates in plants is variable, ranging from 1-10% of dry weight in different tissues (Rosa et al. 1997). Younger rosettes of *Arabidopsis thaliana* have higher glucosinolate concentrations than older leaves (Brown et al. 2003) thus plant age and environmental factors are important in determining glucosionolate content (Fahey et al. 2001). Changes in glucosinolate profiles due to abiotic factors arise from salinity, water availability, temperatures, light cycling and nutritional

deficiencies. In particular, an increased sulfur supply has resulted in higher levels of total glucosinolates in *Brassica rapa* (Li et al. 2007; Martinez-Ballesta et al. 2013).

Isothiocyanates are electrophilic due to the central carbon of the isothiocyante group and are able to bind to the sulfhydryl groups of proteins and glutathione and undergo addition reactions with N, O and S based nucleophiles (Duncan and Milne 1992; Zhang et al. 1995). Isothiocyanates are conjugated with glutathione and excreted in urine as their corresponding mercapturic acids (Vermeulen et al. 2003). In a study done with two hundred and twenty pregnant cows fed *Thlaspi arvense,* one hundred showed signs of colic within four hours of feeding, eight died over the course of five days and four abortions occurred. Through this case study it was calculated that the fatal dose of allyl isothiocyanate (AITC) in cattle was 65 mg/kg body weight (Smith and Crowe 1987).

Isothiocyanates accumulate in cells predominantly as the conjugated glutathione-isothiocyanate whereas free isothiocyanate levels in cells are low. In an experiment to examine if glutathione isothiocyanate conjugates can reach intracellular levels sufficient to cause cytochrome c release from mitochondria, isothiocyanates were added at a concentration of 0.5mmol/L to a concentration of glutathione at 2.5mmol/L. After this incubation for thirty minutes, mitochondria were added and incubated for an additional thirty minutes. All isothiocyanates were converted to the glutathione isothiocyanate conjugate prior to mitochondria even being added, bringing the actual concentration that mitochondria were exposed to down to 0.5mmol/L of glutathione isothiocyanate conjugate with 2mmol/L glutathione. Mitochondria treated under these conditions showed cytochrome c release, indicating apoptosis (Zhang 2001; Tang and Zhang 2005). Isothiocyanates have been considered indirect antioxidants since they readily conjugate with thiol groups such as in glutathione, which lead to a short-term depletion of

glutathione in the cell. This is followed by up-regulation of gamma glutamylcysteine synthase, the rate limiting enzyme in glutathione synthesis, which contributes to a rapid increase in cellular glutathione concentrations (Holst and Williamson 2004). The induction of glutathione within cells is considered to be a protective effect of isothiocyanates.

It should be recognized that the isothiocyanate glutathione conjugate can deconjugate to reach equilibrium with the free isothiocyanate. This reaction is dependent on pH and concentrations of isothiocyanates and thiols. When exposing isothiocyanates to human glutathione transferases, the transferases played an important role in enzymatic cleavage of the glutathione isothiocyanate conjugate. The tendency of the conjugates to dissociate is increased as the pH of the solution is lowered (Zhang et al. 1995; Jiao et al. 1996; Conway et al. 2005). Additionally, isothiocyanates released from their conjugates react with free thiols present (Jiao et al. 1996). Isothiocyanate conjugates can be considered prodrugs of the parent isothiocyanate compounds due to this dissociation (Conaway et al. 2005). This suggests that the higher the intracellular isothiocyanate glutathione conjugate concentration the more free unconjugated isothiocyante is available to bind to other target proteins (Mi et al. 2011). These targets can be cysteine which plays an important role in numerous protein functions for example heme proteins, such as cytochrome P450s, participating in oxidation.

Isothiocyanate chain length is related to its lipophilicity. Increased chain length has shown an increase in binding of the isothiocyanates to cytochrome P450 (Guo et al. 1993, Jiao et al. 1994; Tang and Zhang 2005). Decreased water solubility and higher lipid solubility facilitates the delivery of the isothiocyanates to the mitochondrial membrane (Mike and Chance 1975; Tang and Zhang 2005). Increased glutathione and glutathione S-transferase activity

enhanced the ability of the isothiocyanate to be taken into the cell in addition to the lipophilicity of the individual isothiocyanate (Zhang 2001; Tang and Zhang 2005).

The ability of isothiocyanates to damage carcinogenic cells suggests that it may be a natural chemotherapeutic. Natural and synthetic isothiocyanates are competitive inhibitors of the cytochrome P450 isozymes in mouse lungs responsible for metabolic activation of the tobacco-specific nitrosamine (NNK) (Jiao et al. 1994). This inhibition reduces the formation of $O^6$ methylguanine and reduces neoplasm formation in the lungs of mice treated with NNK (Morse et al. 1991). Isothiocyanates can disrupt the carcinogenic process by: i) increasing phase II enzymes, ii) inducing cell cycle arrest thus preventing cell growth, iii) initiating apoptosis leading to cell death, and iv) inhibition of cytochrome P450 to prevent carcinogen activation (Zhang 2001; Tang and Zhang 2005; Mi et al. 2011; Molina-Vargas 2013).

Isothiocyanate-induced apoptosis is initiated when mitochondria are damaged leading to a caspase cascade and/or Bcl-2 phosphorylation. When the cell undergoes apoptosis the permeability of the mitochondrial membrane increases resulting in the release of cytochrome c and a downstream effect of other apoptotic proteins (Zhang 2001; Zhang et al. 2003; Miyoshi et al. 2004; Tang and Zhang 2005; Zhang and Tang 2005; Satyan et al. 2006; Traka and Mithen 2009; Zhang 2010; Geng et al. 2011; Mi et al. 2011; Molina-Vargas 2013; Bo et al. 2016). For example, in UM-UC-3 cells, the caspase-9 pathway was initiated from mitochondrial damage by exposure to isothiocyanates. Both outer and inner mitochondrial membranes were damaged resulting in the release of cytochrome c into the cytoplasm (Tang and Zhang 2005). Likely, isothiocyanates caused a dissociation of BCL-xl with Bak and Bax in the mitochondria, freeing Bax from Bcl-xl inhibition thus causing the mitochondrial damage (Tang and Zhang 2005). Allyl isothiocyanate significantly decreased Bcl-2 and increased Bax levels in MCF-7 and MDA-

MB-231 cells. This illustrates the involvement of mitochondria in apoptosis due to allyl isothiocyanate exposure (Bo et al. 2016). Exposure to isothiocyanates can cause a time-dependent caspase cascade. After three hours of treatment with 10 μM allyl isothiocyanate and benzyl isothiocyanate, caspases-3, -8, and -9 were activated (Zhang et al. 2003). Benzyl isothiocyanate induced cell cycle arrest, apoptosis and the involvement of mitogen-activated protein kinases (MAPKs). Jurkat cells exposed to benzyl isothiocyanate for 30 minutes at 5 μM showed a rapid increase and sustained activation of p38 MAPK phosphorylation (Miyoshi et al. 2004). Treatment with allyl isothiocyanate induced Bcl-2 phophorylation and further experiments showed that JNK was responsible for allyl isothiocyanate-induced Bcl-2 phosphorylation. There was a dose dependent loss of mitochondrial transmembrane potential, cytoplasmic accumulation of cytochrome c, activation of caspases-9 and -3 with increased allyl isothiocyanate concentrations (Geng et al. 2011). In HL60/S cells, exposure to ally isothiocyanate initiated the rapid loss of the mitochondrial membrane potential disrupting energy production in the cell and resulted in dose dependent caspase-3, -8, -9, and -12 activation (Zhang et al. 2003).

Isothiocyanates also have antiproliferative effects on cells. Phenethyl isothiocyanate has a dose dependent inhibitory effect on OVCAR-3 ovarian cancer cells, shown by a decrease in cell viability over time. Inhibition of DNA synthesis also suggests that phenethyl isothiocyanate is a growth suppressing agent (Satyan et al. 2006). HL60/S cells were exposed to allyl isothiocyanate and benzyl isothiocyanate at 10 μM for three hours and cell cycle progression was blocked. Interestingly, allyl isothiocyanate was determined to arrest cells in the $G_1$ phase while benzyl isothiocyanate blocked cells in $G_2$-M phase (Zhang et al. 2003).

Glucosinolate hydrolysis products were shown to affect morphology and function of different cells and organs following ingestion. They can alter organ mass, cause renal dysfunction, thyroid toxicity, hemolytic anemia and pulmonary emphysema (Holst and Williamson 2004). Isothiocyanates have been demonstrated to result in significant genotoxic effects in kidney, pancreas, and liver. Effects of isothiocyanates in the lung and colon have been seen at lower doses compared to other organs (Kassie and Knasmuller 2000; Holst and Williamson 2004). Unfortunately, the general mechanism through which isothiocyanates exert their genotoxic effect is unknown, however, allyl isothiocyanate has been reported to be cleaved enzymatically to give rise to metabolic intermediates known to induce bacterial mutation, glutathione depletion, lipid peroxidation, and cell death (Kassie and Knasmuller 2000). In particular, isothiocyanates have a profound antithyroid effect by interfering with the synthesis of thyroid hormones. Specifically, studies have shown that radioiodine incorporation is competitively inhibited by isothiocyanates resulting in the inability of the thyroid to concentrate inorganic iodide (Langer and Greer 1968).

In a normal functioning thyroid, activity is controlled by the anterior pituitary through Thyroid Stimulating Hormone (TSH). Release of TSH is determined by level of thyroxine present in the blood through feedback mechanisms and by the hypothalamus through the thyrotopin-releasing hormone (TRH). Thyroid stimulating hormone increases iodine trapping, which is the first stage of thyroid hormone synthesis. Once taken up by the sodium iodide symporter in the follicular cells, iodine is transported to the follicular space where it is oxidized. Thyroid peroxidase then allows the addition of tyrosine units. This form of the thyroid hormone is considered a precursor and are called monoiodotyrosine (MIT) and diiodotyrosine (DIT). After stimulation by thyroid stimulating hormone, the follicular cells move the thyroglobulin via

pinocytosis into the follicle cell where it is cleaved, forming triiodothyronine (T3) and thyroxine (T4) (Davies 1972; Mason and Wilkinson 1973; Doige and McLaughlin 1981; McLaughlin 1986; Oertel et al. 1991; Shi et al. 2016). Triiodothyronine and thyroxine are required for normal fetal development and processes throughout adulthood such as maintenance of metabolic rate, cardiovascular system development, brain development, sexual function, and sleep. During early development any impact to normal thyroid function can alter the production of nuclear ribonucleic acid, mitochondrial activity, and cytoplastic protein synthesis (Mason and Wilkinson 1973), which are critical to growth and differentiation of cells. If the thyroid is not functioning properly a non-inflammatory, non-neoplastic enlargement of the thyroid gland (goiter) can occur (Doige and McLaughlin 1981).

Isothiocyanate exposure also directly causes dose dependent stress to cells. When HL60 cells were exposed to 25 μM of benzyl isothiocyanate, there was a resultant inhibition of superoxide generation by 80% and at 2-10 μM of benzyl isothiocyanate, mitochondrial damage was induced (Zhang et al. 2003; Miyoshi et al. 2004). Additionally, isothiocyanate will conjugate with glutathione; until the cell can upregulate glutathione production (Holst and Williamson 2004), the cell will remain vulnerable and susceptible to oxidative stresses (Zhang and Tang 2005). It has been shown that at low concentrations, isothiocyanates can induce low grade cellular stress which can effectively be cytoprotective. Once the isothiocyanate concentration surpasses a threshold, which is different for each isothiocyanate, the benefits are decreased (Tang et al. 2004). Sulforaphane at a concentration of 10 μM induced activation of antioxidant and heat shock responses and at 30 μM sulforaphane inhibited tubulin polymerization, induced G2/M phase arrest, and subsequently apoptosis in A549 human lung cancer cells (Mi et al. 2008). Isothiocyanates can also cause an increase in reactive oxygen species. Isothiocyanates with

shorter alkyl chains can yield a thiol group resulting in greater DNA damage through superoxide radical anion generation (Murata et al. 2000). Isothiocyanates induce damage at the ACG sequence of cytosine residue, which stimulates tumor production (Levine et al. 1991). Allyl isothiocyanate, as a short chain isthiocyanate, has a greater probability to damage DNA leading to possible mutations and carcinogenensis (Murata et al. 2000). Isothiocyanates can provide a chemoprotective response as a result of initial insult due to increased glutathione; however, this isothiocyanate-induced stress can lead to oxidative damage, mitotic damage, and apoptosis (Zhang and Tang 2005).

These glucosinolate hydrolysis products are responsible for the biting taste of some condiments and contribute to the flavors of many plants consumed by humans. When consumed in small amounts by humans, these flavor products may be desirable, however when consumed by animals in larger amounts, they may be toxic (McGregor et al. 1983).

# 3. MATERIALS AND METHODS

## 3.1 Source of Plants, Autolysis, and Analytical Chemistry

Chorispora tenella, Descurainia sophia and Sisymbrium altissimum were collected near Fort Collins, Colorado during April 2010, April 2016 - June 2016, and April 2017. The above-ground portion of plant was collected randomly by cutting with scissors and then being stored in a sealed polyethylene bag. After collection, samples were chilled immediately and kept at 4°C until analysis. Samples consisted of whole plant, which contained the entire above ground part of the plant (leaves, seeds, flowers, stem); leaves (only leaves), and seeds (only seeds).

### 3.1.1 Sampling Locations

Sampling collection sites are listed in Table 3-1. Collection sites are for *Chorispora tenella, Descurainia sophia,* and *Sisymbrium altissimum.*

Table 3-1

*Plant Collections*

| Location | Latitude (N) | Longitude (W) | Year | Collection |
|---|---|---|---|---|
| 1 | 40.58818 | 104.90797 | 2010 | 1a |
| 1 | 40.58818 | 104.90797 | 2016 | 1b |
| 1 | 40.58818 | 104.90797 | 2017 | 1c |
| 2 | 40.58079 | 104.95024 | 2016 | 2b |
| 3 | 40.56064 | 105.08597 | 2016 | 3b |
| 4 | 40.51171 | 105.09406 | 2016 | 4b |
| 4 | 40.51171 | 105.09406 | 2017 | 4c |
| 5 | 40.56882 | 105.07799 | 2016 | 5b |
| 6 | 40.55295 | 105.04177 | 2016 | 6b |
| 6 | 40.55295 | 105.04177 | 2017 | 6c |

Approximately 10g of plant samples collected in April 2010 were homogenized in a blender with 70mL of pH 7.5 buffer containing 0.1M citric acid and 0.2M sodium phosphate. The homogenate was divided: a 50g portion of the homogenate was transferred to an Erlenmeyer flask with a stopper and a 10g portion of homogenate was poured into an aluminum weigh pan

and placed in an oven set at 30°C for dry weight determination. The sealed Erlenmeyer flask was then placed in an orbital shaker at 100 rpm for a minimum of 12 hours at room temperature (19-21°C) with 20mL of pentane in the sealed flask (Gc Resolv, Fisher Scientific, Pittsburgh, PA). Following autolysis, the pentane layer was centrifuged and collected by decantation. The samples underwent a solvent exchange to hexane, which entailed repetitive addition of hexane and evaporation under a gentle stream of nitrogen at room temperature.

Hexane was dried over sodium sulfate (Fischer Scientific, Pittsburgh, PA). Samples of these extracts were analyzed by gas chromatography (GC) with flame ionization detection (FID) (Table 3-2). Additionally, several samples were analyzed by gas chromatography mass spectrometry (Table 3-3). Isothiocyanates were quantified by an external standard method using GC-FID. Allyl isothiocyanate (AITC) (94% purity (Thermo Fisher Scientific, Pittsburgh, PA), propyl isothiocyanate (PITC) (98% purity, Aldrich Chemistry, St. Louis, MO), butyl isothiocyanate (BITC) (99% purity, Aldrich Chemistry, St. Louis, MO) were used. The limit of detection for AITC was 0.918 µg/mL, PITC was 1.108 µg/mL and the limit of detection for BITC was 0.598 µg/mL.

Table 3-2

*Gas Chromatography Flame Ionization Detection: Conditions for Isothiocyanate Analysis*

| | |
|---|---|
| Gas Chromatography Flame Ionization Detection | HP 5890 Series II |
| Column Flow Rate | Nitrogen, 10 mL/min |
| Column (Restek, Bellefont, PA) | Rtx 5<br>0.53 mmID<br>30M<br>1–µm df<br>5% diphenyl<br>95% dimethyl polysiloxane |
| Injector Temperature | 250°C |
| Detector Temperature | 300°C |
| Splitless Injector | 1 µL |
| Software | Chem Station |
| Oven Initial Temperature | 50 °C |
| Oven Initial Temperature Time | 2.00 min |
| Oven Rate | 7.5 C/min |
| Oven Final Temperature | 140 °C |
| Oven Final Time | 2.00 min |

Table 3-3

*Gas Chromatography Mass Spectrometry Settings for Isothiocyanate Analysis*

| | Model | Column | Temperature Program |
|---|---|---|---|
| GC-MS | Agilent 6890 N Quattro Micro GC-MS | DB5 MS | 50°C for 2 min then increase to 200°C @ 15°C / min<br><br>Inlet & transfer line @ 250°C |

To determine isothiocyanate recovery, homogenates of a plant with no glucosinolates, Taraxacum officinale, were spiked with known amounts of AITC, BITC (94% purity, Thermo Fisher Scientific, Pittsburgh, PA), BITC (99% purity, Aldrich Chemistry, St. Louis, MO) and PITC (98% purity, Aldrich Chemistry, St. Louis, MO). The homogenates were treated under the same conditions used for autolysis described above and extracts were prepared in an identical

fashion. These extracts were analyzed using GC FID. This extraction method had a low recovery rate (Table 3-4).

Table 3-4

Recovery of Isothiocyanates for 2010 Method

| Isothiocyanate | % Recovery | Sample Size (±S.D.[a]) |
|---|---|---|
| Allyl isothiocyanate | 37 | 5 (±19) |
| Propyl isothiocyanate | 36 | 5 (±22) |
| Butyl isothiocyanate | 29 | 5 (±24) |

*Note*. [a] = Standard deviation

The low recovery of isothiocyanates using the original method (above) prompted a revision to eliminate the need for the pentane evaporation step as well as avoid performing the autolysis in the presence of the organic solvent. The revised method required the homogenate shake on the orbital shaker without solvent. After 12 hours, 20 mL of hexane (GC Resolv, Fisher Scientific, Pittsburgh, PA) was added to all samples. The flasks were re-sealed and returned to the orbital shaker with the hexane for a minimum of 30 minutes for extraction. To determine isothiocyanate recovery of the revised method, homogenates of a plant with no glucosinolates, Taraxacum officinale, were spiked with known amounts of AITC, BITC (94% purity, Thermo Fisher Scientific, Pittsburgh, PA), BITC (99% purity, Aldrich Chemistry, St. Louis, MO), and PITC (98% purity, Aldrich Chemistry, St. Louis, MO). The homogenates were treated under the same conditions used for autolysis described above and extracts were prepared in an identical fashion. These extracts were analyzed using GC FID. Recovery rate greatly increased (Table 3-5).

Table 3-5

*Recovery of Isothiocyanates for 2016 Method*

| Isothiocyanate | % Recovery | Sample Size (±S.D.[a]) |
|---|---|---|
| Allyl isothiocyanate | 72 | 9 (±22) |
| Butyl isothiocyanate | 77 | 9 (±9) |
| Propyl isothiocyanate | 86 | 9 (±9) |

Note: [a] = Standard deviation

The total sulfur content of homogenate was determined by a modified AOAC method (923.01) (AOAC, 2005). Briefly, samples were digested on a hot plate at 180°C for one hour with a magnesium nitrate solution (950g/L) (Fisher Scientific, Pittsburgh, PA) and then heated in a muffle furnace (Thermolyne 1500, Thermo Scientific, Pittsburgh, PA) at 400°C for two hours. The resulting ash was dissolved by alternating additions of 2mL concentrated (35%) hydrochloric acid (HCL) (Mallinckrodt, St. Louis, MO) and 2mL water (HPLC Grade; Macron, Center Valley, PA). Aliquots (1mL) of the redissolved ash were mixed with 1mL water, 1M sodium bicarbonate (Fisher Scientific, Pittsburgh, PA) (1mL), 0.4mL seeded sulfate (16 mg $K_2SO_4$/300mL water), and 0.6mL of 10% barium chloride solution (Fisher Scientific, Pittsburgh, PA). Sulfate content was measured turbidimetrically on a Beckman DM 530 UV/VIS Spectrophotometer at a wavelength of 430 nm using anhydrous sodium sulfate (Fisher Scientific, Pittsburgh, PA) to prepare standard solutions.

Thiocyanate ion content was determined by high performance liquid chromatography (HPLC). Triethylamine (0.2%) with acetic acid (pH 5) was used as the mobile phase with a C18 column and absorbance detection at 233nm (Table 3-6). The aqueous samples were filtered through

15cm fast flow filter paper (Scientific Products, Evanston, IL) before injection. The limit of detection, based on analysis of standards prepared using KSCN (Fisher Scientific, Pittsburgh, PA), was 0.112 µg $SCN^-$/mL.

Table 3-6

*Conditions Used for HPLC*

| | |
|---|---|
| HPLC Interface | Hitachi D6000 |
| Pump | Hitachi L-6200 |
| Software | D-7000 HPLC System Manager (HSM) |
| Detector | Hitachi L-4200 UV-Vis 233 nm |
| Autosampler | Hitachi AS 2000 |
| Isocratic Solvent Conditions | 95% of 0.2% Triethylamine buffer with glacial Acetic acid (pH 4.89)<br>5% Methanol<br>1ml/min<br>15 min runtime |
| Alltech Econosphere | C18 5 µm 150mm x 4.6 mm |

### 3.1.2 Cell Culture

**3.1.2.1 Human hepatocellular carcinoma (HepG2) cell line**. A human hepatocellular carcinoma (American Type Culture Collection, Manassas, VA) cell line was maintained in standard incubating conditions (5% $CO_2$, 95% air, 37° C). The culture medium used was Hyclone minimum essential media/Earl's balanced salt solution (EME/EBSS) (GE Healthcare Life Sciences, Logan, UT) supplemented with 10% fetal calf serum (PS-FB1, Peak Serum, Wellington, CO), 1% PSN antibiotics (Thermo Fisher Scientific, Grand Island, NY) and 1mM sodium pyruvate (Millipore Sigma, St. Louis, MO).

**3.1.2.2 Human thyroid carcinoma (K1) cell line**. A human thyroid carcinoma cell line (K-1, Millipore Sigma, St. Louis, MO) was maintained under 5% $CO_2$/95% air at $37^0$ C. The medium used was a combination of Dulbecco's Modification of Eagle's Medium (DMEM) (Corning, Tewksbury, MA.), Ham's F12 (Millipore Sigma, St. Louis, MO), and MCDB 105

(Millipore Sigma, St. Louis, MO). The ratio used was as follows: DMEM:Ham's F12: MCDB 105 (2:1:1 v/v/v). The medium was supplemented with 10% fetal calf serum (PS-FB1, Peak Serum, Wellington, CO), 1% PSN antibiotics (Thermo Fisher Scientific, Grand Island, NY), and 2mM of L-Glutamine (GE Healthcare Life Sciences, Logan, UT).

### 3.1.3 Cell Treatments

Cells were cultured in T75 flasks (Cell Treat Scientific Products, Pepperell, MA) until 80% confluent. Adhered cells were removed with 2mL of Trypsin 0.25% EDTA (Life Technology, Carlsbad, CA) and incubated for 5 minutes at $37^0$C. Trypsin was neutralized with 8 mL of medium described above for each cell line. Cells were counted using an inverted microscope (Nikon TMS, Melville, NY). Cells were grown in a 96 well plate at a density of $2.5x10^4$ for HepG2 cells and at a density of $1.5x10^4$ for K1 cells. Cells were left to grow in appropriate treatment-free medium for 24 hours. At 24 hours, medium was removed and replaced with treatment solutions (Table 3-7). Treatments were prepared in the appropriate medium (which was the vehicle) at various concentrations, however, the final volume of medium was constant. Hydrogen peroxide (3%, Greenbrier International Inc., Chesapeake, VA) was the positive control. Experiments were conducted on passages 8-13 for HepG2 cells and on passages 9-14 for K1 cells.

Cells were treated with isothiocyanates added directly to the medium. A *Chorispora tenella* autolysis product was prepared from 10g of plant (fresh weight) blended in phosphate buffered saline (1xPBS) and placed in a sealed flask mixed for at least 12 hours on an orbital shaker (100 rpm) at room temperature (19-21°C). The mixture was sterile filtered with a 0.22µ filter (Cell Treat Scientific Products, Pepperell, MA) before dilution with medium for cell treatments.

Table 3-7

*Concentrations of Isothiocyanate and Chorispora tenella Treatments in HepG2 and K1 Cells*

| Treatment | Concentration[a] (μM or *Chorispora tenella* %) |
|---|---|
| AITC | 10, 30, 70, 100, 175, 250 |
| BITC | 250, 5000, 1000, 1500, 2000, 1500 |
| PITC | 100, 300, 600, 750, 1000, 1250 |
| *Chorispora tenella* | 1%, 5%, 10%, 12%, 15%, 20% |

*Note*. [a] Treatments were final concentrations. Medium was vehicle. Final volume of medium was constant.

**3.1.3.1 Cytotoxicity assessment: 3-(4,5-dimethylthiazol-2-y.)-2,5-diphenyltetrazolium bromide (MTT).** Various concentrations of AITC, BITC, PITC, and *Chorispora tenella* autolysis product mixed with medium and applied at the concentrations listed below (Table 3-7) to the cells for 24 hours. After incubation, cells were washed with 50 μL1xPBS three times followed by addition of 100 μL phenol free medium (Thermo Fisher Scientific, Grand Island, NY) with 10 μL of the stock concentration of 5mg/mL of MTT (Merck Millipore, Billerica, MA) in 1xPBS and incubated for 1 hour. Medium was aspirated off, 50 μL of Dimethyl Sulfoxide (DMSO) (Fisher Chemical, Pittsburgh, PA) was added and incubated for 30 minutes and absorbance read with a Biotek Synergy 2 Plate reader (Winooski, VT) at 540nm.

**3.1.3.2 Mitochondrial activity: Luciferase assay (ATP)**. Various treatments of AITC, BITC, PITC, and *Chorispora tenella* were mixed with media and applied at the concentrations listed above (Table 3-7) to the cells for 24 hours. After incubation cells were washed with 1xPBS three times followed by addition of 100 μL phenol free medium (Thermo Fisher Scientific, Grand Island, NY) and 100 μL of ATP reagents (lyophilized CellTiter-GLo Substrate and CellTiter-GLo Buffer) (Promega, Madison, WI) and put on a shaker for two minutes then incubated at room temperature for 10 minutes. Luminescence was detected using a Biotek Synergy 2 Plate reader (Winooski, VT).

**3.1.3.3 Cell proliferation: Hoechst dye 33258.** Various treatments of AITC, BITC, PITC, and *Chorispora tenella* were mixed with medium and applied at the following concentrations (Table 3-3) to the cells for 24 hours. After incubation, cells were washed with 100 µL of 1xPBS three times followed by addition of 50 µL of 1xPBS and 50 µL of 2uM Hoechst 33258 dye (Invitrogen, Carlsbad, CA) and incubated at $37^0$C for 15 minutes. Fluorescence was read at the following: excitation 355nm and emission 460nm with a Biotek Synergy 2 Plate reader (Winooski, VT).

**3.1.3.4 Thyroglobulin ELISA.** Treated K1 cell media were measured for free thyroglobulin content per the ELISA (Millipore Sigma, Burlington, MA) instructions. An aliquot (100 µL) of medium sample was incubated at room temperature for two and a half hours. The solution was discarded, and plate was washed four times with 1x Wash Buffer (300 µL). Addition of 100 µL of biotinylated primary antibody was followed by incubation for one hour at room temperature with gentle shaking. The solution was discarded, and the wells were washed four times with 1x Wash buffer (300 µL). Steptavidin–Horseradish Peroxidase solution (100 µL) was added to each well and incubated for 45 minutes. The solution was then discarded, and the wells washed four times with 1x Wash Buffer. The 3,3',5,5'-Tetramethylbenzidine substrate (100 µL) was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking on an orbital shaker. To stop the reaction, 50 µL of the stop solution was added. ELISA was read at 450 nm (Biotek Synergy 2 Plate reader, Winooski, VT).

**3.1.4 Statistics**

The $LC_{50}$ values were calculated from linear regression models with Least Squares Analysis. Proportion data transformed with arcsine square root. ANOVAs using Tukey or Dunn post hoc analysis with Prism 6 software (GraphPad, San Diego, CA).

# 4. PLANT ANALYSIS: RESULTS AND DISCUSSION

## 4.1 2010 Gas Chromatography Mass Spectrometry of allyl isothiocyanate, propyl isothiocyanate, and butyl isothiocyanate in *Chorispora tenella*

The 2010 collections were processed with an autolysis method, which included the addition of 20mL of pentane to the homogenized plant material for overnight shaking. The following day, after decanting the solvent layer, there was a solvent exchange to hexane to avoid problems resulting from the volatility of pentane. This method gave a 37% recovery of allyl isothiocyanate, 36% for propyl isothiocyanate, and 29% for butyl isothiocyanate.

Autolysis extracts from *Chorispora tenella* sampled in June 2010 (collection location 1), were analyzed by Gas Chromatography Mass Spectrometry (GCMS) to characterize the constituents of the plant extracts. Figure 4-1 shows the Total Ion Chromatogram (TIC) for *Chorispora tenella.* Three isothiocyanates were detected: allyl isothiocyanate (AITC), propyl isothiocyante (PITC), and butyl isothiocyanate (BITC). A spectrum for the AITC peak from 2010 collection location 1 is shown in Figure 4-2, the PITC peak spectrum from 2010 is shown in Figure 4-3 and the BITC peak spectrum from 2010 is shown in Figure 4-4.

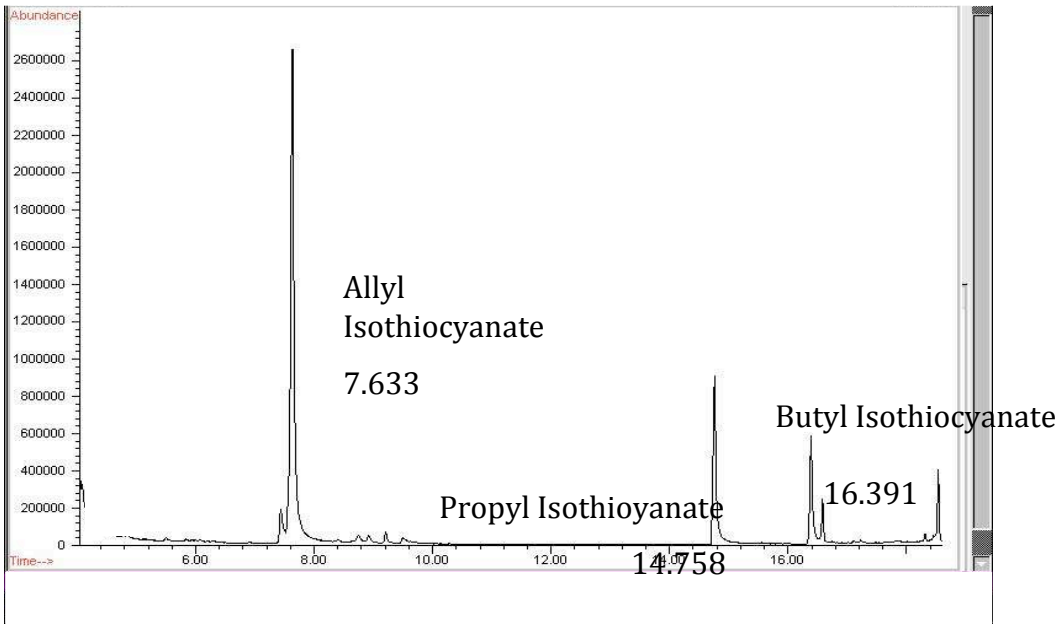


**Figure 4-1.** Total Ion Chromatogram from GCMS Analysis of a *Chorispora tenella* Autolysis Extract from 2010 Collection Location 1

*Note*: Pentane extraction method (refer to Materials and Methods section for details).

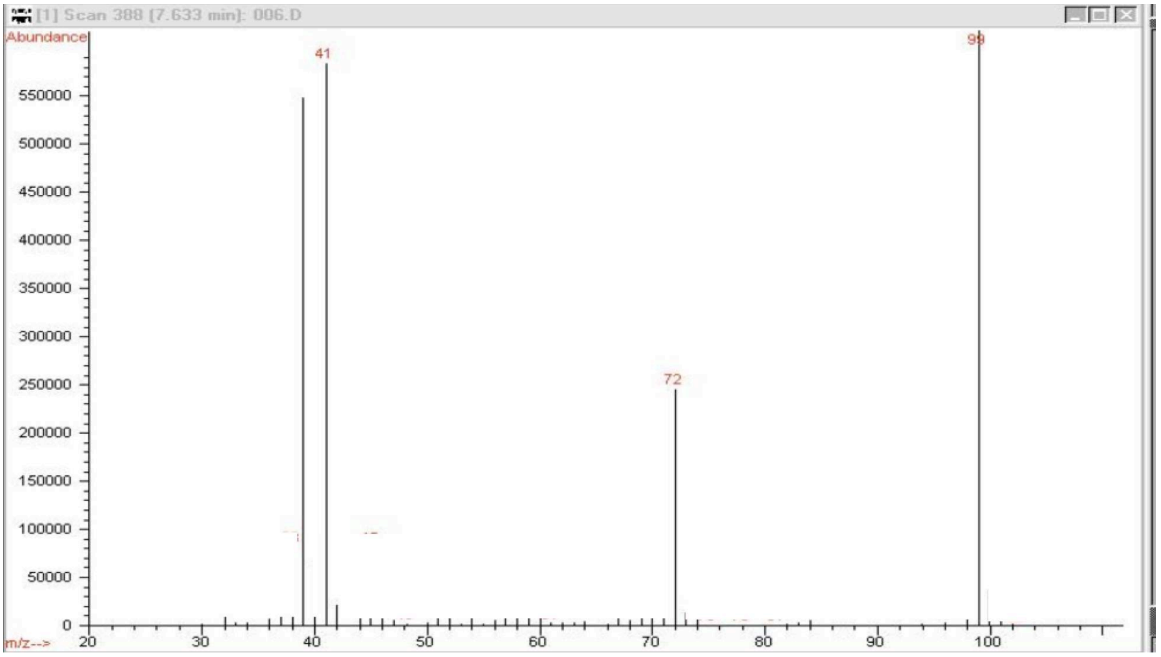


**Figure 4-2.** Allyl isothiocyanate Spectrum from Gas Chromatography Mass Spectrometry Analysis of a *Chorispora tenella* Autolysis Extract from 2010 Collection Location 1

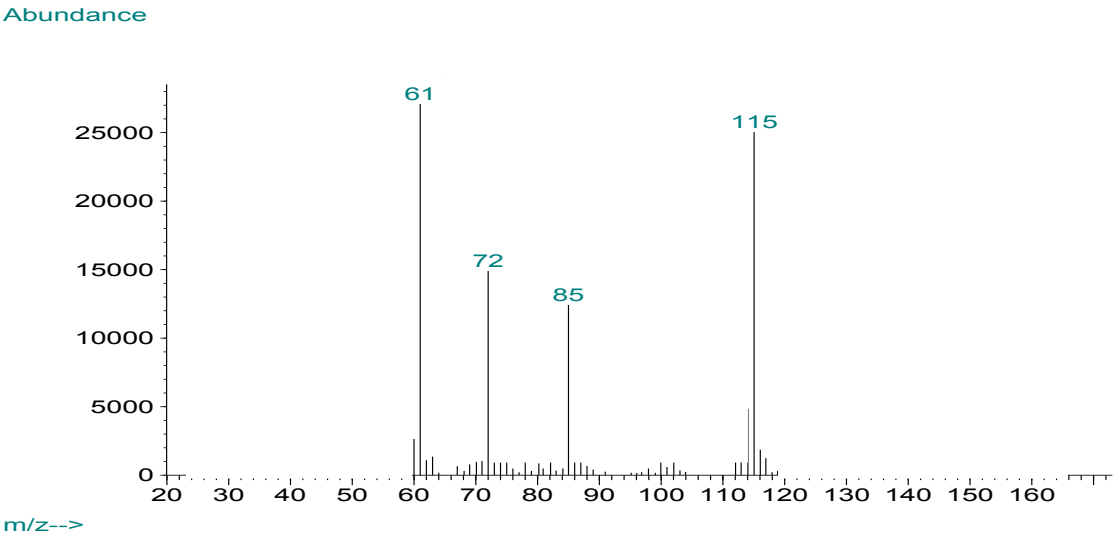


**Figure 4-3.** Propyl isothiocyanate Spectrum from Gas Chromatography Mass Spectrometry from 2010 Collection Location 1

*Note*: Pentane extraction method (refer to Materials and Methods section for details).

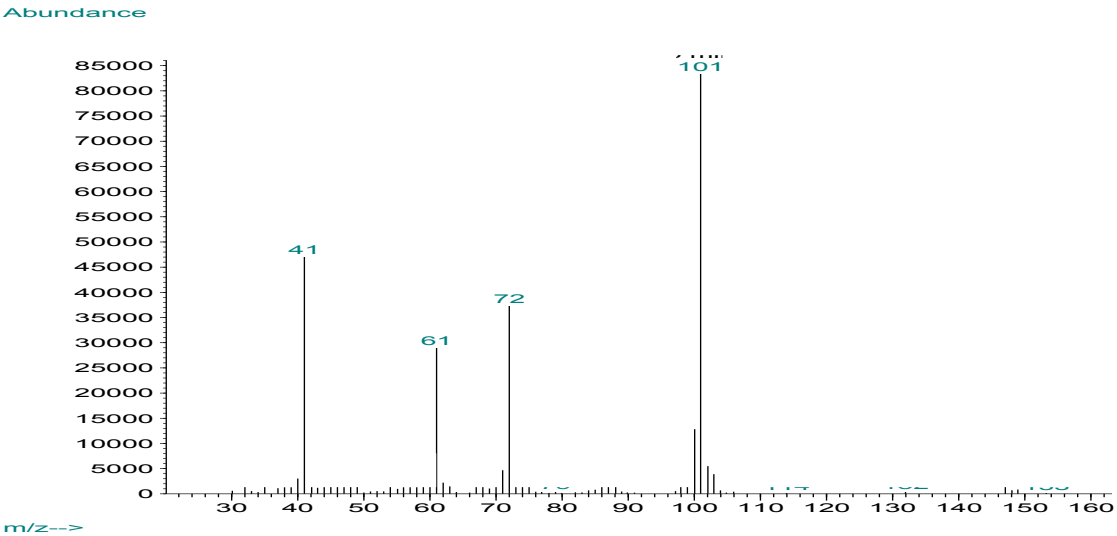


**Figure 4-4**. Butyl isothiocyanate Spectrum from Gas Chromatography Mass Spectrometry Analysis of Chorispora tenella Autolysis Extract from 2010 Collection Location 1

*Note*: Pentane extraction method (refer to Materials and Methods section for details).

### 4.2 2016 Gas Chromatography Mass Spectrometry of allyl isothiocyanate, propyl isothiocyanate and butyl isothiocyanate in *Chorispora tenella*

Analysis of 2016 collection samples used a method that did not include addition of the organic solvent during overnight autolysis and resulted in a recovery of 72% for allyl isothiocyanate, 77% for butyl isothiocyanate, and 86% for propyl isothiocyanate. Standards (Figure 4-5) and autolysis extracts from *Chorispora tenella* were also analyzed in 2016 by GCMS. Figures 4-5, 4-6, 4-7, and 4-8 show the Total Ion Chromatogram (TIC) for the standards and the spectra for allyl isothiocyanate, butyl isothiocyanate, and propyl isothiocyanate, respectively. The TIC for the *Chorispora tenella* (Figure 4-9) shows that allyl isothiocyanate was present, however propyl isothiocyanate, and butyl isothiocyanate were not detected. Figure 4-10 shows the comparison of mass spectrum for allyl isothiocyanate from *Chorispora tenella* 2016 collection 1b (top) to library mass spectrum (bottom). Table 4-1 refers to the total ion chromatogram for the standards of ATIC, PITC, and BITC.

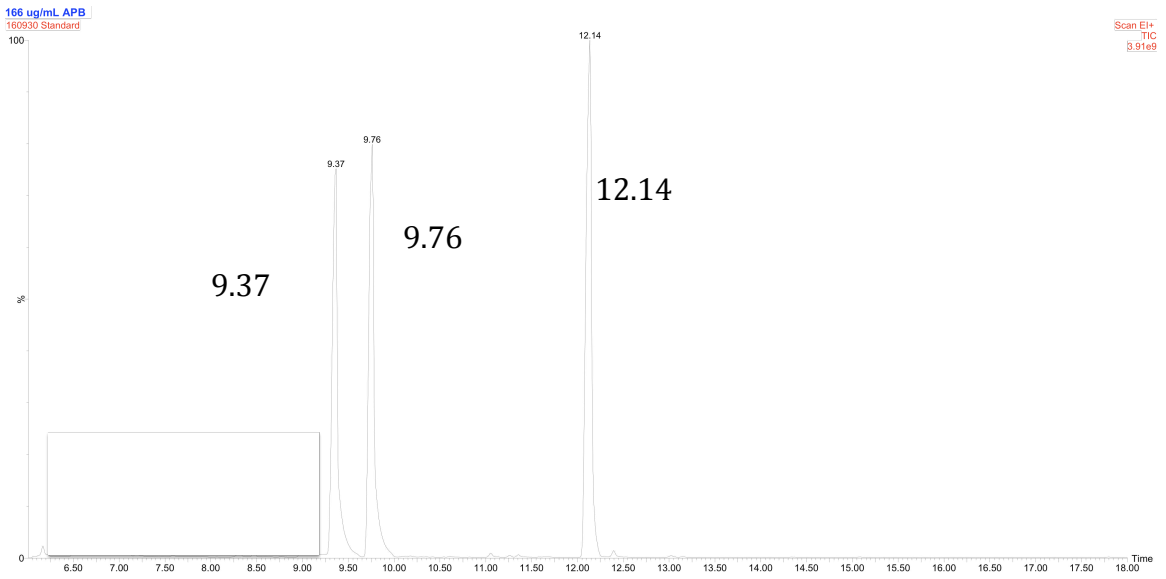


**Figure 4-5**. Total Ion Chromatogram of Standards allyl isothiocyanate, propyl isothiocyanate and butyl isothiocyanate from Gas Chromatography Mass Spectrometry in 2016

*Note*: Hexane extraction method (refer to Materials and Methods section for details).

Table 4-1.

*Standards of allyl isothiocyanate, propyl isothiocyanate, butyl isothiocyanate Analyzed on Gas Chromatography Mass Spectrometry in 2016*

| Isothiocyanate | Retention Time (mins.) |
|---|---|
| Allyl Isothiocyanate | 9.37 |
| Propyl Isothiocyanate | 9.76 |
| Butyl Isothiocyanate | 12.14 |

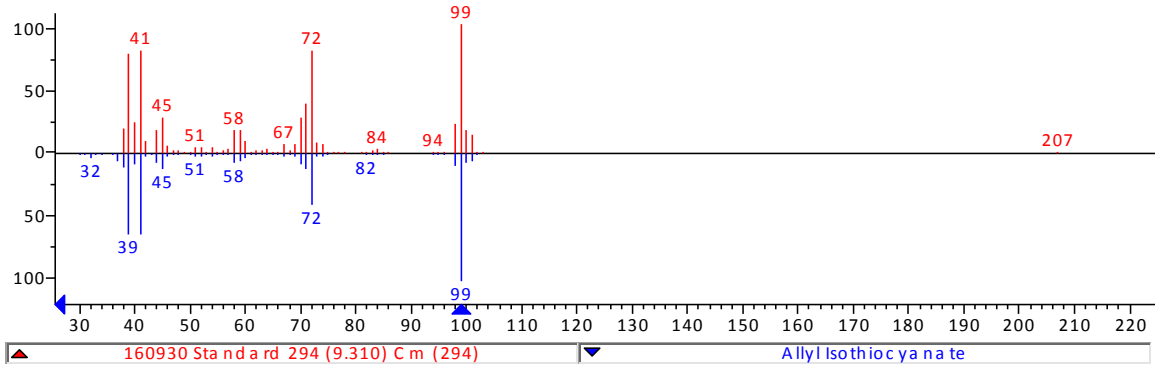


**Figure 4-6.** Comparison of Mass Spectrum from 2016 Standard for allyl isothiocyanate Standard (top) to Library Mass Spectrum (bottom).

*Note*. Hexane extraction method (refer to Materials and Methods section for details).

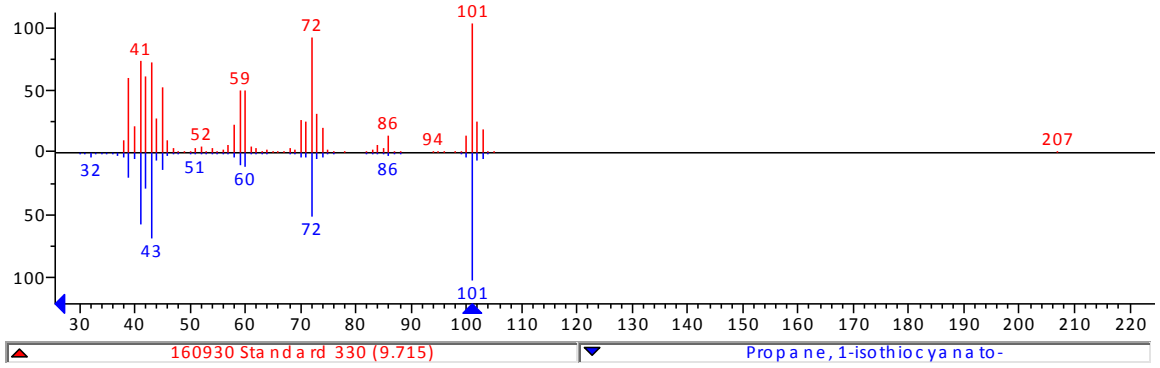


**Figure 4-7.** Comparison of mass spectrum from 2016 standard for propyl isothiocyanate standard (top) to library mass spectrum (bottom).

*Note*. Hexane extraction method (refer to Materials and Methods section for details).

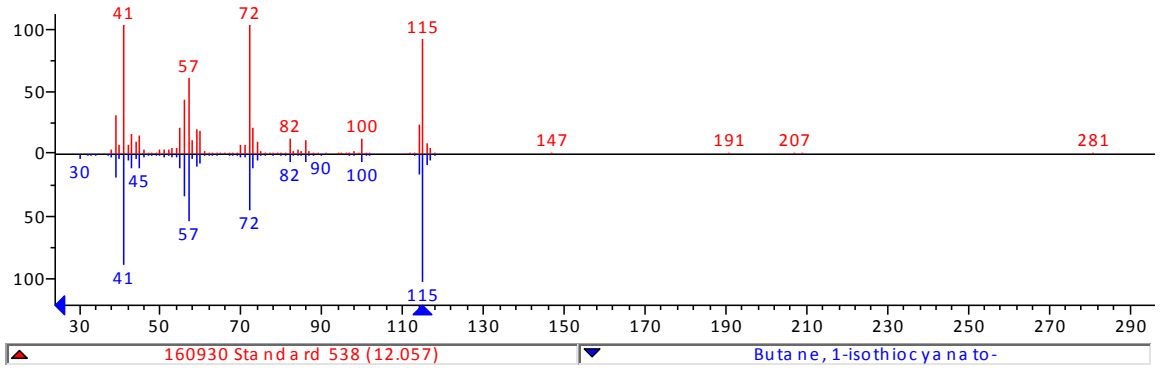


**Figure 4-8.** Comparison of mass spectrum from 2016 standard for butyl isothiocyanate standard (top) to library mass spectrum (bottom).

*Note*. Hexane extraction method (refer to Materials and Methods section for details).

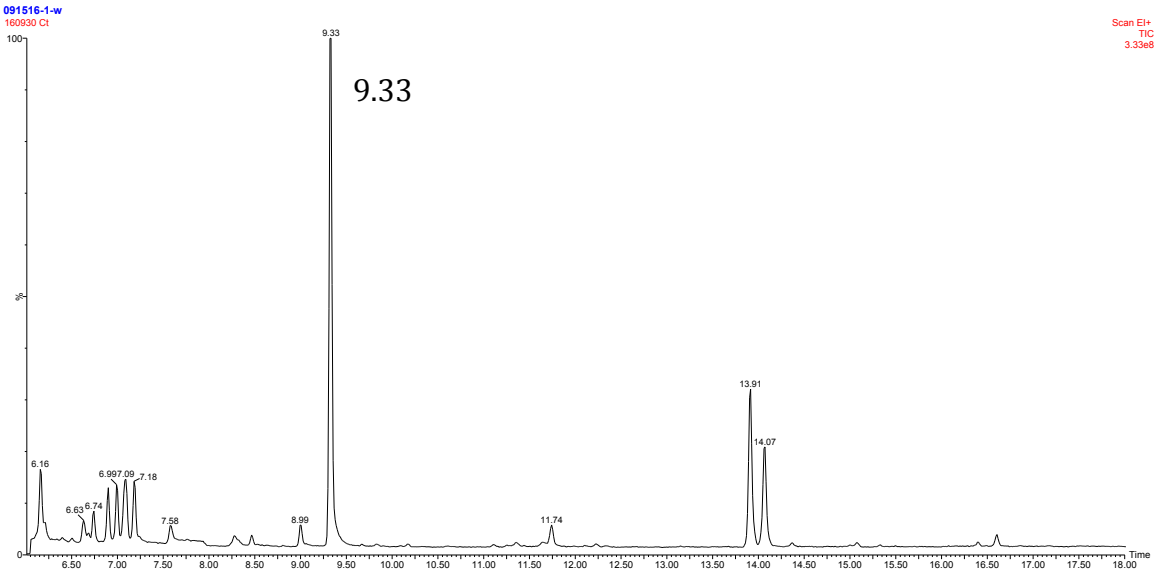


**Figure 4-9.** Total Ion Chromatogram from Gas Chromatrogrphy Mass Spectrometry for *Chorispora tenella* autolysis extract from 2016 collection location 1.

*Note*. Hexane extraction method (refer to Materials and Methods section for details).

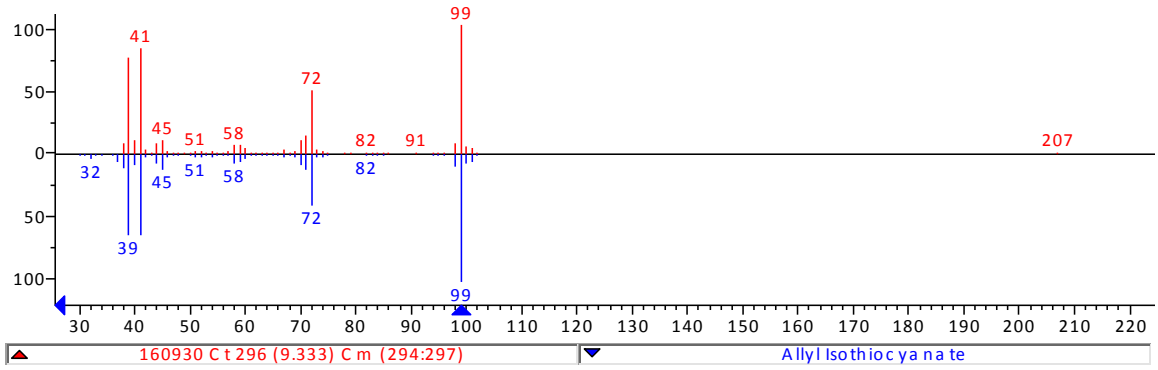


**Figure 4-10.** Comparison of Mass spectrum for allyl isothiocyanate from *Chorispora tenella* 2016 Collection Location 1 (top) to Library Mass Spectrum (bottom)

## 4.3 2010 Gas Chromatography Flame Ionization Detection Quantification of Isothiocyanates in *Chorispora tenella*

### 4.3.1 Allyl Isothiocyanate

Autolysis extracts from *Chorispora tenella* samples in 2010 were analyzed with Gas Chromatography Flame Ionization Detection (GC-FID) to quantify isothiocyanate content. Table 4-2 shows that allyl isothiocyanate in *Chorispora tenella* from 2010 varied in concentration in the various parts of the plant. Whole plant (W), consisting of the entire plant including leaves, flowers, stems, and seeds if applicable; leaves (L), consisting only of leaves; and, seeds (S), consisting only of seeds. Briefly, the seeds (S) had the highest concentration of AITC, followed by the whole plant (W), followed by the leaves (L). Whole plant AITC concentration was significantly different than that of leaves and seeds ($p=0.025$ Tukey). Allyl isothiocyanate concentration was significantly different in seeds compared to that in leaves ($p<0.0001$ Tukey).

Table 4-2

*2010 Chorispora tenella Autolysis Gas Chromatography Flame Ionization Detection of allyl isothiocyanate*

| Plant Part | µg Allyl Isothiocyanate/g Fresh Plant |
|---|---|
| Whole Plant | 756 ± 111[a] |
| Leaves | 389 ± 62 [a,b,c] |
| Seeds | 1123 ± 96 [a,b] |

*Note.* See Materials and Methods section for details. Collection Location 1. Sample size n=5.
[a] Mean ± Standard deviation. [b] Significantly different from whole plant, p=0.025 (Tukey). [c] Significantly different from seeds, p<0.0001 (Tukey).

### 4.3.2 Propyl Isothiocyanate

Table 4-3 shows the results from 2010 *Chorispora tenella* autolysis Gas Chromatography Flame Ionization Detection of propyl isothiocyanate. Propyl isothiocyanate in seeds of *Chorispora tenella* was significantly different from concentration of whole plant (p<0.0001 Tukey) and from leaves (p=0.026 Tukey).

Table 4-3

*2010 Chorispora tenella Autolysis Gas Chromatography Flame Ionization Detection of propyl isothiocyanate*

| Plant Part | µg Propyl Isothiocyanate/g Fresh Plant |
|---|---|
| Whole Plant | 143 ± 21[a] |
| Leaves | 346 ± 26 [a] |
| Seeds | 710 ± 42 [a,b,c] |

*Note.* See Materials and Methods section for details. Collection location 1. Sample size n=5. [a] Mean ± Standard deviation. [b] Significantly different from whole plant, p<0.0001 (Tukey). [c] Significantly different from leaves, p=0.026 (Tukey).

### 4.1.3 Butyl Isothiocyanate

Table 4-4 shows the results from 2010 *Chorispora tenella* autolysis Gas Chromatography Flame Ionization Detection of butyl isothiocyanate. Butyl isothiocyanate in seeds of *Chorispora tenella* were significantly different from whole plant (p=0.001 Tukey) and from leaves (p=0.006 Tukey). Butyl Isothiocyanate in leaves of *Chorispora tenella* was significantly different from whole plant (p=0.001 Tukey).

Table 4-4

*2010 Chorispora tenella autolysis Gas Chromatography Flame Ionization Detection of butyl isothiocyanate*

| Plant Part | µg Butyl Isothiocyanate/g Fresh Plant |
|---|---|
| Whole Plant | 102 ± 13[a] |
| Leaves | 711 ± 45 [a,b] |
| Seeds | 1340 ± 47 [a,b,c] |

*Note*. See Materials and Methods section for details. Collection location 1. Sample size n=5. [a] Mean ± Standard deviation. [b] Significantly different from whole plant, p=0.001 (Tukey). [c] Significantly different from leaves, p=0.006 (Tukey).

### 4.4 2016 Gas Chromatography Flame Ionization Detection Quantification of Isothiocyanates in *Chorispora tenella*

Chromatograms from 2016 of standards (Figure 4-11; Table 4-5) show all three isothiocyanates, AITC, PITC and BITC that were analyzed at the same time as *Chorispora tenella* whole plant (Figure 4-12) and *Chorispora tenella* leaves (Figure 4-13). *Chorispora tenella* seeds were analyzed on a separate date and the standards for AITC, PITC and BITC that correspond to the seed analysis are shown in Figure 4-14 and Table 4-6. *Chorispora tenella* seeds (Figure 4-15) show only allyl isothiocyanate is present; propyl isothiocyanate and butyl isothiocyanate are not detected.

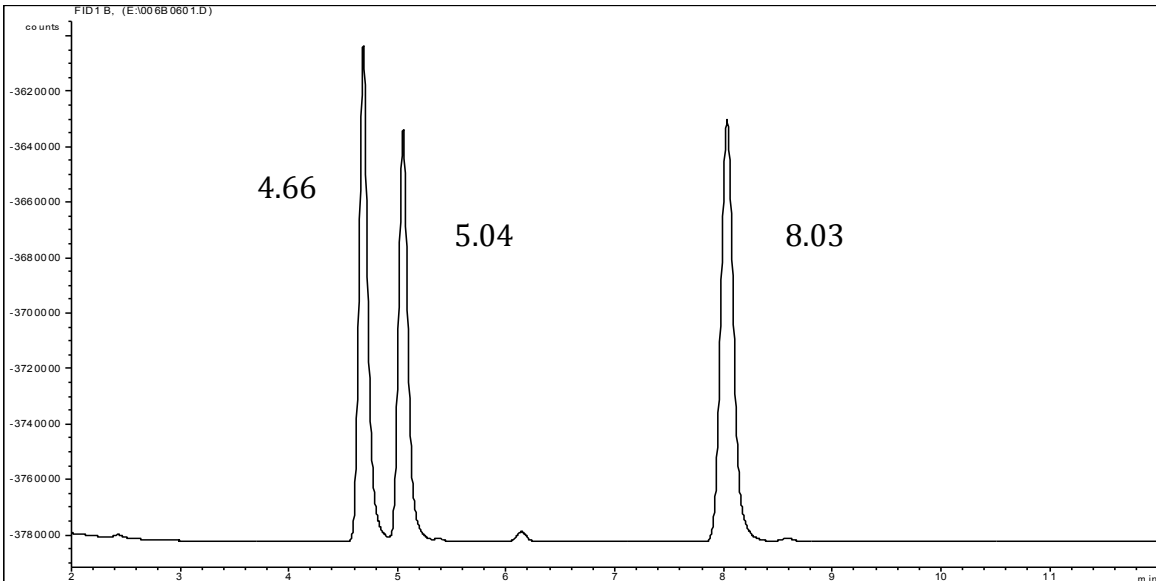


**Figure 4-11.** 2016 Gas Chromatography Flame Ionization Detection of three isothiocyanate standards: allyl isothiocyanate, propyl isothiocyanate and butyl isothiocyanate. *Note*. Hexane extraction method (refer to Materials and Methods section for details).

Table 4-5

*Standards of allyl isothiocyanate, propyl isothiocyanate, butyl isothiocyanate analyzed on Gas Chromatography Flame Ionization Detector for 2016 Chorispora tenella Whole and Chorispora tenella Leaves*

| Isothiocyanate | Retention Time (mins.) |
|---|---|
| Allyl Isothiocyanate | 4.66 |
| Propyl Isothiocyanate | 5.04 |
| Butyl Isothiocyanate | 8.03 |

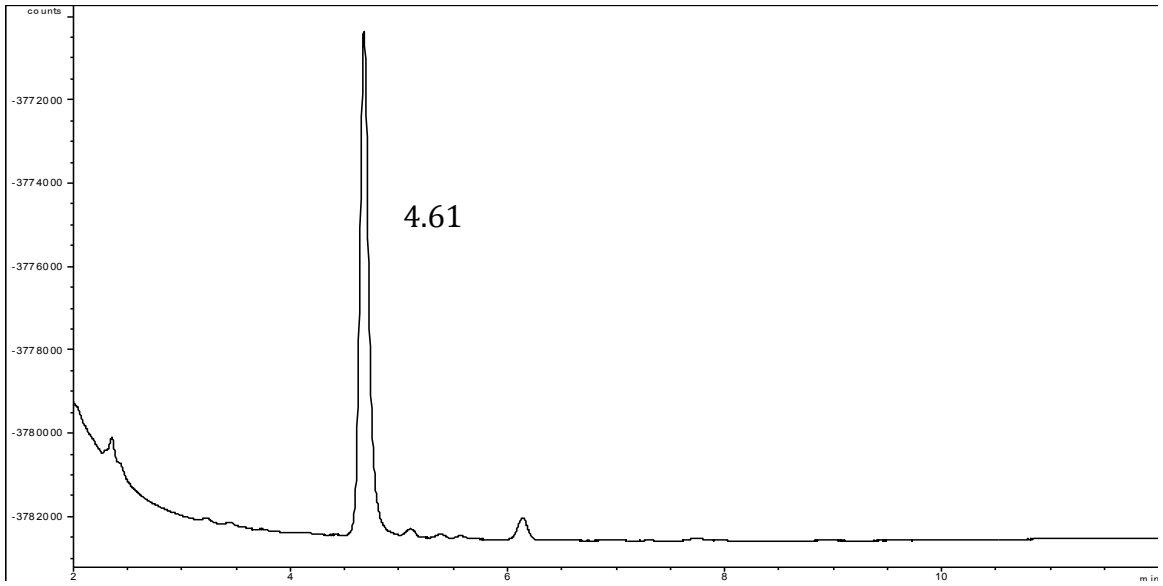


**Figure 4-12.** 2016 Gas Chromatography Flame Ionization Detection of allyl isothiocyanate in *Chorispora tenella* Whole Plant

*Note*. Collection site 1. Hexane extraction method (refer to Materials and Methods section for details).

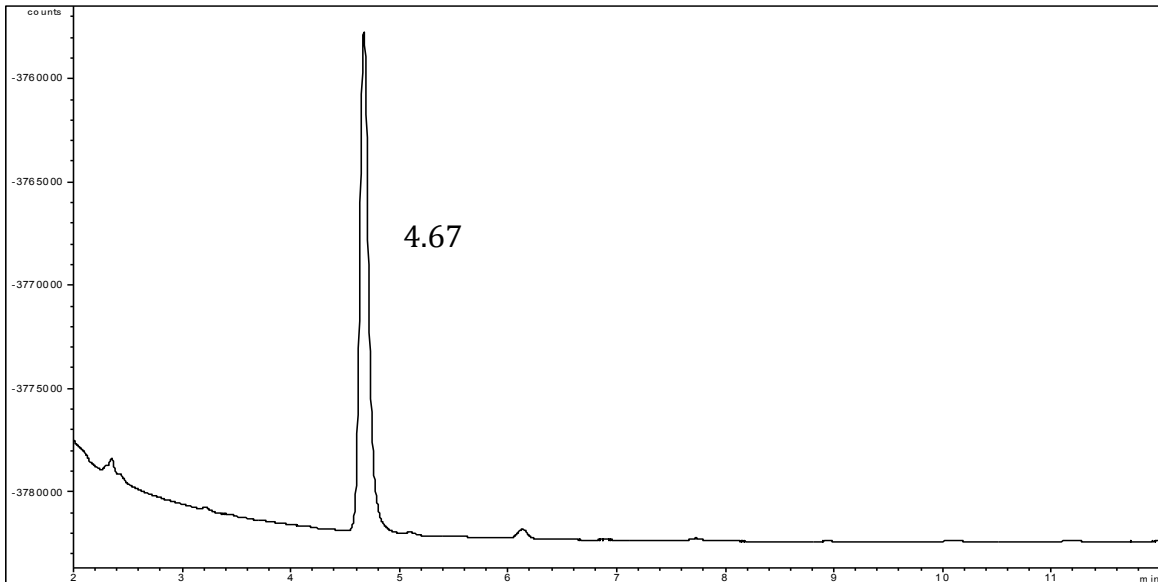


**Figure 4-13.** 2016 Gas Chromatography Flame Ionization Detection of allyl isothiocyanate in *Chorispora tenella* Leaves

*Note*. Collection site 1. Hexane extraction method (refer to Materials and Methods section for details).

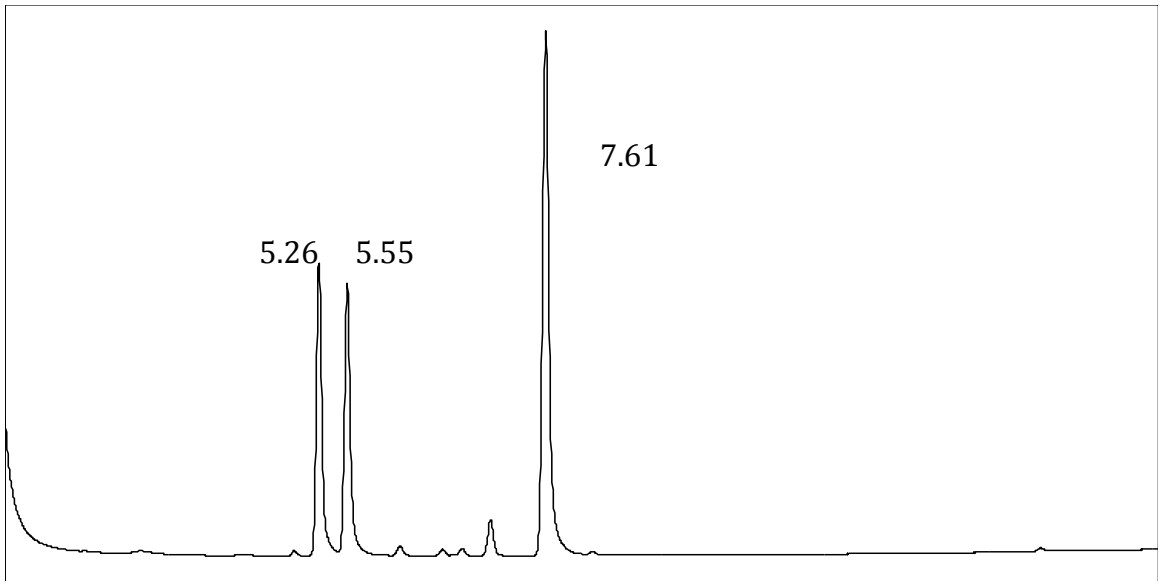


**Figure 4-14.** 2016 Gas Chromatography Flame Ionization Detection of standards allyl isothiocyanate, propyl isothiocyanate and butyl isothiocyanate.

*Note*. Standards for *Chorispora tenella* seeds.

Table 4-6

*Standards of allyl isothiocyanate, propyl isothiocyanate, butyl isothiocyanate Analyzed on Gas Chromatography Flame Ionization Detector for 2016 Chorispora tenella Seeds*

| Isothiocyanate | Retention Time (min) |
|---|---|
| Allyl Isothiocyanate | 5.26 |
| Propyl Isothiocyanate | 5.55 |
| Butyl Isothiocyanate | 7.61 |

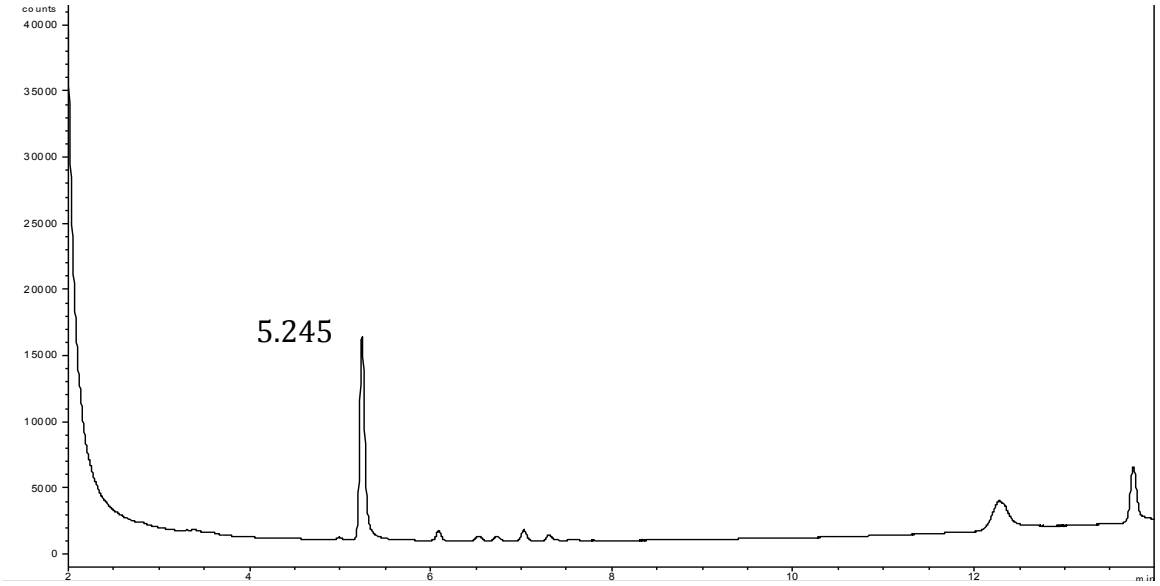


**Figure 4-15**. 2016 Gas Chromatography Flame Ionization Detection of allyl isothiocyanate in *Chorispora tenella* Seeds

*Note*. Collection site 1. Hexane extraction method (refer to Materials and Methods section for details).

Autolysis extracts from 2016 show allyl isothiocyanate in *Chorispora tenella* whole plant (Table 4-7), leaves (Table 4-8), and seeds (Table 4-9). There is no statistical difference between sampling locations for allyl isothiocyanate in whole plant or leaves, however, in seeds (Table 4-9), location 3 is significantly different from 6b ($p=0.00465$ Tukey); location 4b is significantly different from 6b ($p=0.0002$ Tukey); location 6b is significantly different from location 1 ($p=0.0363$ Tukey).

Table 4-7

*2016 Data for allyl isothiocyanate in Chorispora tenella Autolysis Extracts for Whole Plant*

| Sampling Location | µg Allyl Isothiocyanate/g Fresh Plant |
|---|---|
| 1b | 82 ± 19.62[a] |
| 2 | 28 ± 7.89 [a] |
| 3 | 83 ± 8.23 [a] |
| 4b | 69 ± 26.64 [a] |
| 5 | 99 ± 27.20 [a] |
| 6b | 125 ± 90.12 [a] |

*Note.* See Materials and Methods section for details. Sample size n=3 for locations 2, 3, 4, 5. Sample size n=9 for location 1. Sample size n=21 for location 6. No statistical significant between sampling locations (One way ANOVA and Tukey's post hoc test). [a] Mean ± Standard deviation.

Table 4-8

*2016 Data for allyl isothiocyanate in Chorispora tenella Autolysis Extracts for Leaves*

| Sampling Location | µg Allyl Isothiocyanate/g Fresh Plant |
|---|---|
| 1b | 50 ± 12.07 [a] |
| 2 | 41 ± 7.832 [a] |
| 3 | 94 ± 12.08 [a] |
| 5 | 137 ± 7.30 [a] |
| 6b | 101 ± 36.98 [a] |

*Note.* See Materials and Methods section for details. Sample size n=3 for locations 1b, 2, 3, 5. Sample size n=15 for location 6b. No statistical significant between sampling locations (One way ANOVA and Tukey's post hoc test).
[a] Mean ± Standard deviation.

Table 4-9

*2016 Data for allyl isothiocyanate in Chorispora tenella Autolysis Extracts for Seeds*

| Sampling Location | µg Allyl isothiocyanate/g Fresh Plant |
|---|---|
| 1b | 55 ± 8.37 [a] |
| 3 | 97 ± 5.69[a,b] |
| 4b | 120 ± 31.29 [a,c] |
| 6b | 24 ± 6.32 [a.d] |

*Note.* See Materials and Methods section for details. Sample size n=3 for locations 1, 3. Sample size n=5 for location 6b. Sample size n= 14 for location 4b. [a] Mean ± Standard deviation. [b] Location 3 significantly different from 6b, p=0.00465 (Tukey). [c] Location 4b significantly different from 6b, p=0.0002 (Tukey).
[d] Location 6b significantly different form 1, p=0.0363 (Tukey).

Autolysis extracts from 2017 shows allyl isothiocyanate in *Chorispora tenella* whole plant (Table 4-10). At location 4, allyl isothiocyanate content is statistically different from that at sampling locations 6 and 1.

Table 4-10

*2017 data for allyl isothiocyanate in Chorispora tenella Autolysis Extracts for Whole Plant*

| Sampling Location | µg Allyl isothiocyanate/g Fresh Plant |
|---|---|
| 1c | 61 ± 15.59 [a] |
| 4c | 148 ± 15.30 [a,b,c] |
| 6c | 91 ± 18.43 [a] |

*Note.* See Materials and Methods section for details. Sample size n=5. One way ANOVA and Tukey's post hoc test. [a] Mean ± Standard deviation. [b] Location 4c significantly different from 6c, p=0.006 (Tukey). [c] Location 4c significantly different from 1c, p=0.0002 (Tukey).

Comparisons were made between allyl isothiocyanate content in *Chorispora tenella* measured in 2010, 2016, and 2017 collections. Whole plant allyl isothiocyanate content in 2010 was significantly different from 2016 and 2017 (all sites combined) ($p<0.0001$ Tukey) (Table 4-11).

Table 4-11

*Allyl isothiocyanate Content Comparisons Between 2010, 2016 & 2017 in Chorispora tenella Whole Plant*

| Year | µg Allyl isothiocyanate/g Fresh Plant |
|---|---|
| 2010 | 755 ± 111.20 [a,b,c] |
| 2016 | 114 ± 76.76 [a] |
| 2017 | 100 ± 88.40 [a] |

*Note.* See Materials and Methods section for details. Sample size n=5 for 2010, n=30 for 2016, n=15 for 2017. One way ANOVA and Tukey's post hoc test. [a] Mean ± Standard deviation. [b, c] 2010 significantly different from 2016 and 2017, p=0.006 (Tukey).

### 4.4.1 Total Sulfur Analysis

**Chorispora tenella.** Total sulfur analysis was conducted in 2010 on *Chorispora tenella* to determine if excessive quantities of sulfur might cause toxic effects in livestock (Table 4-12).

The total sulfur content in *Chorispora tenella* autolysis extracts were measured turbidimetrically. In 2010, there is no significant difference between sulfur content of various plant parts, based on One way ANOVA and Tukey's post hoc test.

Table 4-12

*Total Sulfur Concentration in Whole Plant, Leaves and Seeds of Chorispora tenella from 2010*

| Plant Part | mg Total S/g Fresh Weight | Range |
|---|---|---|
| Whole | 0.09 ± 0.04 [a] | 0.02 – 0.14 |
| Leaves | 0.07 ± 0.04 [a] | 0.02 – 0.12 |
| Seeds | 0.06 ± 0.05 [a] | 0.00 – 0.13 |

*Note.* Refer to Materials and Methods section for details. N=5. No significant difference between sulfur content of various plant parts. One way ANOVA and Tukey's post hoc test. [a] = Mean ± Standard deviation.

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In 2016, *Chorispora tenella* was analyzed for total sulfur content in the whole plant (Table 4-13). Again, there was no statistical difference in total sulfur at any sampling location for whole plant based on one way ANOVA and Tukey's post hoc test. *Chorispora tenella* leaves (Table 4-14) and seeds (Table 4-15) were also analyzed for total sulfur content. There was no statistical difference in total sulfur at any sampling location for the leaves of *Chorispora tenella* based on one way ANOVA and Tukey's post hoc test.

Table 4-13

*Total Sulfur Concentration in Whole Plant Chorispora tenella 2016*

| Collection | mg S/g Fresh Plant | Range |
|---|---|---|
| 1b | 0.25 ± 0.17[a] | 0.10 – 0.61 |
| 2 | 0.13 ± 0.08[a] | 0.06 – 0.21 |
| 3 | 0.24 ± 0.12[a] | 0.10 – 0.35 |
| 4b | 0.23 ± 0.09[a] | 0.16 – 0.34 |
| 5 | 0.13 ± 0.04[a] | 0.08 – 0.16 |
| 6b | 0.19 ± 0.08[a] | 0.04 – 0.34 |

*Note.* Refer to Materials and Methods section for details. No significant difference between sulfur content of whole plant in different sampling locations. One way ANOVA and Tukey's post hoc test. [a] Mean ± Standard deviation

Table 4-14

*Total Sulfur Concentration in Leaves Chorispora tenella 2016*

| Sampling Location | mg S/g Fresh Plant | Range |
|---|---|---|
| 1b | 0.15 ± 0.06 [a] | 0.13 – 0.22 |
| 2 | 0.13 ± 0.04 [a] | 0.08 – 0.16 |
| 3 | 0.23 ± 0.12 [a] | 0.15 – 0.37 |
| 5 | 0.30 ± 0.03 [a] | 0.27 – 0.33 |
| 6b | 0.28 ± 0.20 [a] | 0.09 – 0.88 |

*Note.* Refer to Materials and Methods section for details. No significant difference between sulfur content of leaves in different sampling locations. One way ANOVA and Tukey's post hoc test. [a] Mean ± Standard deviation.

Table 4-15

*Total Sulfur Concentration in Seeds in Chorispora tenella 2016*

| Collection | mg S/g Fresh Plant | Range |
|---|---|---|
| 1b | 0.14 ± 0.02 [a] | 0.12 – 0.17 |
| 3 | 0.16 ± 0.12 [a] | 0.04 – 0.27 |
| 4a | 0.23 ± 0.10[a] | 0.11 – 0.40 |
| 6a | 0.13 ± 0.04 [a] | 0.07 – 0.15 |

*Notes*. Refer to Materials and Methods section for details. No significant difference between sulfur content of seeds in various sampling locations. One way ANOVA and Tukey's post hoc test. [a] Mean ± Standard deviation.

**Descurainia sophia.** *Descurainia sophia*, another plant in the Brassicaceae family, was analyzed for total sulfur content. In the 2010 sampling year, *Descurainia sophia* was collected at location 1 and analyzed for total sulfur (Table 4-16). In the 2016 sampling season, *Descurainia sophia* was sampled at location 4. There was no statistical difference in total sulfur content between whole plant, leaves, and seeds in *Descurainia sophia* at location 4 in the 2016 sampling season based on One way ANOVA and Tukey's post hoc tests (Table 4-17).

Table 4-16

*Total Sulfur Concentration in Whole Plant of Descurainia sophia in 2010 at Collection Location 1*

| *Descurainia sophia* | mg S/g Fresh Plant | Range |
|---|---|---|
| Whole plant | 0.12 ± 0.03[a] | 0.08 – 0.16 |

*Note.* Refer to Materials and Methods section for details. [a] Mean ± Standard deviation

Table 4-17

*Total Sulfur Concentration in Whole Plant, Leaves, and Seeds of Descurainia sophia in 2016 at Sampling Location 4*

| Plant Part | mg S/g Fresh Plant | Range |
|---|---|---|
| Whole | 0.12 ± 0.11[a] | 0.03 – 0.35 |
| Leaves | 0.12 ± 0.05 [a] | 0.05 – 0.16 |
| Seeds | 0.08 ± 0.02 [a] | 0.06 – 0.10 |

*Note*. Refer to Materials and Methods section for details. No significant difference between sulfur content of whole plant, leaves and seeds at location 4. One way ANOVA and Tukey's post hoc test. [a] Mean ± Standard deviation.

**Sisymbrium altissimum.** The other Brassicaceae species collected in this study was *Sisymbrium altissimum*. The total sulfur content in the 2016 season samples at location 4 showed no significant different between *Sisymbrium altissimum* leaves and seeds (unpaired T test, p=0.458) (Table 4-18).

Table 4-18

*Total Sulfur Concentration in Whole Plant, Leaves and Seeds of Sisymbrium altissimum in 2016 at Sampling Location 4*

| Plant Part | mg S/g Fresh Plant | Range |
|---|---|---|
| Leaves | 0.29 ± 0.10 [a] | 0.21 – 0.39 |
| Seeds | 0.21 ± 0.11 | 0.13 – 0.29 |

*Note*. Refer to Materials and Methods section for details. No significant difference between sulfur content of leaves and seeds. Unpaired T test (p=0.458). [a] Mean ± Standard deviation.

### 4.4.2 Total Sulfur Comparison Between *Chorispora tenella, Descurainia sophia* and *Sisymbrium altissimum*

Total sulfur content was measured in *Chorispora tenella, Descurainia sophia,* and *Sisymbrium altissimum* samples in 2016 to determine if there is a significant difference between these Brassicaceae family plants. All plants were collected at location 4. There was no statistical difference in total sulfur content in the leaves of various plants based on one way ANOVA and Tukey post hoc tests (Table 4-19). There was no statistical difference in total sulfur content in the seeds of different plants based on one way ANOVA and Tukey post hoc tests (Table 4-20).

There was no significant difference between total sulfur in whole plant *Chorispora tenella* and *Descurainia sophia* at location 4 in 2016 (Table 4-21). There was no significant difference between total sulfur in whole *Chorispora tenella* and *Descurainia sophia* at location 1 in 2010 (Table 4-22).

Table 4-19

*Total Sulfur in Leaves of Chorispora tenella, Descurainia sophia and Sisymbrium altissimum in 2016*

| Plant | mg S/g Fresh Plant | Range |
|---|---|---|
| *Chorispora tenella* | 0.28 ± 0.20 [a] | 0.09 – 0.88 |
| *Descurainia sophia* | 0.12 ± 0.05 [a] | 0.06 – 0.16 |
| *Sisymbrium altissimum* | 0.29 ± 0.10 [a] | 0.21 – 0.39 |

*Note.* Refer to Materials and Methods section for details. No significant difference between sulfur content of the plants at location 4. One way ANOVA and Tukey's post hoc test. Minimum of n=3. [a] = Mean ± Standard deviation.

Table 4-20

*Total Sulfur in Seeds of Chorispora tenella, Descurainia sophia, and Sisymbrium altissimum in 2016*

| Plant | mg S/g Fresh Plant | Range |
|---|---|---|
| *Chorispora tenella* | 0.13 ± 0.04[a] | 0.07 – 0.15 |
| *Descurainia sophia* | 0.08 ± 0.02 [a] | 0.06 – 0.10 |
| *Sisymbrium altissimum* | 0.21 ± 0.11 [a] | 0.13 – 0.29 |

*Note*. Refer to Materials and Methods section for details. No significant difference between sulfur content of the different plants at location 4. One way ANOVA and Tukey's post hoc test. Minimum of n=3. [a] Mean ± Standard deviation

Table 4-21

*Total Sulfur in Whole Plant of Chorispora tenella and Descurainia sophia in 2016 from Sampling Location 4*

| Plant | mg S/g Fresh Plant | Range |
|---|---|---|
| *Chorispora tenella* | 0.19 ± 0.08 [a] | 0.04 – 0.34 |
| *Descurainia sophia* | 0.12 ± 0.11 [a] | 0.03 – 0.35 |

*Note.* Refer to Materials and Methods section for details. No significant difference between sulfur content of the different plants at location 4. Unpaired T test shows no significant difference (p=0.065). N=5. [a] Mean ± Standard deviation.

Table 4-22

*Total Sulfur in Whole Plant of Chorispora tenella and Descurainia sophia in 2010 from Sampling Location 1*

| Plant | mg S/g Fresh Plant | Range |
|---|---|---|
| *Chorispora tenella* | 0.09 ± 0.05 [a] | 0.02 – 0.14 |
| *Descurainia sophia* | 0.12 ± 0.03 [a] | 0.09 – 0.17 |

*Note.* Refer to Materials and Methods section for details. No significant difference between sulfur content of the different plants at location 1. Unpaired T test shows no significant difference (p=0.213). N=5. [a] Mean ± Standard deviation.

### 4.4.3 High Performance Liquid Chromatography Analysis of Thiocyanate Anion in *Chorispora tenella*, *Descurainia sophia,* and *Sisymbrium altissimum*

To determine if any glucosinolate parent product is metabolized to thiocyanate anion, high performance liquid chromatography (HPLC) analysis was done on *Chorispora tenella* autolysis aqueous fraction. Standards samples of thiocyanate anion showed a peak with retention of 2.73 minutes (Figure 4-16a). Peaks corresponding to the retention time of the thiocyanate ion standard were not apparent in autolysis product samples of *Chorispora tenella* whole plant (Figure 4-16b), leaves (Figure 4-16c), or seeds (Figure 4-16d).

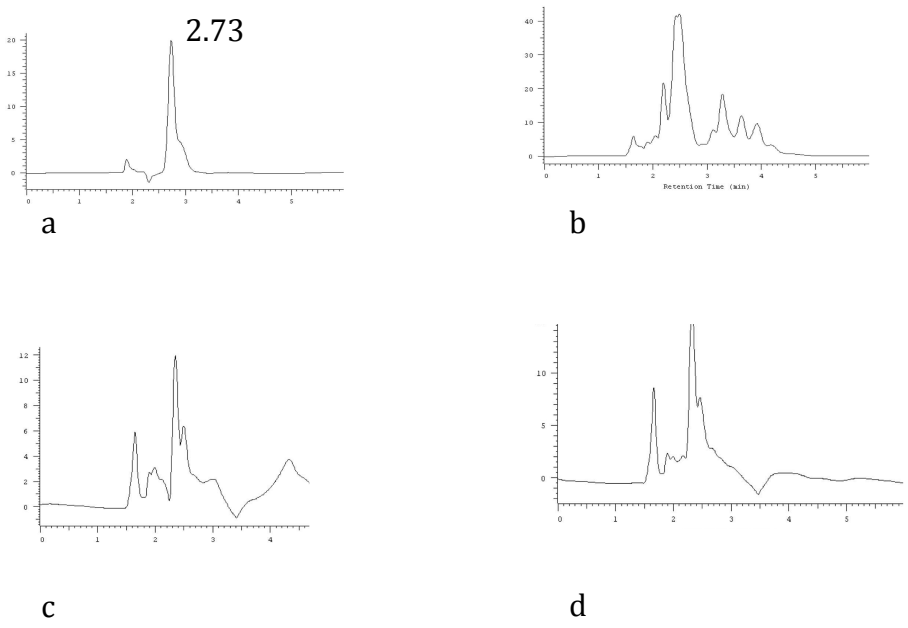



**Figure 4-16**. Thiocyanate Anion Analysis by HPLC

*Note*. a. 50 ng/mL standard; b. Whole plant *Chorispora tenella* autolysis product; c. Seeds; d. Leaves.

The HPLC analysis of *Descurainia sophia* whole plant autolysis product for thiocyanate anion likewise did not show a peak at the retention time of the standard (Figure 4-17). *Sisymbrium altissimum* leaves (Figure 4-18a) and seeds (Figure 4-18b) were also analyzed for thiocyanate anion and there was no peak at the retention time of the thiocyanate anion standard.

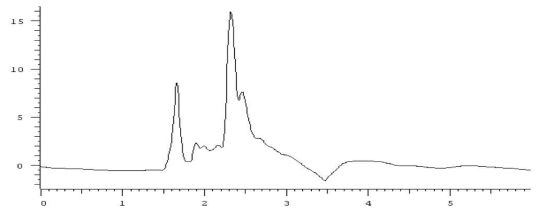
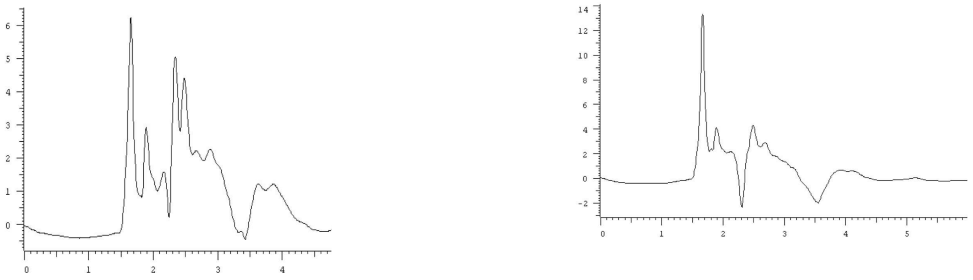


Figure 4-17. HPLC Analysis of thiocyanate anion in *Descurainia sophia* Whole Plant Product



a b

**Figure 4-18.** HPLC Analysis of tnıocyanate anıon in *Sisymbrium altissimum* Autolysis Product.

*Note.* a. leaves; b. seeds

### 4.4.4 Gas Chromatography Flame Ionization Detection Analysis of *Descurainia sophia*

Gas Chromatography Flame Ionization Detection (GC-FID) of three isothiocyanates, AITC, PITC, and BITC (Figure 4-19) was used to analyze *Descurainia sophia* autolysis extracts (Figure 4-20). These results suggested the presence of AITC and PITC in the *Descurainia sophia* autolysis extract; however, this could not be confirmed by GC-MS analysis (see below). In addition, standards (Figure 4-21) of AITC, PITC, and BITC were used to compare leaves and seeds of *Descurainia sophia* autolysis extracts (Figures 4-22 and 4-23) did not indicate the presence of AITC, PITC, or BITC. It is unlikely that a whole plant autolysis extract would contain compounds not found in leaves or seeds.

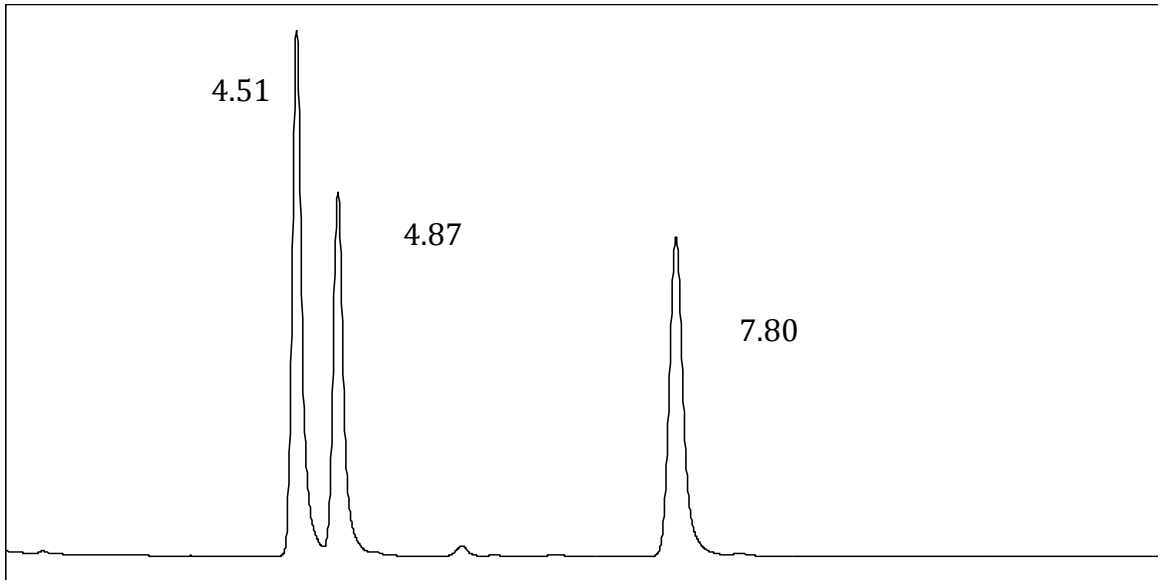



**Figure 4-19.** GC-FID Analysis of allyl isothiocyanate, propyl isothiocyanate and butyl isothiocyanate Standards Analyzed Concurrently with the *Descurainia sophia* Whole Plant Autolysis Extract

*Note*. Refer to Materials and Methods for analysis details.

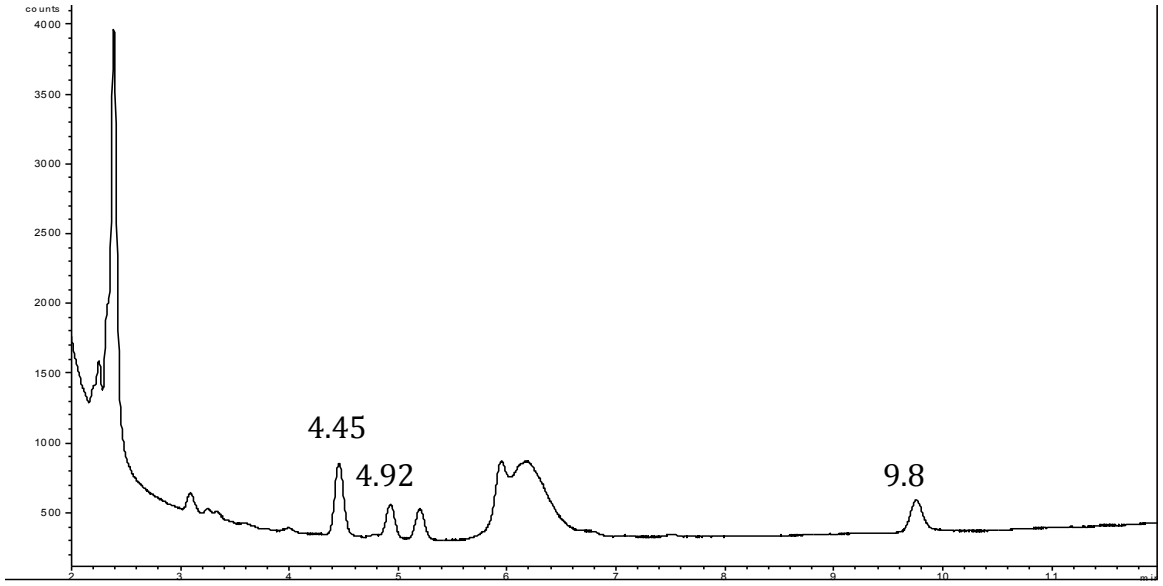



**Figure 4-20**. GC-FID Analysis of *Descurainia sophia* Whole Plant Autolysis Extract.

*Note*. 2016 collection, hexane extraction method.

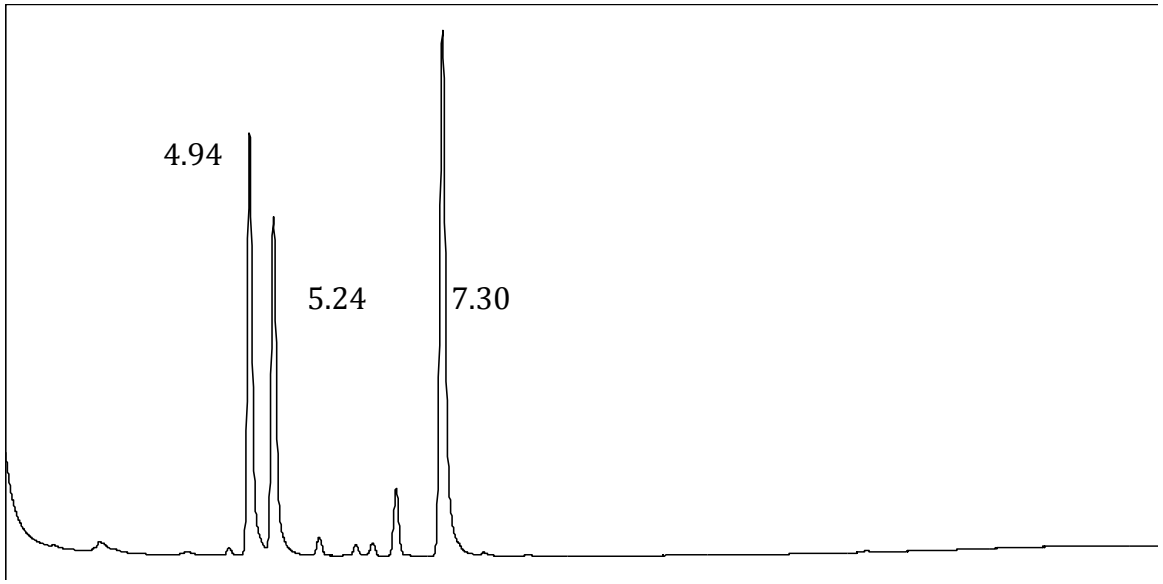



**Figure 4-21.** GC-FID analysis of standards allyl isothiocyanate, propyl isothiocyanate and butyl isothiocyanate concurrently with *Descurainia sophia* leaves and seeds autolysis extracts.

*Note*. Refer to Materials and Methods for analysis details.

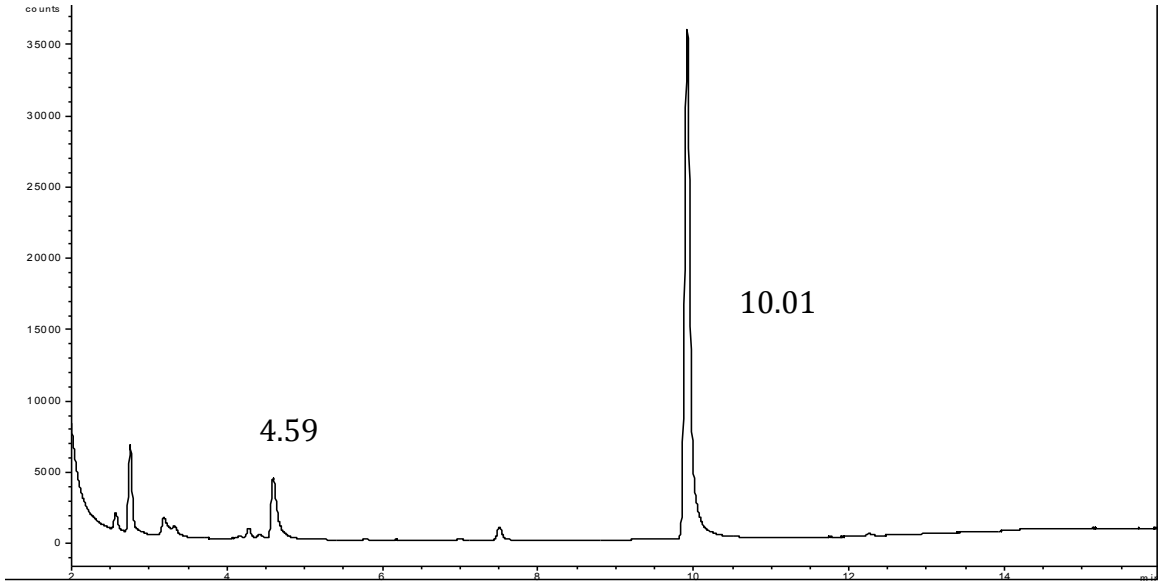



**Figure 4-22**. GC-FID Analysis of *Descurainia sophia* Leaves Autolysis Extract

*Note*. 2016 collection, hexane extraction method.

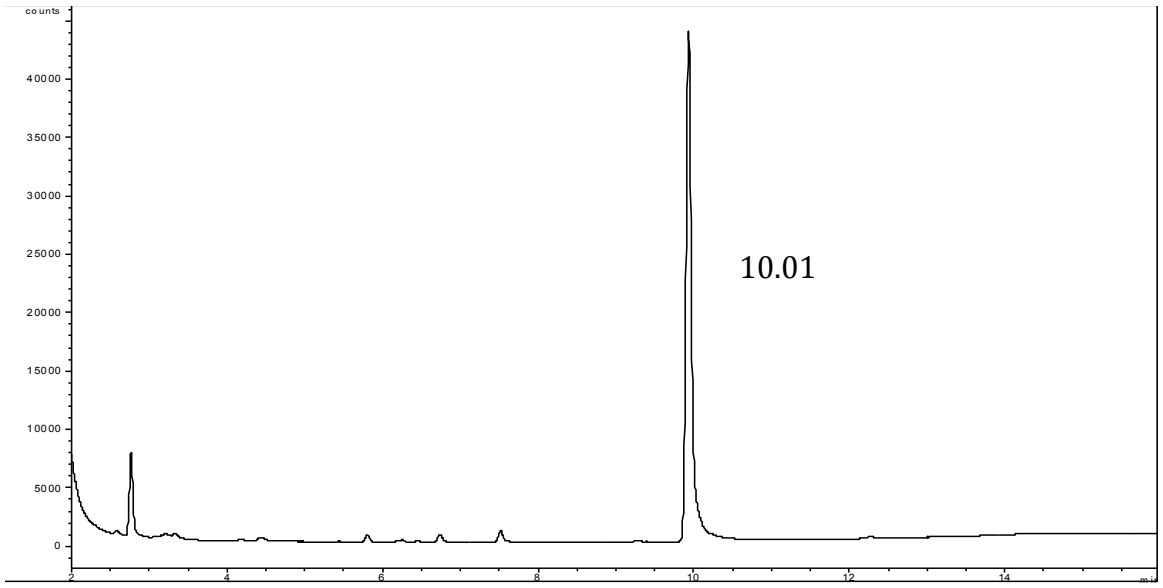



**Figure 4-23**. GC-FID Analysis of *Descurainia sophia* Seeds Autolysis Extract

*Note*. 2016 collection, hexane extraction method.

### 4.4.5 Qualitative Analysis of *Descurainia sophia* Autolysis Products by Gas Chromatography Mass Spectrometry

*Descurainia sophia* autolysis extract was analyzed by Gas Chromatography Mass Spectrometry (GCMS) for qualitative characterization of potential glucosinolate autolysis products (Figure 4-24). Tentative identification of peaks observed in the Total Ion Chromatogram was performed by computer-based comparison with standard spectra in the library provided by the instrument vendor (Table 4-23). Spectra for the peaks in Table 4-23 are shown in Figures 4-24 to 4-27.

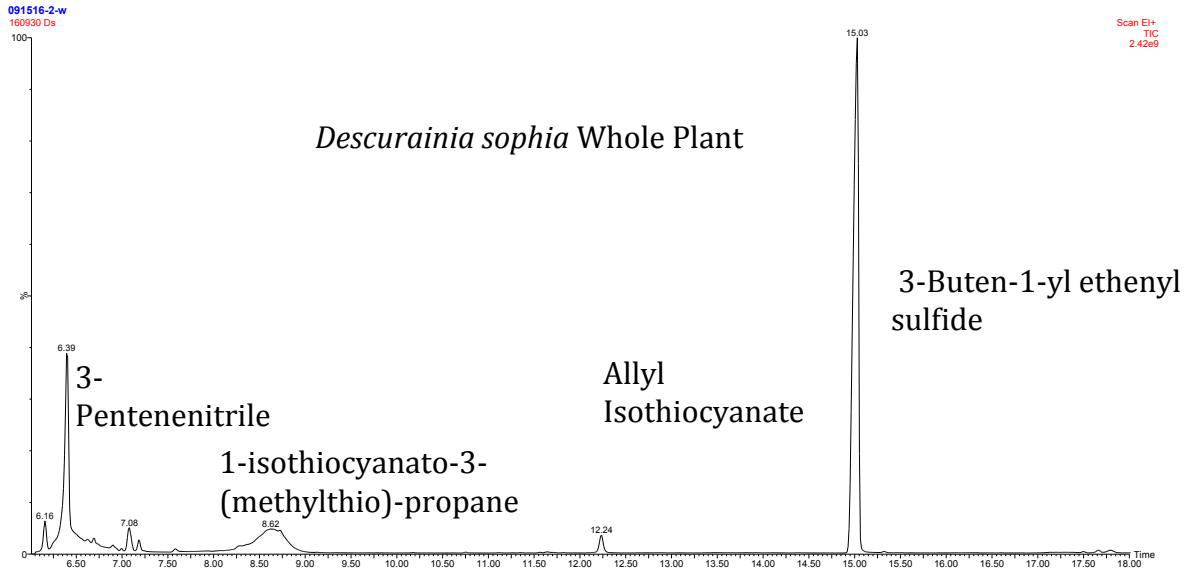



Figure 4-24. Gas Chromatography Mass Spectrometry

*Note*. Total Ion Chromatogram of *Descurainia sophia* whole plant. Hexane extraction method: refer to Materials and Methods section for details.

Table 4-23

*Tentative Identification of Chromatogram Peaks from Descurainia sophia Autolysis Extract*

| Name | Retention Time |
|---|---|
| 3-Pentenenitrile | 6.39 |
| 1-Isothiocyanato-3-(methylthio)-propane | 8.66 |
| Allyl isothiocyanate | 12.24 |
| 2-Propenthioacetonitrile | 15.03 |

The identifications of peaks in Table 4-23 should be regarded as tentative. Inspection of the comparisons of the mass spectra for these compounds indicate that there is a plausible basis for identifications.

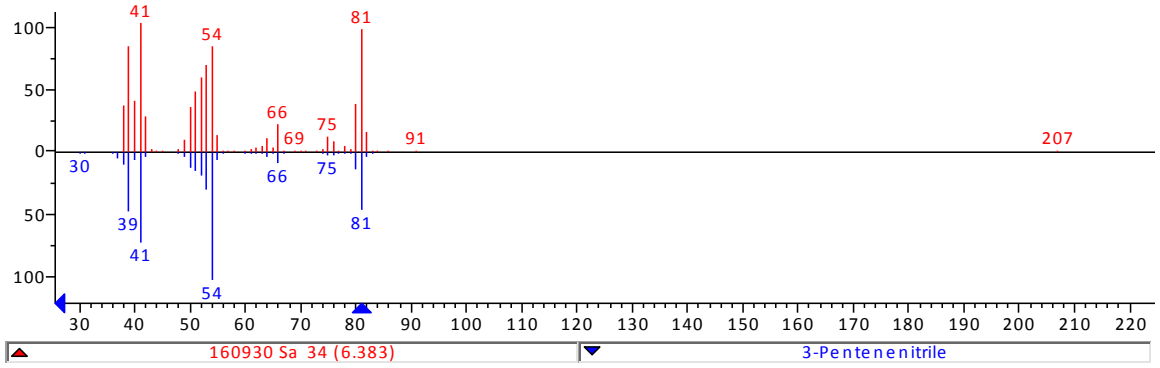



Figure 4-25. Mass Spectral Comparison: *Descurainia sophia* Whole Plant Autolysis Extract

*Note*. Top: 6.39 minute peak (Figure 4-24) Bottom: 3-Pentenenitrile spectrum of library standard. Refer to Materials and Methods section for details.

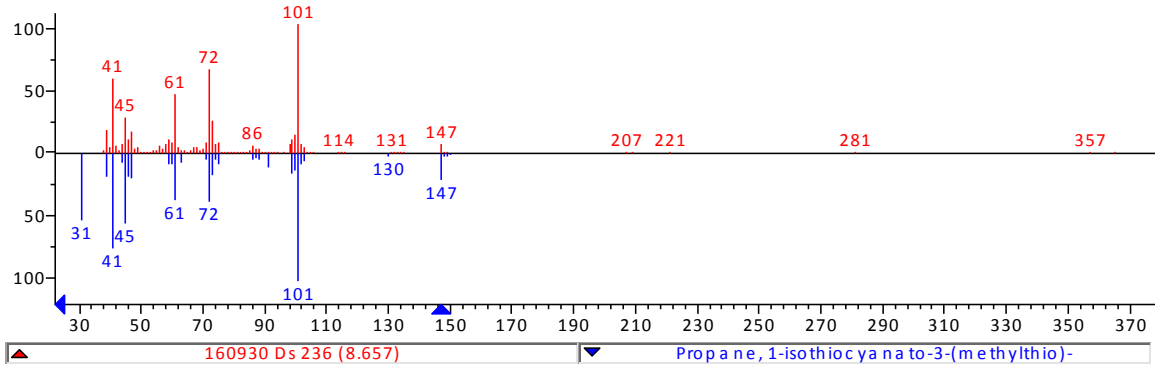



Figure 4-26. Mass spectral comparison: *Descurainia sophia* whole plant autolysis extract.

*Note*. Top: 8.62 minute peak (Figure 4-24) Bottom: 1-isothiocyanato-3-(methylthio)-propane spectrum of library standard. Refer to Materials and Methods section for details.

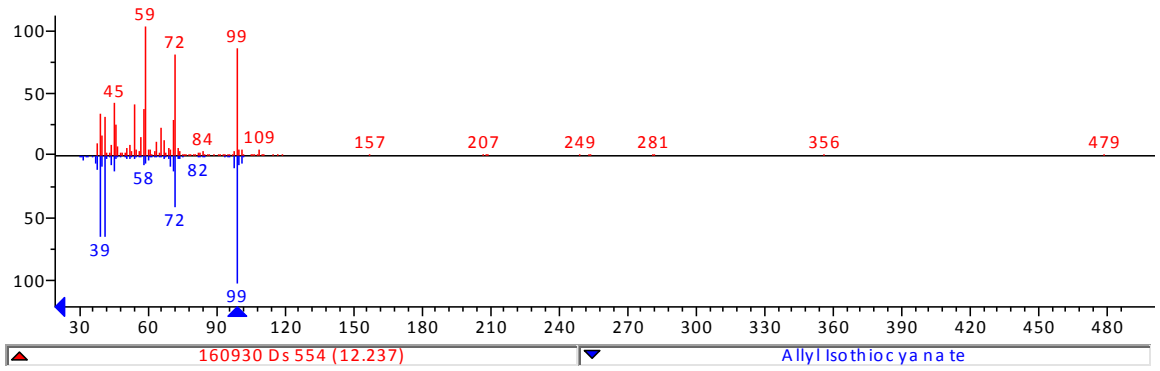



Figure 4-27. Mass spectral comparison: *Descurainia sophia* whole plant autolysis extract.

*Note*: Top: 12.24 minute peak (Figure 4-24) Bottom: Allyl Isothiocyanate spectrum of library standard. Refer to Materials and Methods section for details.

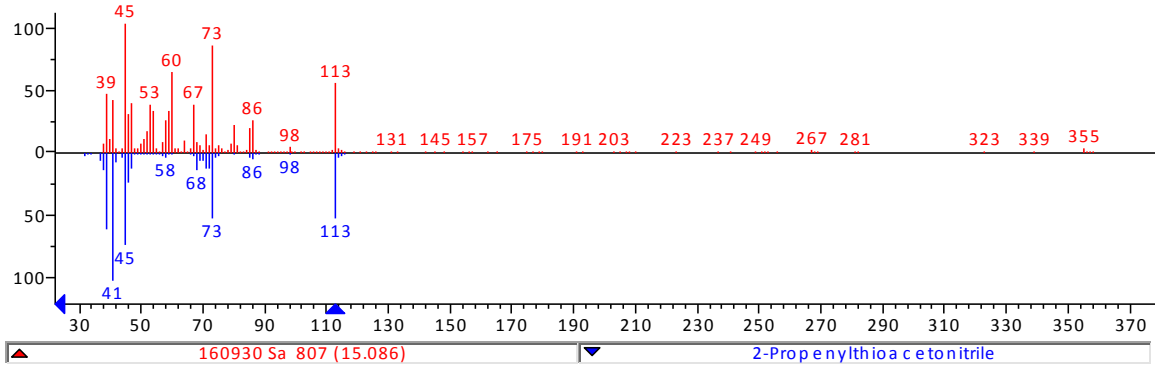



Figure 4-28. Mass Spectral Comparison: *Descurainia sophia* Whole Plant Autolysis Extract

*Note*. Top: 15.03 minute peak (Figure 4-24) Bottom: 2-Propenthioacetonitrile spectrum of library standard. Refer to Materials and Methods section for details.

### 4.4.6 Gas Chromatography Flame Ionization Detection Analysis for *Sisymbrium altissimuim*

Gas Chromatography Flame Ionization Detection was used to analyze three isothiocyanates, AITC, PITC, and BITC in *Sisymbrium altissimum* autolysis extracts, however, AITC, PITC, and BITC were not present in the plant when compared to standards. Figure 4-29 shows AITC, PITC, and BITC standards run when *Sisymbrium altissimum* leaves and seeds were analyzed (Figure 4-30 and Figure 4-31, respectively).

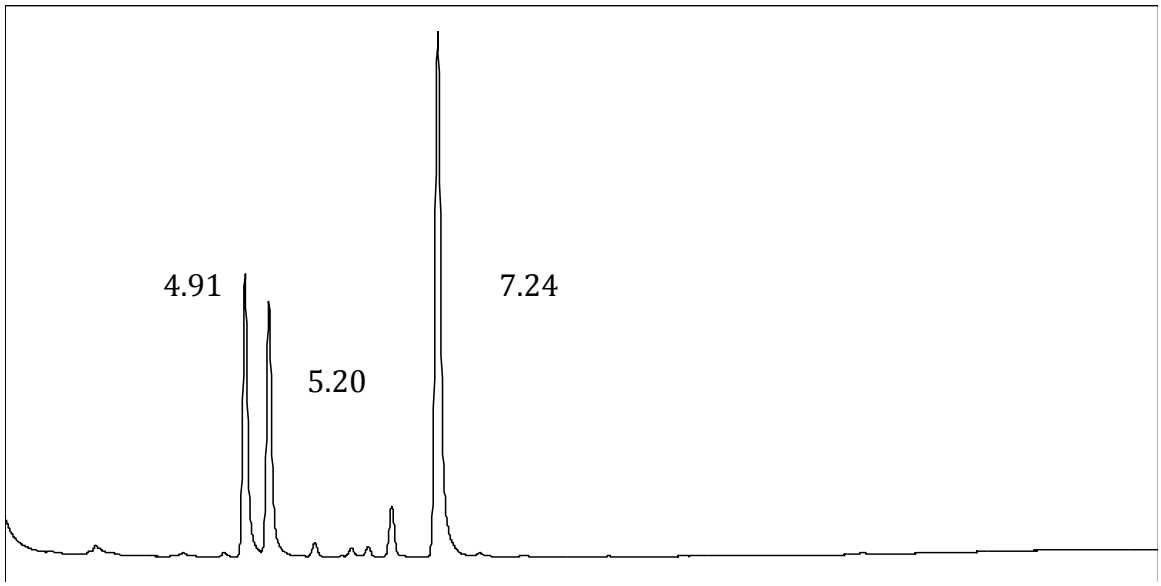



**Figure 4-29.** GC-FID Analysis of Standards allyl isothiocyanate, propyl isothiocyanate and butyl isothiocyanate Concurrent with *Sisymbrium altissimum* Leaves and Seeds Autolysis Extract Analysis

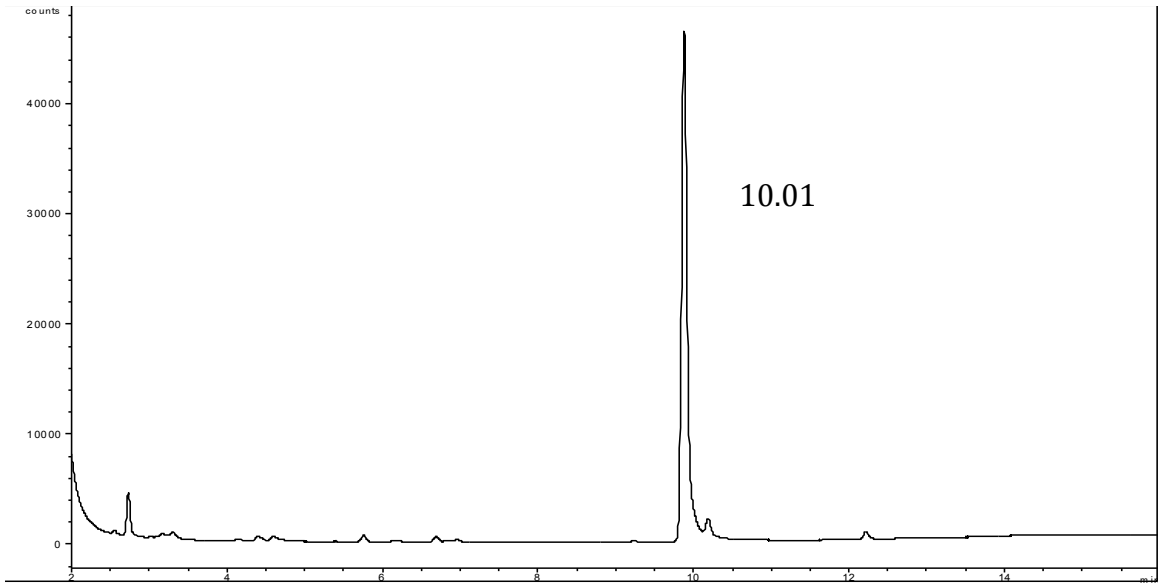



**Figure 4-30**. GC-FID Analysis of *Sisymbrium altissimum* Leaves Autolysis Extract

*Note*. Refer to Materials and Methods for analysis details.

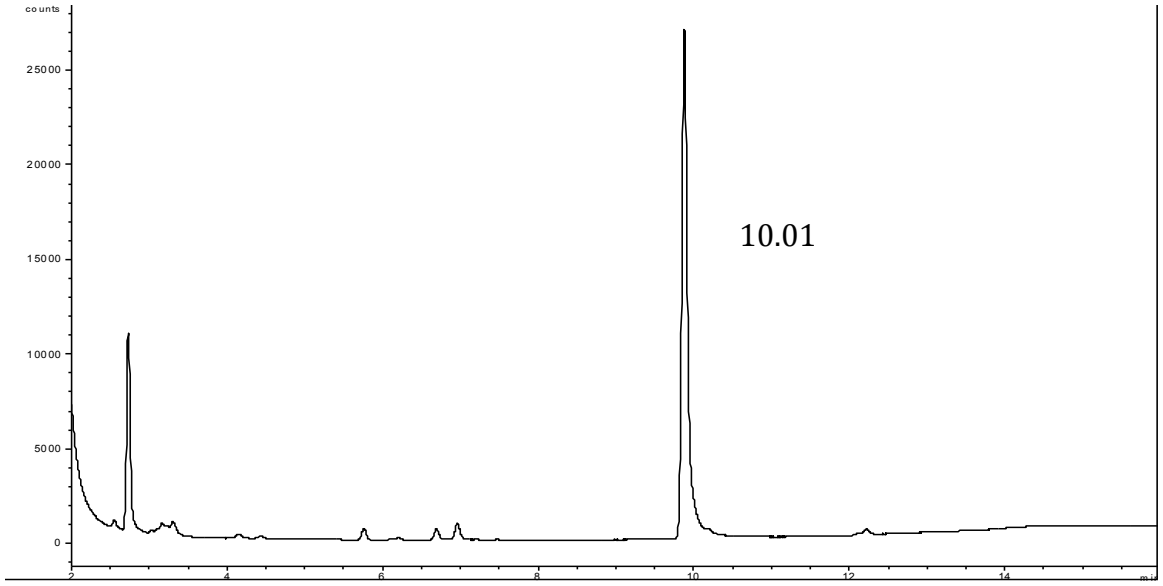



**Figure 4-31.** GC-FID Analysis of *Sisymbrium altissimum* Seeds Autolysis Extract

*Note*. 2016 collection, hexane extraction method.

### 4.4.7 Qualitative Analysis of *Sisymbrium altissimum* by Gas Chromatography Mass Spectometry

A *Sisymbrium altissimum* autolysis extract was analyzed by GC-MS for qualitative characterization (Figure 4-32). The tentative identification of the compounds responsible for the peaks is shown in Table 4-24. Spectra for the peaks in Table 4-24 are shown in Figures 4-33 to 4-37.

Table 4-24

*Tentative Identification of Chromatogram Peaks from Sisymbrium altissimum Autolysis Extract*

| Name | Retention Time |
|---|---|
| 3-Pentenenitrile | 6.41 |
| 5-methyl-Hexanenitrile | 10.70 |
| Heptanonitrile | 11.50 |
| 2-Propenylthioacetonitrile | 15.06 |
| Benzenepropanenitrile | 17.49 |

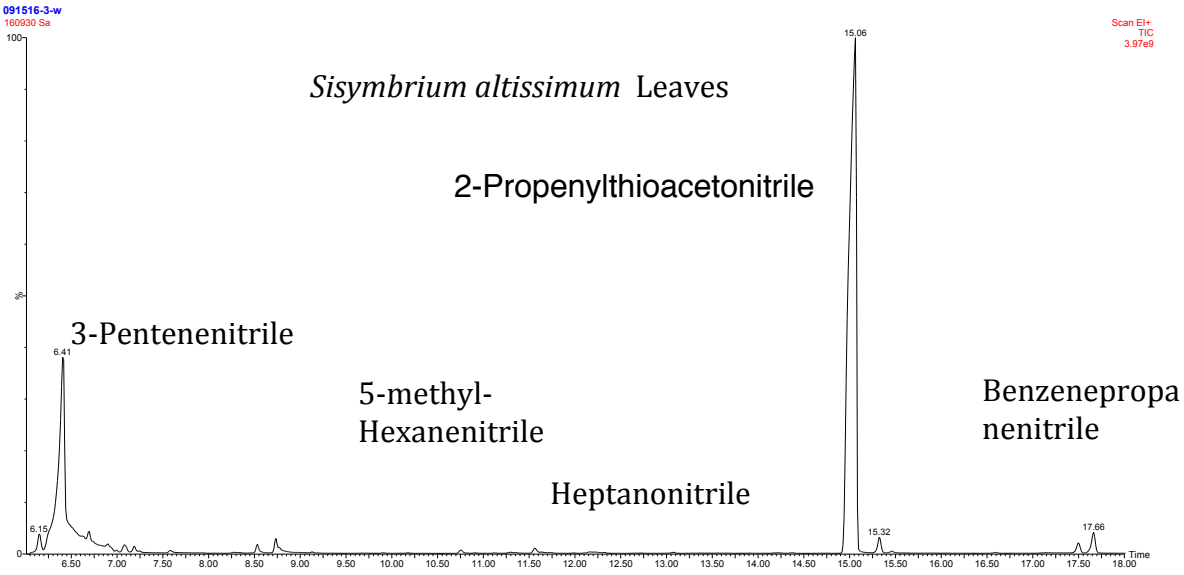



**Figure 4-32**. Gas Chromatography Mass Spectrometry Total Ion Chromatograph of *Sisymbrium altissimum* Leaves

*Note*. 2016 collection, hexane extraction method. Refer to Materials and Methods for details.

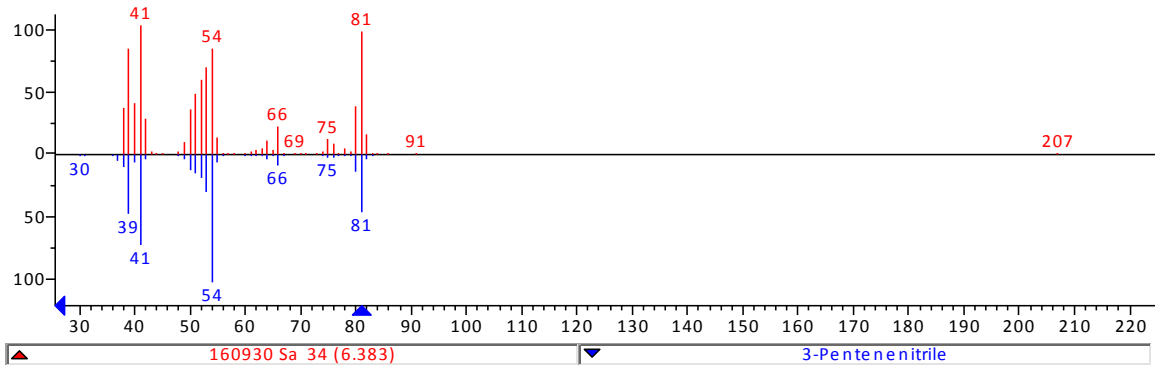



**Figure 4-33.** Mass Spectral Comparison: 2016 *Sisymbrium altissimum* Leaves Autolysis Extract

*Note*. Top: 6.41 minute peak (Figure 4-32) bottom: 3-Pentenitrile spectrum of library standard.

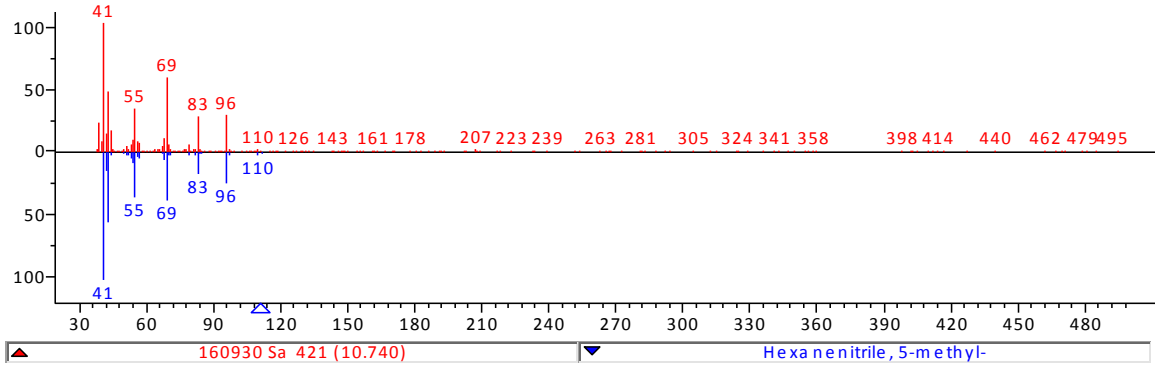



**Figure 4-34.** Mass Spectral Comparison: 2016 *Sisymbrium altissimum* Leaves Autolysis Extract

*Note*. Top: 10.70 minute peak; (Figure 4-32) bottom: 5-methyl-hexanenitrile spectrum of library standard.

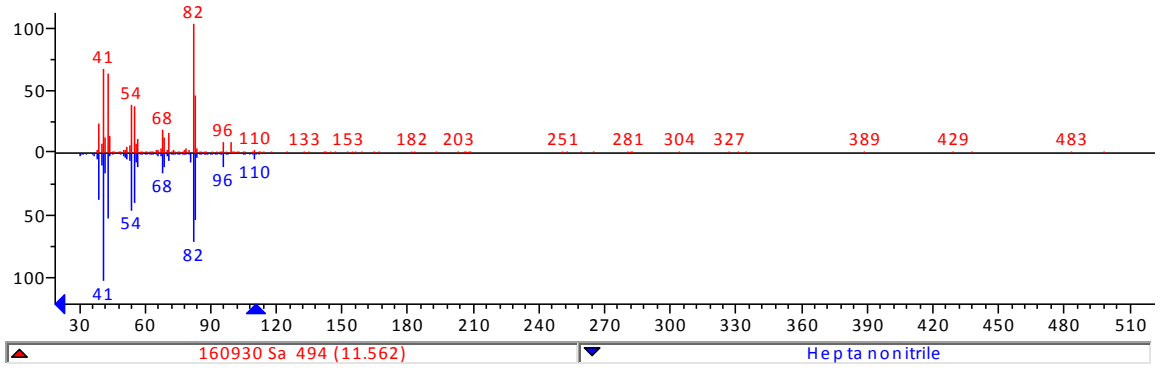



**Figure 4-35.** Mass Spectral Comparison: 2016 *Sisymbrium altissimum* Leaves Autolysis Extract

*Note*. Top: 11.50 minute peak; (Figure 4-32) bottom: 3-Pentenitrile spectrum of library standard.

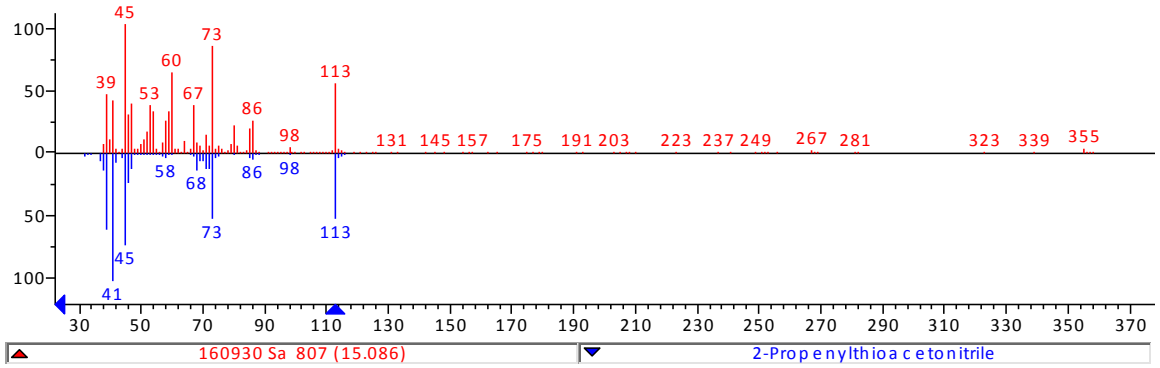



**Figure 4-36.** Mass Spectral Comparison: 2016 *Sisymbrium altissimum* Leaves Autolysis Extract

*Note*. Top: 15.06 minute peak; (Figure 4-32) bottom: 2-Propenylthioacetonitrile spectrum of library standard.

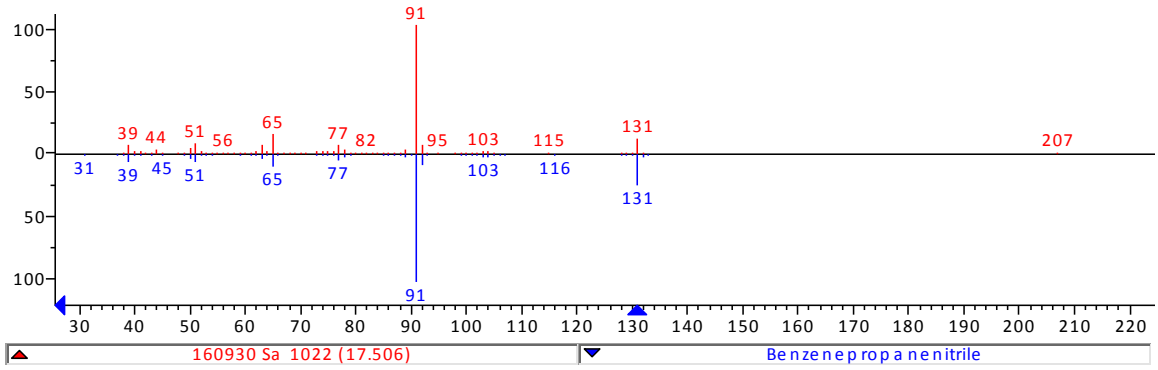



**Figure 4-37**. Mass Spectral Comparison: 2016 *Sisymbrium altissimum* Leaves Autolysis Extract

*Note*. Top: 17.49 minute peak; (Figure 4-32) bottom: Benzenepropanenitrile spectrum of library standard.

## 4.5 Discussion

*Chorispora tenella, Descurainia sophia,* and *Sisymbrium altissimum* are plants all found locally although not native to the Fort Collins, Colorado area. These plants are part of the Brassicaceae family and thus contain glucosinolates. Some glucosinolates have been known to have negative health effects on domesticated livestock when consumed at high concentrations and some have goitrogenic effects (Fenwick et al. 1983; Fahey et al. 2001; Nilsson et al. 2006; Velasco et al. 2008; Sonderby et al. 2010).

Glucosinolates are the precursor to isothiocynates such as allyl isothiocyanate, butyl isothiocyanate, and propyl isothiocyanate. Glucosinolates are believed to help plants defend themselves from biotic stresses from herbivory, fungal pathogens, and insect defense (Winde and Wittstock 2011). After coming in contact with myrosinase, these glucosinolates can be hydrolyzed into isothiocyanates.

Figure 4-1, shows three isothiocyanates, AITC, BITC, and PITC, in *Chorispora tenella* whole plant from the 2010 sampling season. Three different part of the plants --whole plant, leaves, and seeds -- have varying concentrations of the three isothiocyanates but with seeds having the highest for all isothiocyanates (Tables 4-2, 4-3, and 4-4). When comparing glucosinolate profiles, precursors to isothiocyanates, in different tissues of various Brassicaceae

crops, results showed that glucosinolates varied among the different plants, tissues of the plants, and various growth stages of the plants (Bhandari et al. 2015). In particular, the seeds had the highest glucosinolate concentrations in most of the Brassicaceae crops whereas the shoots had the lowest concentration of glucosinolates (Brown et al. 2003; Bhandari et al. 2015). This is duplicated in my data with the BITC and PITC. Whole plant samples, which included the stems, leaves, flowers, and seeds had the lowest overall BITC and PITC content whereas the seeds had the highest isothiocyanate content. Allyl isothiocyanate however, had the second highest concentration in the whole plant samples compared to the other isothiocyanates. This could be due to *de novo* synthesis of glucosinolates and thus their resulting products, isothiocyanates during plant growth. In research done on Portuguese cabbage, Portuguese Kale, and Nabo (common names) glucosinolate content in the seeds and tissues of these Brassicaceae plants can vary independently of each other with seeds having higher content than the leaves, stems, and roots (Rosa 1997).

After the 2010 sampling season, BITC and PITC have not been detected in *Chorispora tenella*. A new method was used for the 2016 sampling season, which was described in Materials and Methods. Recovery of PITC and BITC with this new method was 86% and 77%, respectively. In an experiment conducted comparing the 2010 method and the 2016 method with *Chorispora tenella*, PITC and BITC are absent in both samples (data not shown) confirming that it is not the method but rather a change in glucosinolate profile. The detection limit for PITC was 1.11 µg/mL; for BITC it was 0.59 µg/mL.

It has also been reported that various broccoli cultivars have varying glucosinolate levels. Research showed that glucosinolate content was lower in the spring than in the fall seasons for various broccoli culivars (Bhandari and Kwak 2014). In comparing two sampling seasons, 1999

and 2000, samples of Brassicaceae crops had major glucosinolates differ significantly. The largest difference was observed for neoglucobrassicin, which had a 240% increase in 2000 compared to 1999 and for glucobrassicin, which had a 216% increase in 2000 compared to 1999 (Nilsson et al. 2006). These changes in glucosinolates could be due to differences in genotypes along with environmental factors and with the developmental stage of the plant (Nilsson et al. 2006; Velasco et al. 2008; Bhandari and Kwak 2014). Abiotic environmental factors that can affect glucosinolate profile and thus isothiocyanates can be salinity, drought, extreme temperatures, light cycling, and nutritional deficiencies (del Carmen Martinez-Ballesta et al. 2013). Glucosinolate content varies in response to light and temperature. At moderate temperature, low humidity and high light intensity can induce higher glucosinolate accumulation and thus a higher isothiocyanate concentration (del Carmen Martinez-Ballesta et al. 2013). When comparing 1-methoxyglucobrassicin (1MGB; 1-methoxy-3-ylmethyl glucosinolate in turnip) it was elevated as a result of increased temperature, which resulted in an average increase of 1000% of the glucosinolate (Justen and Fritz 2013).

In 2010 (Table 4-2), allyl isothiocyanate content in *Chorispora tenella* leaves was significantly different from in whole plant ($p=0.025$ Tukey) and significantly different from in seeds ($p<0.0001$ Tukey). In the 2016 sampling season, allyl isothiocyanate content in seeds was significantly different at location 3 compared to location 6 ($p=0.00465$ Tukey), location 4 compared to 6 ($p=0.0002$ Tukey), and location 6 compared to location 1 ($p=0.0363$ Tukey). In the 2017 sampling season, allyl isothiocyanate content in whole plant was significantly different at location 4 compared to 6 ($p=0.006$ Tukey) and location 4 compared to location 1 ($p=0.0002$ Tukey). Comparing all locations during 2010, 2016, and 2017 shows that 2010 allyl isothiocyanate content is significantly higher than in 2016 and 2017 ($p=0.006$ Tukey). These

differences in AITC profiles could be due to environmental factors and a slight change in the glucosinolate profile with various tissues having different amounts of the precursor glucosinolate at collection time, as discussed above.

Characterizations of *Descurainia sophia,* and *Sisymbrium altissimum* show a differing glucosinolate hydrolysis product profile compared to *Chorispora tenella*. Propyl isothiocyanate and butyl isothiocyanate were only observed in *Chorispora tenella* in the 2010 samples. *Descurainia sophia* autolysis extract may contain allyl isothiocyanate based upon the GC-MS analysis but, on the basis of the Total Ion Chromatogram, this compound represents a minor constituent in this species. This is in contrast to *Chorispora tenella,* in which it appears to be the predominant glucosinolate product. The different parent glucosinolate compound in addition to abiotic factors can result in a variety of isothiocyanates.

# 5. TOXICITY OF ISOTHIOCYANATES AND *CHORISPORA TENELLA* TO HEPG2 CELLS

## 5.1 Introduction

HepG2 human hepatocarcinoma cells were used to determine cytotoxicity of three isothiocyanate compounds: allyl isothiocyanate (AITC), butyl isothiocyanate (BITC), and propyl isothiocyanate (PITC) and extracts of autolysis products prepared from the plant Chorispora tenella. HepG2 cell line was chosen for experimentation due to the liver being one of the organs known to be affected by isothiocyanates.

### 5.1.1 Allyl isothiocyanate

To determine metabolic activity of HepG2 cells exposed to AITC for 24 hours (Table 5-1), a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted (Figure 5-1). Allyl isothiocyanate concentrations were based on the results of Kassie and Knasmuller (2000). Cytotoxicity as indicated by the MTT assay was significantly different form control in HepG2 cells treated with 70, 100, 175, and 250 μM AITC. The estimated median lethal concentration ($LC_{50}$) was 116 μM AITC based on a linear regression model ($p<0.0001$ Least Squares Analysis) (Figure 5-1).

A DNA nuclear stain assay using Hoechst 33258 fluorescent dye (Figure 5-2) was conducted to determine DNA content in the HepG2 cells as a metric of cell number at the end of the 24-hour exposure period. The DNA content as indicated by the nuclear stain assay was significantly different from control in HepG2 cells treated with 175 and 250 μM AITC. There was a clear linear decrease in DNA content and, thus, in cell number, with increasing AITC concentration (Figure 5-2). The $LC_{50}$ for inhibition of cell proliferation is 133 μM AITC.

The ATP content of HepG2 cells treated with AITC was determined using a luciferase assay measuring ATP content as an assessment of mitochondrial function (Figure 5-3). HepG2 cells were exposed to AITC (Table 5-1) for 24 hours. The ATP content as indicated by the luciferase assay was significantly different from control in HepG2 cells treated with 250 μM AITC. To determine a potential relationship between cell number (DNA content) at the end of the 24-hour exposure period and ATP content in the HepG2 cells treated with AITC and ATP content was normalized for a DNA content (Figure 5-4). The ATP content normalized for DNA content was significantly different from control in HepG2 cells treated with 175 and 250 μM AITC. The effect of AITC treatment of ATP content of the HepG2 cells did not follow the simple linear decrease as was observed in the MTT and DNA content assays. The measurement of ATP content cannot distinguish between treatment-related impairment of synthesis versus its consumption by the cells. A combination of these phenomena could explain the results shown in Figures 5-3 and 5-4.

Table 5-1

*Allyl isothiocyanate Nominal and Measured Concentrations in HepG2 Cell Culture Medium*

| Nominal Concentration | Measured Concentration | % Nominal |
|---|---|---|
| Control: Medium Only | Control: Medium Only | 0 |
| 10 μM | 91 μM | 910 |
| 30 μM | 98 μM | 31 |
| 70 μM | 105 μM | 150 |
| 100 μM | 111 μM | 111 |
| 175 μM | 149 μM | 85 |
| 250 μM | 337 μM | 135 |

*Note*. Measured concentrations were determined by GC-FID. Isothiocyanates were extracted from medium with hexane within two hours after initiation of treatment to HepG2 cells.

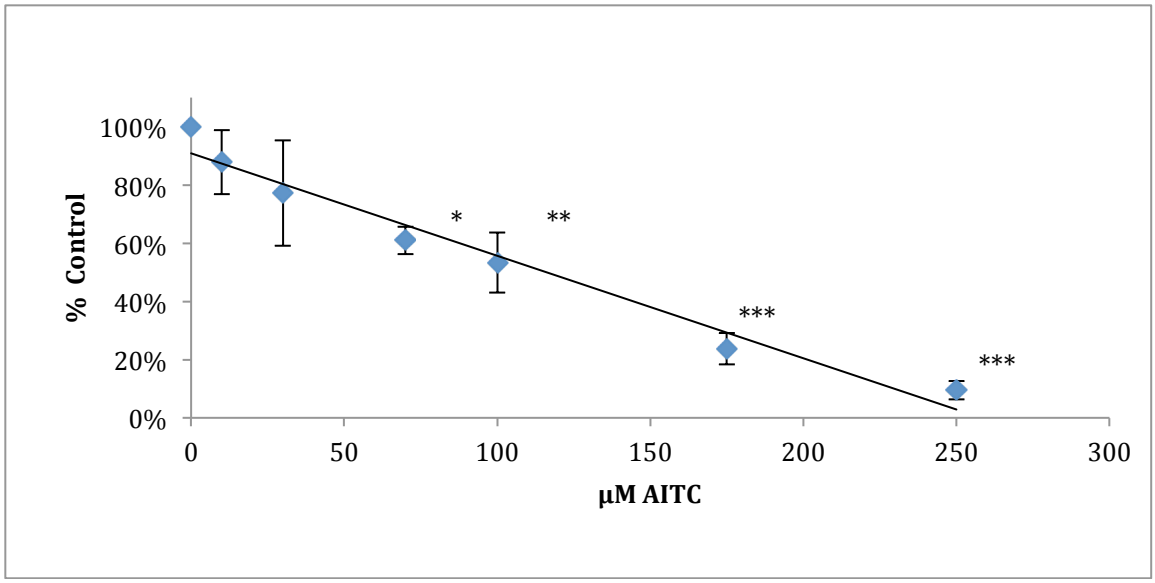



**Figure 5-1**. HepG2 Cells Treated with allyl isothiocyanate for 24 hours Followed by an MTT Cytotoxicity Assay to Determine Cell Metabolism

*Note*. Points represent the mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's test were conducted for parametric data expressed as a proportion transformed with arcsine square root. $R^2$ = 0.969. *Significantly different from control p=0.0012 (Tukey). **Significantly different from control p=0.0002 (Tukey). ***Significantly different from control p<0.0001 (Tukey).

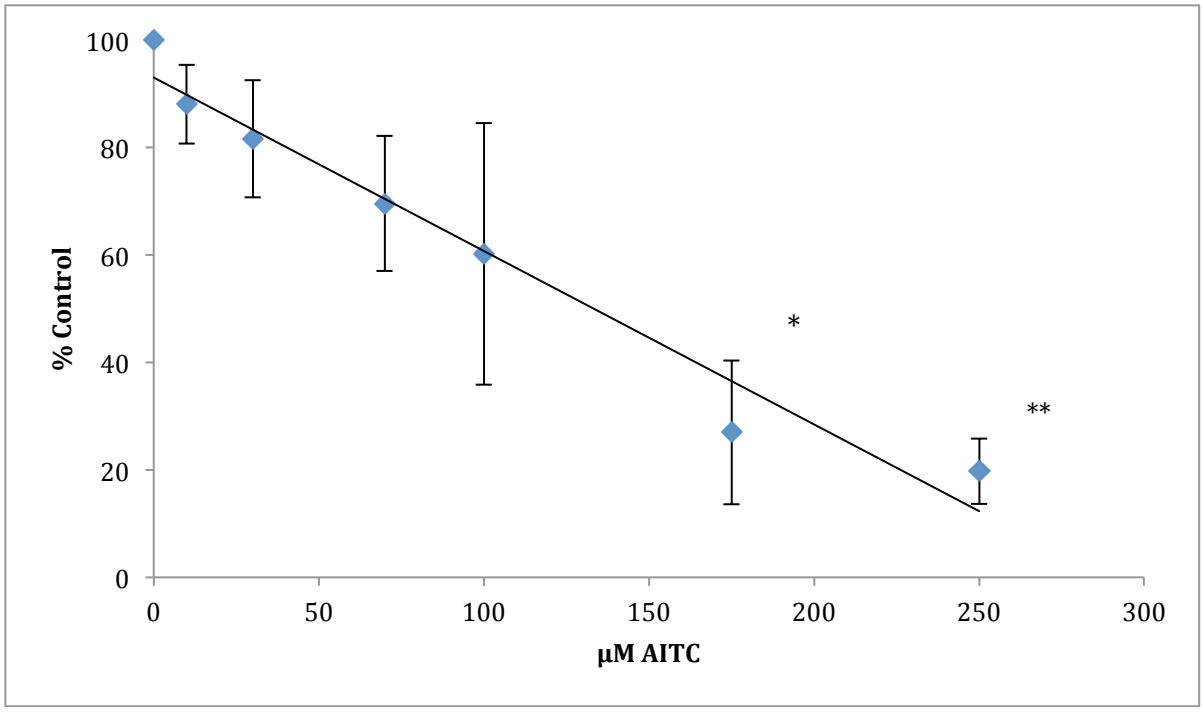



**Figure 5-2.** HepG2 Cells Treated with allyl isothiocyanate for 24 hours Followed by a DNA Nuclear Stain Assay with Hoecsht Dye 33258 to Determine DNA Content

*Note*. Percent of control is DNA content expressed as arbitrary fluorescence units using Hoecsht 33258. Nominal concentrations are shown. Points represent a mean value based on three separate plates with four replicates per plate. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test was conducted for nonparametric data expressed as a proportion transformed with arcsin square root. $R^2 = 0.963$. * Significantly different from control p=0.0002 (Dunn). ** Significantly different from control p<0.0001 (Dunn).

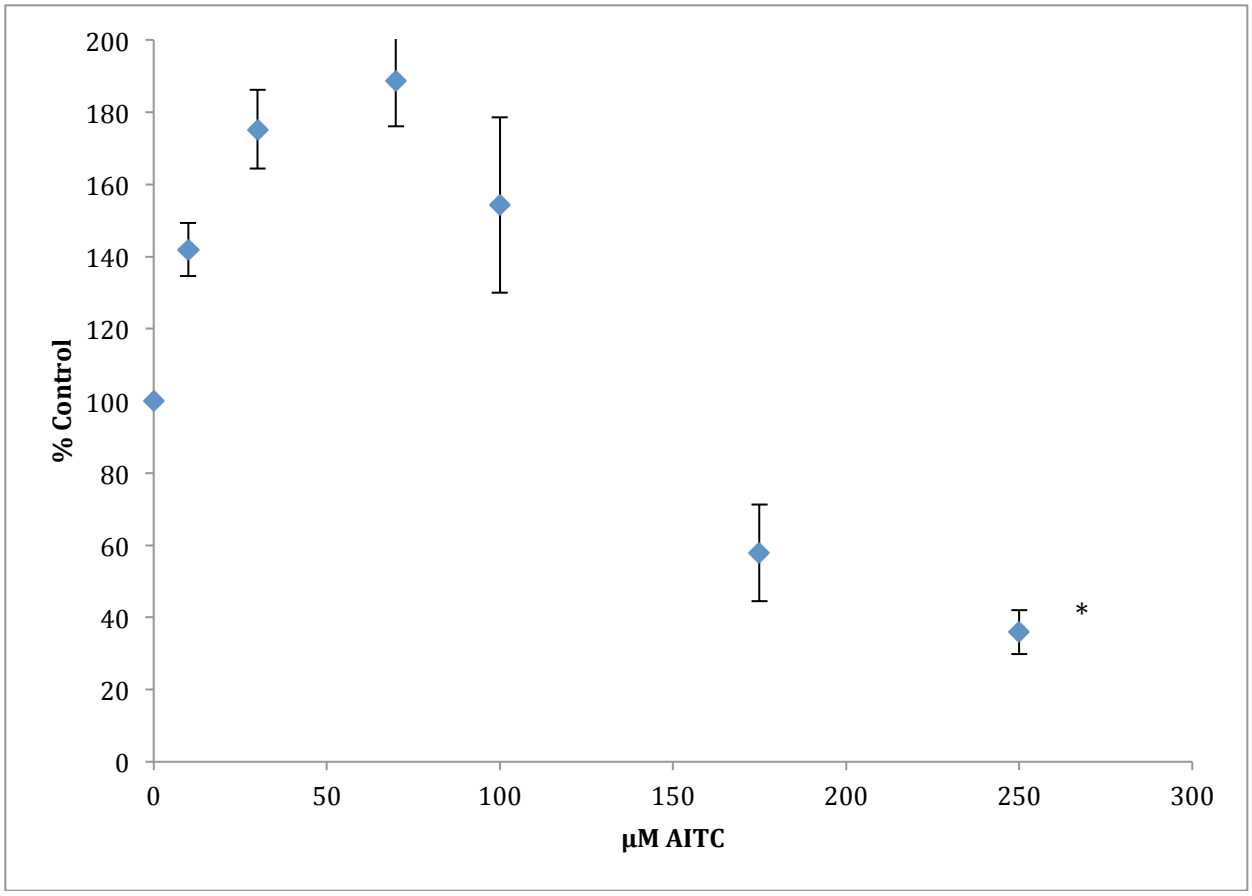



**Figure 5-3.** HepG2 Cells Treated with allyl isothiocyanate for 24 hours Followed by a Luciferase Assay to Determine ATP Content (Mitochondrial Function)

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test was conducted for nonparametric data expressed as a proportion transformed with arcsine square root.
*Significantly different from control p=0.0460 (Dunn).

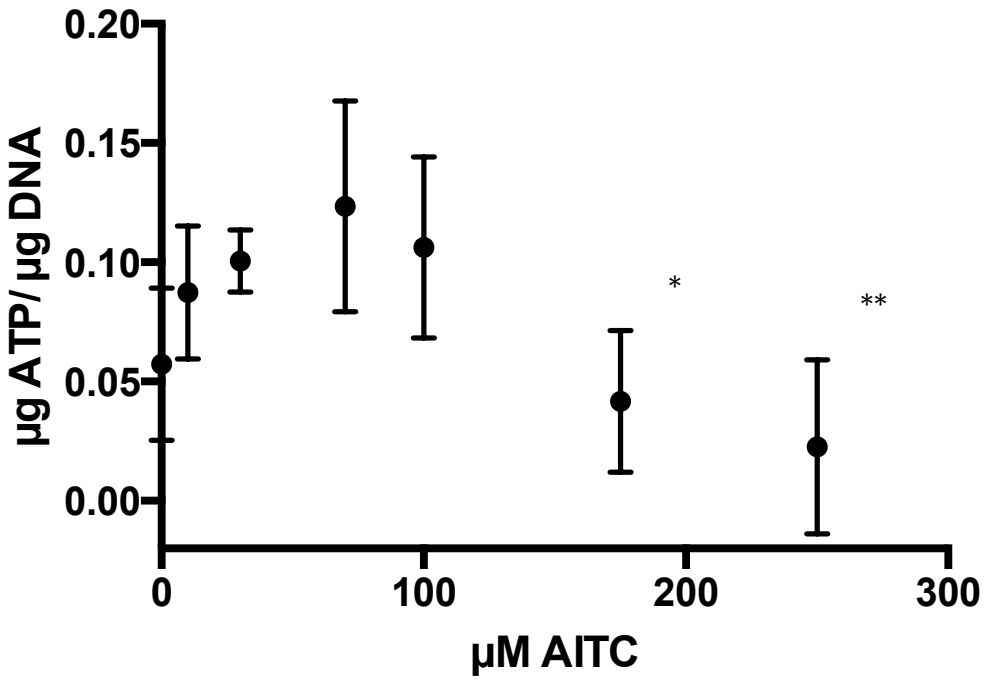



**Figure 5-4.** ATP Content Normalized to DNA Content in HepG2 Cells Exposed to allyl isothiocyanate for 24 hours

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's test was conducted for parametric data. *Significantly different from control p=0.0315 (Tukey). **Significantly different from control p=0.0021 (Tukey).

**Propyl Isothiocyanate**

HepG2 cells were assessed for metabolic activity by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after a 24 hour exposure to propyl isothiocyanate (PITC) (Table 5-2). Treatment concentrations were selected based on structure and experimentation. Cytotoxicity, as indicated by the MTT assay (Figure 5-5), was significantly different from control in HepG2 cells treated with 1000 and 1250 µM PITC. The estimated median lethal concentration ($LC_{50}$) was 1152 µM PITC based on a linear regression model (p=0.0013 Least Squares Analysis) (Figure 5-5). A DNA nuclear stain assay using Hoechst 33258 fluorescent dye (Figure 5-6) was conducted to determine DNA content in the HepG2 cells

as a metric of cell number at the end of the 24-hour exposure period. The DNA content as indicated by the nuclear stain assay was significantly different from control in HepG2 cells treated with 1000 and 1250 μM PITC.

The ATP content of HepG2 cells treated with PITC was determined using a luciferase assay measuring ATP content as an assessment of mitochondrial function. HepG2 cells were exposed to PITC (Table 5-2) for 24 hours. The ATP content as indicated by the luciferase assay was significantly different from control in HepG2 cells treated with 750, 1000, and 1250 μM PITC (Figure 5-7). The $LC_{50}$ for ATP content is 708 μM PITC. To determine a potential relationship between cell number (DNA content) at the end of the 24-hour exposure period and ATP content in the HepG2 cells treated with AITC, ATP content was normalized for a DNA content (Figure 5-8). The ATP content normalized for DNA content was significantly different from control in HepG2 cells treated with 1000 and 1250 μM PITC.

Table 5-2

*Propyl isothiocyanate Nominal and Measured Concentrations in Cell Culture Medium*

| Nominal Concentration | Measured Concentration | % Nominal |
|---|---|---|
| Control: Medium Only | Control: Medium Only | 0 |
| 100 μM | 35 μM | 35 |
| 300 μM | 56 μM | 19 |
| 600 μM | 73 μM | 12 |
| 750 μM | 257 μM | 34 |
| 1000 μM | 306 μM | 31 |
| 1250 μM | 356 μM | 28 |

*Note*. Measured concentrations were analyzed by GC-FID. Isothiocyanates were extracted from medium with hexane within two hours after initiation of treatment of HepG2 cells.

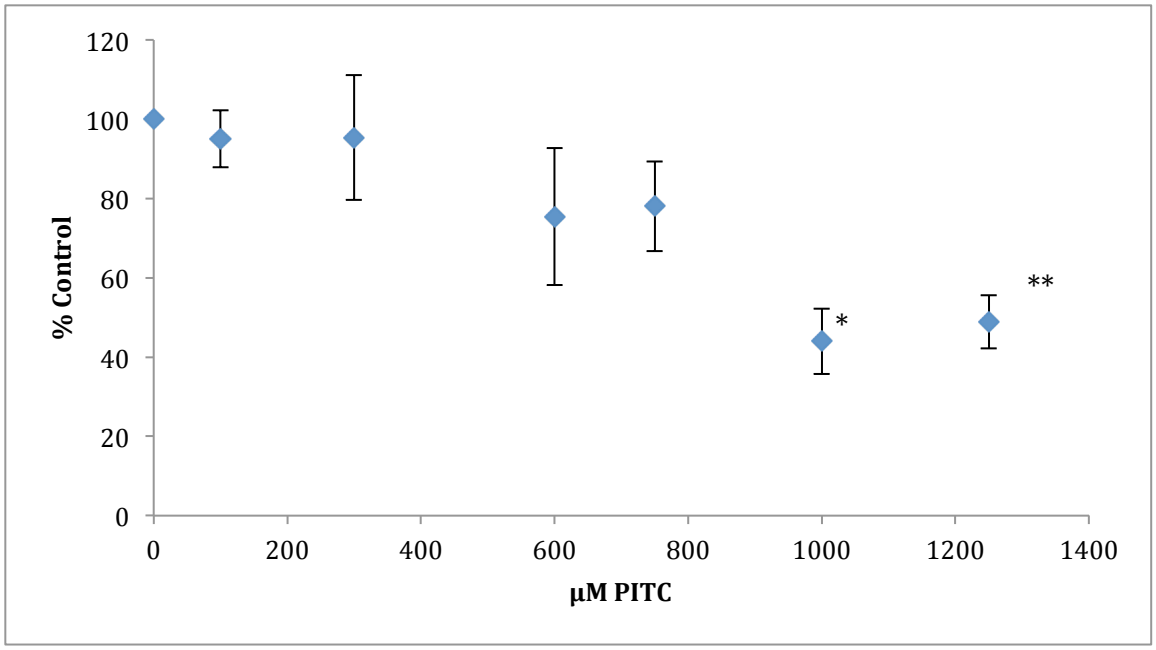



**Figure 5-5.** HepG2 Cells Treated with propyl isothiocyanate Treatments for 24 hours Followed by an MTT Cytotoxicity Assay to Determine Cell Metabolism

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test were conducted for nonparametric data expressed as a proportion transformed with arcsine square root. $R^2$= 0.892.
* Significantly different from control p<0.0017 (Dunn). ** Significantly different from control p<0.0045 (Dunn).

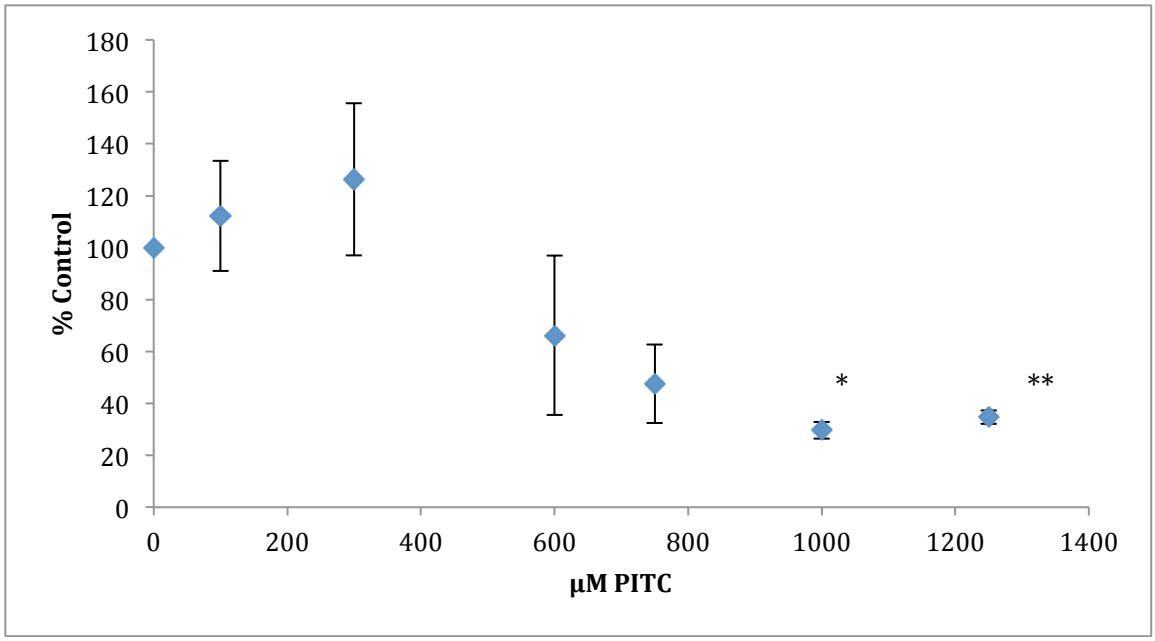



**Figure 5-6.** HepG2 Cells Treated with propyl isothiocyanate Treatments for 24 hours Followed by a DNA Nuclear Stain Assay with Hoecsht Dye 33258 to Determine DNA Content

*Note*. Percent of control is DNA content expressed as arbitrary fluorescence units using Hoecsht 33258. Nominal concentrations are shown. Points represent a mean value based on three separate plates with four replicates per plate. The error bars shown represent one standard deviation. One way ANOVA and post hoc Tukey's test was conducted for parametric data expressed as a proportion transformed with arcsine square root. * Significantly different from control p=0.0002 (Tukey). ** Significantly different from control p=0.0033 (Tukey).

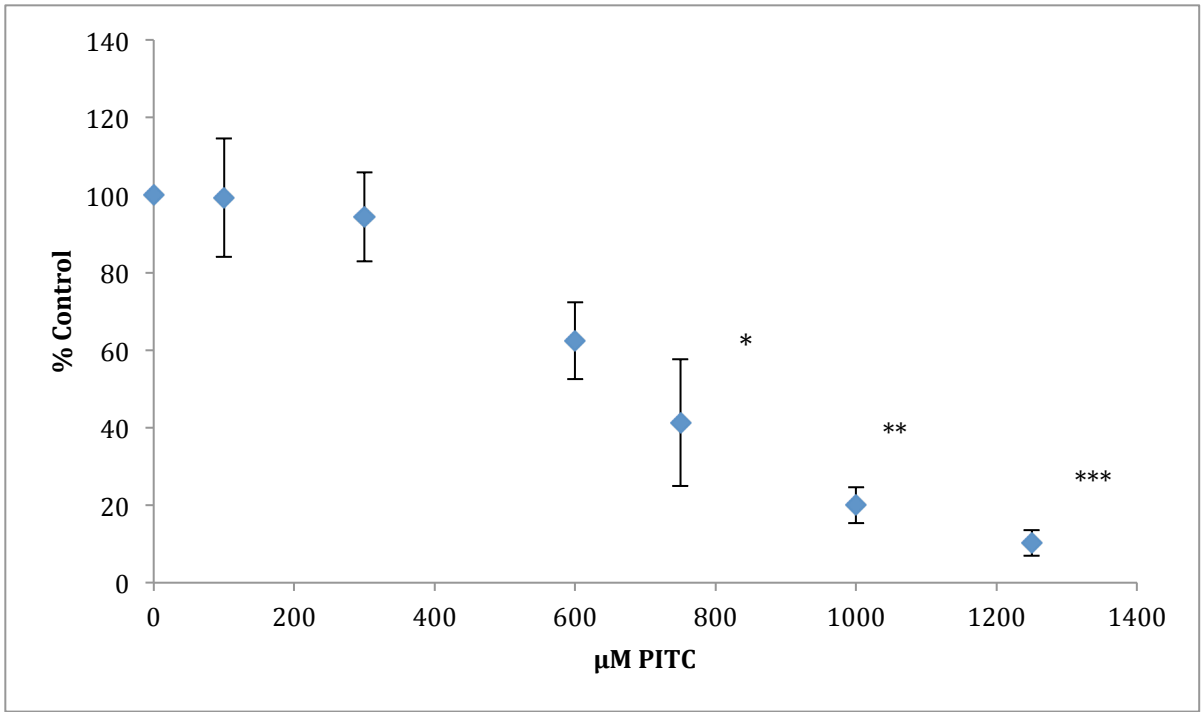



**Figure 5-7.** HepG2 Cells Treated with propyl isothiocyanate for 24 hours Followed by a Luciferase Assay to Determine ATP Content (Mitochondrial Function)

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test was conducted for nonparametric data expressed as a proportion transformed with arcsine square root. $R^2$=0.976. * Significantly different from control p=0.0208 (Dunn). ** Significantly different from control p<0.0003 (Dunn). *** Significantly different from control p<0.0001 (Dunn).

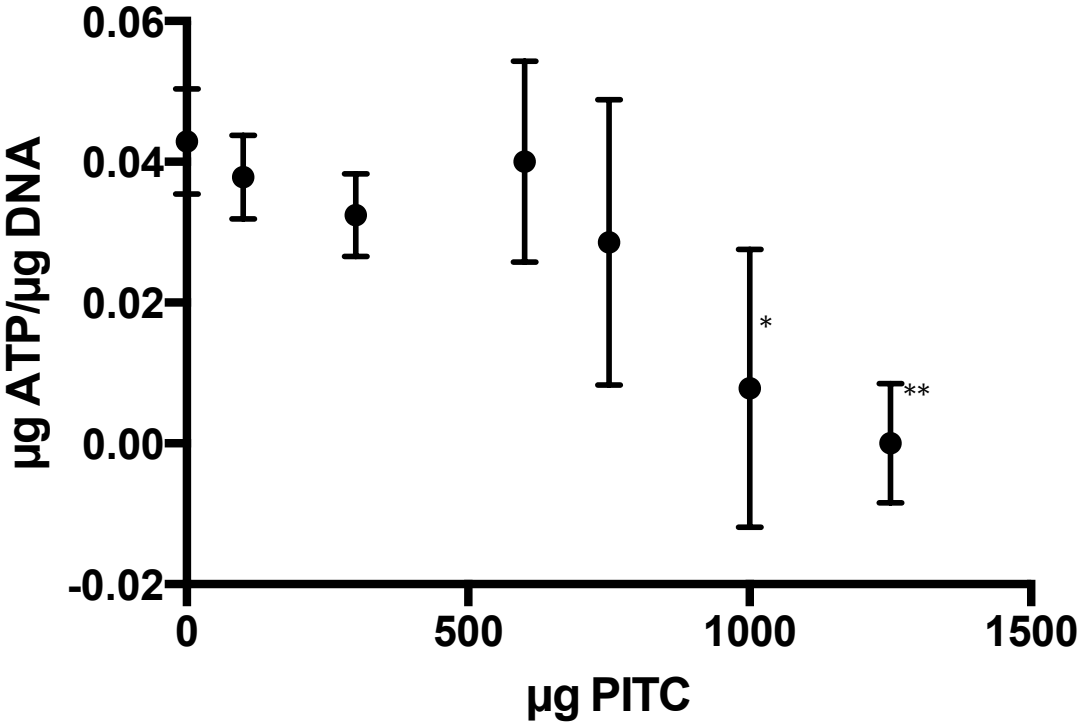



**Figure 5-8.** ATP Content Normalized to DNA Content in HepG2 Cells Exposed to propyl isothiocyanate for 24 hours.

Note. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's test conducted for parametric data. * Significantly different from control p=0.0189 (Tukey). ** Significantly different from control p=0.0008 (Tukey).

### 5.1.3 Butyl Isothiocyanate

HepG2 cells were assessed for metabolic activity by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after a 24 hour exposure to butyl isothiocyanate (BITC) (Table 5-3). Treatment concentrations were identified based on chemical structure and preliminary experiments (data not shown). Cytotoxicity as indicated by the MTT assay showed that there is no significant effect of BITC treatment compared with control. The estimated median lethal concentration ($LC_{50}$) was greater than 2500 µM BITC based on inspection of the data (Figure 5-9). A DNA nuclear stain assay using Hoechst 33258 fluorescent dye (Figure 5-10) was conducted to determine DNA content in the HepG2 cells as a metric of cell number at

the end of the 24-hour exposure period. The DNA content as indicated by the nuclear stain assay shows no significant differences compared to control, again indicating a lack of effect of BITC of the range of concentrations tested. The ATP content of HepG2 cells treated with BITC was determined using a luciferase assay measuring ATP content as an assessment of mitochondrial function (Figure 5-11). HepG2 cells were exposed to AITC (Table 5-3) for 24 hours. The ATP content as indicated by the luciferase assay shows no significant difference between control and treatments.

To determine a potential relationship between cell number (DNA content) at the end of the 24-hour exposure period and ATP content in the HepG2 cells treated with BITC, ATP content was normalized for a DNA content (Figure 5-2). The ATP content normalized for DNA content was not significantly different from control in HepG2 cells.

Table 5-3.

*Butyl isothiocyanate Nominal and Measured Concentrations in Cell Culture Medium*

| Nominal Concentration | Measured Concentration | % Control |
|---|---|---|
| Control: Medium Only | Control: Medium Only | Control: Medium Only |
| 250 µM | 0 µM | 0 |
| 500 µM | 0 µM | 0 |
| 1000 µM | 1.3 µM | 0.13 |
| 1500 µM | 71 µM | 5 |
| 2000 µM | 135 µM | 7 |
| 2500 µM | 152 µM | 6 |

*Note*. Measured concentrations analyzed by GC-FID. Isothiocyanates were extracted from medium with hexane within two hours after initiation of treatment to HepG2 cells.

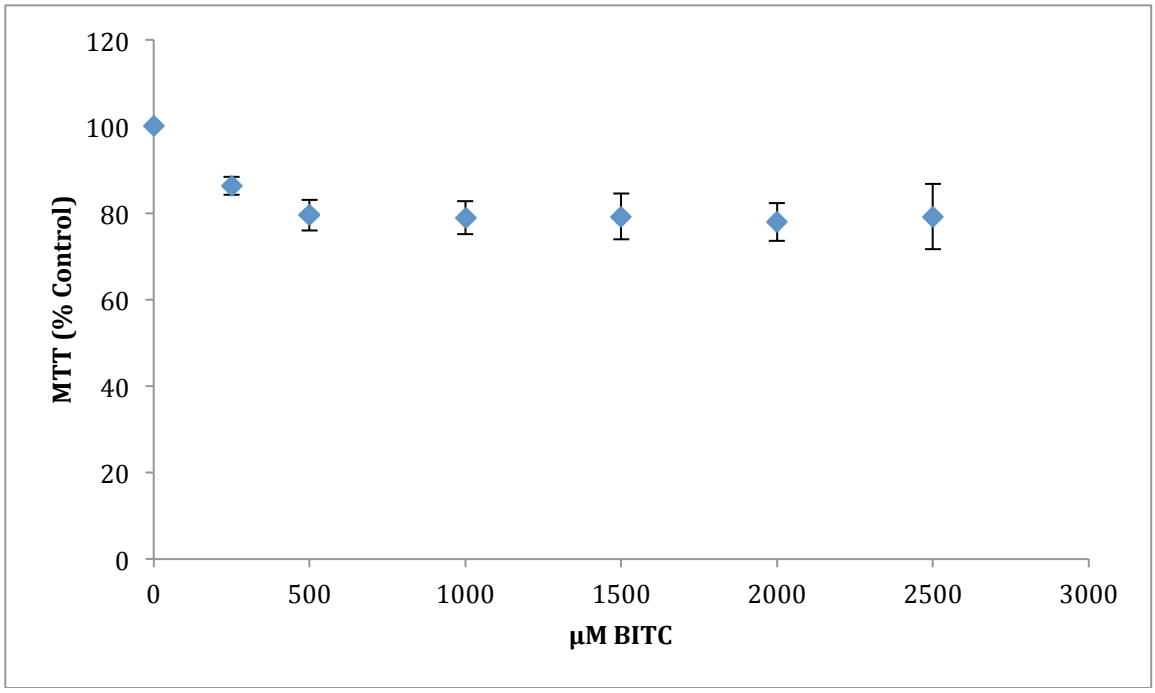



**Figure 5-9.** HepG2 Cells Treated with Butyl Isothiocyanate Treatments for 24 hours Followed by an MTT Cytotoxicity Assay to Determine Cell Metabolism

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test conducted for nonparametric data expressed as a proportion transformed with arcsine square root. Data show no significant difference compared to control.

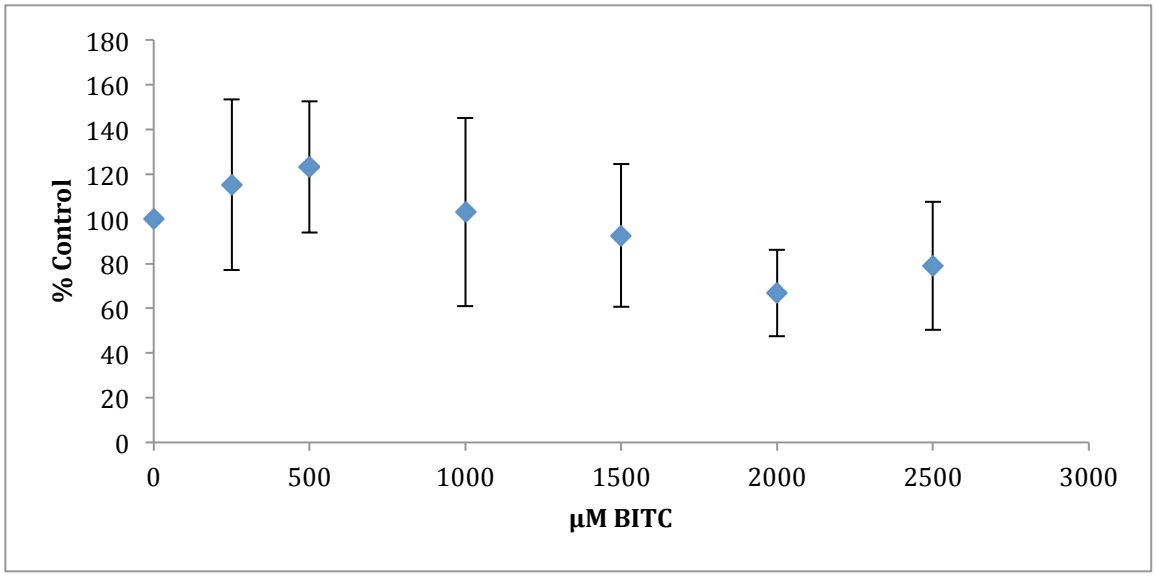



Figure 5-10. HepG2 Cells Treated with Butyl Isothiocyanate Treatments for 24 hours Followed by a DNA Nuclear Stain Assay with Hoecsht Dye 33258 to Determine DNA Content

*Note*. Percent of control is DNA content expressed as arbitrary fluorescence units using Hoecsht 33258. Nominal concentrations are shown. Points represent a mean value based on three separate plates with four replicates per plate. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's test conducted for parametric data expressed as a proportion transformed with arcsine square root. Data show no significant difference compared to control.

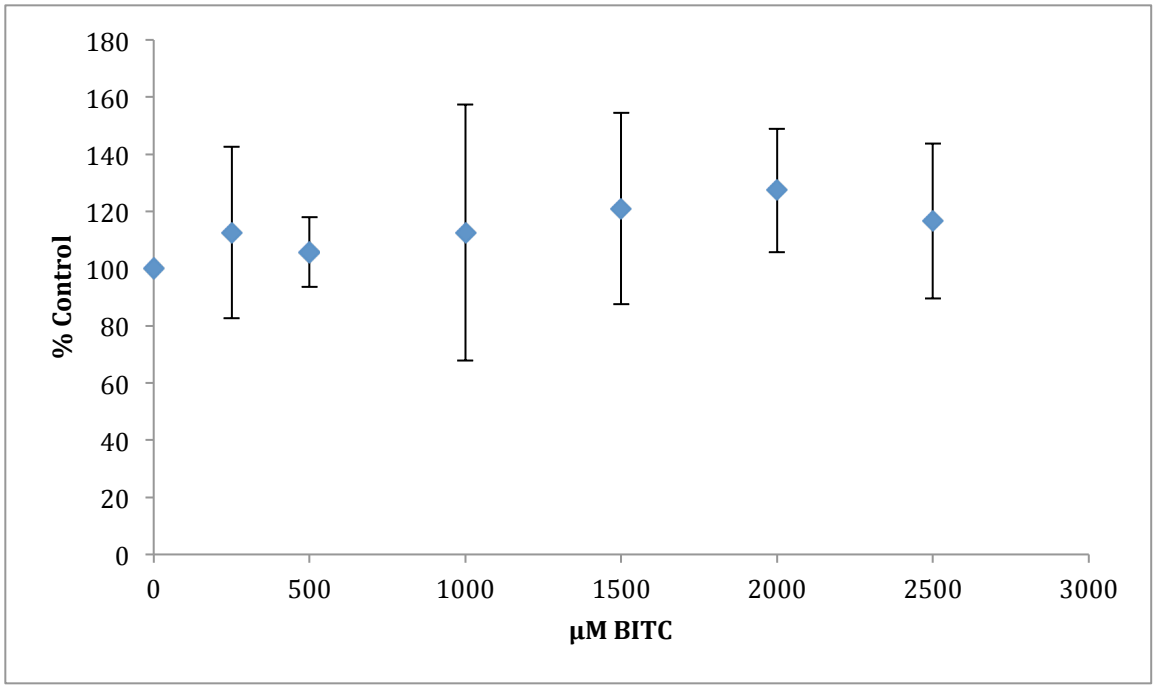



**Figure 5-11.** HepG2 Cells Treated with Butyl Isothiocyanate for 24 hours Followed by a Luciferase Assay to Determine ATP Content (Mitochondrial Function)

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's test conducted for parametric data expressed as a proportion transformed with arcsine square root. Data show no significant effect compared to control.

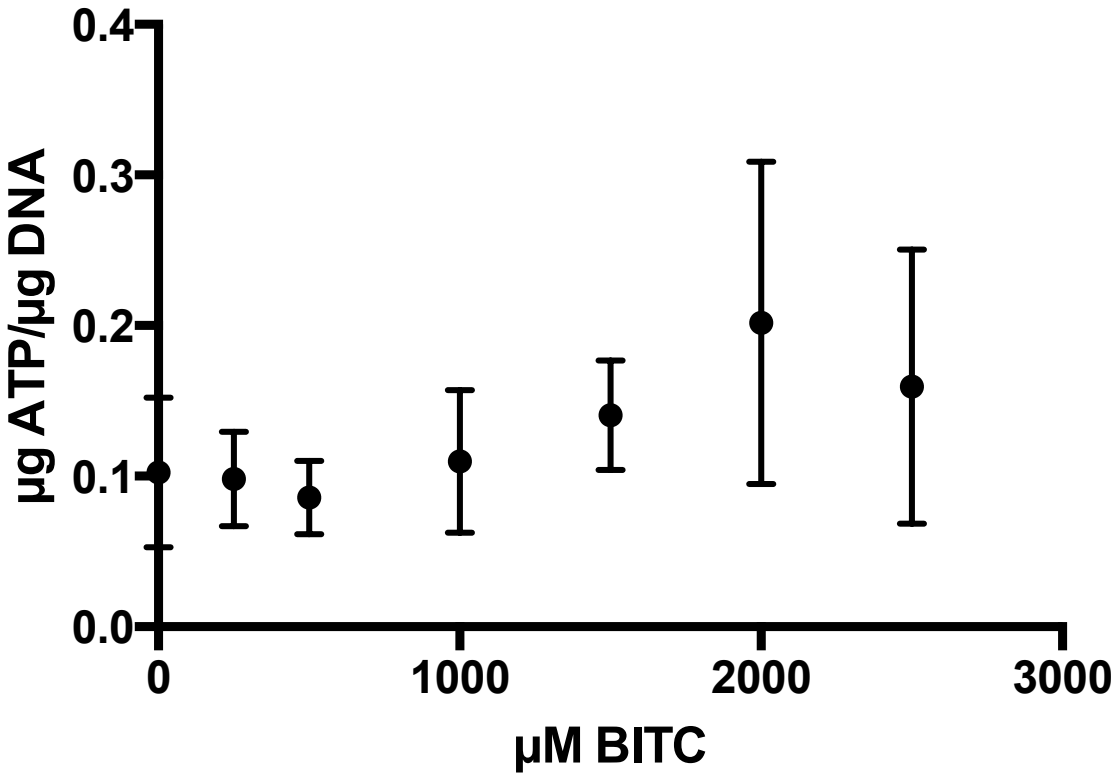



**Figure 5-12.** ATP Content Normalized to DNA Content in HepG2 Cells Exposed to Butyl Isothiocyanate for 24 hours

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's test conducted for parametric data. Data shows no significant difference from control.

### 5.1.4 Chorispora

HepG2 cells were assessed for metabolic activity by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after a 24-hour exposure to *Chorispora tenella* plant autolysis extract mixed in medium at varied percentages (Table 5-4). Treatment concentrations were picked based on preliminary experiments (data not shown). Cytotoxicity as indicated by the MTT assay was significantly different from control in HepG2 cells treated with 15% and 20% *Chorispora tenella* plant autolysis extract. The estimated median lethal concentration ($LC_{50}$) was 10% *Chorispora tenella* autolysis extract based on a linear regression model (p= 0.0001

Least Squares Analysis) (Figure 5-13), corresponding to a measured AITC concentration of 37 μM AITC tenella. A DNA nuclear stain assay using Hoechst 33258 fluorescent dye (Figure 5-14) was conducted to determine DNA content in the HepG2 cells as a metric of cell number at the end of the 24-hour exposure period. The DNA content as indicated by the nuclear stain assay was significantly different from control in HepG2 cells treated with 15 % and 20% of *Chorispora* tenella autolysis extract, corresponding to measured concentrations of AITC of 88 and 90 μM AITC, respectively.

The ATP content of HepG2 cells treated with *Chorispora tenella* autolysis extract was determined using a luciferase assay measuring ATP content as an assessment of mitochondrial function. HepG2 cells were exposed to *Chorispora tenella* autolysis extract (Table 5-4) for 24 hours. The ATP content as indicated by the luciferase assay was significantly different from control in HepG2 cells treated with 15% and 20% *Chorispora tenella* autolysis extract (Figure 5-15). The measured AITC concentrations in the treatment media were 88 and 90 μM, respectively

To determine a potential relationship between cell number (DNA content) at the end of the 24-hour exposure period and ATP content in the HepG2 cells treated with *Chorispora tenella* autolysis extract, ATP content was normalized to DNA content (Figure 5-16). The ATP content normalized for DNA content was significantly different from control in HepG2 cells treated with 15% and 20% *Chorispora tenella* autolysis extract.

Table 5-4

*Chorispora Tenella Autolysis Extract Percentages in Medium and Measured Allyl Isothiocyanate Concentrations in Medium*

| Percentage of Chorispora tenella autolysis extract | Measured Allyl isothiocyanate from Chorispora tenella autolysis extract | Equivalent fresh weight plant mass compared to autolysis extract percentage |
|---|---|---|
| 1% | 29 µM | 9 mg |
| 5% | 34 µM | 46 mg |
| 10% | 37 µM | 93 mg |
| 12% | 85 µM | 111 mg |
| 15% | 88 µM | 139 mg |
| 20% | 90 µM | 186 mg |

*Note*. Concentrations were analyzed by GC-FID. Isothiocyanates were extracted from medium with hexane within two hours after initiation of treatment of the HepG2 cells.

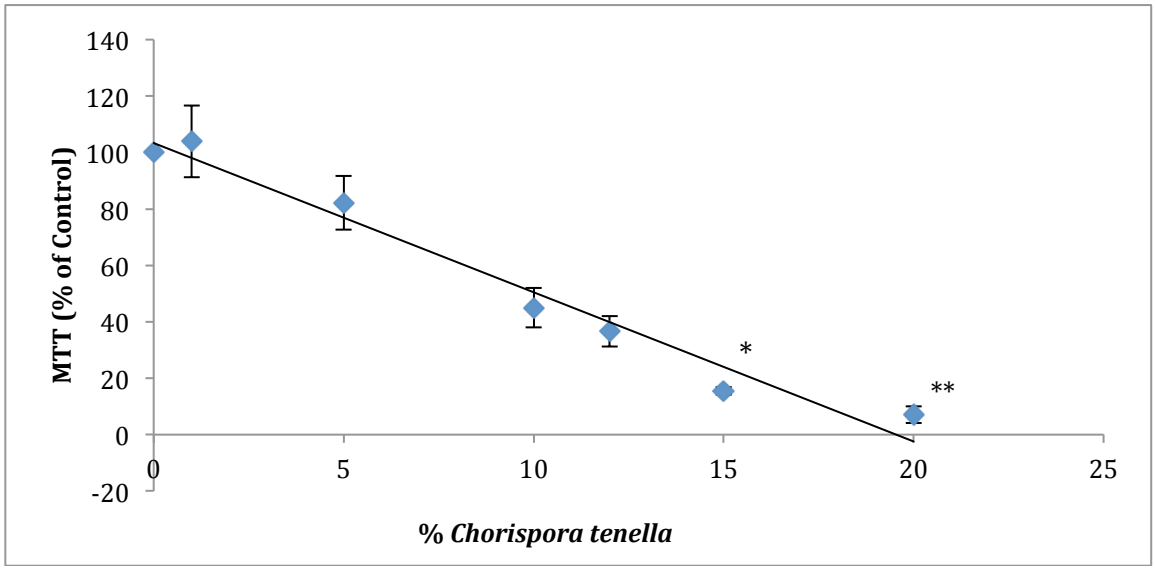



Figure 5-13. HepG2 Cells Treated with Chorispora Tenella Autolysis Extracts at Various Percentages in the Medium for 24 hours Followed by an MTT Cytotoxicity Assay to Determine Cell Metabolism

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test conducted for nonparametric data expressed as a proportion transformed with arcsine square root. $R^2$=0.970. *Significantly different from control p=0.0006 (Dunn). **Significantly different from control p<0.0001 (Dunn).

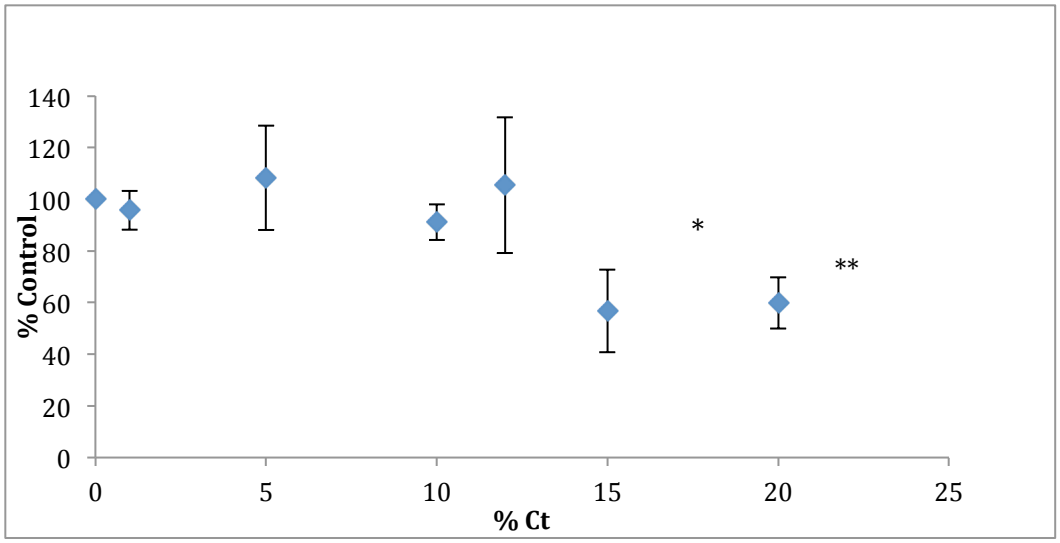



Figure 5-14. HepG2 Cells Treated with Chorispora Tenella Autolysis Extract in Medium for 24 hours. Followed by a DNA Nuclear Stain Assay with Hoecsht Dye 33258 to Determine DNA Content

*Note*. Percent of control is DNA content expressed as arbitrary fluorescence units using Hoecsht 33258. Points represent a mean value based on three separate plates with four replicates per plate. The error bars represent one standard deviation. One way ANOVA with post hoc Tukey's test conducted for parametric data expressed as a proportion transformed with arcsine square root. * Significantly different from control p=0.0014 (Tukey). ** Significantly different from control p=0.0056 (Tukey).

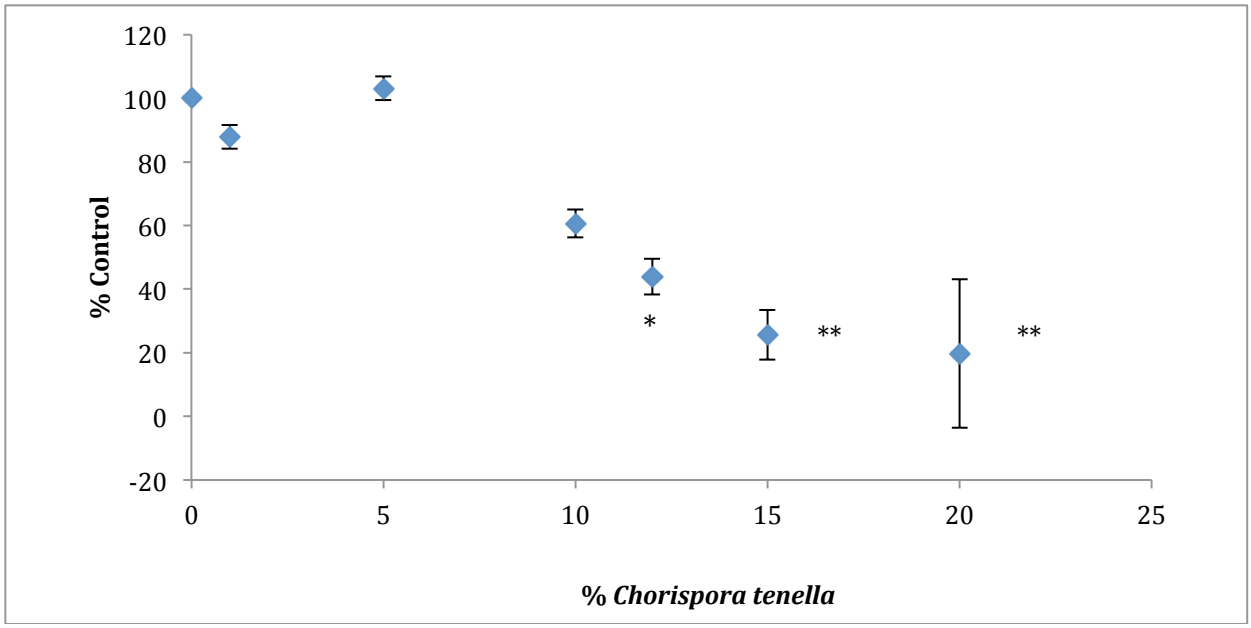



Figure 5-15. HepG2 Cells Treated with Chorispora Tenella Plant Autolysis Extract in Medium for 24 hours Followed by a Luciferase Assay to Determine ATP Content (Mitochondrial Function)

Note. Points represent a mean value based on three separate with four replicates. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test conducted for nonparametric data expressed as a proportion transformed with arcsine square root. * Significantly different from control p=0.0050 (Dunn).
** Significantly different from control p<0.0001 (Dunn).

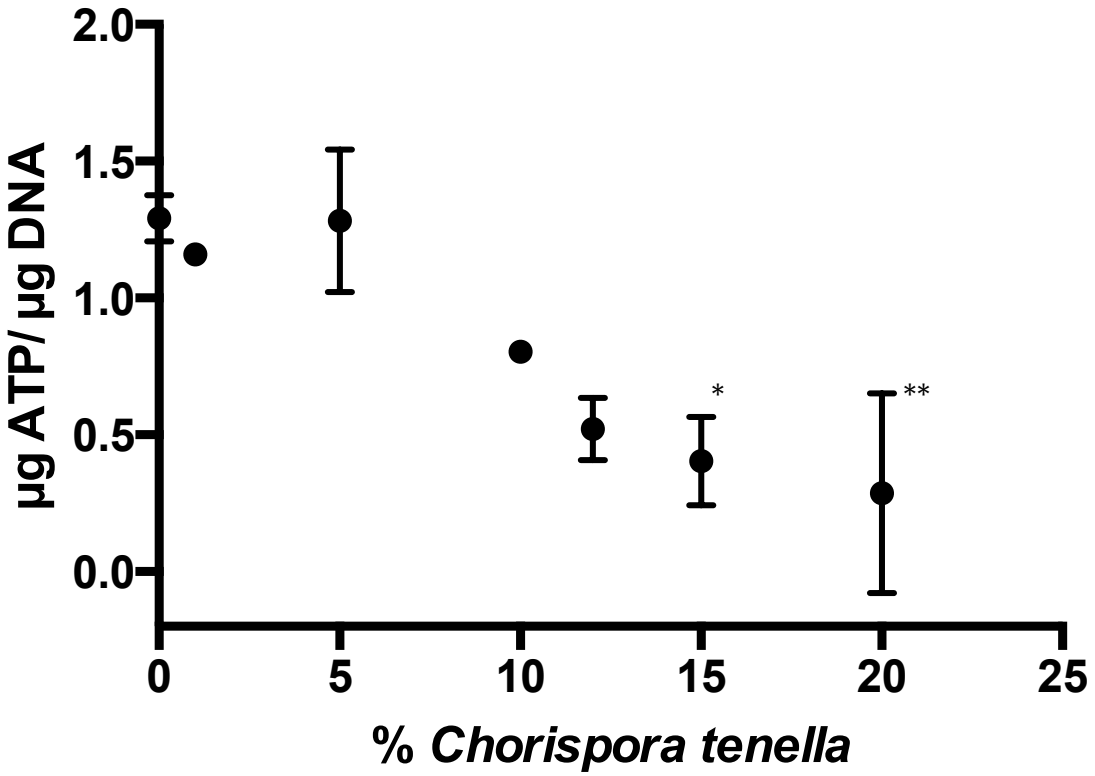



Figure 5-16. ATP Content Normalized to DNA Content in HepG2 Cells Exposed to Chorispora Tenella Autolysis Extract in Medium for 24 hours

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's conducted for parametric data. * Significantly different from control p=0.0315 (Tukey). ** Significantly different from control p=0.0021 (Tukey).

### 5.1.5 Comparison of Isothiocyanates and Chorispora tenella Autolysis Extract

The $LC_{50}$ for HepG2 cells with AITC, BITC, PITC and *Chorispora tenella* show that AITC has an effect on HepG2 cells at the lowest concentration compared to the other isothiocyanates (Table 5-5). The relative toxic potency and the isothiocyanate percentage in *Chorispora tenella* are the predicted potential contributions to toxicity based on the analysis of plants collected in 2010. Data from 2010 collection was used because there was no detection of PITC and BITC in the *Chorispora tenella* autolysis samples from 2017, used for cell treatments.

Table 5-5

*Table of Comparison of Median Lethal Concentration for HepG2 Cells*

| Isothiocyanate or Plant | Concentration μM, Percentage of *Chorispora tenella* Autolysis Extract or Equivalent Fresh Weight Plant Mass |
|---|---|
| AITC | 116 μM |
| PITC | 1152 μM |
| BITC | >2500 μM |
| *Chorispora tenella* | 37 μM AITC equivalent or 10% or 93 mg |

The relative toxicity of isothiocyanates to HepG2 cells as determined by the MTT assay is shown in Table 5-6 below. Allyl isothiocyanate is most toxic followed by PITC and finally BITC. Additionally, Table 5-7 shows the isothiocyanate percentage within *Chorispora tenella*. Allyl isothiocyanate has the highest percentage in *Chorispora tenella* followed by PITC then BITC.

Table 5-6

*Relative Toxic Potency for Isothiocyanates in HepG2 Cells*

| Isothiocyanate | Relative Potency |
|---|---|
| AITC | 1 |
| PITC | 0.10 |
| BITC | < 0.05 |

Table 5-7

*Isothiocyanate Percentage in Chorispora tenella Autolysis Extracts*

| Isothiocyanate | % in *Chorispora tenella* |
|---|---|
| AITC | 98 |
| PITC | 2 |
| BITC | 0.6 |

## 5.2 Discussion

The three isothiocyanates and *Chorispora tenella* plant autolysis extract have varying impacts on the HepG2 cell line. Allyl isothiocyanate has the greatest toxicity to the cells based on the $LC_{50}$ values among the three isothiocyanates tested (Table 5-5). This could be related to

the structure of AITC. Isothiocyanates are generally electrophilic in nature (Dunnick et al. 1982; Kawakishi and Kaneko, 1987; Duncan and Milne, 1993) and AITC is inherently so since it is an alkene compared to BITC and PITC which are alkanes.

In the Hepa 1c1c7 murine hepatoma cell line, isothiocyanates accumulated predominantly as the glutathione-isothiocyanate conjugate and the free isothiocyanate concentrations were low in cells (Zhang 2000; Mi et al. 2011). There is, however, some deconjugation that occurs intracellularly to achieve equilibrium with free isothiocycnates. This proportion is dependent on various factors such as pH and concentrations of isothiocyanates and thiols (Zhang 2000). Intracellularly, once the isothiocyanates reach the mitochondrial membrane, they can cause a rapid decrease of membrane potential. When human bladder cancer cells, UM-UC-3, were exposed to benzyl isothiocyanate and to phenethyl isothiocycnate, it was observed that both compounds caused a loss of membrane potential in a dose dependent manner (Tang and Zhang 2005). Damage to the mitochondrial membrane is associated with apoptosis of the cell (Conaway et al. 2002; Zhang et al, 2003; Tang and Zhang 2005; Zhang 2010) and ATP is required for apoptosis.

Induction of apoptosis has not been demonstrated with BITC and PITC, however AITC has been shown to induce apoptosis by significantly decreasing Bcl-2 (anti-apoptotic protein) and increasing Bax (pro-apoptotic protein) in breast cancer cell lines, indicating mitochondrial involvement (Zhang et al. 2003; Tang and Zhang 2005; Bo et al. 2016). Exposure to isothiocyanates causes the reduction of the mitochondrial membrane potential causing a release of cytochrome c, which triggers the caspase cascade, in particular activation of caspase-9 and subsequently caspase-3 (Tang and Zhang 2005; Zhang et al. 2003; Bo et al. 2016). Specific caspases activated in isothiocyanate induced apoptosis may be cell line-specific (Tang and Zhang

2005). HepG2 cells exposed to an isothiocyanate, sulfophorane, had activation of caspases 12 and 3, which induced apoptosis (Park et al. 2007; Zou et al. 2016). Additionally, AITC causes cell cycle arrest at the $G_2$/M stage, which then leads to apoptosis (Smith et al. 2004; Geng et al. 2011).

Allyl isothiocyanate has low $LC_{50}$ values, into the low micromolar range, 10 μM, representing the highly electrophilic nature of the compound and its ability to cause metabolic disruption (measured by MTT) at low micromolar concentrations in human promyelocytic acute leukemia (HL60/S) cells (Zhang et al. 2003; Zhang 2010). *Chorispora tenella*, collected in the 2016 and 2017 plant season contains AITC (data shown in previous chapter) and shows an $LC_{50}$ value 10% *Chorispora tenella* autolysis extract, found to contain 37 μM AITC by GC-FID analysis (Table 5-4). The difference in $LC_{50}$ values between AITC alone (116 μM AITC) compared to the AITC content in the *Chorispora tenella* autolysis extract (37 μM AITC) may be attributed to the sum of different components formed from the plant during autolysis. The influence of other constituents of the autolysis product was not characterized in this study.

Tables 5-1, 5-2, and 5-3 show nominal concentrations compared to measured concentrations of isothiocyanates in exposure medium. This difference could be due to the length of time from making the exposure treatments and performing all the isothiocyanate extraction with hexane. Treatments were prepared immediately prior to use. Commercially obtained synthetic isothiocyanates were added directly to medium. Remaining exposure treatment underwent analysis within two hours of preparation. Hexane (1mL) was added to each sample and allowed to shake on an orbital shaker for a minimum of 30 minutes. Upon completion of shaking, samples were centrifuged and decanted. Solvent was analyzed on GCFID. Discrepancies between nominal and measured concentrations could be due to the length

of time after preparation that samples were analyzed. Additionally, fetal bovine serum was present in the treatment medium potentially resulting in binding of the isothiocyanates. It is possible that the apparent lack of toxicity of BITC in HepG2 cells could be related to the very low measured concentration of the compound in hexane extracts of the culture medium used for cell treatments. This might indicate low bioavailability of BITC to the cells under the culture conditions.

Calculating the relative potency for the isothiocyanates based on the $LC_{50}$ observed for allyl isothiocyanate, the most toxic isothiocyanate found in *Chorispora tenella* autolysis extract (assigned a value of 1) propyl isothiocyanate had a relative potency value of 0.1 and that of butyl isothiocyanate was less than 0.05. When compared to the percentage that these isothiocyanates make up in the plant, (based on the 2010 collection (Figures 4-2, 4-3, and 4-4) because PITC and BITC were not found in autolysate of plants collected in 2017), it would be expected that liver toxicity would be primarily due to allyl isothiocyanate (98%), followed by propyl isothiocyanate (2%) and then butyl isothiocyanate (<0.6%). Since allyl isothiocyanate is the most abundant isothiocyanate in *Chorispora tenella* and the most toxic isothiocyanate, the possibility of any adverse effects is likely due to allyl isothiocyanate.

# 6. TOXICITY OF ISOTHIOCYANATES AND *CHORISPORA TENELLA* TO K1 THYROID CELLS

## 6.1 Introduction

K1 human thyroid carcinoma cells were used to determine cytotoxicity of three isothiocyanate compounds: allyl isothiocyanate (AITC), butyl isothiocyanate (BITC) and propyl isothiocyanate (PITC) and extracts of the autolysis products from the plant Choripsora tenella. The K1 cell line was chosen for experimentation since the thyroid was hypothesized as being a potential target of the isothiocyanates. Very few thyroid cell lines are available and the K1 line has been reported to retain the function of thyroid follicular cells (American Type Culture Collection, Personal Communication).

### 6.1.1 Allyl Isothiocyanate

To determine metabolic activity of K1 cells exposed to AITC for 24 hours (Table 6-1, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted (Figure 6-1). Allyl isothiocyanate concentrations were based on the results of Kassie and Knasmuller (2000). Cytotoxicity as indicated by the MTT assay was significantly different from control in HepG2 cells treated with 30, 70, 100, 175, and 250 μM AITC. The estimated lethal concentration of 50% of the cell population ($LC_{50}$) was 235 μM AITC, based on linear regression model ($p<0.0034$ Least Squares Analysis) (Figure 6-1).

A DNA nuclear stain assay using Hoechst 33258 fluorescent dye (Figure 6-2) was conducted to determine DNA content in the K1 cells as a metric of cell number at the end of the 24-hour exposure to AITC (Table 6-1). The DNA content as indicated by the nuclear stain assay was significantly different from control in K1 cells treated with 30, 100, and 175 μM AITC.

The ATP content of HepG2 cells treated with AITC was determined using a luciferase assay measuring ATP content as an assessment of mitochondrial function. K1 cells were exposed to AITC (Table 6-1) for 24 hours. The ATP content as indicated by the luciferase assay was significantly different from control in K1 cells treated with 70, 100, 175, and 250 μM AITC (Figure 6-3). To determine a potential relationship between cell number (DNA content) at the end of the 24-hour exposure period and ATP content in the HepG2 cells treated with AITC and ATP content was normalized for DNA content (Figure 6-4). The ATP content normalized for DNA content was significantly different from control in K1 cells treated with 70, 100, 175, and 250 μM AITC.

Table 6-1

*Allyl Isothiocyanate Nominal and Measured Concentrations in K1 Cell Culture Medium*

| Nominal Concentration | Measured Concentration | % Nominal |
|---|---|---|
| Control: medium only | Control: medium only | 0 |
| 10 μM | 9 μM | 90 |
| 30 μM | 10 μM | 33 |
| 70 μM | 15 μM | 21 |
| 100 μM | 37 μM | 37 |
| 175 μM | 47 μM | 27 |
| 250 μM | 74 μM | 30 |

*Note*. Concentrations were analyzed by GC-FID. Isothiocyanates were extracted from medium with hexane within two hours after initiation of treatment to K1 cells.

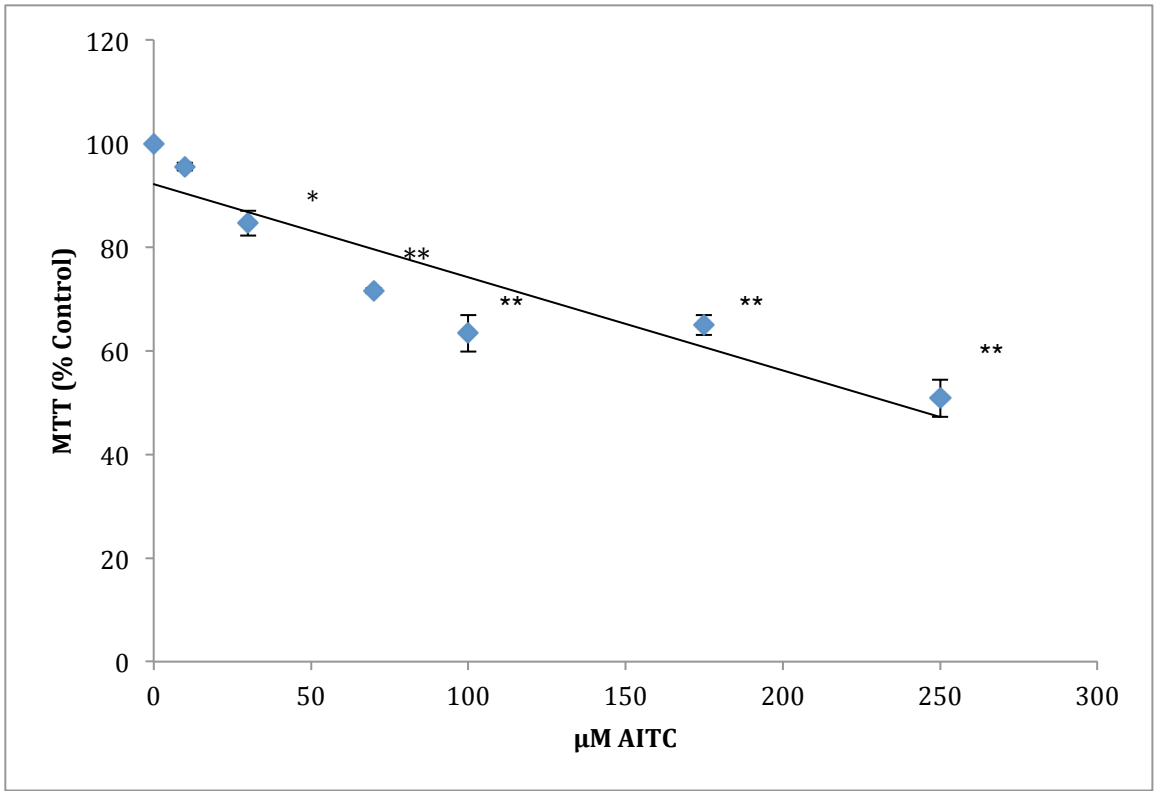



**Figure 6-1**. K1 cells treated with allyl isothiocyanate treatments for 24 hours followed by MTT cytotoxicity assay to determine cell metabolism.

*Note*. Points represent the mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's for parametric data. Data transformed with arcsine square root. $R^2$=0.845. * Significantly different from control $p<0.0153$ (Tukey). ** Significantly different from control $p<0.0001$ (Tukey).

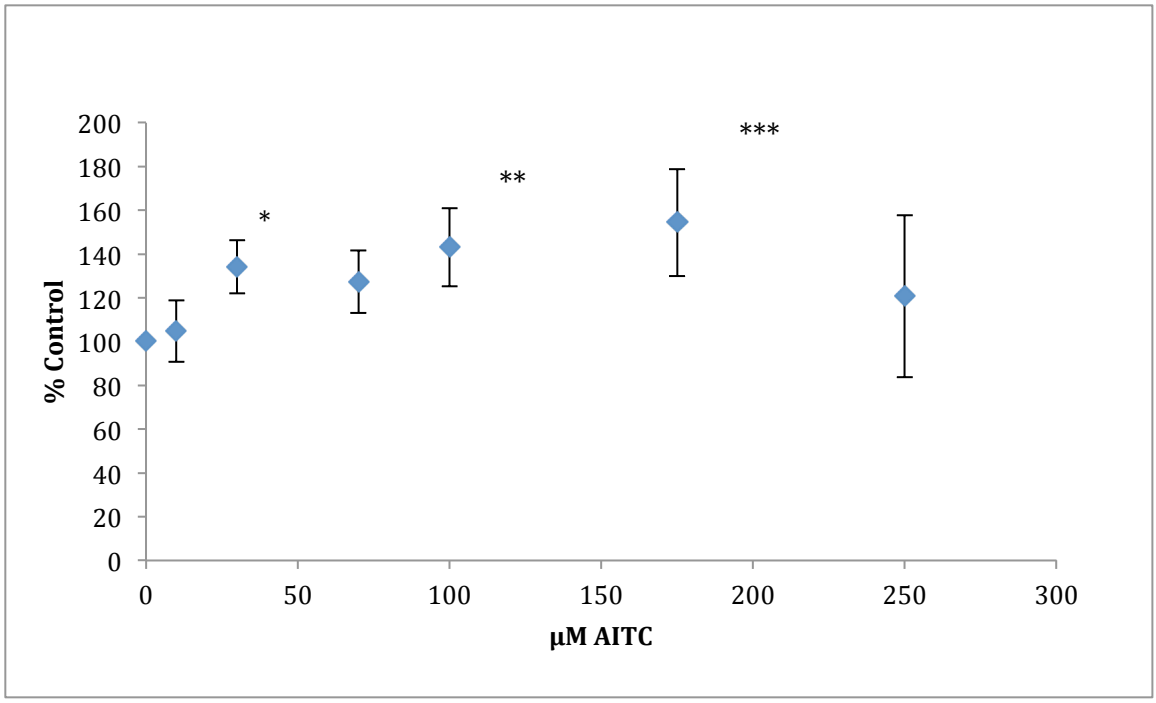



**Figure 6-2.** K1 cells treated with allyl isothiocyanate for 24 hours followed by a DNA nuclear stain assay with Hoecsht dye 33258 to determine DNA content.

*Note*. Percent of control is DNA content expressed as arbitrary fluorescence units using Hoecsht 33258. Nominal concentrations are shown. Points represent a mean value based on three separate plates with four replicates per plate. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test was conducted for nonparametric data expressed as a proportion transformed with arcsine square root. * Significantly different from control $p<0.0435$ (Dunn). ** Significantly different from control $p<0.0010$ (Dunn). *** Significantly different from control $p<0.0002$ (Dunn).

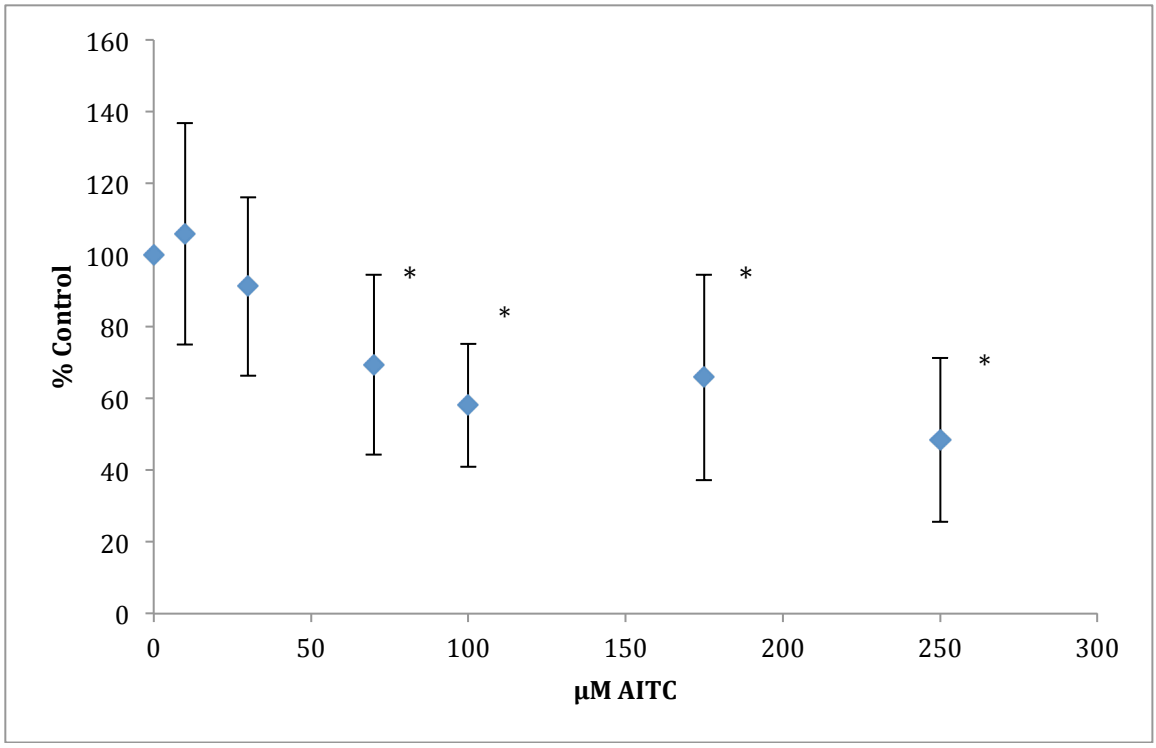



**Figure 6-3.** K1 Cells Treated with Allyl Isothiocyanate for 24 hours Followed by a Luciferase Assay to Determine ATP Content (Mitochondrial Function)

Note. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA with post hoc Tukey's test was conducted for parametric data expressed as a proportion transformed with arcsine square root.
*Significantly different from control $p<0.0001$ (Tukey).

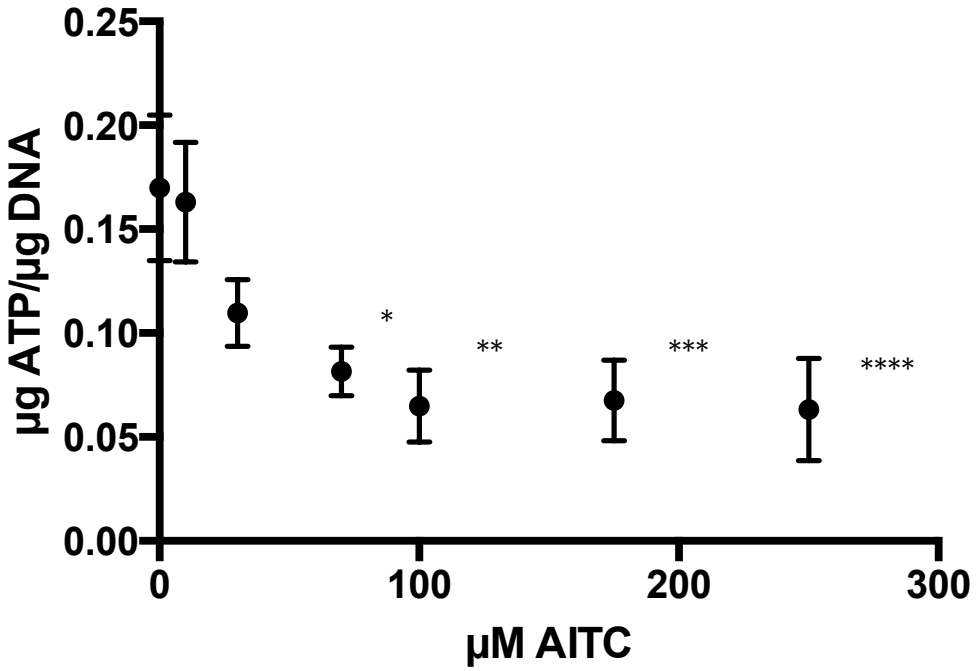



**Figure 6-4.** ATP Content (Mitochondrial Function) Normalized to DNA Content in K1 Cells Exposed to allyl isothiocyanate for 24 hours

Note. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's test was conducted for parametric data. * Significantly different from control p<0.0101 (Tukey). ** Significantly different from control p<0.0015 (Tukey). *** Significantly different from control p<0.0020 (Tukey). **** Significantly different from control p<0.0012 (Tukey).

### 6.1.2 Propyl Isothiocyanate

K1 cells were assessed for metabolic activity by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after a 24-hour exposure to propyl isothiocyanate (PITC) for 24 hours (Table 6-2). Treatment concentrations were selected based on structure and preliminary experiments (data not shown). Cytotoxicity as indicated by the MTT assay was significantly different from control in K1 cells treated with 600, 750, 1000, and 1250 µM PITC. The estimated median lethal concentration ($LC_{50}$) was 530 µM PITC based on a linear regression model (p=0.0012 Least Squares Analysis) (Figure 6-5).

A DNA nuclear stain assay using Hoechst 33258 fluorescent dye (Figure 6-6) was conducted to determine DNA content in the HepG2 cells as a metric of cell number at the end of the 24-hour exposure period. The DNA content as indicated by the nuclear stain assay was not significantly different from control in K1 cells treated with PITC.

The ATP content of K1 cells treated with PITC was determined using a luciferase assay as an assessment of mitochondrial function. K1 cells were exposed to PITC (Table 6-2) for 24 hours. The ATP content as indicated by the luciferase assay was significantly different from control in K1 cells treated with 600, 800, 1000, and 1250 µM PITC (Figure 6-7). The $LC_{50}$ of K1 cells exposed to PITC was 524 µM.

To determine a potential relationship between cell number (DNA content) at the end of the 24-hour exposure period and ATP content in the K1 cells treated with AITC, ATP content was normalized for a DNA content (Figure 6-8). The ATP content normalized for DNA content was significantly different from control in K1 cells treated with 300, 600, 750, 1000, and 1250 µM PITC.

Table 6-2

*Propyl Isothiocyanate Nominal and Measured Concentrations in Cell Culture Medium*

| Nominal Concentration | Measured Concentration | % Nominal |
|---|---|---|
| Control: medium only | Control: medium only | 0 |
| 100 µM | 85 µM | 85 |
| 300 µM | 107 µM | 36 |
| 600 µM | 139 µM | 23 |
| 750 µM | 461 µM | 61 |
| 1000 µM | 497 µM | 50 |
| 1250 µM | 563 µM | 45 |

*Note*. Measured concentrations were analyzed by GC-FID. Isothiocyanates were extracted from medium with hexane within two hours after initiation of treatment to K1 cells.

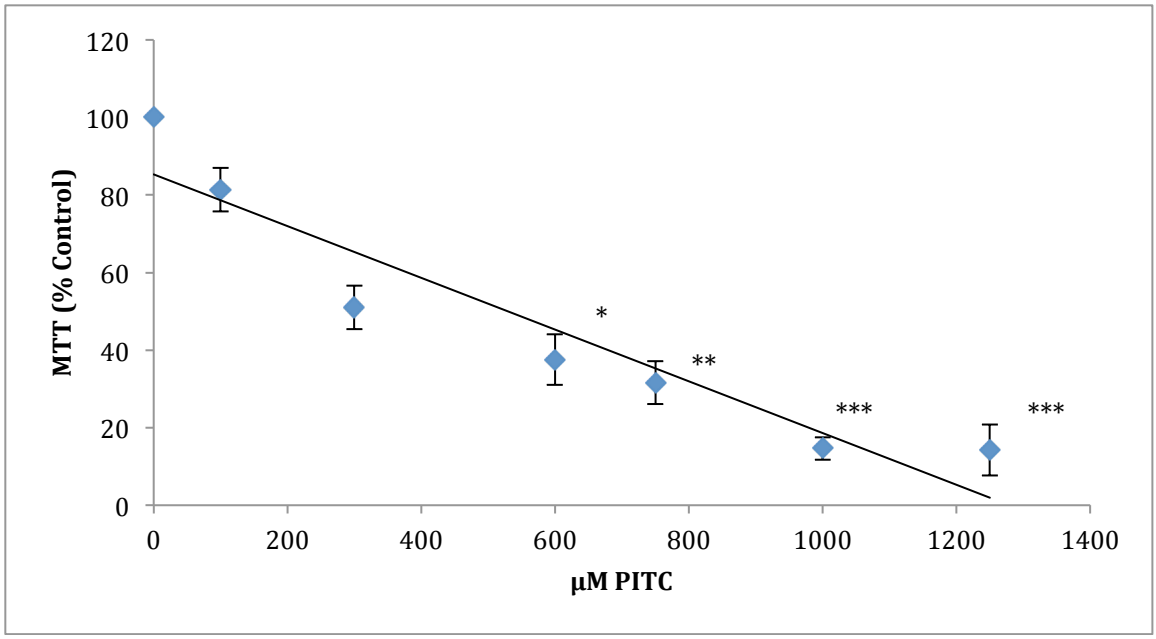



**Figure 6-5.** K1 cells treated with propyl isothiocyanate for 24 hours followed by an MTT cytotoxicity assay to determine cell metabolism

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test were conducted for nonparametric data expressed as a proportion transformed with arcsine square root. $R^2 = 0.90$.
* Significantly different from control p=0.0319 (Dunn). ** Significantly different from control p=0.0038 (Dunn).
*** Significantly different from control p<0.0001 (Dunn).

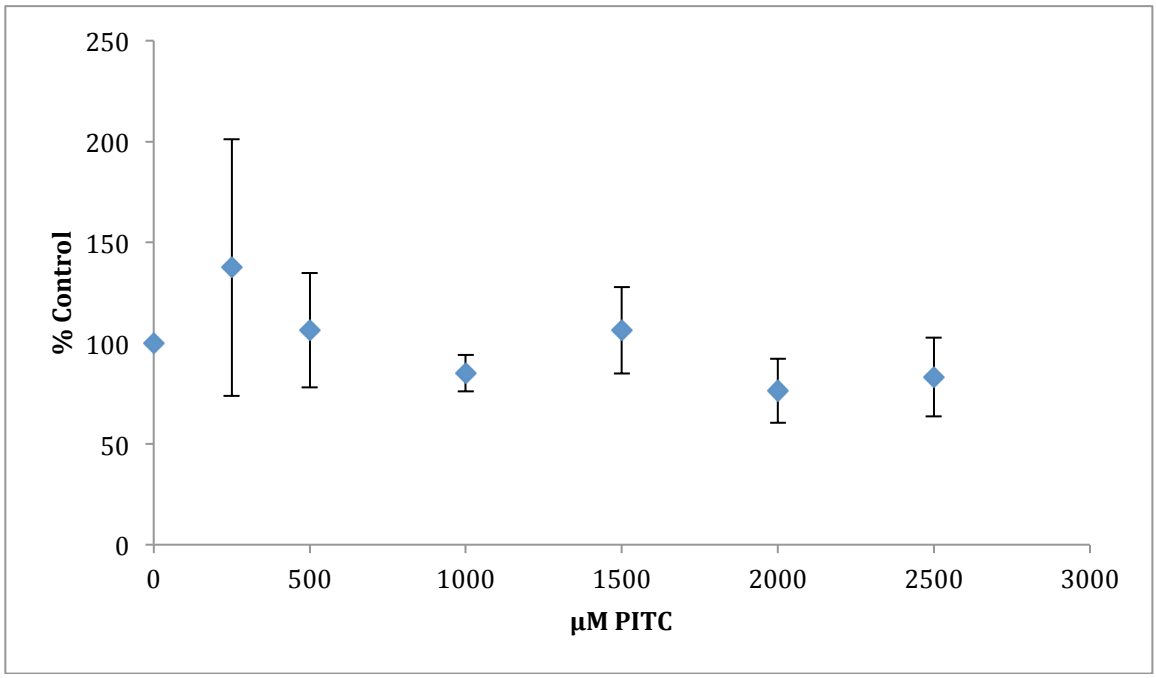



**Figure 6-6.** K1 Cells Treated with Propyl Isothiocyanate for 24 hours Followed by a DNA Nuclear Stain Assay with Hoecsht Dye 33258 to Determine DNA Content.

*Note*. Percent of control is DNA content expressed as arbitrary fluorescence units using Hoecsht 33258. Nominal concentrations are shown. Points represent a mean value based on three separate plates with four replicates per plate. The error bars shown represent one standard deviation. Kruskal – Wallis and post hoc Dunn's test was conducted for nonparametric data expressed as a proportion transformed with arcsine square root. Data shows no significant difference between treatments and control.

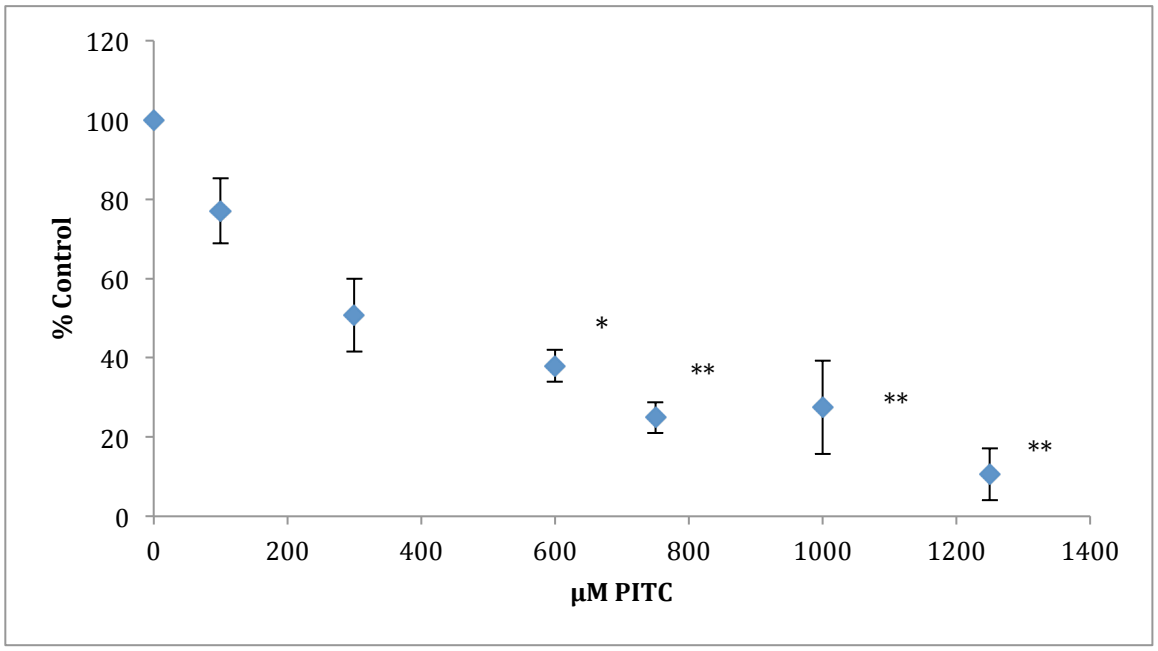



Figure 6-7. K1 cells treated with propyl isothiocyanate for 24 hours followed by a luciferase assay to determine ATP content (mitochondrial function).

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test was conducted for nonparametric data expressed as a proportion transformed with arcsine square root. $R^2$=0.88. * Significantly different from control p=0.0130 (Dunn). ** Significantly different from control p<0.0001 (Dunn).

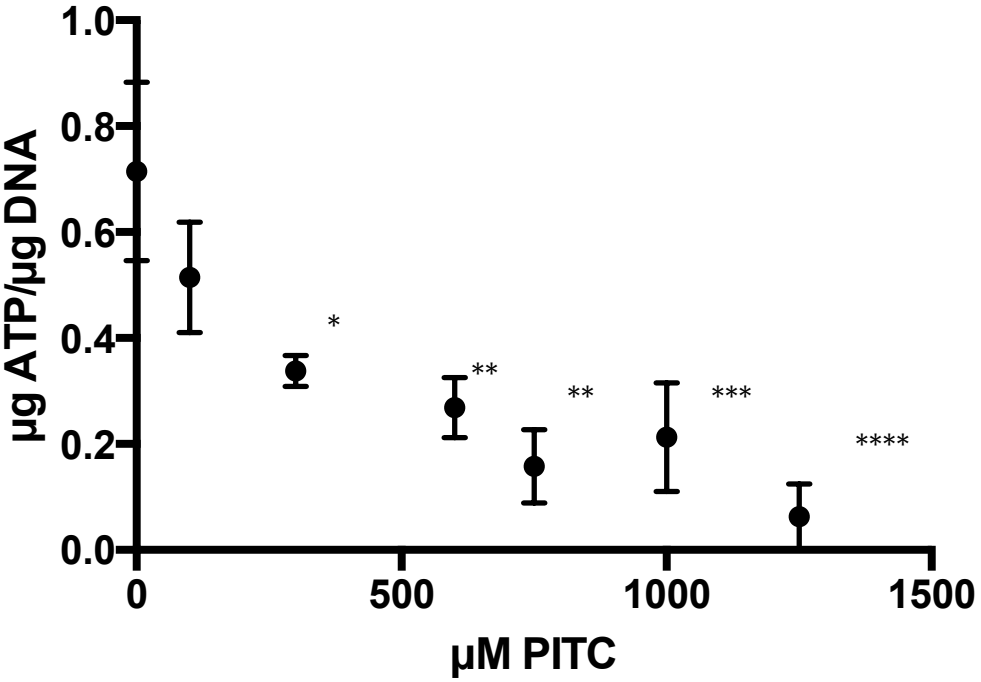



**Figure 6-8.** ATP Content (Mitochondrial Function) Normalized to DNA Content in K1 cells Exposed to Propyl Isothiocyanate for 24 hours

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's test conducted for parametric data. * Significantly different from control $p<0.0215$ (Tukey). ** Significantly different from control $p<0.0055$ (Tukey). *** Significantly different from control $p<0.0016$ (Tukey). **** Significantly different from control $p<0.0001$ (Tukey).

### 6.1.3 Butyl Isothiocyanate

K1 cells were assessed for metabolic activity by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after a 24-hour exposure to butyl isothiocyanate (BITC) (Table 6-3). Treatment concentrations were identified based on chemical structure and preliminary experiments (data not shown). Cytotoxicity as indicated by the MTT assay was significantly different from control in K1 cells treated with 1000, 1500, 2000, and 2500 µM AITC. The estimated median lethal concentration ($LC_{50}$) was 1086 µM BITC based on a linear regression model ($p<0.0012$ Least Squares Analysis) (Figure 6-9).

A DNA nuclear stain assay using Hoechst 33258 fluorescent dye (Figure 6-10) was conducted to determine DNA content in the K1 cells as a metric of cell number at the end of the 24-hour exposure period. The DNA content as indicated by the nuclear stain assay showed no significant difference compared to control.

The ATP content of K1 cells treated with BITC was determined using a luciferase assay as an assessment of mitochondrial function (Figure 6-11). K1 cells were exposed to AITC (Table 6-3) for 24 hours. The ATP content as indicated by the luciferase assay was significantly different from control in K1 cells treated with 1500, 2000, and 2500 µM BITC. The $EC_{50}$ was estimated to be 931 µM BITC.

To determine a potential relationship between cell number (DNA content) at the end of the 24-hour exposure period and ATP content in the K1 cells treated with BITC, ATP content was normalized to DNA content (Figure 6-12). The ATP content normalized for DNA content was significantly different from control in K1 cells treated with 2500 µM BITC.

Table 6-3

*Butyl Isothiocyanate Nominal and Measured Concentrations in Cell Culture Medium*

| Nominal Concentration | Measured Concentration | % Nominal |
|---|---|---|
| Control: medium only | Control: medium only | 0 |
| 250 µM | 62 | 25 |
| 500 µM | 66 | 13 |
| 1000 µM | 68 | 7 |
| 1500 µM | 207 | 14 |
| 2000 µM | 146 | 7 |
| 2500 µM | 165 | 7 |

*Note*. Concentrations analyzed by GC-FID. Isothiocyanates were extracted from medium with hexane within two hours after initiation of treatment to K1 cells.

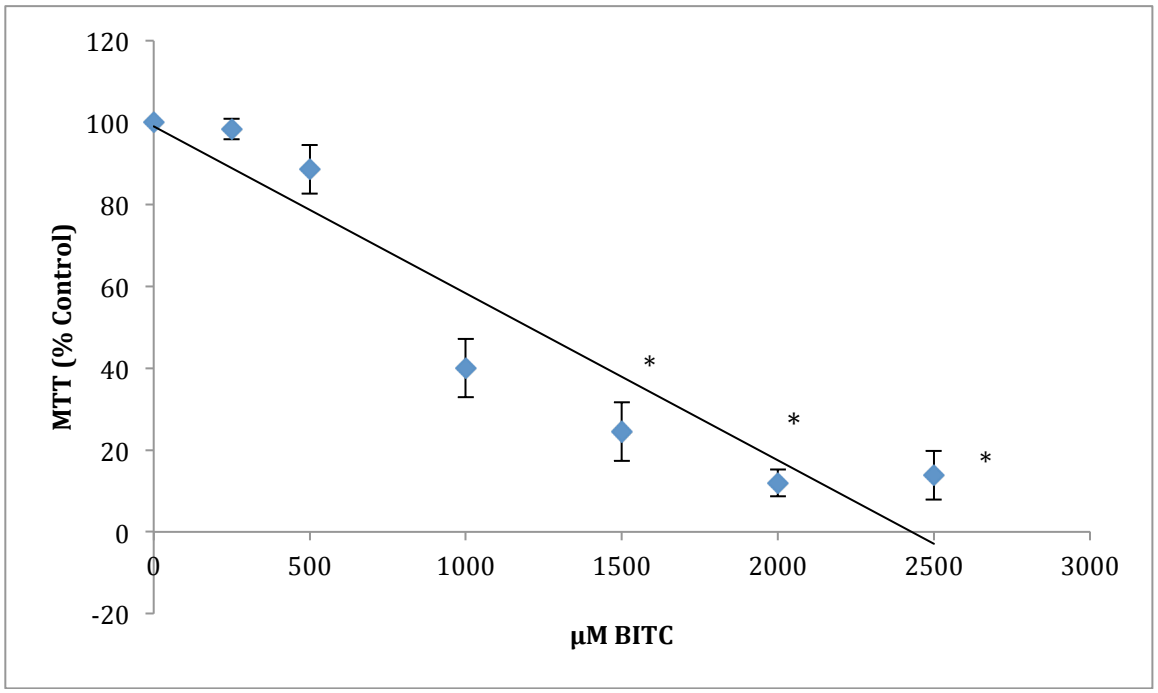



**Figure 6-9.** K1 cells treated with butyl isothiocyanate for 24 hours followed by an MTT cytotoxicity assay to determine cell metabolism.

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA with post hoc Tukey's test data conducted for parametric data expressed as a proportion transformed with arcsine square root. $R^2$ =0.896. * Significantly different from control $p<0.01$ (Dunn).

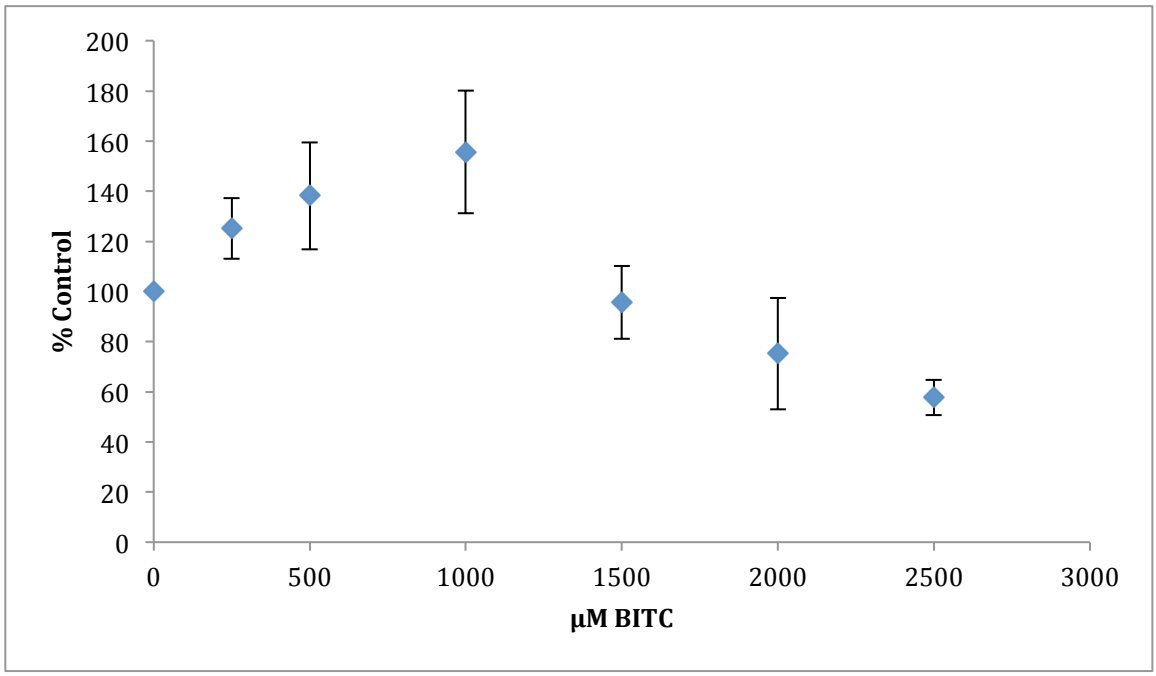



Figure 6-10. K1 Cells Treated with Butyl Isothiocyanate Treatments for 24 Hours Followed by a DNA Nuclear Stain Assay with Hoecsht dye 33258 to Determine DNA Content

*Note*. Percent of control is DNA content expressed as arbitrary fluorescence units using Hoecsht 33258. Nominal concentrations are shown. Points represent a mean value based on three separate plates with four replicates per plate. The error bars represent one standard deviation. Kruskal-Wallis and post hoc Dunn's test conducted for nonparametric data expressed as a proportion transformed with arcsine square root. Data shows no significant difference compared to control.

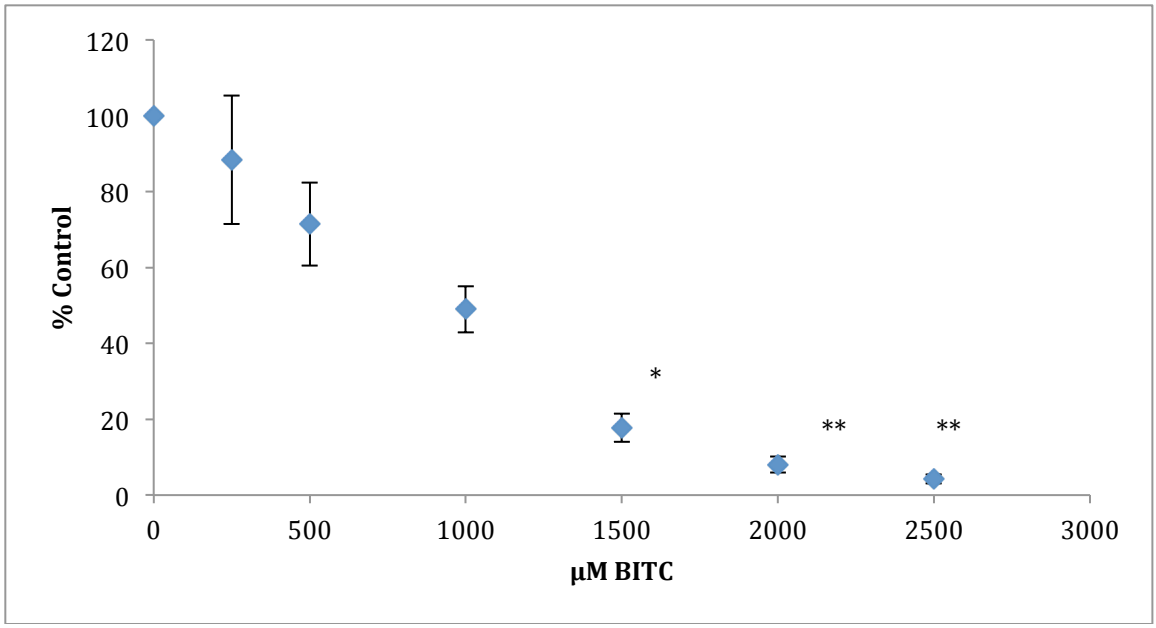



**Figure 6-11.** K1 Cells Treated with Butyl Isothiocyanate for 24 Hours Followed by a Luciferase Assay to Determine ATP Content (Mitochondrial Function)

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. Kruskal-Wallis and post hoc Dunn's test conducted for nonparametric data expressed as a proportion transformed with arcsine square root. * Significantly different from control $p<0.0063$ (Dunn). ** Significantly different from control $p<0.0002$ (Dunn).

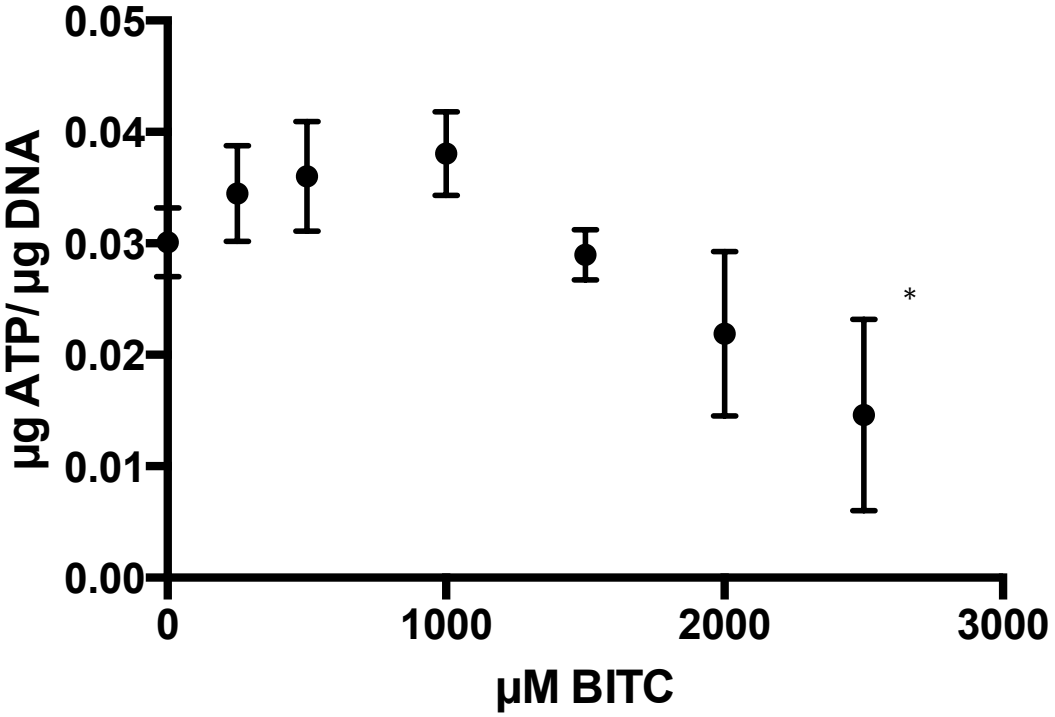



**Figure 6-12.** ATP Content (Mitochondrial Function) Normalized to DNA Content in HepG2 Cells Exposed to Butyl Isothiocyanate for 24 hours.

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. Nominal concentrations are shown. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's test conducted for parametric data. * Significantly different from control $p<0.0352$ (Tukey).

### 6.1.4 Chorispora tenella

The metabolic activity of K1 cells was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after a 24-hour exposure to Chorispora tenella plant autolysis extract mixed in medium at varied percentages (Table 6-4). Treatment concentrations were identified based on experimentation (data not shown). Cytotoxicity as indicated by the MTT assay was significantly different form control in K1 cells treated with 5%, 10%, 12%, 15%, and 20% Chorispora tenella plant autolysis extract. The estimated median lethal concentration ($LC_{50}$) was 8.6% plant autolysis extract based on a linear regression model ($p= 0.007$ Least Squares Analysis) corresponding to 119 µM AITC (Figure 6-13).

A DNA nuclear stain assay using Hoechst 33258 fluorescent dye (Figure 6-14) was conducted to determine DNA content in the K1 cells as a metric of cell number at the end of the 24-hour exposure period. The DNA content as indicated by the nuclear stain assay was not significantly different from control in K1 cells treated with *Chorispora* tenella autolysis extract.

The ATP content of K1 cells treated with *Chorispora tenella* autolysis extract was determined using a luciferase assay as an assessment of mitochondrial function. K1 cells were exposed to *Chorispora tenella* autolysis extract (Table 6-4) for 24 hours. The ATP content as indicated by the luciferase assay was significantly different from control in K1 cells treated with 10%, 12%, 15%, and 20% *Chorispora tenella* autolysis extract which correspond to 138, 274, 289, and 296 μM AITC.

To determine a potential relationship between cell number (DNA content) at the end of the 24-hour exposure period and ATP content in the K1 cells treated with *Chorispora tenella* autolysis extract, ATP content was normalized to DNA content (Figure 4-16). The ATP content normalized for DNA content was significantly different from control in K1 cells treated with 15% and 20% *Chorispora tenella* autolysis extract, corresponding to 289 and 296 μM AITC.

Table 6-4.

*Chorispora tenella Autolysis Extract Percentages in Medium and Measured allyl isothiocyanate Concentration in Medium*

| Percentage of Chorispora tenella autolysis extract | Measured Allyl isothiocyanate from Chorispora tenella autolysis extract | Equivalent fresh weight plant mass compared to autolysis extract percentage |
|---|---|---|
| 1% | 97 μM | 9 mg |
| 5% | 109 μM | 46 mg |
| 10% | 138 μM | 93 mg |
| 12% | 274 μM | 111 mg |
| 15% | 289 μM | 139 mg |
| 20% | 296 μM | 186 mg |

*Note*. AITC concentrations were analyzed by GC-FID. Isothiocyanates were extracted from medium with hexane within two hours after initiation of treatment to K1 cells.

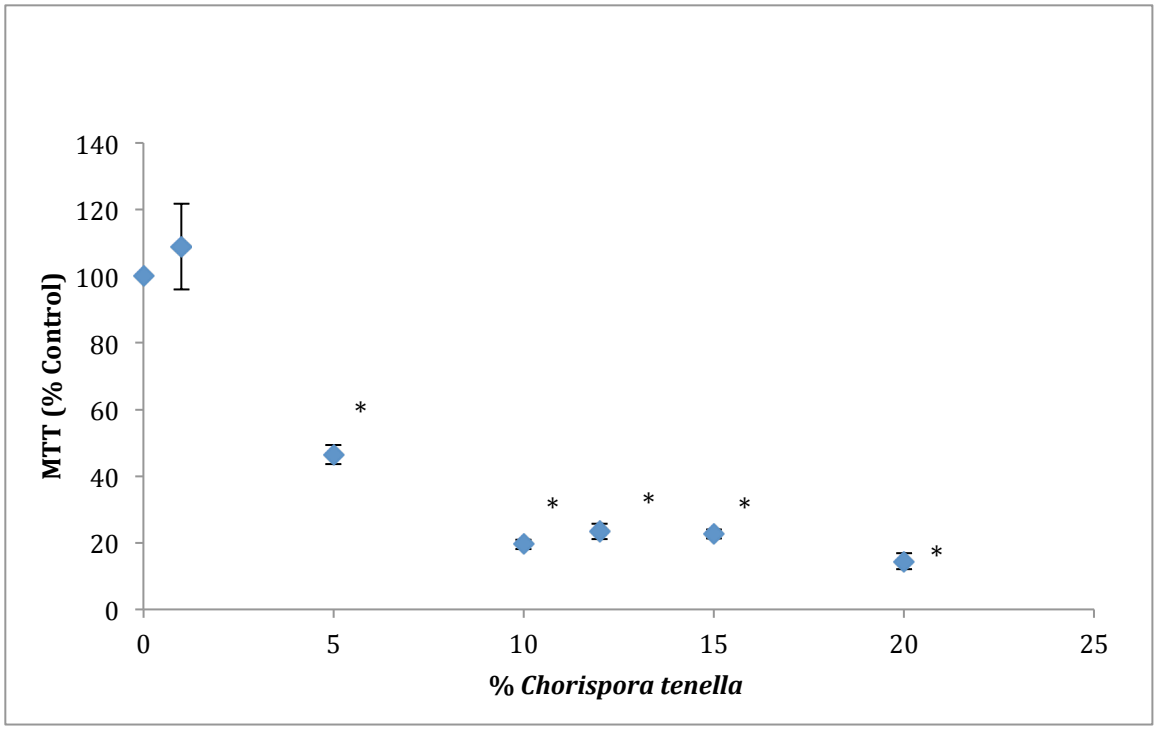



**Figure 6-13.** K1 cells treated with Chorispora tenella autolysis extracts at various percentages in the medium for 24 hours followed by an MTT cytotoxicity assay to determine cell metabolism.

*Note*. Points represent a mean value based on three separate plates with four replicates per plate. The error bars represent one standard deviation. One way ANOVA and post hoc Tukey's test conducted for parametric data expressed as a proportion transformed with arcsine square root. * Significantly different from control $p<0.0001$ (Tukey).

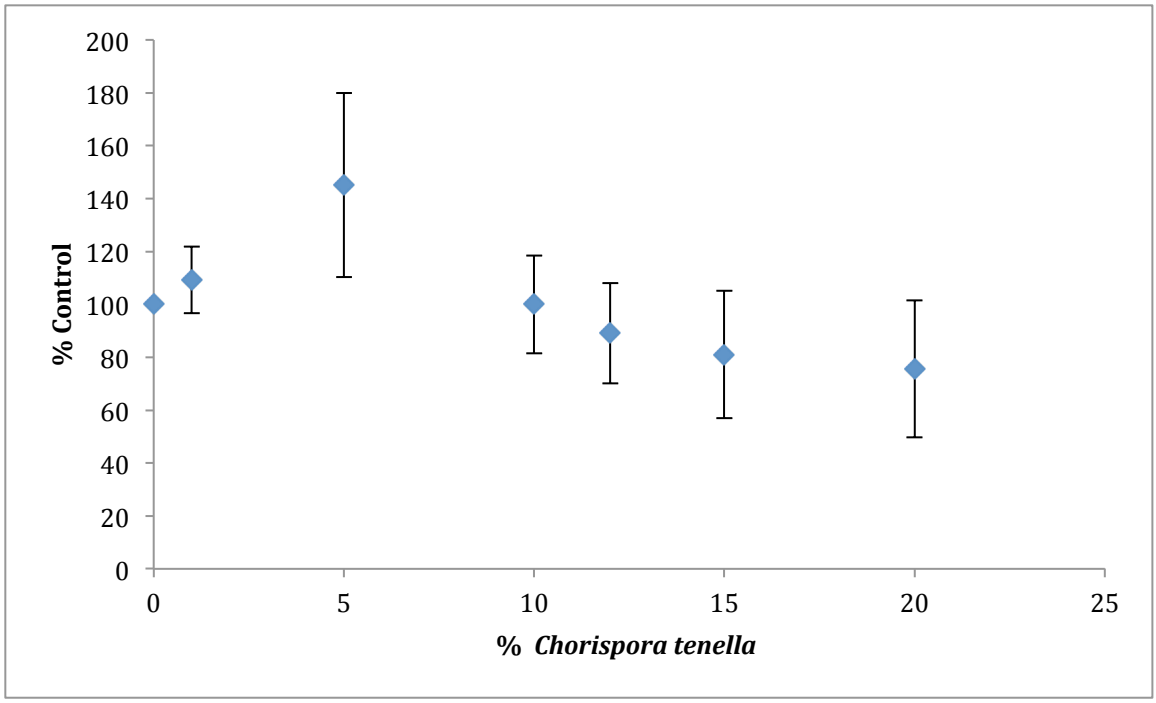



**Figure 6-14.** K1 Cells Treated with *Chorispora tenella* Autolysis Extract in Medium for 24 Hours Followed by a DNA Nuclear Stain Assay with Hoecsht Dye 33258 to Determine DNA Content

*Note*. Percent of control is DNA content expressed as arbitrary fluorescence units using Hoecsht 33258. Points represent a mean value based on three separate plates with four replicates per plate. The error bars represent one standard deviation. Kruskal-Wallis and post hoc Dunn's test conducted for nonparametric data expressed as a proportion transformed with arcsine square root. No significant differences were observed compared to control.

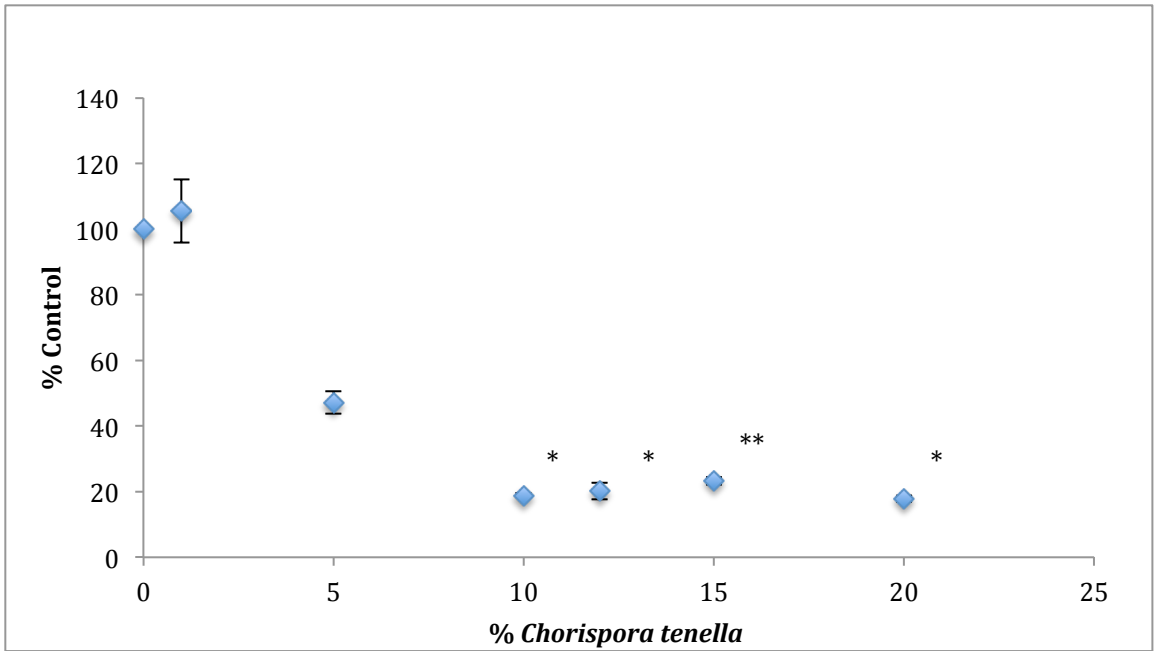



**Figure 6-15.** K1 Cells Treated with Chorispora tenella Plant Autolysis Extract in Medium For 24 Hours Followed by a Luciferase Assay to Determine ATP Content (Mitochondrial Function)

*Note*. Points represent a mean value based on three separate with four replicates. The error bars represent one standard deviation. Kruskal Wallis and post hoc Dunn's test conducted for nonparametric data expressed as a proportion transformed with arcsine square root. * Significantly different from control $p<0.0001$ (Dunn). ** Significantly different from control $p=0.0067$ (Dunn).

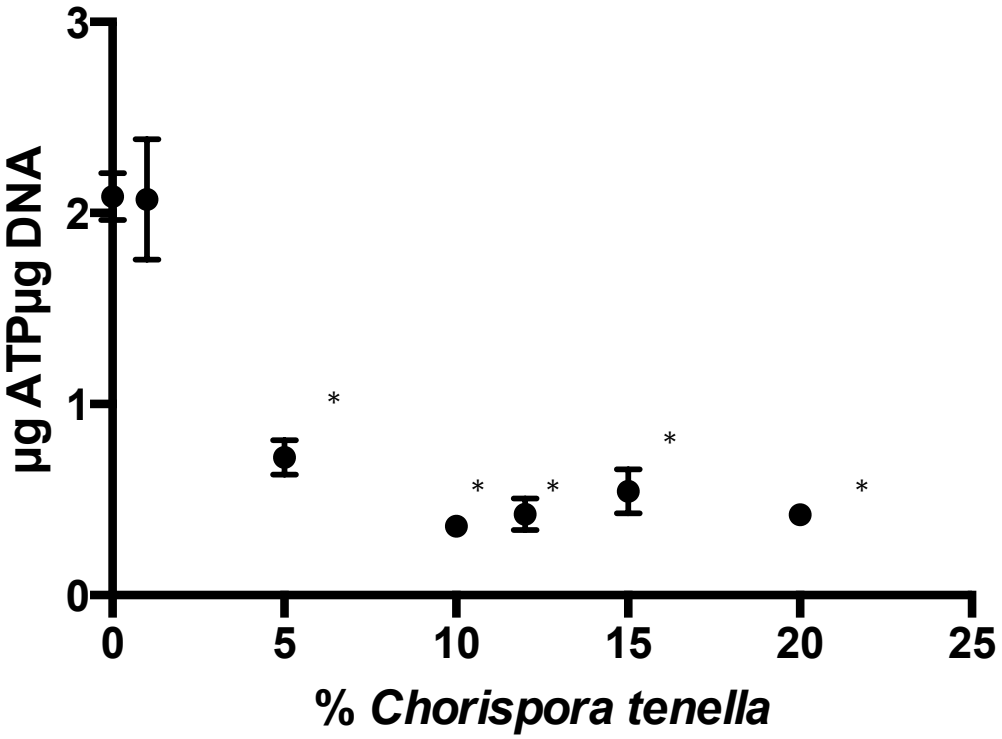



**Figure 6-16.** ATP Content Normalized to DNA Content in K1 Cells Exposed to *Chorispora tenella* Autolysis Extract in Medium for 24 Hours

*Note*. Points represent a mean value based on three separate plates each averaged from four replicates. The error bars shown show one standard deviation. Data transformed with arcsin square root. One way ANOVA and post hoc Tukey's test conducted for parametric data. * Significantly different from control $p<0.0001$ (Tukey).

### 6.1.5 Comparison of Isothiocyanates and Chorispora tenella Autolysis Extract

The $LC_{50}$ for K1 cells with AITC, BITC, PITC and *Chorispora tenella* show that AITC has an effect on K1 cells at the lowest concentration compared to the other isothiocyanates (Table 6-5).

Table 6-5

*Table of Comparisons of Lethal Concentration of Fifty Percent of K1 Cell Population*

| Isothiocyanate or plant | Concentration μM, percentage of *Chorispora tenella* autolysis extract or equivalent fresh weight plant mass |
|---|---|
| AITC | 235 μM |
| PITC | 530 μM |
| BITC | 1086 μM |
| *Chorispora tenella* | 119 μM, 8.6% or 77 mg |

Toxic potency and the isothiocyanate percentage in *Chorispora tenella* are the predicted potential contributions to toxicity based on the 2010 collection data. Data from 2010 collection were used because there was no detection of PITC and BITC in the *Chorispora tenella* samples from 2017, which were used to prepare autolysis product for cell treatments.

Toxicity potency based on the MTT assay in K1 cells is shown in Table 6-6, below. Allyl isothiocyanate is most toxic, followed by PITC and finally BITC. Additionally, Table 6-7 shows the isothiocyanate percentage within *Chorispora tenella*. Allyl isothiocyanate has the highest percentage in *Chorispora tenella,* followed by PITC then BITC.

Table 6-6

*Toxicity Potency for isothiocyanates in K1 Cells*

| Isothiocyanate | Relative Toxic Potency |
|---|---|
| AITC | 1 |
| PITC | 0.4 |
| BITC | 0.2 |

Table 6-7

*Isothiocyanate Percentage Within Chorispora tenella*

| Isothiocyanate | % in *Chorispora tenella* |
|---|---|
| AITC | 90 |
| PITC | 8 |
| BITC | 2 |

### 6.1.6 Thyroglobulin ELISA

K1 cells were exposed to allyl isothiocyanate, propyl isothiocyanate, butyl isothiocyanate, and *Chorispora tenella* in K1 cell medium. A thyroglobulin ELISA was conducted to quantify thyroglobulin produced by K1 cells (Figure 6-17). None of the isothiocyanates or the *Chorispora tenella* autolysis extract had an effect on the amount of thyroglobulin detected.

Table 6-6

*Nominal and Measured Concentrations in Cell Culture Medium for Various Isothiocyanates and Chorispora tenella Autolysis Extract Percentages*

| Nominal Concentration of isothiocyanate or percentage of *Chorispora tenella* autolysis extract | Measured Concentration of isothiocyanate µM or measured Allyl isothiocyanate from *Chorispora tenella* autolysis extract | % Nominal Concentration or equivalent fresh weight plant mass compared to autolysis extract percentage |
|---|---|---|
| AITC 10 µM | 13 µM | 130 |
| AITC 30 µM | 17 µM | 57 |
| AITC 100 µM | 49 µM | 49 |
| AITC 175 µM | 54 µM | 31 |
| AITC 250 µM | 76 µM | 30 |
| PITC 100 µM | 92 µM | 92 |
| PITC 600 µM | 180 µM | 30 |
| PITC 1000 µM | 215 µM | 22 |
| PITC 1250 µM | 285 µM | 23 |
| BITC 250 µM | 127 µM | 51 |
| BITC 1000 µM | 180 µM | 18 |
| BITC 2000 µM | 236 µM | 12 |
| BITC 2500 µM | 237 µM | 9 |
| 1% | 8 µM | 9 mg |
| 5% | 9 µM | 46 mg |
| 10% | 12 µM | 93 mg |
| 15% | 26 µM | 139 mg |
| 20% | 29 µM | 186 mg |

*Note*. Measured concentrations analyzed by GC-FID. Isothiocyanates were extracted from medium with hexane within two hours after initiation of treatment to K1 cells.

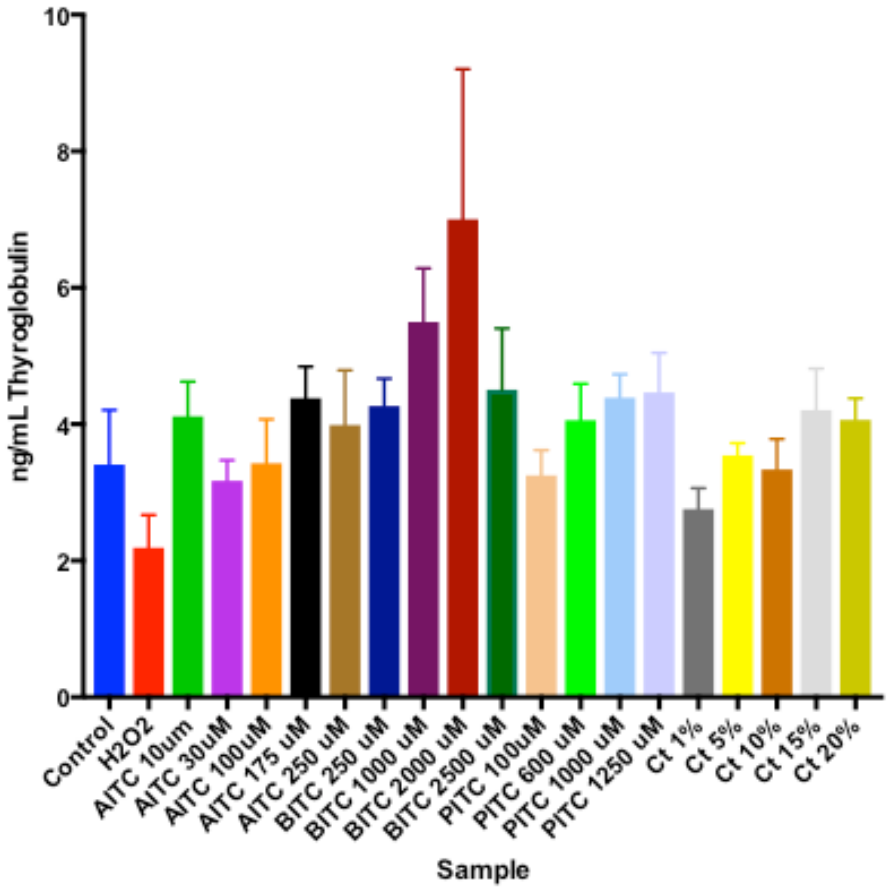



**Figure 6-17.** Thyroglobulin concentration was measured by ELISA in K1 cells exposed to allyl isothiocyanate, butyl isothiocyanate, propyl isothiocyanate and *Chorispora tenella* autolysis extract in medium for 24 hours.

*Note*. Points represent a mean value based on four replicates. Nominal concentrations are indicated. The error bars represent one standard deviation. Kruskal-Wallis and post hoc Dunn's test conducted for nonparametric data. No significant differences were found compared to the control.

## 6.2 Discussion

The three isothiocyanates and *Chorispora tenella* plant autolysis extract have varying impacts on the K1 cell line. Allyl isothiocyanate has the greatest toxicity to the cells based on the $LC_{50}$ values of the various isothiocyanates (Table 6-5). This could be related to the structure of AITC. Isothiocyanates are generally electrophilic in nature (Dunnick et al. 1982; Kawakishi and Kaneko, 1987; Duncan and Milne, 1993) and AITC is inherently so since it is an alkene compared to BITC and PITC which are alkanes.

The MTT assays for AITC (Figure 6-1), PITC (Figure 6-5), BITC (Figure 6-9), and *Chorispora tenella* autolysis extract (Figure 6-13) show a dose dependent decrease in cell viability. Allyl isothiocyanate was significantly different from control at 30 μM, PITC was significantly different at 600 μM, BITC was significantly different at 1500 μM, and *Chorispora tenella* autolysis extract was significantly different at 109 μM AITC which resulted in 5% autolysis extract in medium. K1 cells exposed to allyl isothiocyanate had highest toxicity followed by PITC then BITC (Table 5). The DNA content in K1 cells only was significantly different when compared to control at the AITC of 100 μM and 175 μM. Mitochondrial function, ATP content, showed a dose dependent decrease compared to control. For AITC starting at 70 μM, PITC at 300 μM, BITC at 1500 μM and *Chorispora tenella* at 138 μM AITC which represents 10% autolysis extract. When ATP was normalized for DNA content, AITC was significantly different from control at 70 μM, PITC at 300 μM, BITC at 1500 μM, and *Chorispora tenella* autolysis extract at 109 μM AITC which represents 5% autolysis extract. These data emphasize that the structure of AITC is more reactive than PITC or BITC.

Tables 6-1, 6-2, and 6-3 show nominal concentrations compared to measured concentrations of isothiocyanates in exposure medium. This difference could be due to the length of time between making the exposure treatments and performing the isothiocyanate extraction with hexane. Additionally, fetal bovine serum was present in the treatment medium potentially binding the isothiocyanates. The influence on bioavailability of the compounds was not assessed so nominal concentrations have been used in reporting the results.

Exposure to isothiocyanates initiates the apoptotic pathway resulting in cell death. Apoptosis is believed to be the ultimate cause of the decline of metabolic activity in the cell. Specific mechanisms of apoptosis for BITC and PITC are not known, however, AITC is

regularly studied. Regulation of apoptosis by isothiocyanates is performed by release of cytochrome c, Bcl-2 family regulation, MAPK signaling and followed by activation of caspases, which initiate apoptosis (Traka and Mithen 2009).

For example, allyl isothiocyanate induced Bcl-2 phosphorylation and was associated with JNK activation. JNK inhibition prevented AITC from inducing Bcl-2 phosphorylation, thus showing that AITC causes Bcl-2 phosphorylation by activating JNK (Geng et al. 2011). Bladder cancer cells, UM-UC-3, were exposed to ATIC at 15 μM for 24 hours and the cellular activity of caspase-3 and -7 was elevated compared to control. Treating the cells with 15 μM of benzyl isothiocyanate and phenethyl isothiocyanate for 24 hours significantly increased the number of apoptotic cells (Tang and Zhang 2004). Additionally, when exposed to isothiocyanates the mitochondrial membrane potential decreases causing a release of cytochrome c, subsequently triggering a caspase cascade (Zhang et al. 2003; Tang and Zhang 2004; Tang and Zhang 2005; Bo et al. 2016).

Reactive Oxygen Species (ROS) production may also contribute to AITC induced apoptosis in MCF-7 and MDS-MD-231 breast cancer cell lines. Allyl isothiocyanate was found to induce dose dependent DNA damage in the cells by increasing the levels of ROS and calcium. This ROS production in cells resulted in endoplasmic reticulum stress and triggered an endoplasmic reticulum calcium release resulting in apoptosis (Bo et al. 2016).

Cell cycle arrest resulting in reduced cell proliferation and decreased DNA content are content common with isothiocyanates. Phenyl isothiocyanate induced cell cycle arrest in PC-3 cells (human prostate cells) due to proteasome mediated degradation of Cdc25c and Cdk1 proteins and resulting in apoptosis (Xiao et al. 2004). Research shows that AITC is a mitotic blocking agent, after a single treatment of AITC at either 7.5, 15, or 30 μM concentration, 90%

of the UM-UC-3 cells arrested. Allyl isothiocyanate down regulated alpha and beta tubulin, however not gamma tubulin, showing that AITC has a different mechanism causing cell cycle arrest that other isothiocyanates (Geng et al. 2011).

Isothiocyanates could be affecting other parts of the thyroid hormone synthesis pathway. For example, if isothiocyanates inhibit iodide uptake into the follicular cell they would inhibit T3 and T4 synthesis downstream since there would be no iodine present to bind to thyroglobulin to make the precursor monoiodothyronine (MIT) and diiodothyronine (DIT). However, thyroglobulin would not be affected since it does not require iodine for synthesis in the rough endoplasmic reticulum, which could explain our results (Langer and Greer 1968; Davies 1972; Mason and Wilkinson 1973; Oertel et al. 1991). An ELISA determining T4 concentration would be beneficial to determine antithyroid effects.

The relative toxic potency values for K1 cells show that allyl isothiocyanate is the most toxic isothiocyanate present in *Chorispora tenella*, followed by propyl isothiocyanate and butyl isothiocyanate. When combined with the percentage that these isothiocyanates make up in the plant material analyzed from the 2010 collection, it is likely that toxicity would be mostly attributable to allyl isothiocyanate (90%) followed by propyl isothiocyanate (8%), and then butyl isothiocyanate (2%).

# 7. CONCLUSION

*Chorispora tenella* plants in the Fort Collins vicinity show varying isothiocyanates throughout different collection years. In the 2010 growing season, *Chorispora tenella* had three isothiocyanates present in the plant: allyl isothiocyanate, propyl isothiocyanate, and butyl isothiocyanate. However, in the 2016 and 2017 collection years propyl isothiocyanate and butyl isothiocyanate were absent from *Chorispora tenella*.

After exposing two cell lines to pure standards of the isothiocyanates, it was shown that in the HepG2 liver cell line allyl isothiocyanate is the most toxic of the three with an $LC_{50}$ of 116 μM AITC compared to 1152 μM PITC and >2500 μM BITC. When K1 thyroid cells were exposed to the synthetic isothiocyanates, allyl isothiocyanate was again the most toxic with an $LC_{50}$ of 235 μM AITC compared to 530 μM PITC and 1086 μM BITC. Thus, K1 cells are somewhat less sensitive to AITC than HepG2 cells but more sensitive to PITC and BITC. The $LC_{50}$ values correspond with expected toxicity based on chemical structure. The toxicity of allyl isothiocyanate is consistent with its more electrophilic structure. Propyl isothiocyanate was the next most potent followed by BITC.

In comparing the abundance of the three isothiocyanates present in the 2010 *Chorispora tenella* plant and their toxic potency, the effects on both HepG2 and K1 cells demonstrate that allyl isothiocyanate would account for the majority of the toxicity. Livestock consuming *Chorispora tenella* are thus going to be at risk of toxic responses regardless of the presence of propyl or butyl isothiocyanates.

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