Antiproliferative activity of Ontario grown onions against colorectal adenocarcinoma cells

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1. Introduction

Increasing evidence from clinical and epidemiological studies supports an association between a diet rich in fruits and vegetables and a reduced incidence of chronic diseases, such as cancer (Hui et al., 2013; He, Jin, Gong, Zhang, & Zhou, 2014; Liu et al., 2014; Guercio, Turati, La Vecchia, Galeone, & Tavani, 2016). In 2012, there were 14.1 million newly reported cases of cancer reported globally (Ferlay et al., 2015), of which malignant neoplasms were the main leading cause of mortality in Canada (Statistics Canada, 2012). After resection, mainstay cancer therapeutics remain radiation and chemotherapy, despite their often severe side effects and mixed successes. Therefore, researchers continue to search for alternative therapeutics, which ideally may include lifestyle changes or other non-invasive mitigation strategies for fighting this prevalent and chronic disease. One reason a diet rich in fruits and vegetables may be beneficial in preventing cancer is that these foods are an excellent source of phytochemicals. Phytochemicals are bioactive compounds, classified by their chemical structures and divided into several classes, some of which are more prevalent in a wider range of fruits and vegetables (Schreiner & Huyskens-Keil, 2006). Phytochemicals commonly found in plant-based foods include carotenoids, phenolic acids, organosulfides, polyphenols and flavonoids.

Flavonoids represent the most prevalent phytochemical in plants. The health-promoting effects of dietary flavonoids continue to be an active area of research (Caridi et al., 2007). Flavonoids can exert antioxidant radical scavenging activities, limiting the lipid peroxidation of cell membranes (Peng & Kuo, 2003). Additionally, flavonoids have been demonstrated to have anti-cancer properties against several cancer types, including colorectal (He et al., 2014; Linsalata, Orlando, Messa, Refolo, & Russo, 2010), upper digestive tract (Guercio et al., 2016), breast (Yamazaki, Miyoshi, Kawabata, Yasuda, & Shimoi, 2014), and liver cancers (Yang, Meyers, Heide, & Liu, 2004). Dietary sources of flavonoids include berries, onions, garlic, citrus fruits, apples, and leeks (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Onions are one of the most widely produced and consumed vegetables (Sellappan & Akoh, 2002; Slimestad, Fossen, & Vågen, 2007), representing one of the most common sources of flavonoids in the human diet (Suleria, Butt, Anjum, Saeed, & Khalid, 2015). Three well studied flavonoids, kaempferol, myricetin, and quercetin, are found in onions, while quercetin is the most abundant among these in onions (Sellappan & Akoh, 2002).

Agronomic and genetic factors can influence the quality of phytochemicals among cultivars (Tomas-Bareberan & Espin, 2001),...
negatively impacting the health-promoting benefits that these nutrients provide. Therefore, it is possible that the benefits reported in onions grown outside of Canada may not necessarily apply to the locally grown varieties, as the cultivars and growing conditions are different between locals. Accordingly, an investigation on the health promoting properties of Ontario grown onions, specifically in regards to their cytotoxic and cytostatic bioactivity, is merited. Caco-2 as our cell line of interest because it is extensively used as a model for human intestinal epithelium and is the work horse of the pharmaceutical industry for in-vitro toxicology studies (Sambuy et al., 2005; Yamazaki et al., 2014). Therefore, we investigated the bioactive properties of five Ontario grown varieties of onion and assessed their capacity to exert cytostatic/cytotoxic effects on a relevant human colorectal cancer cell line.

2. Materials and methods

2.1. Materials

Quercetin (purity: pharmaceutical secondary standard), myricetin (purity ≥96%), kaempferol (purity ≥ 98%), MEM (minimum essential medium), FBS (fetal bovine serum), l-glutamine, and d-glucose were purchased from Sigma Aldrich (St Louis, MO). Phenol free DMEM (Dulbecco’s modified eagle’s medium) and 100× penicillin-streptomycin solution were obtained from GE Healthcare Life Sciences (Logan, UT). Lactate dehydrogenase (LDH) cytotoxicity and MTS cell proliferation assay kits were purchased from BioVision Inc. (Milipitas, CA). A TrypLE express and Image-IT® lipid peroxidation kit was purchased from Invitrogen (Carlsbad, CA). The ORIS™ cell migration assay kit was acquired from Platypus Technologies (Madison, WI). Five Ontario grown onion varieties were graciously donated by the Holland Marsh Growers’ Association (Bradford, Ontario).

2.2. Phytochemical extraction using pressurized low polarity water

Low polarity water extractions of five Ontario grown onion varieties (Lasalle, Fortress, Safrane, Stanely, and Ruby Ring) were performed. Chopped onions were lyophilized, and 5 g of freeze-dried onion powder was mixed with 80 mL of 0.1% formic acid in milliQ water (v/v). An automated Speed SFE NP model 7100 instrument (Applied Separation Inc., Allentown, PA) equipped with a module 7100 pump and a 10 mL thick-walled stainless cylindrical extractor vessel was used for the extractions. Phytochemical extraction of the onions using pressurized low polarity water extraction was performed under the following conditions: 60 °C at 150 bar for 60 min.

2.3. Total flavonoid content

The total flavonoid content assay was performed by aluminum chloride colorimetric assay. 0.5 mL of the total extracted onion test samples were mixed with 0.1 mL of 10% aluminum chloride and 0.1 mL of 1 M potassium acetate. Followed by the addition of 2.8 mL of deionized water. The test samples were then kept for incubation at room temperature for 30 min. The optical density (OD) was then measured using 96 well plate by a plate reader (ELISA plate reader (Amersham Biosciences Corp., USA) at 415 nm. The total flavonoid content was expressed as mg quercetin equivalent (QE)/g dry plant sample.

2.4. Cell culture

The Caco-2 strain, a human colorectal adenocarcinoma cell line, was purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in MEM media supplemented with 4 mM l-glutamine, 1% non-essential amino acids, 10% heat inactivated fetal bovine serum, and 1% 100× penstrep solution (final concentration of 100 IU/mL penicillin and 100 μg/mL streptomycin), d-glucose was supplemented at a final concentration of 4 g/L. Caco-2 cells were propagated in a 75-cm² culture flask at 37 °C and 5% CO₂ in a humidified atmosphere. Media was refreshed three times per week, and cells were passaged at approximately 80% confluency. Cells were harvested using Tryple Express and then collected using centrifugation at 200 × g for 5 min. Cells at passage numbers 18–20 were used for the experiments.

2.5. Colorimetric quantification of Caco-2 cell proliferation

The cytostatic capacities of the onion extracts were determined by measuring Caco-2 proliferation using the MTS colorimetric assay. Briefly, Caco-2 cells were seeded in a tissue culture-treated 96-well plate at a density of 1.5 × 10⁴ cells per well. Cells were left undisturbed for 24 h to allow for attachment. After 24 h, a total of 100 μL of the sample extracts containing the growth media were subsequently added. Flavonoids quercetin, myricetin, and kaempferol were used as positive controls to assess the cytostatic properties of the onion extracts. The concentration of pure flavonoids used for the present study was 100 μM. The flavonoids were dissolved in DMSO and further diluted in cell culture media. The final concentration of DMSO was 0.5% (v/v). The 96-well plate was incubated for 72 h and was gently washed with PBS prior to the addition of the MTS dye. Absorbance was measured at 490 nm using an xMark spectrophotometer (Bio-rad; Hercules, CA) after a 3 h incubation period with MTS dye.

2.6. Determination of cytotoxicity and cytolysis

Lactate dehydrogenase (LDH) supernatant levels were assessed as an indicator of Caco-2 cell death and were used as a measure of cytotoxicity activity. Caco-2 cells were seeded into a tissue culture–treated, 96-well plate at a density of 1.5 × 10⁴ cells per well. The plate was undisturbed for 24 h to allow for cell attachment. The media was then aspirated and replaced with media containing onion extracts at different concentrations. LDH activity was determined 72 h post-treatment. The plate was centrifuged at 600 × g for 10 min to precipitate any cells and cellular debris. Ten μL of the debris free medium was transferred to a 96-well plate. To assess LDH levels, 100 μL of a reaction mixture solution containing a water soluble tetrazolium salt dye was added to the supernatant samples. Absorbance was read using an xMark spectrophotometer (Bio-rad; Hercules, CA) after a 30 min incubation period at 450 nm.

2.7. Induction of apoptosis in Caco-2 cells

Expression of the executioner caspases, caspases 3 and 7, was used to determine the presence of bioactive properties capable of inducing apoptosis. Briefly, cells were seeded in a black, clear bottom 96 well plate at 1.5 × 10⁴ cells per well. The plate was left undisturbed for 48 h, after which the media was aspirated. Media containing the various extract treatments was added as previously described. After 24 h treatment incubation, the treatment medium was replaced with 100 μL of phenol free RPMI:F12 (1:1 ratio) medium supplemented with 5% FBS, 2 mM l-glutamine, and the CellEvent™ Caspase-3/7 Green Detection Agent at a final concentration of 7.5 μM. Cells were incubated for 30 min. High content imaging was performed using a Cytation 5 multi-mode plate reader (Bioteck, Winooski, VT).

2.8. Cell migration

Measurements of cell motility were used to determine the efficacy of extract treatments in inhibiting the invasive progression of Caco-2 cells. The Oris™ cell migration assay kit consists of a 96-well plate with round bottom silicone stoppers which create a 2 mm diameter exclusion zone. Caco-2 cells were seeded at 1 × 10⁵ cells/well and were allowed to attach for 24 h. After the incubation period, the silicone stoppers were gently removed, and the plate was imaged to capture the exclusion zone at time 0. Media containing the extract treatments was then added to the growth media. The plate was incubated for another 72 h,
after which high content imaging of the exclusion zones was performed using the Cytation 5 multimode plate reader.

2.9. Statistical analysis

One-way ANOVA using R Open Source Statistical Programming software was performed to assess the difference between the groups. Comparisons among treatments were calculated using Tukey’s honestly significant differences as a post hoc test. P-values < 0.05 were considered significant. The data was reported as mean ± standard deviation.

3. Results and discussion

3.1. Total flavonoid content assay

We have recently characterized the flavonoid profiles as well as antioxidant activities of each of the five onion varieties (Manohar, Xue, Murayyan, Neethirajan, & Shi, 2017). We found that the Stanley and Ruby Red varieties contained the highest flavonoid content, while all varieties contained readily detectable amounts of flavonoids (Fig. 1). Fortress, a yellow onion variety, showed the highest total flavonoid content at 0.33 mg of quercetin equivalent per gram of dried onion (P < 0.05). A 2-fold difference was observed in the total flavonoid content between the tested onion varieties Stanley (highest ranked) and Lasalle (lowest ranked). These results are consistent with prior observations that onions are rich in flavonoid compounds.

3.2. MTS cell proliferation assay

To then assess the potential anti-cancer properties of these onion flavonoids, we treated a relevant and commonly used immortalized human cancer cell line (Caco-2) with our prepared onion extracts. Because unchecked proliferation is a hallmark of cancer and a chief property contributing to the spread of and damage caused by human cancers, we first assessed the ability of the onion extracts to slow or inhibit cellular proliferation by Caco-2 cells. We tested three concentrations of onion extract (a 1:10, 1:50, and 1:100 dilutions of each extract), because it was unclear what concentration may be necessary to inhibit proliferation. We found that higher dilutions (1:50 and 1:100) were unsuccessful in inhibiting Caco-2 cell proliferation (data not shown). However, a 1:10 dilution (10 μL of sample extract with 90 μL of growth media) of each extract showed a significant effect of proliferation as determined by MTS assay (Fig. 2). A 69–80% decrease in absorbance was observed compared to the untreated control wells, indicating a strong antiproliferative effect of the onion extracts against Caco-2 cells. All onion extracts (at a 1:10 dilution) exerted a strong and significant anti-proliferative effect on Caco-2 cells (>65% reduction in absorbance). Furthermore, we determined that DMSO alone (the flavonoid delivery vehicle) had no impact on proliferation, indicating the effects of the onion extracts were due to the compounds present in the onions themselves. Despite the differences in phytochemical composition (e.g. varying flavonoid contents), no statistical differences in proliferation inhibition among the five onion varieties were observed. Furthermore, the crude onion extracts were as successful in inhibiting proliferation as the commercially obtained purified flavonoids (e.g. myricetin). Isolation of pure compounds from food products can be extremely costly and time consuming, generating large volumes of waste materials, the end result of which is high production costs and a great expense to patients (Ovadje et al., 2015). Thus, an ideal natural product-derived agent would require minimal processing to exert its impact. Our results suggest that these onion extracts may be successful in inhibiting proliferation without further refinement, while further exploration into diet modification may reveal other effective strategies for reducing cancer risk.

Our findings that onion extracts impede cellular proliferation in Caco-2 cells are consistent with previous reports. It is highly likely that treatment with onion extracts impacts cell cycle progression, thus accounting for the cytostatic properties of these extracts. Bioactive flavonoid and organosulfur compounds found in onions have been reported to impact signal transduction pathways, leading to cell cycle arrest in the G1 and G2/M phases when used to treat various cancer cell lines (Herman-antosiewicz & Singh, 2004; Nicastro, Ross, & Milner, 2015). Several studies have elucidated the different mechanisms causing cell cycle arrest for different cancer cell lines, which include the down-regulation of cyclin (Jeong, An, Kwon, Rhee, & Lee, 2009) and M-phase inducer protein expression (Xiao, Pinto, Gundersen, & Weinstein, 2005), the disruption of microtubule formation (Xiao et al., 2005), and the decreased polyamine synthesis necessary for cellular proliferation (Linsalata et al., 2010).

3.3. Cytotoxic assay

We went on to explore the potential cytotoxic effects of our onion extracts using lactate dehydrogenase assays. The detection of lactate dehydrogenase (LDH), an enzyme intrinsic to the cytosol, is commonly used as an indicator of compromised membrane integrity and
ultimately cell viability. In the MTS proliferation assay, MTS is converted by viable cells into a compound measureable by absorbance reading. Therefore, a reduction in absorbance in the MTS assay with onion extract treatment could also denote changes in cell viability. As expected, we found that LDH levels increased with onion extract treatment (versus untreated controls), indicating that these extracts also possess cytotoxic effects. Similar to results from the proliferation assays, the extracts were most effective at a 1:10 dilution (Fig. 3), showing no significant differences at a 1:50 or 1:100 dilutions (data not shown). A 125–228% increase in LDH release was observed for treatments prepared at a 1:10 dilution. However, unlike the proliferation assays, we found measureable differences in the cytotoxic effects among the five onion varieties, which may reflect extract composition. A 228% increase in the ratio of LDH release was induced by Fortress and Stanley extracts, which represented the most potent cytotoxic extracts. This ratio was similar to that exhibited by the control flavonoids quercetin and kaempferol. The remaining three onion variety extracts exhibited a lower LDH release ratio, which was similar to the myricetin-treated cells, although all extracts induced a significant amount of cytotoxicity compared to untreated controls.

The differences in total flavonoid content among the tested onion varieties cannot fully account for why the Fortress variety, with contains only half as much total flavonoid content as the Stanley variety, has a similar effect on cell viability as assessed by LDH assay. Furthermore, the Ruby Ring extract performed similarly to varieties with the lowest total flavonoid content, although it contains one of the highest concentrations of flavonoids. Therefore, it is very likely that other bioactive compounds, such as organosulfur and/or phenolic compounds contribute to flavonoid effects or may have a more pronounced effect than the flavonoids themselves. Due to the heterogeneous nature of the extracts, it may also be possible that the presence of a currently unidentified phytochemical may play a role in exerting these effects. Future studies could be performed to isolate the active components in these extracts, which may have more potent effects when administered in higher concentrations as pure compounds. However, as far as a therapeutic is concerned, isolated compounds may be significantly less cost effective and may not show greater efficacy; some natural product extracts are more effective as mixtures, due to the potential synergistic or additive effects of the individual compounds present within the mixture (Liu et al., 2014).

3.4. Induction of apoptosis in Caco-2 cells

Although LDH assays are reasonable measures of cell viability, they do not directly quantify the number of apoptotic cells/events. Apoptosis, or programmed cell death, is a highly organized and tightly regulated sequence of biochemical events leading to morphological changes and eventually cell death. The activation of executioner protease caspases, namely caspases 3 and 7, is key path shared by both the intrinsic and extrinsic apoptotic pathways (Reed, 2003; Boatright & Salvesen, 2003). The evasion of programmed cell death, due to oncogenic mutations in malignant cells, is one of the hallmarks of cancer (Hanahan & Weinberg, 2011). These mutations result in defects in the signaling transduction pathway of the apoptosis signaling cascade, giving rise to the chemotherapeutic resistance of malignant cells (Johnstone, Ruefl, & Lowe, 2002; Lowe & Lin, 2000). Consequently, induction of apoptosis by a therapeutic agent would likely be instrumental in controlling the proliferation and metastasis of cancer cells. If a greater number of cancerous cells are pushed down the apoptotic pathway, this would help to stop proliferation and neoplastic growth. Therefore, we also assessed the ability of our onion extracts to induce the apoptosis of Caco-2 cells. We found that all extracts were successful in inducing apoptosis at a dilution of 1:10 compared to the untreated or DMSO control (Fig. 4). Consistent with previous assays, 1:50 and 1:100 dilutions of our extracts did not significantly induce apoptosis (data not shown). Overall, a 3.5–4-fold increase in the number of apoptotic cells was observed for nearly all treatments (versus untreated control). The Stanley extract was most potent at inducing cell death and was followed by the Lasalle, Ruby Ring, Fortress, and Safrane extracts. A very promising finding was that on average, the number of apoptotic events induced by the extract treatments was higher than the number of events induced by our positive controls quercetin and myricetin.

Several proposed mechanisms of actions for phytochemicals, which affect the transduction signaling pathways involved in apoptosis, have been documented in literature and are dependent not only on the cellular model, but on the phytochemical involved in the study. Dietary flavonoids found in onions have been shown to down-regulate the elevated levels of fatty acid synthase expression in cancer cells, compromising their ability to convert acetyl-CoA to fatty acids and negatively impacting cell membrane functions leading to cell death.
(Brusselms, Vrolix, Verhoeven, & Swinnen, 2005). In other studies, flavonoids have been shown to promote the expression of pro-apoptotic genes (Wenzel, Kunz, Brendel, & Daniel, 2000) as well as down-regulate the expression and activation of nuclear transcription factor-κB, essential for inhibiting apoptosis (Wenzel et al., 2000; Ban et al., 2007). A review by Herman-Antosiewicz and Singh (2004) on allium-derived organosulfur compounds nicely summarizes the possible modes of apoptosis induction, including those suggested by flavonoid studies. The authors cited the disruption of intracellular calcium homeostasis, the down-regulation of the anti-apoptotic Bcl-2 family of genes, increased expression of pro-apoptotic genes, and the generation of reactive oxygen species facilitating the release of cytochrome c (and subsequent activation of cytochrome c and caspace 3) as possible modes of apoptosis induction by organosulfur compounds. Further characterization of our extracts would be necessary to determine the precise modes of actions of their constituent parts. Here, we provide clear evidence that our onion extracts induce apoptosis, but additional study would be warranted to determine the mechanisms of action. Because these onion extracts are a heterogeneous mixture of different bioactive molecules (e.g. flavones, organosulfur compounds, and phytoesters), it is possible that any 1 or a combination of these is responsible for triggering apoptosis.

Another biological hallmark of cancer cells is their ability to invade and metastasize from primary neoplasms, which can go on to colonize other (even, more distant) body sites (Hanahan & Weinberg, 2011). Ninety percent of all cancer-related mortalities are a consequence of the metastasis of primary tumors to other parts of the body (Spano, De Antonellis, Christofori, & Zollo, 2012; Monteiro & Fodde, 2010). The series of events where primary neoplasms colonize distal sites in the body is called the metastatic cascade (Nguyen, Bos, & Massaque, 2009; Mehlen & Puisieux, 2006). While some phytochemicals have been shown to possess cytostatic and cytotoxic properties in vitro, not all exert a bioactive effect in modulating the mechanisms responsible for the metastasis of cancer cells (Caltagirone et al., 2000). Limiting the ability of cancer cells to migrate within their microenvironment, as well as from their primary sites, would greatly reduce the risk of mortality associated with metastasis. Therefore, we investigated the abilities of our onion extracts to impair the cellular motility of Caco-2 cells.

3.5. Anti-migratory assay

We found that the onion extracts elicited different anti-migratory effects against Caco-2 cells. Fig. 5 shows the number of cancer cells detected in the exclusion zone after a 72 h dosing period. Stanley extracts performed the best at inhibiting cell migration with a 60% decrease in the number of cells in the exclusion zone compared to the untreated control. This was followed by Lasalle (56% decrease), Fortress (49% decrease), Ruby Ring (47% decrease), and Safrane (40% decrease). Myricetin did not inhibit Caco-2 cell migration into the exclusion zone. Factors including cell cycle arrest/decreased proliferation and cytotoxicity/apoptosis may have contributed to reductions in migration, as it counts the number of cells migrating to the exclusion zone, which means non-viable cells will not be counted. Representative images of Caco-2 cells treated with Lasalle extracts and quercetin are shown in Fig. 6. This data correlates with our apoptosis data, showing the same order of efficacy among the different extracts. In support of our observation, flavonoids have an established role in disrupting cancer cell migration/metastasis; flavonoids, such as quercetin, have been shown to disrupt microtubule formation (Gupta & Panda, 2002), rearrange actin microfilaments in the cytoskeleton (Psahoulia et al., 2007), and modulate enzymes and signaling pathways associated with the metastasis cascade (Weng & Yen, 2012; Pan, Lai, Wu, & Ho, 2011; Park et al., 2005). The complex mixture of compounds present in the onion extracts, with varying effects on proliferation and viability, likely contributes to their observed anti-migratory effects. The anti-migratory effect of Safrane on Caco-2 cells was similar to the effect of kaempferol, but was statistically different than the effect of quercetin. However, the other varieties were more effective in impeding Caco-2 migration, with a 23–42% and 13–34% reduction in the number of observed cells compared to quercetin and kaempferol, respectively. Interestingly, myricetin did not inhibit Caco-2 migration, even though it exerted cytostatic and cytotoxic effects. Therefore, it is plausible that myricetin cannot modulate the signaling pathways associated with the metastasis cascade and/or disrupt the cytoskeletal microstructures responsible for structural support and cellular motility. It is recognized that the results of this study is limited to colonic cell line only. Absorption of flavonoids may be poor in an in vivo setting and hence the relevance of transformed cells in peripheral tissues would be unknown. Unabsorbed compounds may enter large intestine where that may be bio-transformed by microbiota. Such metabolites may be active against transformed cells in colon and absorbed where they may have similar activity.

4. Conclusions

Here we show that extracts created from five different Ontario grown onion varieties are effective in inducing cytotoxic, cytostatic, and anti-migratory activities in a human colorectal adenocarcinoma cell line. These extracts significantly induced apoptosis, reduced the rate of proliferation, and slowed the migration of cancer cells into an exclusion zone. Importantly, these effects were at least comparable, and often stronger, than those of the commercially available pure flavonoid compounds. Moving forward, we are continuing to conduct studies to elucidate the possible mode of action of these extracts against Caco-2 cells and to determine which active agents are most important for these properties. These data represent an important step in characterizing the anti-cancer properties of flavonoids and provide further evidence for their inclusion in dietary treatments. Although positive results were observed at the highest tested concentration (1:10 dilution of onion extracts), the marked success of our crude onion extract supports the notion that dietary supplementation or even the use of a fairly inexpensive onion extract could have potent anti-cancer effects. Further in vivo and clinical trials however, would be required to confirm the efficacy of the onion extracts in preventing tumor development.
Fig. 6. Cell migration assay. Figure shows cells (green) migrating the exclusion zone A) Untreated A, B) Caco2 cells treated with Lasalle at 0 h C) Caco2 cells treated with Quercetin at 0 h D) Caco2 cells treated with Quercetin at 0 h E) untreated cells at 72 h F) Caco2 cells treated with Lasalle at 72 h G) Caco2 cells treated with Quercetin at 72 h.

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