

## Cytotoxic activity and anti-cancer potential of Ontario grown onion extracts against breast cancer cell lines

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### ABSTRACT

**Background:** Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer deaths among Canadian women. Cancer management through changes in lifestyle, such as increased intake of foods rich in dietary flavonoids, have been shown to decrease the risk associated with breast, liver, colorectal, and upper-digestive cancers in epidemiologic studies. Onions are high in flavonoid content and one of the most common vegetables. Additionally, onions are used in most Canadian cuisines. This study aimed to investigate the extracts synthesized from Ontario-grown onion varieties in inducing anti-migratory, cytostatic, and cytotoxic activities in two sub-types of human breast cancer cell lines.

**Methods:** We investigated the effect of five prominent Ontario grown onion (Stanley, Ruby Ring, LaSalle, Fortress, and Safrane) extracts on two subtypes of breast cancer cell lines: a triple negative breast cancer line MDA-MB-231 and an ER+ breast cancer line MCF-7.

**Results:** These onion extracts elicited strong anti-proliferative, anti-migratory, and cytotoxic activities on both the cancer cell lines. Flavonoids present in these onion extracts induced apoptosis, cell cycle arrest in the G2/M phase, and a reduction in mitochondrial membrane potential at dose-dependent concentrations. Onion extracts were more effective against MDA-MB-231 compared to the MCF-7 cell line.

**Conclusion:** Anti-tumor activity of these extracts depends upon the varietal and can be formulated into nutraceuticals and functional foods for the wellbeing of cancer patients. Overall, the results suggest that onion extracts are a good source of flavonoids with anti-cancerous properties.

**Keywords:** onion extracts, flavonoids, anti-proliferative, breast cancer, cytotoxic activity

## INTRODUCTION

Epidemiological studies have shown a decreased risk of various chronic diseases, such as cardiovascular disease, diabetes, and cancers, from increased intake of fruits and vegetables. Plant-based foods are rich sources of dietary phytochemicals and can be subdivided into different classes based on their chemical structure, which include carotenoids, phenolic acids, organosulfides, polyphenols, and flavonoids. Flavonoids have strong anti-oxidant properties including free radical scavenging capacity, reduction of membrane lipid peroxidation, and are a source of cation chelating agents [1]. Dietary flavonoids also have strong anti-tumor activity through the induction of apoptosis in liver [2], colorectal [3], upper digestive tract [4], and breast cancer cells [5]. Fruits and vegetables are rich dietary sources of phytochemicals, including high amounts of flavonoids. Onions are among the richest sources of flavonoids, and are one of the most widely produced and consumed vegetables worldwide [1]. However, the widespread global production of onions results in cultivars with different compositions of flavonoids. Accordingly, these differences may have a strong impact on the health-promoting benefits from onions grown outside of Canada compared to those grown in Ontario.

In 2012, breast cancer was the most commonly diagnosed cancer and the fifth leading cause of cancer deaths in women worldwide [6]. Overall, survival rates are increasing, but cancer cell metastasis to secondary sites may lead to complications which need to be controlled and can be prevented with effective cancer management through lifestyle changes. Tumor metastasis depends on a complex set of events that includes cell adhesion, motility, proliferation, and vessel formation. Interruption or inhibition of one or more of these steps is important for anti-metastatic therapy. Considerable progress has been made in the field of cancer prevention and treatment over the past few decades. However, a high morbidity rate persists. Currently, radiation and chemotherapy is used to treat cancer with the use of natural, synthetic, or biological agents to suppress, reverse, or prevent either the initial stages of carcinogenesis or progression into further stages of the disease. While this is a common method for breast cancer patients undergoing chemotherapy, there are also several treatment-limiting side effects such as cardiovascular toxicity and neuropathy. Thus, dietary phytochemicals with the potential to target breast cancer cells may be a promising, safer, and natural alternative to chemotherapy drugs. Consumption of fruits and vegetables high in phytochemicals like flavonoids may help decrease the risk of cancer, including breast cancer. As a result, research has focused on the search for new anti-tumor flavonoids with the highest bioactivities.

Breast cancers have multiple phenotypically different subtypes and are treated differently based on the presence or absence of receptors for progesterone, estrogen, and human epidermal growth factor. Estrogen-receptor-positive breast cancer (ER+) means that estrogen is influencing the rate of cancer cell expression [7]. This leads to therapeutic options that include hormone therapy as an alternative treatment or in conjunction with chemotherapy [7]. Receptor-positive breast cancers are often treated with targeted therapies through the use of these receptors. In contrast, triple negative breast cancer (TNBC) lack these receptors and is often a more aggressive subtype that cannot be subdued through targeted therapy [7]. For this reason, it is important to find alternative cancer therapies, like flavonoids, for different types of breast cancers, especially because breast cancers often produce heterogeneous tumors [7].

Onions are exceptionally rich in flavonoid content, especially the flavonoid quercetin. In our earlier study [8], we established an environmentally friendly, pressurized low polarity water technology to extract polyphenols from five Ontario grown onion varieties and thoroughly characterized their anti-oxidant properties. A previous study [8] demonstrated that five Ontario-grown varieties – Stanley, Ruby Ring, LaSalle, Fortress, and Safrane – contain high flavonoid content, and exhibit a wide range of efficacies and antioxidant activities. The extracted onion polyphenols using low polarity water technology have been previously demonstrated to have anti-tumor activity on human colorectal adenocarcinoma [3, 8]. Along with determining the total polyphenolic and flavonoid content, the experimental results from DPPH and ABTS radical scavenging assays; Ferric reducing antioxidant power assay; total antioxidant capacity colorimetric assay; lipid peroxidation assay and oxygen radical absorbance capacity assays thoroughly characterized the antioxidant activity of polyphenols from Ontario grown onion varieties [8].

In this study, we sought to determine the effects of onion extracts on the viability, proliferation, and motility of two subtypes of breast cancer cells (MDA-MB-231 representing the triple negative subtype and MCF-7 representing the estrogen-receptor-positive (ER+) subtype) and a control healthy cell line (MCF-10A). Because the molecules from polyphenols of onions have a natural origin and are commonly ingested, they were expected to have more effect on cancer cell lines than a normal breast epithelial cell line MCF-10A. We further investigated the mode of action to understand the biological and biochemical differences between cancerous and normal cells in order to improve the treatment selectivity.

## **MATERIALS AND METHODS**

### ***Materials***

Quercetin (purity: pharmaceutical secondary standard), kaempferol (purity  $\geq 90\%$ ), FBS (fetal bovine serum), PBS (phosphate buffered saline), thiazolyl blue tetrazolium bromide (MTT reagent), mitochondria staining kit, cholera toxin, and hydrocortisone were purchased from Sigma Aldrich (St. Louis, MO). DMEM (Dulbecco's modified eagle's medium), phenol-free DMEM, and human recombinant EGF (epidermal growth factor) were acquired from Gibco by Life Technologies (Carlsbad, CA). L-glutamine and 100 $\times$  penicillin-streptomycin were obtained from GE Healthcare Life Sciences (Logan, UT). The MTS cell proliferation assay kit was obtained from BioVision Inc. (Milipitas, CA). The Oris<sup>TM</sup> cell migration assay kit was purchased from Platypus Technologies (Madison, WI). Ribonuclease A and propidium iodide dye for cell cycle analysis were purchased from BioShop (Burlington, ON). Annexin-V FITC apoptosis detection kit was purchased from eBioscience (San Diego, CA). Five Ontario-grown onion varieties were donated by the Holland Marsh Growers' Association (Bradford, Ontario).

### ***Pressurized Low Polarity Water Extraction***

Pressurized low polarity water extractions of five Ontario grown onion varieties, namely Stanley, Ruby Ring, Lasalle, Safrane, and Fortress respectively, were performed as described in our previous work [8]. Onions of the same variety were chopped, grounded, and freeze-dried for 72 hours. A total of 5 g of freeze-dried onion powder were mixed in 80 mL of 0.1% formic acid in milliQ water (v/v) for each variety. Extraction of the mixtures was carried out using an automated

Speed SFE NP model 7100 instrument (Applied Separation Inc., Allentown, PA, USA), equipped with a pump (Module 7100) and 10 mL thick-walled stainless cylindrical extractor vessel. The extraction of onion phytochemicals into water was executed using the following parameters: temperature at 60°C, pressure at 150 bar, and extraction time of 60 min.

### ***Cell Culture***

The MDA-MB-231, MCF-7, and MCF-10A cell lines were a triple negative breast cancer cell line, an estrogen-receptor-positive (ER+) breast cancer cell line, and an epithelial fibrocystic non-cancerous breast cell line (control) respectively. The 3 cell lines were purchased from the American Type Culture Collection (Rockville, MD). MDA-MB-231 and MCF-7 cells were maintained in DMEM media supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. MCF-10A cells were used as a negative control (normal breast cells) and were maintained in DMEM media supplemented with 5% heat inactivated FBS, 2.5 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin, 0.5 µg/mL hydrocortisone, 20 ng/mL human recombinant EGF, and 100 ng/mL cholera toxin. All three cell lines were grown in 75 cm<sup>2</sup> culture flasks at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere. Media was renewed three times per week and cells were passaged nearing 90% confluency. Cells were harvested using trypsin and collected using centrifugation at 200 × g for 5 min. Cells at passage number 168–182 (MDA-MB-231), O+4–O+8 (MCF-7), and 96 (MCF-10A) were used for experiments.

### ***Colorimetric Quantification of Breast Cancer Cell Proliferation***

The anti-proliferative effects of five Ontario onion extracts were assessed by quantifying the inhibition of proliferation of the MDA-MB-231, MCF-7, and MCF-10A cell lines. Anti-proliferative activity was determined by a MTS colorimetric assay. Cells were seeded onto a 96-well flat-bottom plate at a density of  $1.5 \times 10^4$  cells per well for MDA-MB-231 and  $3.0 \times 10^4$  cells per well for MCF-7 and MCF-10A in triplicate. An incubation period of 24 h was allowed for cell adherence. Following adherence, media was removed and replaced with 100 µL of 1:10 dilutions of the onion extract in media. Pure flavonoids, kaempferol and quercetin, were used as positive controls at concentrations of 100 µM in DMSO diluted in media to 0.5%. The treatments were administered over a 72 h time period and then removed. The wells were washed with PBS before the addition of 10% MTS dye in phenol-free media. Absorbance was measured at 490 nm using an xMark spectrophotometer (Bio-rad; Hercules, CA) after a 3 h incubation period for each of the cell lines.

### ***Cell Migration***

Cell motility was investigated to assess the efficacy of onion extract treatments on the inhibition of aggressive advancement and dispersion of breast cancer cells. The Oris™ cell migration assay kit consists of a 96-well plate with round bottom silicone stoppers that create a 2 mm diameter exclusion zone. MDA-MB-231 and MCF-7 cells were seeded in triplicate at cell densities of  $1.0 \times 10^5$  cells per well and allowed a 24 h time period to adhere to the wells. Afterwards, the stoppers were gently removed and the plates were imaged to capture the exclusion zone at time 0. Media in

the wells were then replaced with the extract treatments and incubated for 72 h to allow for migration to occur. The exclusion zones were imaged at 72 h using a Cytation 5 multimode plate reader.

### ***Mitochondrial Membrane Potential***

Lowered mitochondrial membrane potential is often linked to apoptotic events. A mitochondrial staining assay kit was used to assess the efficacy of onion extracts in interfering with the optimal mitochondrial potential. Changes in potential were detected by a mitochondrial staining kit that used JC-1 dye. Cells were seeded onto 27 mm diameter glass bottomed petri dishes at a density of  $1.0 \times 10^5$  cells per dish. An incubation time of 24 h was allotted for adherence and then media was replaced with 2.0 mL of extract treatment (1:10 and 1:5 dilutions in media). After another 24 h incubation period, the cells were dyed according to the manufacturer's protocol and imaged using a Diskovery Spinning Disk confocal microscope (63 × oil lens). The excitation of JC-1 was measured using laser light (488 nm for green and 560 nm for red), and emission was recorded at 525 nm for green monomer fluorescence and 620 nm for aggregate red fluorescence.

### ***Cell Cycle Analysis***

The timing of cell cycle arrest provides insight into the mode and time of action of the onion extracts. Flow cytometry was used for analyzing the cell cycle after a 24 h treatment period (1:10 dilutions). MDA-MB-231 cells were grown in T-75 cm<sup>2</sup> flasks until they neared 90% confluency, after which the media was replaced with the diluted onion extract-containing media. After 24 h, cells were collected through trypsinization and washed in PBS. Cells were pelleted and fixed in 70% ethanol at 4 °C for 1–4 h. After the fixation period, cells were washed twice in PBS, resuspended in 500 µL of PBS containing 50 µL of 100 µg/mL RNase and 10 µL of 1 mg/mL propidium iodide, and incubated in the dark at 24 °C for 30 min. Samples were then analyzed by flow cytometry.

### ***Annexin-V FITC Apoptosis Detection***

To show that onion extracts could induce apoptosis in breast cancer cells, annexin-V FITC and propidium iodide dyes were used for early and late apoptotic cell detection. MDA-MB-231 cells were grown in T-75 cm<sup>2</sup> flasks and treated for 24 h with onion extracts when nearing 80% confluency. After the treatment period, cells were harvested and suspended in 1× binding buffer prior to the addition of annexin-V FITC and propidium iodide according to the manufacturer's protocol. Prepared samples were then analyzed by flow cytometry.

### ***Statistical Analysis***

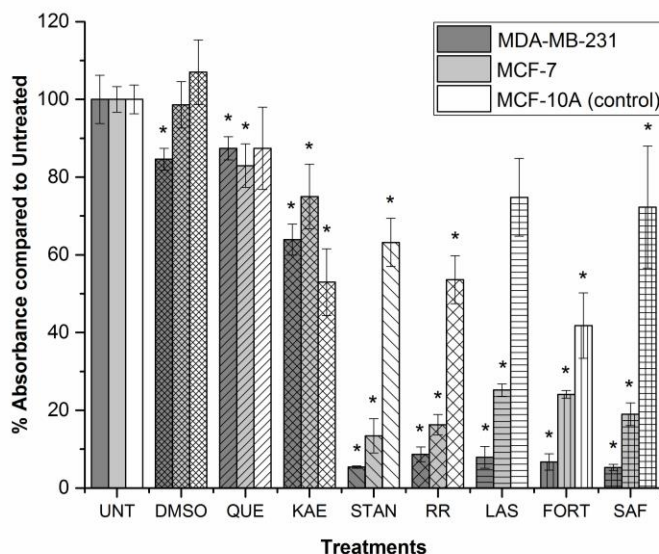
One-way ANOVA using R Open Source Statistical programming software was performed to evaluate the difference between treatment groups within the respective cell lines. Comparisons between treatments were computed using Tukey's post hoc test. P values less than 0.05 were

considered significant. The data were reported as mean  $\pm$  standard deviation. All the experiments were performed in triplicate and the representative results are shown.

## RESULTS AND DISCUSSION

### Cell Proliferation Analysis

To determine the potential anti-cancer properties of flavonoids from the five onion varieties, we treated two commonly used human breast cancer cell lines (MCF-7 and MDA-MB-231) with prepared onion extracts. Increased and unregulated cell proliferation is a hallmark symptom of breast cancer and being able to slow or inhibit uncontrolled cellular division will aid cancer treatments. Previous work with these onion extracts revealed an inhibition effect at dilutions of 1:10 (10  $\mu$ L of extract in 90  $\mu$ L of growth media) on colorectal adenocarcinoma (Caco-2) cells [3]. Therefore, this dilution was used with the breast cancer cell lines. Similar to the previous results, 1:10 diluted extracts had a strong inhibitory effect on cellular proliferation of both breast cancer cell lines as determined by MTS assay (Fig. 1). A 74–86% decrease in absorbance was observed for MCF-7 cells and a 91–94% decrease was observed for MDA-MB-231 cells compared to that of the untreated controls. All five onion extracts demonstrated anti-proliferative activity on both the breast cancer cell lines (>74% reduction in absorbance). It was also observed that DMSO alone (the commercially purchased purified flavonoid delivery vehicle) had no impact on the proliferation for MCF-7 and little impact on MDA-MB-231 ( $P < 0.05$ ,  $P = 0.0336$ ). Despite the differences in phytochemical composition among the five varieties, no statistical differences were observed in the ability to inhibit proliferation in either of the cancer cell lines. Crude onion extracts were more potent than the commercially obtained purified flavonoids (e.g., quercetin) in exhibiting cytotoxic activity.

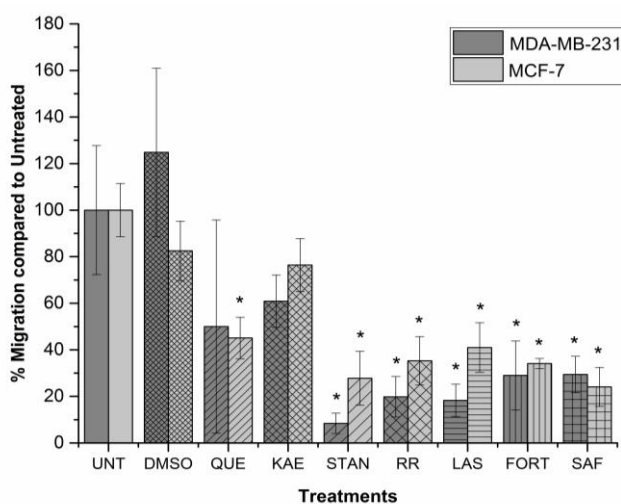


**Figure 1.** Cell proliferation assay. Comparison of three different cell lines; MDA-MB-231, MCF-7 and MCF-10A. UNT: Untreated; QUE: Quercetin (100  $\mu$ M); KAE: Kaempferol (100  $\mu$ M); STAN: Stanley; RR: Ruby Ring; LAS: LaSalle; FORT: Fortress; SAF: Safrane; Data shown as means  $\pm$  S.D. ( $n = 3$ ). Treatments with an asterisk differ significantly from respective untreated cells ( $P < 0.05$ ) by one-way ANOVA followed by Tukey's post hoc test.

Isolation and purification processes from complex matrices of food can be extremely time consuming, produces waste material, involves high production expenses, and costs consumers [9]. Thus, an ideal naturally derived agent would bypass these additional expenses and processes while maintaining efficacy. Our results suggest that flavonoids in combination with other biomolecules (e.g., organosulfur compounds) found in raw onion extracts may aid anti-proliferative activity [10]. These results further suggest that diet modification may be effective in reducing disease risk, which is consistent with other reports, and may reveal additional biomolecules for disease control [11, 12].

Our findings that onion extracts interfere with cellular proliferation in MDA-MB-231 and MCF-7 cells are consistent with previous reports on flavonoid effects [13, 14]. It is likely that the treatment with onion extracts have an impact on cell cycle progression, either through halting cells in senescence, initiating apoptosis, or via cytotoxicity [13, 15]. Our findings demonstrated an apparent difference in the efficacy between the two subtypes of breast cancer cells. The onions were more effective against TNBC MDA-MB-231 cells compared to ER+ MCF-7 cells.

MCF-10A, a non-cancerous fibrocystic breast cell line was used as a control to demonstrate the effects of onion extracts on non-cancerous breast cells. The MTS results depicted in Figure 1 demonstrate that onion extracts had an effect on cell proliferation as measured by decreased absorbance in treated cells. Fig. 1 shows all three cell lines in comparison with each other as an absorbance percentage compared to their respective untreated controls. There was a large difference in the anti-proliferative effect between cancerous and non-cancerous cell lines. Although these results do not show that there is no effect on non-cancerous breast cells, the effect on proliferation is less compared to the effect in cancerous breast cells. Studies have investigated the suitability of MCF-10A as a model for ‘normal’ breast cells and suggested that MCF-10A has phenotypic characteristics of both luminal and basal breast tissue, which suggests this cell line may be a potential hybrid different from true ‘normal’ breast tissue cells [16]. Additional studies have shown that the bioavailability of flavonoids in breast tissue is limited, warranting the design of new drug delivery systems [17]. Studies have examined the addition of an analogue to the flavonoid backbone structure to improve cell membrane permeability, mitochondrial selectivity, and overall bioavailability [17]. Therefore, if onion extracts are toxic to normal cells, improved selectivity in the drug delivery system would be required in order to use onion flavonoids pharmaceutically.



**Figure 2.** Anti-migratory activity of the onion extracts in a comparison of MDA-MB-231 and MCF-7. UNT: Untreated; QUE: Quercetin (100  $\mu$ M); KAE: Kaempferol (100  $\mu$ M); STAN: Stanley; RR: Ruby Ring; LAS: LaSalle; FORT: Fortress; SAF: Safrane; Data shown as means  $\pm$  S.D. (n = 3). Treatments

with an asterisk differ significantly from respective untreated cells ( $P < 0.05$ ) by one-way ANOVA followed by Tukey's post hoc test.

### ***Anti-migratory Effects on Cancer Lines***

We found that the five different onion extracts followed the MTS assay results in that varying compositions among the varieties had statistically similar anti-migratory effects on both cancer lines. Fig. 2 shows the number of cancer cells detected in the exclusion zone after a 72 h treatment period. Safrane extracts inhibited cell migration the most with a 76% decrease in cells within the exclusion zone for MCF-7 cells compared to that of the untreated control. Stanley (72% decrease), Fortress (66% decrease), Ruby Ring (65% decrease), and LaSalle (59% decrease) extracts also had an anti-migratory effect. Kaempferol did not inhibit MCF-7 cell migration into the exclusion zone. The results for MDA-MB-231 migration showed that Stanley extracts inhibited cell migration the most with a 92% decrease in cells within the exclusion zone after a 72 h treatment period followed by LaSalle (82% decrease), Ruby Ring (80% decrease), Fortress (71% decrease), and Safrane (71% decrease) (Fig. 2). Both quercetin and kaempferol did not significantly affect the migration compared to that of the untreated controls, but the effect of quercetin was not statistically different than that of the onion extracts. DMSO had no effect on migration in either cell line. Factors including cell cycle arrest, decreased proliferation, cytotoxicity, and apoptosis may contribute to the reduction of migratory cells because the experiment counts the number of cells in the exclusion zone, which means non-viable cells are not included in the calculations.

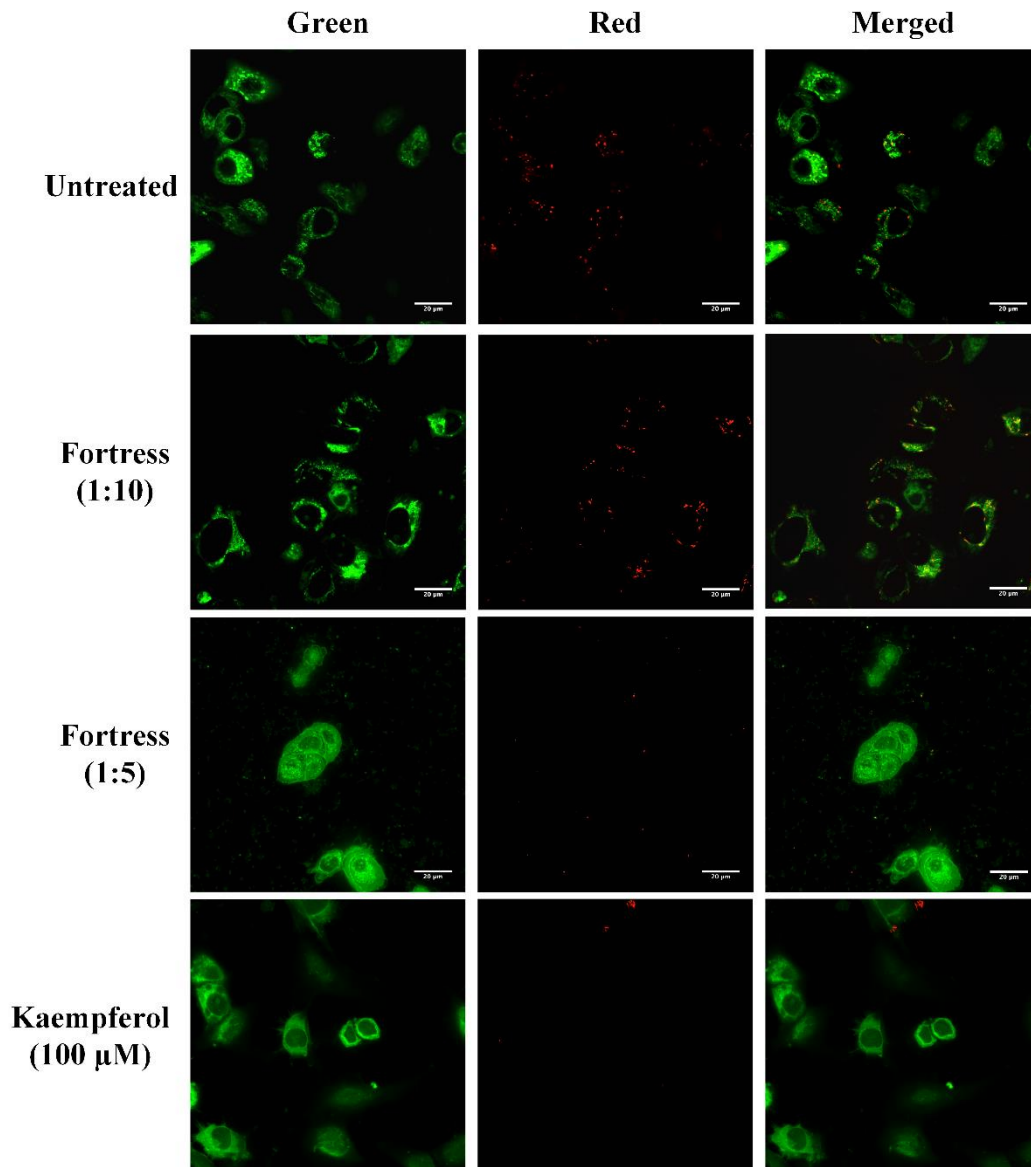
The results of the migration assay correlate with the MTS assay results, indicating that the reduction in migratory cells is potentially correlated to decreased cell proliferation. Migration and metastasis abilities are dependent on more than just the rate of cellular proliferation; the ability of cells to detach from surrounding cells and tissues, migrate through tissue barriers into blood circulation to relocate, and adhere in new areas of the body to synthesize new blood vessels in a secondary tumor are all phases of the metastatic cascade. The design of this migration assay allowed observation of the cell detachment, mobility, and adherence stages into a lesser-populated area. Cell-cell detachment is regulated by the epithelial-mesenchymal transition (EMT) process, which allows cells to detach from tissue and become singular mobile cells. This process is the major mechanism that initiates the metastatic cascade, and is an ideal mechanism to interfere with cancer cell metastasis. Flavonoids affect the EMT process by altering the expression of intercellular adhesion-related proteins. If the cells are unable to detach from tissue, metastasis is perturbed [13]. The potential effect on the metastatic cascade in conjunction with decreased cellular proliferation may explain the anti-migratory effects observed with onion extract treatment.

### ***Investigation of Mitochondrial Dysfunction***

Mitochondrial membrane potential ( $\Delta\Psi_m$ ) is vital in maintaining the electrochemical gradient that stabilizes ATP production, while also regulating redox status, biomolecular synthesis, and metabolism [18, 19]. Reduction in this potential is a common event in the mitochondrial-dependent intrinsic signaling pathway of apoptosis [20]. To investigate whether mitochondrial dysfunction is an event induced by onion extract treatment, we imaged cells with a membrane potential-sensitive dye JC-1 after a 24 h treatment period. Fluorescent red "J-aggregates" indicate that the mitochondrion has high potential and green fluorescent monomers in the cytosol indicate a low



potential. Under normal, untreated physiological conditions both red and green fluorescence were observed within the cells, and red aggregates were prominent.



**Figure 3.** Evaluation of mitochondrial membrane potential ( $\Delta\psi_m$ ) in untreated and Fortress-treated MCF-7 (1:10 and 1:5 dilutions) by confocal spinning disk microscopy. After treatment, cells were dyed with 2.5  $\mu\text{g}/\text{mL}$  JC-1 according to the manufacturer's protocol and incubated for 20 min. Green fluorescence was measured at 488 nm excitation/525 nm emission, and red fluorescence was measured at 560 nm excitation/620 nm emission. The data is representative of experiments with all other onion varieties for both cell lines, MDA-MB-231 and MCF-7.

The population of mitochondria in untreated samples of MCF-7 and MDA-MB-231 cells had a combination of both red fluorescence (high redox potential) and green fluorescence (low redox potential). After a treatment period of 24 h with the onion extracts (1:10 dilution) both red and green fluorescence were again observed. However, green fluorescence increased in prominence while the amount of red aggregates remained similar to that of the untreated cells. Because

quantitative comparison is complex between samples through multiple confocal planes, the experiment was repeated with a more concentrated (1:5 dilution) onion extract solution. This concentrated treatment caused a significant decrease in red aggregate fluorescence and an increase in green fluorescence, suggesting the effect is dose-dependent. Similar results were observed after 24 h treatment with quercetin and kaempferol (Fig. 3).

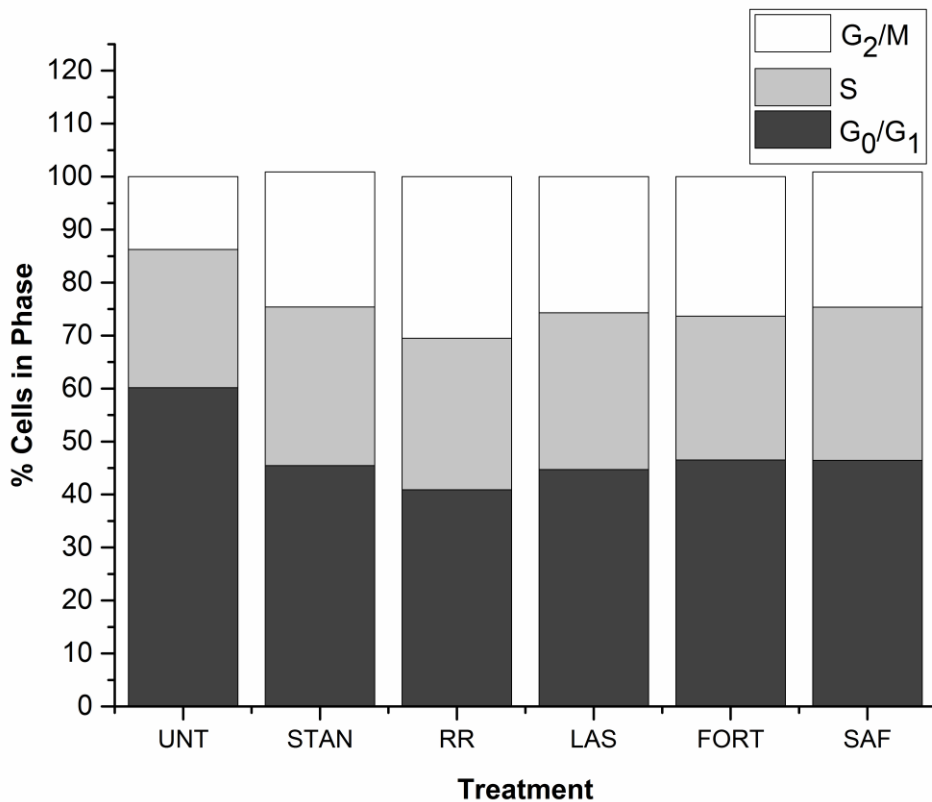
The results from the JC-1 dye demonstrated that the flavonoid positive controls were different compared to that of the onion extracts, which suggests the possibility that the onion extracts may induce a different primary apoptotic pathway from the isolated flavonoid controls. This may be due to the presence of other biomolecules within the extracts such as organosulfur compounds or anthocyanin. The 1:5 onion extract dilution has increased flavonoid content, which may induce a reduction in mitochondrial membrane potential similar to that of the control flavonoids. Furthermore, a lower concentration of extract may induce apoptosis via a mitochondrial-independent pathway. Onion extracts may induce apoptosis via the death receptor-mediated (extrinsic) pathway through the activation of death receptor ligands of the tumor necrosis factor (TNF) family, like the FAS ligand that activates caspase 8, and may bypass the mitochondrial intrinsic pathway that reduces membrane potential and releases cytochrome c [21]. Because a membrane potential reduction is observed at a higher concentration of extract, the higher amount of flavonoids may contribute to the mitochondrial-dependent pathway in conjunction with the extrinsic pathway. In some cases, apoptosis may be induced through the death receptor-mediated pathway and then amplified by the mitochondrial intrinsic pathway as by-products of the extrinsic pathway (i.e., tBID generated by Caspase 8-mediated proteolysis of BID can initiate intrinsic mitochondrial apoptosis) [21]. The MTS assay results showed that 1:10 treatments reduce proliferation more strongly than the control flavonoids; it is plausible that the extracts induce apoptosis through a different pathway and are further amplified through additional pathways as treatment exposure lengthens.

### ***Cell Cycle Analysis***

To investigate potential alteration of the cell cycle from onion extracts, DNA content was measured by flow cytometry. A shift in cell cycle phase in breast cancer cell lines based on onion extracts may be an area of focus for further mechanistic research. DNA content was analyzed only for the MDA-MB-231 cell line because the natural aggregation of MCF-7 cells made it difficult to obtain meaningful data via flow cytometry. Propidium iodide was used to quantify the DNA. A summary of the data obtained can be seen in Fig. 4. The results of the analysis using FC Express 4, show a 12-17% increase in onion extract-treated cells in the G2/M phase compared to the untreated cells after a 24 h treatment period. Ruby Ring had the largest increase with 16.75%, followed by Fortress (26.32%), LaSalle (25.71%), Safrane (25.54%), and Stanley (25.49%). The proportion of cells in S phase from the extract with the largest increase by Stanley (29.95%) increased only slightly compared to the untreated cells (26.09%).

There was a decrease of 14-19% in treated cells in the G0/G1 phase compared to that of untreated MDA-MB-231 cells. More cells collected in the G2/M phase and the proportion of cells

in the S phase remained consistent, which resulted in the loss of cells in the G<sub>0</sub>/G<sub>1</sub> phase. This indicates a potential cell cycle arrest at the G<sub>2</sub> checkpoint that prevents cells from entering mitosis and proliferating, which caused cells to re-enter the G<sub>0</sub>/G<sub>1</sub> phase. The cell cycle has a crucial role in regulating cell growth and proliferation through maintaining cellular integrity and inducing cell division [22].



**Figure 4.** Effects of raw onion extract treatment on the cell cycle. Cell cycle profiles of MDA-MB-231 cells after 24 h treatment with five Ontario-grown onion varieties and untreated cells by flow cytometry using FC Express 4 for data analysis. UNT: Untreated; STAN: Stanley; RR: Ruby Ring; LAS: LaSalle; FORT: Fortress; SAF: Safrane; Quantitative estimation of the percent of cells in G<sub>1</sub>, S, and G<sub>2</sub>/M phases compared to that of untreated cells. MDA-MB-231 treated with onion extract cells had an increase in G<sub>2</sub>/M cells ranging from 25.49-30.50% compared to 13.75% in untreated cells.

Therefore, targets for new anti-cancer drugs aim to disrupt the cancer cell cycle [23]. An increase in cells in the G<sub>2</sub>/M phase demonstrates the timing at which the flavonoids disrupt the cell cycle for the MDA-MB-231 cancer cell line. This provides insight into the potential mechanisms of flavonoid activity and limits dysfunction to processes involved in the replication of DNA in S phase which would lead to arrest at the G<sub>2</sub> checkpoint or to dysfunction in cells entering mitosis [24]. The cell cycle is regulated by three main families of proteins: Cyclin-

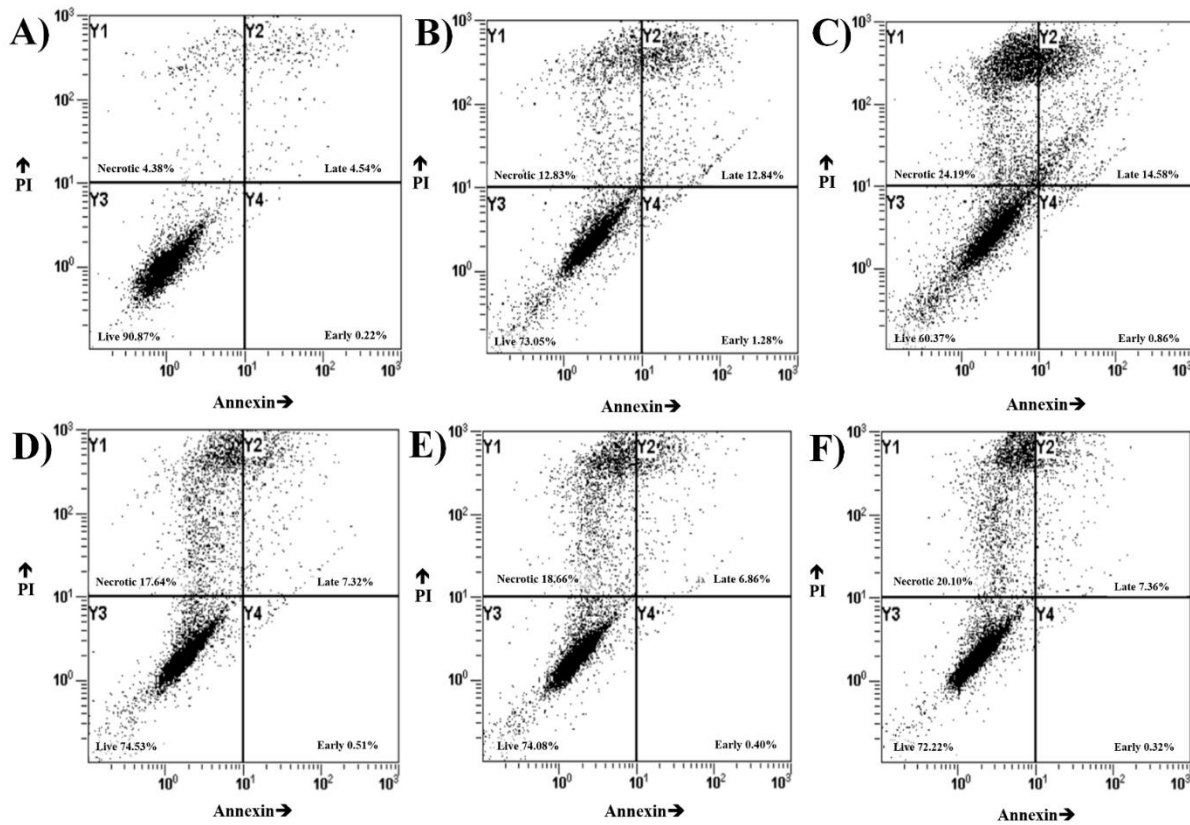
dependent kinases (CDKs), Cyclins that are activated by CDKs, and CDK inhibitor proteins [25]. Cyclins function during periods of cell proliferation and CDK inhibitors function in response to anti-proliferative signals and induce cell cycle arrest. The normal physiological expression of these proteins allow cells to grow, pass through the G1 and G2 checkpoints, and enter mitosis to divide. Therefore, the regulation and presence of these proteins is critical for normal cell progression and further studies to determine their rate of expression after onion extract treatment may reveal information about cell cycle arrest mechanisms [25]. Flavonoids are also known to exert anti-proliferative activity by affecting microtubule binding, which prevents cells from successfully entering mitosis and thereby inducing cell cycle arrest at the G2/M phase [24]. Microtubule formation is another area to be investigated that may be affected by flavonoids in onion extracts.

Ultimately, the increase in G2/M cells provides a window to focus further research on the mechanisms and pathways in this section of the cell cycle. Cell cycle arrest in the G2/M phase may provide clues as to why flavonoids are more effective against the triple negative breast cancer line. This cell line is more aggressive than other breast cancer subtypes and our cultures grew faster than the ER+ cell line (MCF-7) which is in agreement with similar studies by Chang et al. [22]. This faster growth rate may cause a higher percentage of cells to be in the G2/M phase, which are thereby more affected by flavonoid activity in that stage of the cell cycle.

### ***Annexin-V FITC Apoptosis Detection***

Because flavonoids exhibited anti-proliferative activity and a potential cell cycle arrest, we examined the presence of apoptotic cells to distinguish cytotoxic versus cytostatic activity using annexin-V FITC and propidium iodide dyes for early stage apoptosis (annexin-positive, PI-negative) and late stage apoptosis (annexin-positive, PI-positive) in conjunction with deceased cells (annexin-negative, PI-positive). MDA-MB-231 cells treated with each of the five onion varietal extracts demonstrated apoptosis induction through a strong increase in late stage apoptotic and deceased cells that can be seen in Fig. 5. The strongest effects were observed from Ruby Ring extract, which resulted in 24.19% deceased cells and an increase of 19.81% from the untreated control. Ruby Ring also increased by 10.04% late stage apoptotic cells and resulted in the highest decrease in live cells with 30.50% followed by Safrane (18.65% decrease), Stanley (17.82%), Fortress (16.79%), and LaSalle (16.34%). The increase in apoptotic cells indicates that the anti-proliferative and anti-migratory effects observed were partially due to an increase in cytotoxicity. Furthermore, it suggests that the cell cycle arrest at G2/M likely induces cell death instead of merely halting cell cycle progression through cytostatic activity. Further studies are warranted to determine mechanistically the mode of action by which the onion extracts stimulate cell death. This data signifies the potential use of onion extracts not only for breast cancer prevention but also for potential treatment, as the onion polyphenol extracts induced a cytotoxic effect. Onion extracts

interfered with normal breast cancer cell progression, inhibited key processes in disease advancement, and terminated cancer cells in vitro.



**Figure 5.** Onion extracts induced cell death in MDA-MB-231 (A) Untreated control, (B) Stanley-treated, (C) Ruby Ring-treated, (D) LaSalle-treated, (E) Fortress-treated, and (F) Safrane-treated. Cells were grown in T-75 cm<sup>2</sup> flasks in growth media until nearly 80% confluency. Media was replaced with 1:10 dilutions of onion extracts for a 24 h treatment period. Cells were harvested, suspended in 1× binding buffer, and stained with annexin-V FITC and propidium iodide. After analysis of flow cytometry, a strong increase in late stage apoptotic cells was observed in addition to an increase in deceased cells compared to the untreated control.

## CONCLUSION

Extracts synthesized from Ontario-grown onion varieties were effective in inducing anti-migratory, cytostatic, and cytotoxic activities in two sub-types of human breast cancer cell lines. These extracts strongly slowed the migration of cancer cells and reduced the rate of proliferation into an exclusion zone. Importantly, these extracts were consistently more effective than the commercially available pure flavonoid counterparts. The extracts were also more effective against the TNBC cell line MDA-MB-231 compared to the ER+ breast cancer cell line MCF-7. Increased concentrations (1:5 dilutions in media) of onion extracts significantly reduced mitochondrial membrane potential compared to standard concentrations (1:10 dilutions in media). This

information in conjunction with a potential cell cycle arrest at G2/M phase provides details about the potential mechanisms of the onion flavonoids. Further studies are warranted to understand the pathways and mechanisms behind these anti-tumor effects.

**List of Abbreviations:** ER+, estrogen-receptor-positive breast cancer; TNBC, triple negative breast cancer; FBS, fetal bovine serum; PBS, phosphate buffered saline; DMEM, Dulbecco's modified eagle's medium; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition;  $\Delta\Psi_m$ , mitochondrial membrane potential; TNF, tumor necrosis factor; CDKs, cyclin-dependent kinases.

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**Competing Interests:** The authors have no potential conflicts of interest to report.

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