THE EFFECTS OF YELLOW PASSION FRUIT, *Passiflora Edulis Flavicarpa*,
PHYTOCHEMICALS ON CELL CYCLE ARREST AND APOPTOSIS OF
LEUKEMIA LYMPHOMA MOLT-4 CELL LINE

By

CINDY MARIE DE NEIRA

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2003

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Abstract of Thesis Presented to the Graduate School
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Cindy Marie De Neira

August 2003

Chair: Susan S. Percival
Major Department: Food Science and Human Nutrition

Yellow passion fruit, *Passiflora edulis flavicarpa*, has become increasingly popular

in the United States as a tropical fruit juice commodity, and is mainly imported from

Brazil and Ecuador. The fruit, known for its natural sweet taste and medicinal purposes,

is mainly consumed as juices, jellies, and jams. The nutrient content of passion fruit,

which consists mainly of Vitamin A, Vitamin C, potassium, carotenoids and

polyphenolics. However, little research has been conducted on the health benefits of

passion fruit, especially its anticancer properties. Certain non-nutritive phytochemicals,

also present in passion fruit, carotenoids and polyphenols have been found to inhibit cell

proliferation of leukemia and induce apoptosis. The objective of this study was to

investigate the effect of stock passion fruit juice and its carotenoid and polyphenolic

components on cell cycle, apoptosis, and cell viability of the MOLT 4 lymphoma cell

line. The cell cycle was analyzed by flow cytometry, apoptosis by caspase-3 activity and

cell viability by MTT activity. Stock passion fruit juice and the unbound aqueous

polyphenolic fraction, at a concentration of 2%, showed a significant inhibition and induced arrest at the G_0/G_1 phase of the cell cycle at 48 hours. The collective carotenoid/aqueous polyphenolic fraction at 4-8% inhibited G_0/G_1 phase and induced arrest at 24 hours. In addition, the DMSO, carotenoid, aqueous polyphenolic, and collective carotenoid/aqueous polyphenolic fractions increased caspase-3 activity and decreased cell viability after 4 hours of incubation. These results, arrest of the cell cycle, induction of apoptosis, and a reduction of cell viability, showed that phytochemicals present in passion fruit may have beneficial anticancer effects.

CHAPTER 1
INTRODUCTION

Cancer is the leading cause of the death in the world and the second in the United States. It is projected that 14.7 million people globally will be diagnosed with cancer by the year 2020 (World Cancer Research Fund/American Instiute for Cancer Research [WCRF/AICR] 1997). Some cancers can be prevented through lifestyle modifications; dietary measures have been estimated to have the potential to prevent about 30-40% of all cancers worldwide (WCRF/AICR 1997). The World Cancer Research Fund now recommends a plant-based diet rich in a variety of fruits, vegetables, and legumes along with reduction in the amount of processed starchy staple foods (WCRF/AICR 1997).

Strong epidemiological and experimental evidence inversely correlate plant-based diets with the incidence of several types of cancers (WCRF/AICR 1997). Specifically, the North American diet, associated with the highest incidence of cancer, correlated to high levels of saturated fat and low levels of plant-based foods. The lowest incidences of cancer were observed in populations with Mediterranean and Asian diets that were characterized as having a lower content of saturated fats and a higher rate of consumption of plant based foods such as legumes, vegetables and fruits (Simopoulos 2001). It has been suggested that several distinctive phytochemicals (vitamins, fibers and micronutrients) may be responsible for the protective effect of fruits and vegetables (reviewed in Steinmetz & Potter 1996). More recently, scientists have been interested in investigating micronutrients, carotenoids and polyphenolic compounds, because of their potential as inhibitors of tumor growth.

In in-vitro studies, these phytochemicals have been found to inhibit leukemia cancer cell growth and induce apoptosis at different phases. Carotenoids have been shown to cause a G_0/G_1 arrest in HL-60 myeloid leukemia cells (Palozza et al. 2002, Palozza et al. 2001, Amir et al. 1999). In addition, certain polyphenols have been responsible for a G_0/G_1 arrest in CEM leukemic T-cells (Yoshida et al. 1992) and a G_2 arrest in HL-60 myeloid leukemic cells (Kang & Liang 1997). Palozza et al. (2002) demonstrated that carotenoids can induce apoptosis in HL-60 cells at the same concentration required for cell cycle arrest. For polyphenols, in general, there is a dose and time dependent induction of apoptosis in human leukemia cells (Csokay et al. 1997, Ferry-Dumazet 2002).

In this study, the total carotenoid and polyphenolic fractions of passion fruit juice were compared to the stock fruit juice, to better understand the effects of food sources in the diet. Study of the individual carotenoid and polyphenolic compounds was not deemed as important, because when food is consumed, there is an interaction of all the components. The hypothesis stated that passion fruit would prevent cancer cell cycle progression and increase apoptosis activity, due to its rich phytochemical composition. This study had three main objectives:

- To use stock passion fruit juice to characterize the appropriate time and dose for the evaluation of cell cycle arrest.

- Use passion fruit juice fractions to assess which components, carotenoids or aqueous polyphenolics, had the greatest effect on cell cycle kinetics.

- Use passion fruit fractions to assess if apoptosis was stimulated after arresting the cell cycle.

CHAPTER 2
LITERATURE REVIEW

Cell Cycle

The cell cycle has five stages, Gap 1 (G_1) phase, Synthesis (S) phase, Gap 2 (G_2) phase, Mitosis (M) phase and Gap 0 (G_0) phase. During the G_1 phase, protein synthesis occurs to ready the cell for the subsequent phase. During the S_1 phase, the DNA is replicated, and in the G_2 phase proteins are synthesized to make sufficient proteins for two daughter cells. In the final stage of the cell cycle, the M phase, mitosis or cell division occurs and the cell divides into two cells. At this point, the cell has the choice of returning to the cell cycle for proliferation or entering the G_0 phase. This phase is called the "quiescent" phase because the cell no longer proliferates, but is a fully functional cell until it undergoes apoptosis or programmed cell death (as reviewed in Flatt & Pietenpol 2000; Grana & Reddy 1995). During the cell progression, there are several different checkpoints, at the G_1/S transition, S, G_2/M transition and M phases, where the cell is checked for any abnormalities (Hartwell & Weinhert 1989). If abnormalities are detected, then cellular repair or apoptosis occurs.

Since cancer is characterized by rapid, uncontrolled cell cycle, it would be ideal to study the cell cycle a possible cancer preventative effect of passion fruit phytochemicals. After the relationship between passion fruit phytochemicals is investigated, the health benefits of passion fruit can be further understood by exploring the effects of the phytochemicals on apoptosis, cell growth and viability.

Apoptosis

As reviewed in King and Cidowski (1998) after cell cycle arrest, the cell can repair itself and re-enter the cell cycle, or if damage is too extensive programmed cell death or apoptosis occurs. The purpose of apoptosis is to rid the body of unwanted cells without producing an inflammatory response. During the apoptotic response morphological changes occur including cell shrinkage, lose substrate attachment, condensing of the chromatin, display of rapid membrane blebbing, and DNA fragmentation (reviewed by Schwartzman and Cidowski 1993).

Some inducers of the apoptotic response are: growth factor withdrawal, ionizing radiation, Ca^{+2} influx, tumor necrosis factor, viral infection and glucocorticoids (reviewed by Schwartzman and Cidowski 1993). After the cell is signaled by one of the inducers, a cascade is generated, which ultimately leads to the destruction of the cell. Two main catalytic reactions are stimulated, protease activation, which includes the destruction of cellular proteins and nuclease activation, leading to DNA fragmentation, and RNA degradation (reviewed by Schwartzman and Cidowski 1993; Thornberry and Lazebnik 1998).

There are three main protein signals for the apoptosis cascade, Bcl-2 family proteins, caspases, and Apaf-1/CED-4 protein (Adams and Cory 1998). This research will focus on the caspases, a family of cysteine proteases that function to cleave proteins after aspartic acid residues (Cohen 1997). Caspases remain inactive in the cells until apoptosis is activated after which they are cleaved into their active enzyme (Samali et al. 1999; Thornberry and Lazebnik 1998). The activation of the caspases is responsible for the morphological changes that occur during apoptosis (Goldstein 1997).

There are several different caspases. Caspase- 1 and -11 are involved in cytokine processing (Li et al. 1995; Kuida et al. 1995), while -2, -3, -6, -7, -8, -9, and -10 are involved in apoptotic regulation and implementation (Kuida et al. 1996, 1998; Varfolomeev et al. 1998; Bergeron et al. 1998). Specifically, caspase-3 is an important protease because it is a "point of no return" caspase, which means that once it is activated apoptosis is definitely induced. This makes caspase-3 an ideal marker to study for apoptotic activity.

Cancer

Cancer is the second leading cause of death in the United States, with one out of four deaths being cancer related (WCRF/AICR 1997). Cancer is characterized by the abnormal proliferation of cells causing a total loss of cell function. Cancer is considered to be an unpredictable disease because treatments, even aggressive invasive procedures, often come with unpredictable outcome and reoccurrence (reviewed by Gerster 1993). For this reason it is important to emphasize the implications of education to prevent cancer.

One important factor in the prevention on cancer is to assess the risk for cancer, which can be influenced by exposure to radiation, chemicals, hormones, infectious agents, chemicals, genetics and nutrition. It is expected that about one- third of the number of cancer deaths will be associated with poor nutrition, physical inactivity and other lifestyle factors that can be changed (WCRF/AICR 1997).

MOLT-4

The MOLT-4 cell line, derived from a 19-year-old male with acute lymphocytic leukemia, was chosen for this study. Blood cells are important as they are first to make contact with the absorbed phytochemicals, before they are degradation by other organs.

In addition, this model can potentially lead to future research with human studies, where phytochemical effects may be investigated using less complicated procedures.

Health Benefits of Fruits and Vegetables

The results from different studies of fruits and vegetables regarding the incidence of chronic diseases have been debatable. Some epidemiological studies have suggested that the consumption of fruits and vegetables have been associated with reduced incidences of cancer and cardiovascular disease. High intakes of fruits and vegetables have been correlated with the decrease incidence of certain cancers, such as colon, endometrial and pharyngeal (Flood et al. 2002;Uzcudun et al. 2002; Littman et al 2001). Specifically, in a study of female American nurses, increased fruit and vegetable consumption had significantly reduced the risk of certain cancers (reviewed in Terry et al. 2001). The results also concluded that approximately two servings of fruit per day significantly reduced the risk of cancer (Terry et al. 2001). However, not all epidemiological studies are consistent, for example, the Nurse's Health Study and the Health Professionals Follow-up Study showed no association between the intake of fruits and vegetables and colon cancer (Kolonel et al. 2000; Michels et al. 2000), breast cancer (Sauvaget et al. 2003) and bladder cancer risk (Michaud et al. 1999).

Studies have also showed that some yellow/orange and dark green colored fruits and vegetables were found to be very beneficial in reducing the risk of some cancers such as oesphageal, lung, stomach, liver, prostate and total cancer (reviewed by Committee on Medical Aspects of Food, Nutrition Policy [COMA] 1998; Malin et al. 2003; Sauvaget et al. 2003; Kolonel et al. 2000). It has also been shown that these yellow/orange and dark green fruits and vegetables that are associated with reduced cancer risk are rich in carotenoids and polyphenols (reviewed by Gerster 1993; Steinmetz & Potter 1996; Le

Marchand et al. 1989;1993). These phytochemicals have been thought to have the anticancer properties that may contribute to the protective effect of fruits and vegetables (Gerster 1993; Knekt et al. 1997).

In a review article, by Steinmetz & Potter (1996), several cohort studies suggested that an increase in the amount of fruits and vegetables inversely correlated with the incidence of cancer. However, of the 174 case-controlled studies that were reviewed, cancers of the lung, stomach and esophagus supported this premise, while other cancer were often not well studied or led to contradictory results. In this same review article, in animal studies, results showed that cancerous mice fed more fruits and vegetables were likely to have fewer tumors, less DNA damage, higher enzymes involved in the detoxification of carcinogens, and other factors involved in lowering the risk of cancer (Steinmetz & Potter 1996).

Passion Fruit

Passion fruit has become increasingly popular in the U.S., where it is mainly used in jams, jellies, and fruit juices. Regions in the US where it is cultivated are in south Florida, California and Hawaii, however, there is a demand for it in Europe and Asia (California Rare Fruit Growers [CRFG] 1996; Knight & Sauls 1994; Regional Agribusiness Project [RAP] 1998). This fruit is native to Brazil and Ecuador, where it is used for medicinal purposes as a sedative, as well as a food source (CRFG 1996). As an edible fruit, it contains several components such as acids and sugars, nutrients, and non-nutritive phytochemicals that make passion fruit a tasteful and healthy addition to the diet.

Acids and Sugars

Passion fruit is a high acid food (pH~ 3.2) due to the predominance of two acids, citric (~93-96 % of total) and malic (3-6 % of total) acid (United States Department of Agriculture [USDA], 2002). Passion fruit also contains about 14.45 g sugar/100g of edible portion, including fructose, glucose and sucrose, along with seven others in trace amounts (USDA 2002). The acids and sugars add to the unique taste and serve as a preservative nature for the tropical fruit.

Nutrients and Phytochemicals

Passion fruit provides a good source of nutrients such as Vitamin C (18.2 mg/100g of edible portion), Vitamin A (2410 IU/100g of edible portion) and potassium (278mg/100g of edible portion) (USDA, 2002) and non-nutritive phytochemicals, carotenoids (9.25 mg/L) and polyphenols (435 mg/L) (Talcott et al., 2003). Vitamin C function as an antioxidant that reduce free radical damage by scavenging oxyradicals (Fischer-Nielsen 1992). Vitamin A has important roles in the maintenance of vision and skin care, cell growth, and reproduction (reviewed by Olson, 1996; Gerster 1997). Vitamin A can be obtained directly from the diet or derived from non-nutritive phytochemicals called carotenoids. In addition to being a vitamin A precursors, certain carotenoids such as β-carotene were found to have anticancer properties (Palozza et al. 2001). In passion fruit, thirteen different carotenoids have been identified, including zeta-, beta- and alpha-carotene, b-cryptoxanthin, and lycopene (Mercandente et al. 1998). Other non-nutritive phytochemicals found in passion fruit are polyphenolic compounds, which have been found to have antioxidant activity (Rice-Evans & Miller. 1996; Salah et al. 1995) as well as anticancer properties (Yoshida et al. 1992; Kang & Liang 1997).

Only some of the polyphenolics such as the phenolic acids have been identified in passion fruit, however, the flavonoids have not (Talcott et al. 2003).

Carotenoids

Characteristics

Carotenoids are lipid-soluble pigments that are responsible for the red, yellow and orange colors of fruits and vegetables. They are found in foods like carrots, tomatoes, oranges, mangos, papayas, bananas and passion fruit. Some of the identified carotenoids in passion fruit include ζ- α- and β-carotene, lycopene and β-cryptoxanthin (Mercadante et al. 1998). The total carotenoid concentration of passion fruit is 9.32 mg/L, which only three individual carotenoids, α-carotene (35g/100g of edible portion), β-carotene (525 g/100g of edible portion), and β-cryptoxanthin (46g/100g edible portion) had been quantified (USDA 1998). Table 1 shows the comparative carotenoid content of some popular fruits. Passion fruit provides a richer source of α-carotene and β-carotene, than other popular tropical fruits such as papaya, mango and banana (Table 1). However, when compared to other rich sources of carotenoids like tomatoes and carrots, passion fruit only provides more α-carotene than tomatoes and carrots provided a richer source of α-carotene and β-carotene than passion fruit (USDA 1998).

Table 1: The carotenoid (α-carotene & β-carotene) content of comparative fruits

	α-carotene (g/100g of edible portion)	β-carotene (g/100g of edible portion)
Passion fruit	35	525
Papaya	0	276
Mango	17	445
Banana	5	21
Tomatoes	112	393
Carrots	4,649	8,836
Oranges	16	51

Effect on Chronic Disease

In humans, carotenoids have an important role in the prevention of chronic disease e.g. cardiovascular disease and cancer. Several different mechanisms for carotenoids exist that show the prevention of chronic disease: pro-vitamin A activity, antioxidant activity, and cell cycle arrest and apoptosis activity. Carotenoids such as α-, β-, ζ - carotene and β-cryptoxanthin are converted to vitamin A (retinol form), which plays an important role in cell differentiation and proliferation (reviewed in Ross and Gartner 1994; Rock 1997). Of the pro-vitamin A carotenoids, β-carotene is most effectively converted to vitamin A with α- and ζ-carotene and β-cryptoxanthin being half as efficient (reviewed in Rock 1997). As reviewed in Aruoma (1998) carotenoids also act as antioxidants, specifically α-carotene, β-carotene, and β- cryptoxanthin and other non-vitamin A active compounds such as lutein, lycopene, canthaxanthin, and zeaxanthin by quenching of singlet oxygen species and scavenging free radicals (Pavia & Russell 1999, Foote et al. 1970). Carotenoids such as lycopene and β-carotene also have been shown to inhibit the cell cycle progression and induce apoptosis activity, which is beneficial for the prevention of tumor growth (Murakoshi et al. 1989; Palozza et al. 2001).

Effect on Cell Cycle

Some of the well-known carotenoids in the literature are beta-carotene, alpha-carotene and lycopene, because they have been studied for their anticancer properties. More specifically, the carotenoids have been found to induce cell cycle arrest and apoptosis. With respect to leukemia, only beta-carotene and lycopene have been found to arrest the cell cycle. β-carotene causes a G_0/G_1 arrest in HL-60 myeloid leukemic cells at low concentrations and a G_2/M arrest at high concentrations ($>20\mu M$) and induces

apoptosis at a concentration of 10 μM (Palozza et. al. 2002). Lycopene has been found to cause a G_O/G_1 arrest in HL-60 myeloid leukemic cells (Amir et al. 1999). Both carotenoids showed dose dependent activity, however, no time course was evaluated in the articles. No literature was found on the effects of carotenoids on the cell cycle of lymphomic leukemic (MOLT-4) cells so the further studies must be performed.

Polyphenolic Compounds

Characteristics

Polyphenolic compounds are non-nutritive phytochemicals that contribute to the flavor, pigmentation and health benefits of certain plants. Some dietary sources of polyphenols include onions, red wine, green tea, soy, and other vegetables and fruits. Epidemiological studies have associated foods rich in polyphenols provide prevention against diseases such as cancer (Steinmetz & Potter 1996).

The two main classes of polyphenols found in the diet are phenolic acids and flavonoids. Flavonoids represent the larger class of polyphenolics accounting for approximately 2/3 of the identified polyphenolics (Birt et al. 2001). The main flavonoid families include flavones, flavonols, flavanones, catechins (flavanols), anthocyanidins and isoflavones. Fruits in general have been found to contain several flavonoids including flavonols (quercetin), flavonones, catechins and anthocyanidins. In passion fruit, the total flavonoid content has been quantified (435 mg/L) and the phenolic acids have been identified, however, individual flavonoids have not (Talcott et al. 2003).

Effect on Chronic Disease

Polyphenolic compounds have an important role in the prevention of chronic disease such as cardiovascular disease and cancer. Several different mechanisms for polyphenolics exist that show the prevention of chronic disease: antioxidant activity, cell

cycle arrest and apoptosis activity. Certain polyphenols, such as quercetin, catechins, resveratrol, gallic acid, and anthocyanins exhibit antioxidant activity by inhibiting lipid perioxidation and scavenging oxygen radicals (Terao et al. 1994; Miura et al. 1995; Salah, et al. 1995), thus potentially lowering the risk of coronary heart disease and cancer. Polyphenols such as catechins and quercetin can also induce cell cycle arrest and apoptosis, which can be beneficial for the inhibition of cancer cell growth (Kang & Liang 1997; Csokay et al. 1997).

Effect on Cell Cycle

Flavonoids have been widely studied because of their potential health benefits as anticancer phytochemicals. They have been found to inhibit cancer cell growth by preventing the progression of the cancer cell cycle. With respect to several leukemic cell lines, the specific flavonoids, quercetin and catechin were shown to have an effect on cell proliferation. Quercetin has been known to cause a late G_1 arrest in CEM leukemic T-cells (Yoshida et al. 1992) however in HL-60 myeloid leukemic cells it caused a G_2/M arrest (Kang & Liang 1997). Quercetin has also been found to induce apoptosis at $5.5\mu M$ in K562 human leukemia cells (Csokay et al. 1997). Catechins, they were found to cause a growth inhibition of MOLT-4 leukemia lymphoma but the cell cycle had not been analyzed (Kuroda & Hara 1999). No specific mechanism has been determined for the cell cycle arrest in leukemic cells so further studies are needed. Since the total flavonoid content have not been studied in MOLT-4 leukemic cells, two common flavonoids in fruits, quercetin and catechins, along with similar leukemic cell lines were reviewed above to develop the hypothesis of this thesis.

CHAPTER 3
MATERIALS AND METHODS

Materials

The MOLT 4 cell line was purchased from the American Type Culture Collection (ATCC) in Rockville, Maryland. RPMI-1640 and 10% Fetal Bovine Serum (FBS) + PSF (Penicillin Streptomycin Fungicide) was purchased from Sigma Chemical Co (St. Louis, MO). The passion fruit juice was obtained from iTi Tropicals, Inc. (Lawrenceville, NJ)/ Quicornac (Ecuador). Propidium Iodide (PI), RNase, Trypan Blue, and MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) were purchased from Sigma Chemical Co. Cell Lysis Buffer [10mM HEPES, 2mM EDTA, 0.1% CHAPS, 1mM PMSF, 10μg/ml pepstatin A, 20 μg/ml leupeptin, 10 μg/ml aprotinin, 5mM DTT (dithiothreitol)], 4mM DEVD-pNA (N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide), and Cell Reaction Buffer (100mM HEPES, 20% v/v glycerol, 0.5 mM EDTA, 5mM DTT) were purchased from Sigma Chemical Co. FACScan (Fluorescence Activated Cell Sorting Scan) flow cytometer (Becton Dickinson, San Jose, CA) and UV Max Spectrophotometer (SpectraMax 340 C, Molecular Devices, Sunnyvale, CA) was used to analyze the samples.

Methods

Maintenance of Cells

The MOLT 4 cells were cultured in the RPMI-1640 medium with 10% FBS + PSF and incubated in a humidified atmosphere at 37°C at 5% CO_2 atmosphere. The cells were centrifuged, the pellet was resuspended in 5mL of fresh medium and viable cells

counted using trypan blue (0.4%) on a hemocytometer. Cells in culture were passed every two days at a concentration of 5.0×10^5 cells/mL in 30 mL of RPMI-1640 with 10% FBS, following recommended guidelines for cell line maintenance provided by ATCC.

Unbound and Methanol Fractions

A C_{18} Sep-Pak cartridge is a non-polar, reverse phase that was used to separate the phytochemicals in the passion fruit juice, either by molecular weight, affinity, functional group or polarity. Passion fruit juice was loaded onto the C_{18} column and the polar non-retained compounds, which may have included vitamin C, sugar, acids, proteins, amino acids, and low molecular weight polyphenols. This sample was labeled the "unbound fraction". The non-polar retained compounds, which may have included the carotenoids and polyphenolic compounds, were partitioned from the cartridge with methanol in two separate partitions with one at 50% and the other at 100%. The sample of the bound compounds eluded with 50% methanol was labeled as "50% MeOH fraction," which included polar compounds and low molecular weight polyphenols. Similarly, the bound compounds eluded with 100% methanol was labeled "100% MeOH fraction," which consists of the carotenoids and polyphenolics. The methanol was evaporated under reduced pressure and residue sample was redissolved in a known volume of DMSO.

Individual Carotenoid and Aqueous Polyphenolic Fractions

Passion fruit juice (50 mL) was extracted in a solution of 50:50 ethanol and acetone (E:A) to solubilize both carotenoids and polyphenolics. Following filtration and subsequent washing off the insoluble residues with E:A, the solvent was pooled and evaporated at 50°C under reduced pressure. The remaining solution (water, carotenoids and polyphenolics) was poured into a separatory funnel after which petroleum ether was

added. Carotenoids and other lipophilic compounds were partitioned into the upper petroleum ether phase, while more of the polar compounds, such as polyphenolics, acids and sugars, remained in the lower, aqueous solution. The carotenoid fraction was removed and filtered through sodium sulfate to remove water residues and evaporated under reduced pressure. The residue obtained was then resuspended in a known volume of DMSO for subsequent application to the cell line.

The residual aqueous extract was then extracted with 3 volumes of ethyl acetate to separate compounds such as polyphenolics from organic acids, amino acids, vitamin C, or sugars. Ethyl acetate extracts were pooled, filtered through sodium sulfate, evaporated under reduced pressure, and finally redissolved in a known volume of DMSO for treatment.

Collective Carotenoid/Aqueous Polyphenol Fraction

To 200 grams of passion fruit juice, 600mL of 95% ethanol was added to extract the desired compounds and precipitate the polysaccharides (pectin). Whatman # 4 filter paper was used to remove the insoluble matter and the solvent was evaporated by rotary evaporation at 45°C. Water remained and DMSO was added to help solubilized the carotenoids, which was loaded onto a C_{18} Sep-Pak cartridge. Following elution of the extract through the cartridge, the bound isolate was washed with water to remove the soluble residue. This removed most of the acids and sugars. The C_{18} bound compounds were eluted with 20mL of 100% methanol. Methanol was then evaporated by reduced pressure and extract was redissolved in 5.5 mL of DMSO with 1.1 mL of 100% ethanol. The ethanol was added to the sample to sterilize the extract. The extract was brought to 55mL with DMSO, warmed slightly in the 37°C water bath and sonicated for 15 minutes.

Whole PF, Carotenoid, Polyphenol Fractions for Caspase and MTT

To 300 grams of passion fruit juice, 1200mL of ethanol and acetone (E:A) was added to extract the desired compounds and precipitate the polysaccharides (pectin). Whatman # 4 filter paper was used to remove the insoluble matter and the solvent was evaporated by rotary evaporation at 45°C. Water remained and DMSO was added to help solubilized the carotenoids, which was loaded onto a C_{18} Sep-Pak cartridge. Following elution of the extract through the cartridge, the bound isolate was washed with water to remove the soluble residue. This removed most of the acids and sugars. The C_{18} bound compounds were eluted with 20mL of 100% methanol and acetone. Methanol and acetone isolate was frozen overnight, which removed more polysaccharides (pectin). Then it was filtered and the solvent was evaporated by reduced pressure. Then the solution was dissolved in 25mL of deionized water, plus 125mL of DMSO to make a 2X concentrated solution. The first 75mL was retained as a "stock" solution of carotenoids and polyphenolics. To the remaining 75mL of "stock" extract, hexane was added to partition the carotenoids into the upper phase. This solvent layer was removed and evaporated. Then dry carotenoid residue was dissolved in DMSO and water (16.6% water) equaling the solvent concentration of the "stock" solution. Then the aqueous layer was evaporated by reduced pressure to remove the hexane solvent, filtered and the polyphenolic residue remained in 16.6% water in DMSO. The carotenoid and polyphenolic concentrations were equivalent to the "stock" extraction.

Cell Cycle Kinetics

Treatment design

The MOLT-4 cancer cells were diluted to a concentration of 2.5×10^5 cells/mL and 12 mL of the cell suspension was placed in each $25cm^2$ flask. One flask was used as

a control and treatments were added to individual flasks. Two mL aliquots were removed at 24, 48, and 72 time points from each of the flasks. The aliquots were stored at room temperature in cylindrical labeled tubes until assayed. The specific treatments used were Stock passion fruit juice, unbound, 50% MeOH, 100% MeOH, individual carotenoid, individual flavonoid, and collective carotenoid/polyphenol fractions.

Stock passion fruit juice was added to cells at different concentrations 0% (0 μL), 0.5% (60μL), 1% (120μL), and 2% (240μL). A second experiment used the unbound, 50% MeOH, and 100% MeOH fractions at a concentration of 2% of total volume. A third experiment used individual carotenoid and polyphenolics fractions at a concentration of 2% of total volume. The fourth experiment used collective carotenoid and polyphenolic fraction at a concentration of 2%, 4%, and 8% of total volume.

Cell counts

Cells from each aliquot were counted to compare the number of viable cells for the different concentrations and time. To 20 μL each aliquot, 60 μL of 0.4% trypan blue dye was added in a 1.5 mL microcentrifuge tube for a 1:4 dilution. The solution was vortexed and a 20 μL sample from the middle of the microcentrifuge tube was taken and 10 μL loaded onto each side of the hemocytometer. The number of viable and dead cells was counted in each of five squares on the two sides of the hemocytometer. If the two sides counts were within 10% of each other, the numbers of viable and dead cells were multiplied by 4000 to obtain the concentration of cells/mL.

Flow Cytomtery

Fixation

The cells from the aliquots were washed twice (1050 rpm x 5 min x 24° C) with phosphate buffered saline (PBS) and the pellet resuspended in 500 μL of PBS. Then this cell suspension was vortexed while 500 μL of ice-cold 70% ethanol were added. The ethanol caused the cell membrane to become more permeable for easy incorporation of the stain. The sample was placed on ice for 15 minutes (Braylan et al., 1982; Crissman et al., 1973).

DNA staining

The cells were then washed twice with PBS to remove the ethanol, and 125μL of RNase was added to the cell pellet and vortexed. The RNase solution denatured the RNA in the cancer cells, so that the RNA would not be stained and not interfere with the assay results. This solution was incubated in a 37°C water bath for 15 minutes. Then 125 μL of 0.05 mg/mL Propidium iodide (PI) was added and vortexed. PI is a fluorochrome that stains the DNA by intercalcating into the double helix. The PI stained cells were incubated at room temperature for 30 minutes before the FACScan flow cytometer measured the fluorescence. The WinMDI software was used to process the data from the flow cytometer, a histogram produced (number of cells vs fluorescence) and the area under the peaks calculated. This data was used to calculate the percentage of cells in each of the cell cycle stages.

Statistics

Each experiment was repeated at least three times and the mean percentages of dead cells, cells arrested in the G_0/G_1, G_2, and S_1 were calculated from data obtained

using the flow cytometer and software WinMDI. SigmaStat was used to perform a two-way ANOVA test (Student-Newman Keuls test) to test time and dose effects of passion fruit puree treatments.

Caspase-3 Activity Assay (for Apoptosis)

The treatments included media control, DMSO control, whole passionfruit, carotenoid fraction, polyphenolic fraction and a collective polyphenolic and carotenoid fractions. To make serial dilutions, 50 μL were added to the first well of a 96-well flat-bottom microtiter (tissue culture treated) plate, 50 μL was taken out and passed to the second well, and so on for a total of four dilutions. After the dilutions were made in the plate, then 150 μL of cells (1.3×10^{7} cells/mL) were added, mixed and incubated for four hours. After incubation, 100 μL of these cell cultures were added to a new microtiter plate for the caspase-3 activity assay. The rest of the cells in the original plate were used for the MTT assay. After aliquoting, the cells were centrifuged in the plate at 1400 rpm for 10 minutes at 23°C. The cell medium was removed and the plate was washed two more times with PBS. After the last wash was removed from the plate, the cells were resuspended in Cell Lysis Buffer (10mM HEPES, 2mM EDTA, 0.1% Triton X-100) with 0.1 M PMSF, pepstatin A (10mg/mL), leupeptin (20 mg/mL), aprotinin (10 mg/mL) and 5mM DTT. The plate was put at -85°C overnight. After thawing, 50 μL of Reaction Buffer (100mM HEPES, 20% v/v glycerol, 0.5mM EDTA) with 5mM DTT and 4mM DEVD-pNA substrate was added to the wells of the plate. Then plate was covered with aluminum foil and incubated at 37°C for 3 hours. The absorbance was read with the UV Max at 405-450nm.

MTT (Formazan) Assay (for Cell Viability)

      The original plate was incubated at 37°C for a total of 24 hours. For the assay, 10

μL of 5mg/mL MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)

was added to the wells and incubated for 4 hours at 37°C. Then 100 μL of acid

isopropanol (0.04N HCl in isopropanol 1:300) was added to the wells and using a pipette,

mix the solution to dissolve any dark crystals. The plate was read using the UV Max

Spectrophotometer within one hour of adding the isopropanol at 490-540nm.

CHAPTER 4
RESULTS

To investigate the effects of whole passion fruit juice and various fractions (unbound, MeOH, individual carotenoid, individual polyphenolic, carotenoid/aqueous polyphenolic fractionated passion fruit juice) on MOLT-4 cells, the phases of the cell cycle, cell growth, cell viability, caspase-3 and MTT activity were evaluated.

Effects of Stock Passion Fruit Juice (PFJ) on MOLT-4 Cells

Cell Cycle Analysis

Analysis of G_0/G_1, G_2/M and S phases of stock PFJ

The purpose of stock passion fruit juice treatment was to investigate a dose and time response for the administration of whole passion fruit juice for 24, 48 and 72 hours onto MOLT-4 cells. Figure 1 shows the percentages of cells in the cell cycle phases with passion fruit juice after 24, 48, and 72 hours. After treatment with various doses (0, 0.25, 0.5, 1, and 2%) at 48 hours, a significant accumulation of cells in the G_1 phase at 1% (~3.5% increase; p=0.037) and 2% (~5.2% increase; p=0.033) and a significant decrease (~4.7%) in the G_2 phase (p=0.036) at 2% were shown. However, no statistical significance was reported for any of the other fraction groups (p>0.05). When comparing the percentage of cells at the three time points, there was a no significant difference seen among the treatments, except for 2% PF which was significantly different among 24, 48 and 72 hours (p<0.019). This data demonstrated a dose dependent G_1 phase arrest for 2% PF at 48 hours as the G_1 phase increased and G_2/M phase decreased. The MOLT-4 cells

had a time dependency, as 48 hours showed a significant effect in the cell cycle however after 72 hours the cells have recovered from the passion fruit juice.

Analysis of subploidy phase of stock passion fruit juice

Figure 2 shows the percent of cells in the subploidy or sub-G_0/G_1 phase after passion fruit juice was administered for 24, 48 and 72 hours. At 24 hours, there was no significant difference among the various concentrations ($p > 0.05$). At 48 hours, there was significant increased difference at 1% (~ 12.2) and 2% (~ 50.4) concentrations ($p < 0.05$). At 72 hours, there was a significant accumulation at 2% (~ 81.8) passion fruit juice ($p < 0.001$). This data showed that at 2% passion fruit juice after 48 and 72 hours, a significant number of cells that have been degraded in the sub-G_0/G_1 phase in a dose dependent manner. This was indicative of DNA degradation and cell death.

Cell Growth and Viability Analysis of Stock Passion Fruit Juice

Cell growth and viability were measured to assess the effect of stock passion fruit juice on the proliferation of MOLT-4 cells (Figure 3 & 4). When passion fruit juice was administered at 2%, hours there was a significant $\sim 15.7\%$ decrease in viability ($p < 0.001$) after 48 hours. At the same concentration, after 72 hours there was both a significant $\sim 55.3\%$ decrease in viability ($p < 0.001$) and a 9 fold reduction in the number of cells ($p < 0.001$) as compared to the control ($p = 0.001$). Results of all other treatment doses were not significant ($p > 0.05$). At 48 hours, passion fruit juice has the capacity to induce cell death but only after 72 hours does passion fruit juice inhibit cell growth.

Effects of Unbound and Methanol Fractions

Cell Cycle Analysis

Analysis of G_0/G_1, G_2/M and S phases of unbound and methanol fractions

The purpose of evaluating the effects of unbound and 50% and 100% methanol fractions on the cell cycle was to established if this partition was adequate in investigating which compounds were responsible for the cell cycle arrest. The unbound fraction contained very polar, small molecules such as sugars, organic acids and amino acids. The 50% methanol fraction included polar compounds and small molecular polyphenolics. The 100% methanol fractions consist of the non-polar compounds, large molecular weight polyphenolics and carotenoids. Since a significant difference was observed at 2% stock passion fruit juice (Fig. 1), this was the concentration used for these fractions.

The effect of unbound, 50% methanol and 100% methanol fractions on the cell cycle of MOLT-4 cells is illustrated in Figure 5. After treatment with the DMSO control, 50% and 100% MeOH after 24 hours, there was no statistical significance (p>0.05) for any of the cell cycle phases. In contrast, after 48 hours, the unbound fraction had a significant accumulation (~4%) of cells in the G_1 phase (p=0.023) and a significant decrease (~5.4) in the G_2 phase (p=0.02). Results of the S phase were not statistically significant (p>0.05). Results of the other doses were not significant (p=0.662). After 72 hours, the unbound fraction had shown statistical significance with a decrease in G_1, G_2, and S phases (p<0.05). When the percentage of cells between the three time points were compared, there was a significant difference (p<0.05) after 72 hours with the unbound and unbound/bound fractions, but not after 24 or 48 hours (p>0.05). When the unbound fraction of passion fruit juice was administered, there was a significant reduction in the

percentage of cells at 48 and 72 hours (Figure 5) compared to the control. This indicates that after 48 hours, the unbound fraction was effective in arresting the cell cycle at 2% concentration.

Analysis of subploidy phase of unbound and methanol (MeOH) fractions

Figure 6 shows the percent of cells in the subploidy region after unbound, 50% MeOH, and 100% MeOH fractions were administered for 24, 48 and 72 hours. At 24 hours, there was significance for unbound fraction ($p<0.001$). At 48 hours, there was significance at unbound fraction and DMSO control ($p<0.05$). After 72 hours, there was a significant difference from the control in the unbound fraction ($p<0.001$). This graph suggests that after 48 and 72 hours, the unbound fractions with the organic acids, amino acids and proteins show that there are a significant amount of cells in the sub-G_0/G_1 phase, which is indicative of DNA degradation and cell death.

Cell Growth and Viability Analysis of Unbound and Methanol (MeOH) Fractions

Cell growth and viability was measured to assess the effect of unbound/methanol fractions on the proliferation of MOLT-4 cells (Figure 7). When passion fruit juice was administered with the unbound fraction, there was a significant reduction in the number of viable cells at 48 (71.2% reduction) and 72 hours (25.4% reduction) as compared to the control ($p<0.001$). Also in 72 hours there was a significant increase in number of cells for the 50% MeOH fraction (1.5 fold; $p<0.001$). All other dose treatments were not significant ($p>0.05$).

Effect of Individual Carotenoid and Aqueous Polyphenolic Fraction

Cell Cycle Analysis

Analysis of G_0/G_1, G_2/M and S phases of carotenoids and aqueous polyphenol

The effect of aqueous carotenoid and polyphenolic fractions on the cell cycle of MOLT-4 cells is illustrated in Figure 8. After adding carotenoid and aqueous polyphenolic fractions at 2%, the percentages of cells in the phases were compared to the control group. After 24, 48 or 72 hours, there was no statistical difference seen in any of the phases (p>0.05). Individual carotenoid and aqueous polyphenolic fractions had no effect on the leukemia cell cycle at 2% of total volume.

Analysis of subploidy phase of carotenoid and aqueous polyphenol

Figure 9 shows the percent of cells in the subploidy region after carotenoid and aqueous polyphenolic fractions were administered for 24, 48 and 72 hours. The results showed that at 24, 48, and 72 hours, there was no significant difference among any of the treatments (P>0.05). The carotenoid and aqueous polyphenolic fractions had no effect on the subploidy phase.

Cell Growth and Viability Analysis of Carotenoid and Aqueous Polyphenolic

Cell growth and viability was measured to assess the effect of carotenoid and aqueous polyphenolic passion fruit juice fractions on the proliferation of MOLT-4 cells (Figure 10 & 11). When passion fruit juice carotenoid and aqueous polyphenolic fractions were administered, there was a significant 1.3 fold increase in cell growth for the aqueous polyphenolic fraction at 72 hours (p=0.001). All other fractions for the cell growth and viability at 24, 48 and 72 hours as compared to the control were not significantly different (p>0.05).

Effect of Collective Carotenoid/Aqueous Polyphenolic Fraction

Cell Cycle Analysis

Analysis of G_0/G_1, G_2/M and S phases of carotenoid/aq. polyphenolic fraction

The effect of collective carotenoid/polyphenolic passion fruit juice fraction on the cell cycle of MOLT-4 Cells is illustrated in Figure 12. The purpose of using a fraction that has both the carotenoid/polyphenolic passion fruit juice components together was to evaluate its effect of the fractionated fruit on MOLT-4 cells after 24, 48, and 72 hours. The treatments were the control, DMSO/EtOH, 2%, 4%, and 8% PF. After 24 hours, the DMSO/EtOH (Control), 4%, 8% treatments showed a significant statistical increase in the G_0/G_1 phase as compared to the control (DMSO/EtOH & 8%=1.4 fold increase; 4%=1.2 fold increase; $p \leq 0.001$). However, for the G_2/M phase DMSO/EtOH (1.6 fold) and 4% (1.7 fold) showed a significant decrease and 8% showed a significant 1.7 fold accumulation of cells as compared to the control ($p<0.05$). In the S phase, there was a significant reduction in the number of cells for the DMSO/EtOH (control) and 8% fractionated PFJ samples (DMSO/EtOH= 1. fold, and 8%= 5 fold decrease; $p<0.05$). In contrast, after 48 hours, for the DMSO/EtOH (control) and 8% PF treatments had a significant accumulation of cells in G_0/G_1 phase (DMSO/EtOH= 1. fold and 8%=1.3 fold increase; $p=0.007$). The DMSO/EtOH control showed a significant 2 fold decrease in the percentage of cells in the G_2/M phase ($p<0.001$). The fraction at 8% concentration had a significant 1.6 fold decrease in the S phase ($p=0.002$). All other doses did not show significance ($p>0.05$). After 72 hours, the concentrations were not significant; except for the 8% concentration because there was not enough cells alive to be analyzed ($p>0.05$). The collective carotenoid/aqueous polyphenolic fraction was effective in inducing a cell cycle arrest at a concentration of 4% and 8% at 24 hours. By 48 hours the cells from the

4% concentration have recovered, but the 8% was still showed a G_0/G_1 and G_2/M phase arrest. However the DMSO/EtOH solvent could have induced the effects and not the phytochemicals, due to the significant differences from the control.

Analysis of subploidy phase of carotenoid/aqueous polyphenolic fraction

Figure 13 shows the percent of cells in the subploidy region after carotenoid/aqueous polyphenolic fraction was administered for 24, 48 and 72 hours. The treatments used were the control, DMSO, 2%, 4%, 8% fractionated PFJ. At 24, 48, and 72 hours, there was significance at 8% passion fruit juice ($p < 0.05$). This graph suggests that after 48 and 72 hours, at 8% concentration showed that a significant amount of cells have been degraded, indicating cell death.

Cell Growth and Viability Analysis of Carotenoid/Polyphenolic Fraction

Cell growth and viability were measured to assess the effect of a mixture of collective carotenoid/polyphenolic passion fruit juice fraction on the proliferation of MOLT-4 cells (Figure 14 & 15). When PFJ carotenoid/aqueous polyphenolic fraction was administered at 8% concentrations, there was a significant ~13.4 reduction in the number of cells after 48 hours of incubation as compared to the control ($p < 0.001$). For the DMSO/EtOH, 2%, 4%, and 8% concentrations, after 72 hours there were significant differences in the number of cells as compared to the controls (DMSO/EtOH= 1.92 fold, 2%= 1 fold, 4%= 1.9 fold, and 8%=66.6 fold decrease; $p < 0.05$). For the cell viability, after 24 hours, there was a significant decrease in the percentage of viable cells for the 8% (3.4 fold) concentration ($p < 0.001$). After 48 hours, at the 8% concentration, there was a significant 5.2 fold decrease from the control ($p < 0.001$). After 72 hours, there was a significant decrease in the viability for the DMSO/EtOH (1.2 fold) and the 8% (10.4 fold) concentration ($p < 0.001$). All other treatments did not show any significance as

compared the control at any time point (p>0.05). At the 8% concentration, for 48 and 72 hours, the carotenoid/polyphenolic may be effective in decreasing the number of live cells and inducing cell death, however according to the data, the 4% concentration is capable of only inhibiting cell growth.

Effect of Passion Fruit Juice on Caspase-3 Activity

The effect of passion fruit juice fractions on caspase-3 activity of MOLT-4 cells is illustrated in Figure 16. The purpose of the caspase-3 assay was to establish whether passion fruit juice extracts affect the expression of caspase-3 thereby inducing apoptosis. Four hours after various treatments were administered (whole passion fruit juice, DMSO, carotenoid, aqueous polyphenolic, and carotenoid + aqueous polyphenolic fractions) there was a significant increase in caspase-3 activity among the fractions. Especially at the 6.25 μL dosage, a peak of caspase-3 activity was seen, indicating an increase in apoptosis due to the addition of passion fruit juice. Among the treatments at the 6.25 μL dosage, the DMSO, carotenoid, aqueous polyphenolic, and carotenoid + aqueous polyphenolic were significantly different from the control (p<0.001), except whole passion fruit juice (p=0.133). This data shows that DMSO, carotenoid, aqueous polyphenolic, and carotenoid + polyphenolic have the capacity to induce caspase-3 activity thereby inducing apoptosis.

Effect of Passion Fruit on Juice MTT Activity

The effect of passion fruit juice fractions on cell viability of MOLT-4 cells is illustrated in Figure 14. The purpose of the MTT assay was to establish whether passion fruit juice extracts affect the viability of cancer cells. Various treatments were administered (whole passion fruit juice, DMSO (control) carotenoid, polyphenolic, and

mixture of carotenoid/polyphenolic fractions). After 24 hours there was a dramatic

decrease in absorbance in all treatment groups, reflecting a decrease in the cell viability

or percentage of live cells. Specifically a 25μL dosage resulted in a three-fold decrease

in the amount of live cells in all treatments as compared to the control ($p>0.05$). This

shows that as more passion fruit juice fraction is added, there is a decrease in cell

viability. At 6.25 μL dosage, when caspase-3 activity has peaked, there is a significant

decrease in the cell viability for the carotenoid, polyphenolic, and collective

carotenoid/polyphenolic fractions, compared to the control ($p<0.05$). This indicates the

correlation between induction of the caspase-3, or apoptosis and the decrease in cell
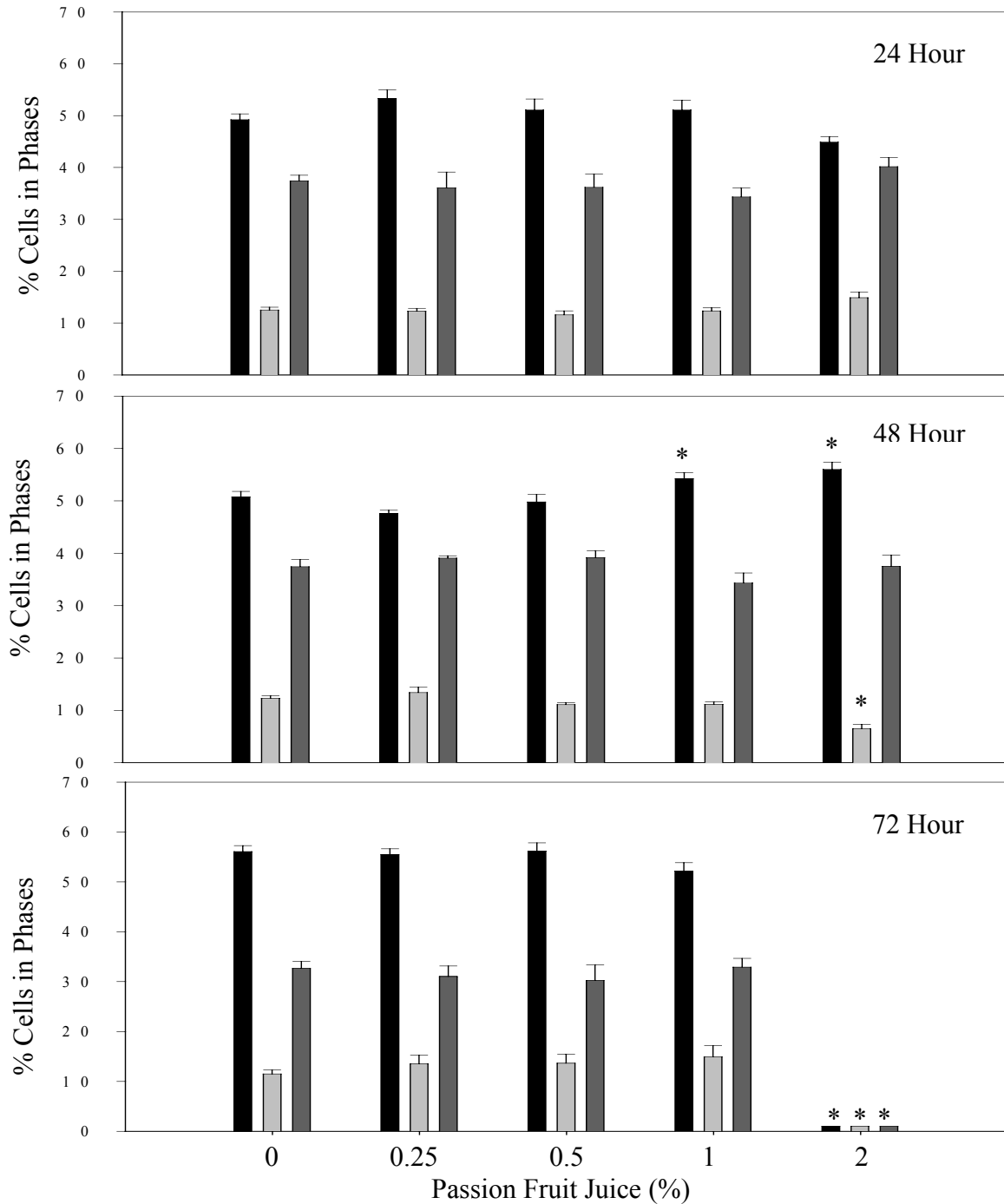
viability.

Figure 1. Cell cycle analysis of passion fruit juice treated MOLT-4 cells. Passion fruit juice was added as a percent of total volume of cells in μL. 0%= 0 μL (0 mg/L carotenoid; 0 mg/L aqueous polyphenolic), 0.25%= 30 μL (23.12 mg/L carotenoid; 1087.5 mg/L aqueous polyphenolic) 0.5%= 60 μL (46.25 mg/L carotenoid; 2175.0 mg/L aqueous polyphenolic) 1%= 120 μL (92.5 mg/L carotenoid; 4350.0 mg/L aqueous polyphenolic) 2%= 240 μL (185 mg/L carotenoid 8700.0 mg/L aqueous polyphenolic) Significant difference from control (p<0.05) designated by *. ■:G1 Phase, ▨:G2 Phase, ▥:S Phase
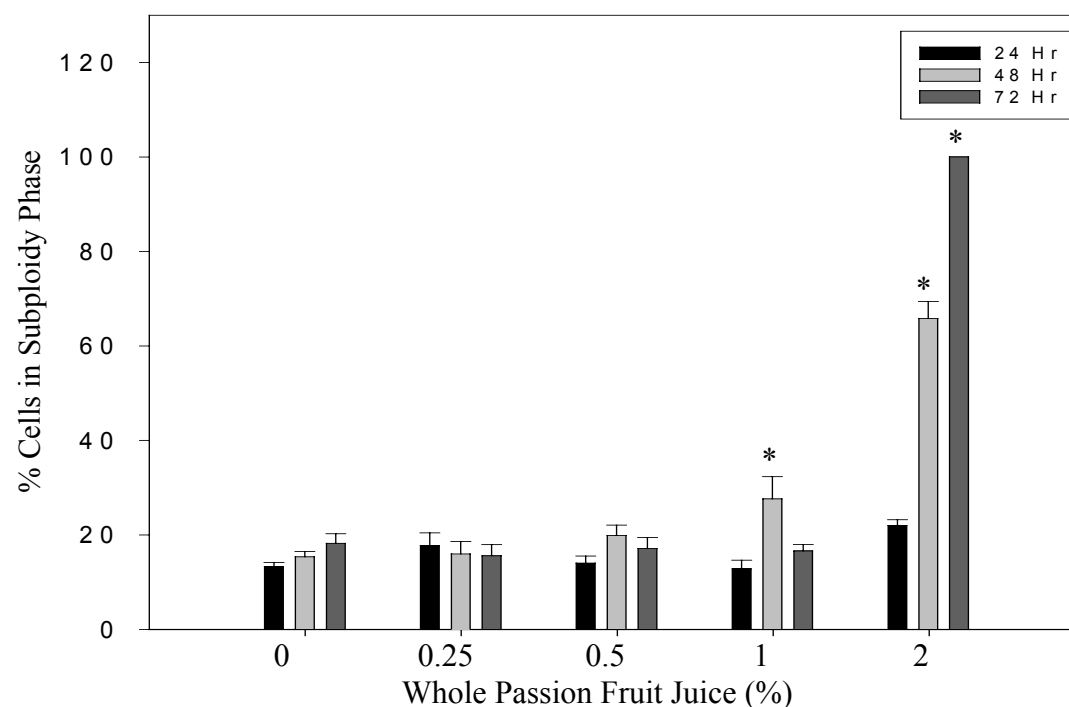
Figure 2. Percentage of cells in subploidy phase of passion fruit juice treated MOLT-4 cells. Whole passion fruit juice (9.25 mg/L carotenoid; 435 mg/L aqueous polyphenolic) was added as a percent of total volume of cells in μL. 0%= 0 μL (0 mg/L carotenoid; 0 mg/L aqueous polyphenolic), 0.25%= 30 μL (23.12 mg/L carotenoid; 1087.5 mg/L aqueous polyphenolic) 0.5%= 60 μL (46.25 mg/L carotenoid; 2175.0 mg/L aqueous polyphenolic) 1%= 120 μL (92.5 mg/L carotenoid; 4350.0 mg/L aqueous polyphenolic) 2%= 240 μL (185 mg/L carotenoid 8700.0 mg/L aqueous polyphenolic) Significant difference from control ($p<0.05$) designated by *.
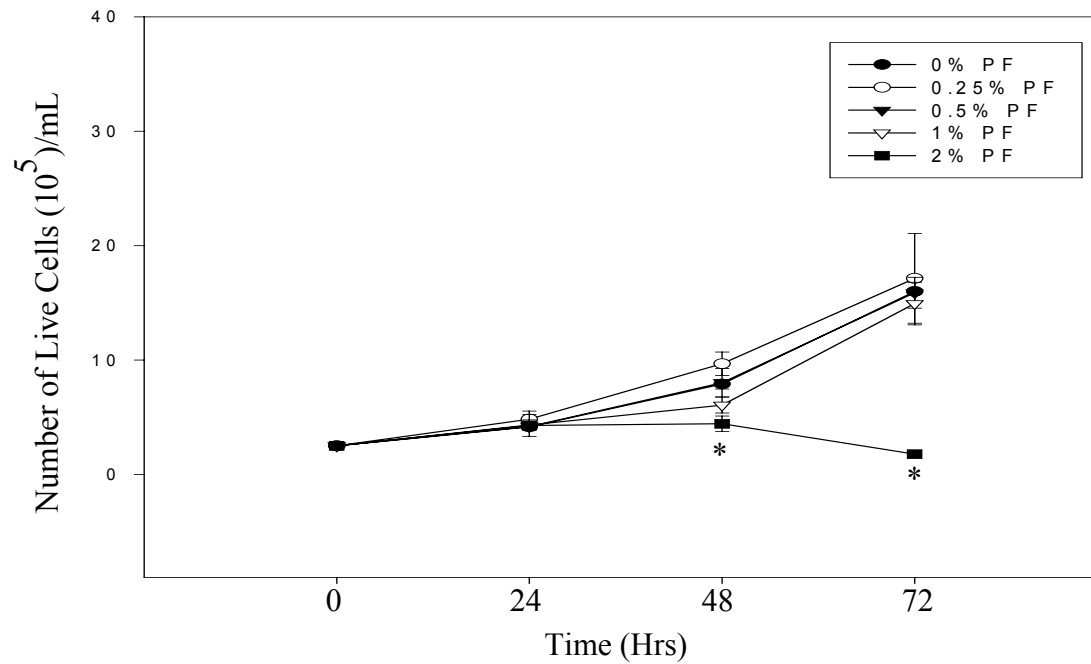
Figure 3. Cell growth (10^5 cells/mL) of whole passion fruit juice treated MOLT-4 cells over time. Passion fruit was added as a percent of total volume of cells in μL. 0%= 0 μL= 0 mg/L carotenoid & aqueous polyphenolic, 0.25%= 30 μL= 23.12 mg/L carotenoid & 1087.5 mg/L aqueous polyphenolic, 0.5%= 60 μL= 46.25 mg/L carotenoid & 2175.0 mg/L aqueous polyphenolic, 1%= 120 μL= 92.5 mg/L carotenoid & 4350.0 mg/L aqueous polyphenolic, 2%= 240 μL= 185 mg/L carotenoid= 8700.0 mg/L Significant difference from control (p<0.05) designated by *.
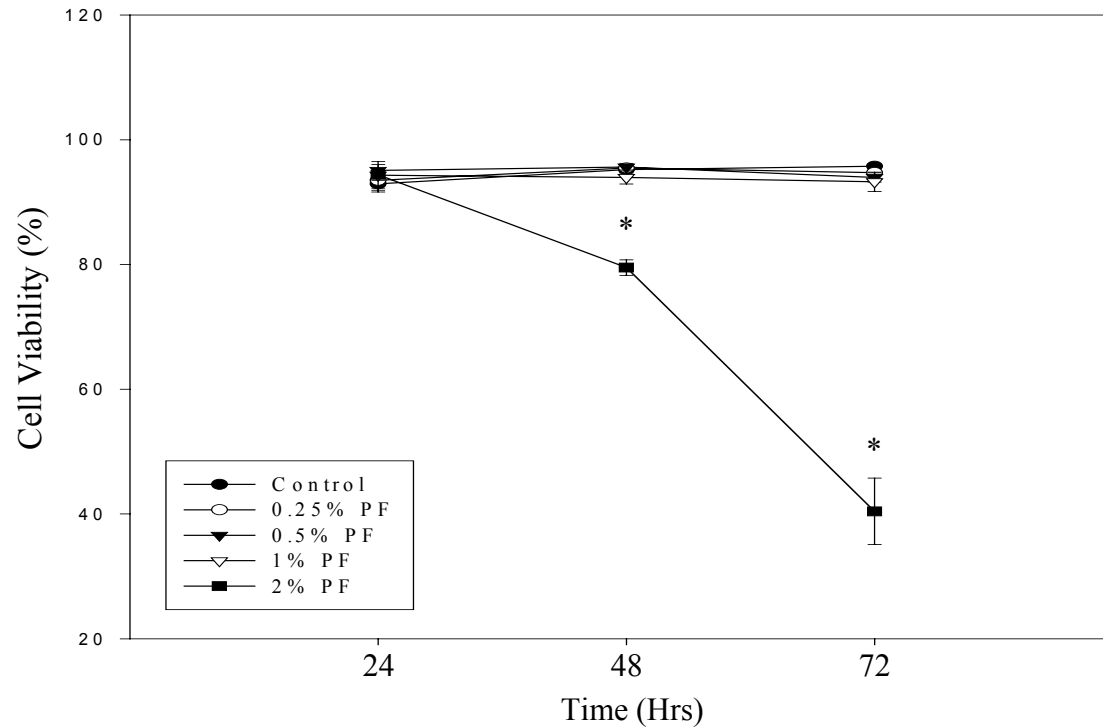
Figure 4. Cell Viability (% live cells /total cells) of passion fruit treated MOLT-4 cells over time. Passion fruit juice was added as a percent of total volume of cells in μL. 0%= 0 μL= 0 mg/L carotenoid & aqueous polyphenolic, 0.25%= 30 μL= 23.12 mg/L carotenoid & 1087.5 mg/L aqueous polyphenolic, 0.5%= 60 μL= 46.25 mg/L carotenoid & 2175.0 mg/L aqueous polyphenolic, 1%= 120 μL= 92.5 mg/L carotenoid & 4350.0 mg/L aqueous polyphenolic, 2%= 240 μL= 185 mg/L carotenoid= 8700.0 mg/L Significant difference from the control ($p < 0.05$) designated by *.
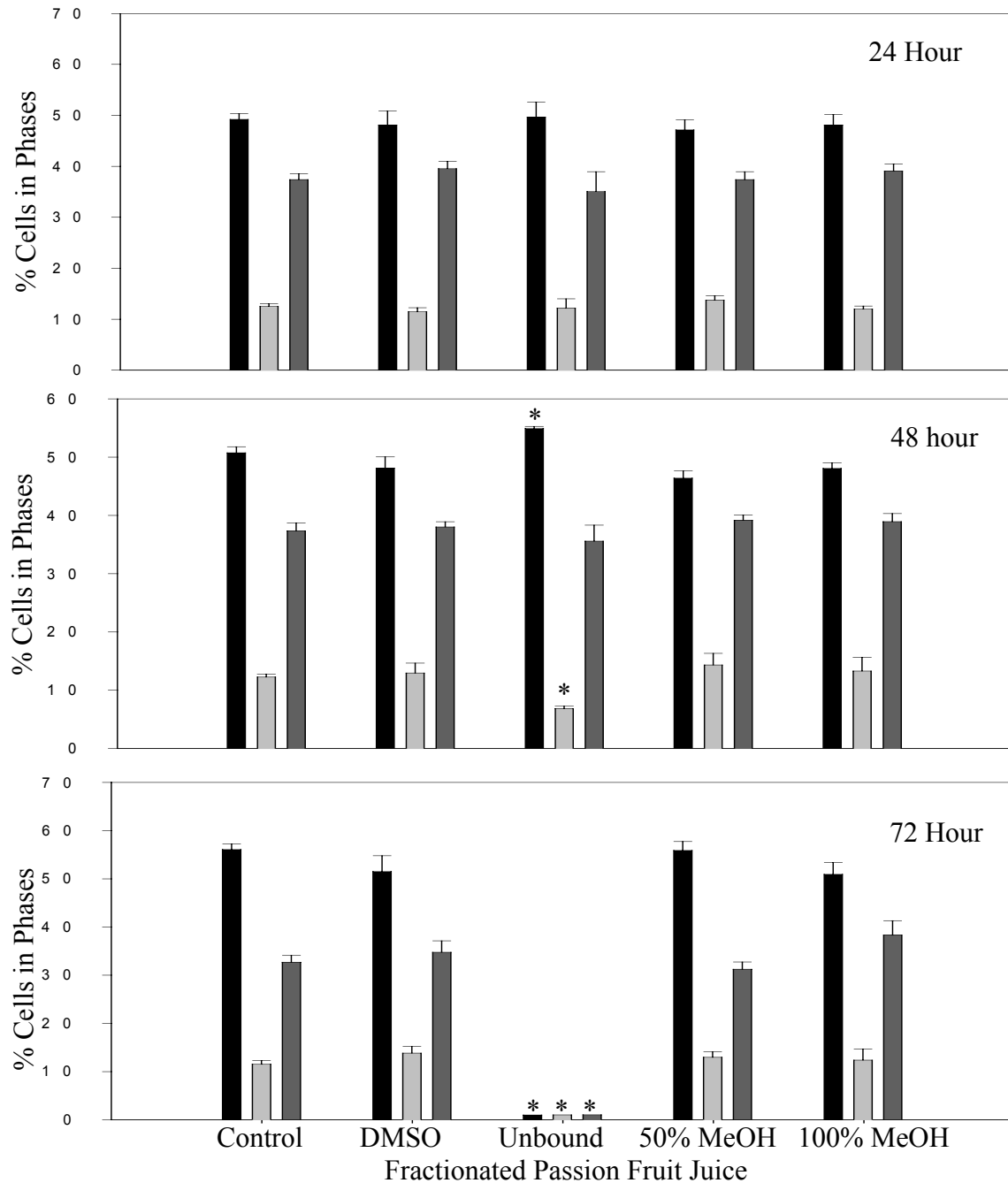
Figure 5. Cell cycle analysis of unbound and bound fraction juice treated MOLT-4 cells. The unbound fraction consisted of compounds not bound to the C18 column. The 50% MeOH fraction were compounds from C18 column eluted with 1:1 ratio of methanol: deionized water and 100% MeOH fraction were compounds eluted from the C18 column with 1:0 ratio of methanol: deionized water. DMSO was a control for the 100% MeOH fraction only. Fractions were equivalent to 2% of stock PFJ. Significant difference from the control (p<0.05) designated by *.. ▉ :G1 Phase, ▉ :G2 Phase, ■ :S Phase
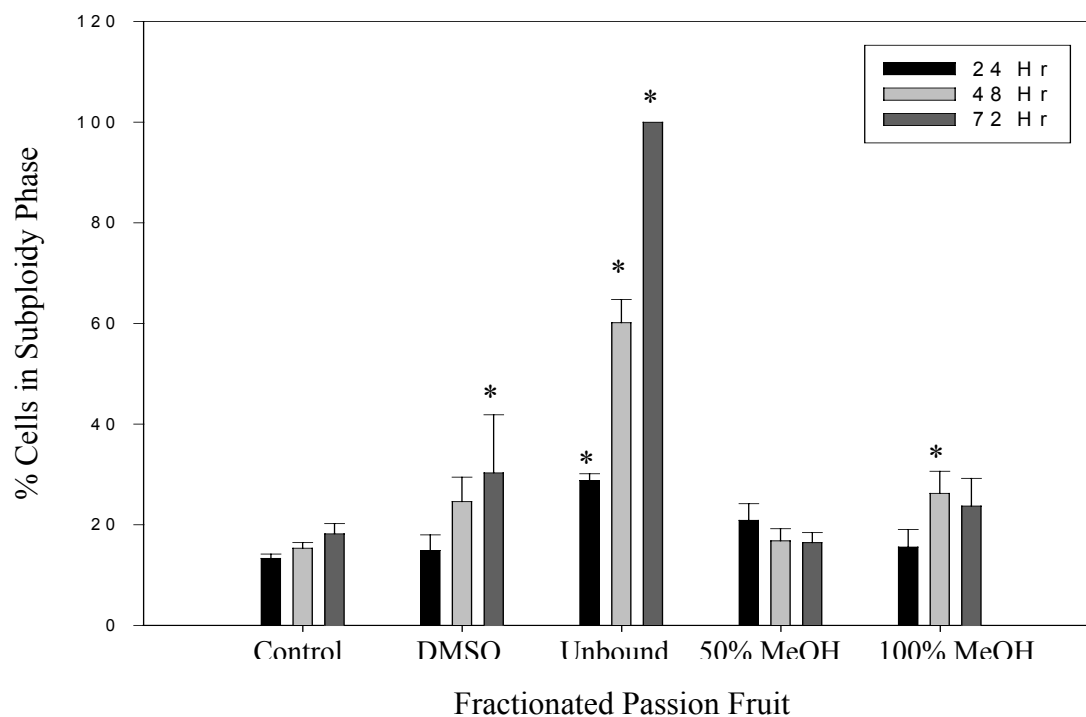
Figure 6. Percentage of cells in subploidy region of unbound and methanol fractionated passion fruit juice treated MOLT-4 cells. The unbound fraction consisted of compounds not bound to the C_{18} column. The 50% MeOH fraction were compounds from C_{18} column eluted with 1:1 ratio of methanol: deionized water and 100% MeOH fraction were compounds eluted from the C_{18} column with 1:0 ratio of methanol: deionized water. DMSO was a control for the 100% MeOH fraction only. Fractions added as 2% concentration, equivalent to 2% of stock PFJ. Significant difference from the control (p<0.05) designated by *.
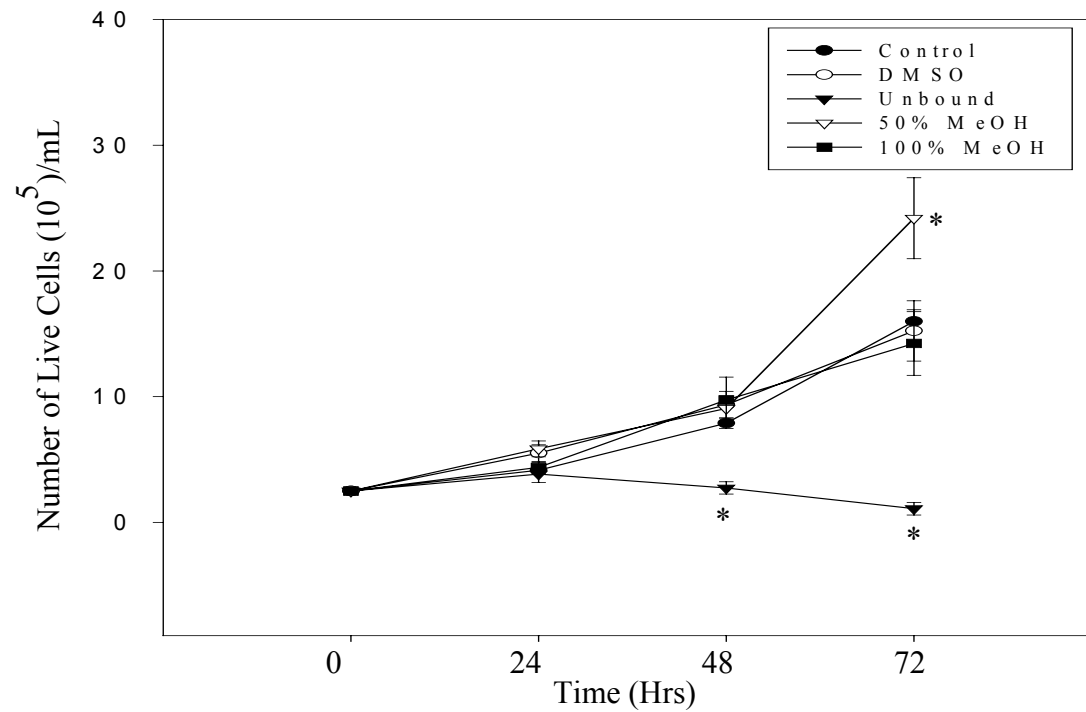
Figure 7. Cell Growth (10^5 cells/mL) of passion fruit juice treated MOLT-4 cells over time. The unbound fraction contained compounds not bound to the C_{18} column. The 50% MeOH fraction were compounds from C_{18} column eluted with 1:1 ratio of methanol: deionized water and 100% MeOH fraction were compounds eluted from the C_{18} column with 1:0 ratio of methanol: deionized water. DMSO was a control for the 100% MeOH only. Fractions added as 2% concentration, equivalent to 2% of stock PFJ. Significant difference from the control ($p<0.05$) designated by *.
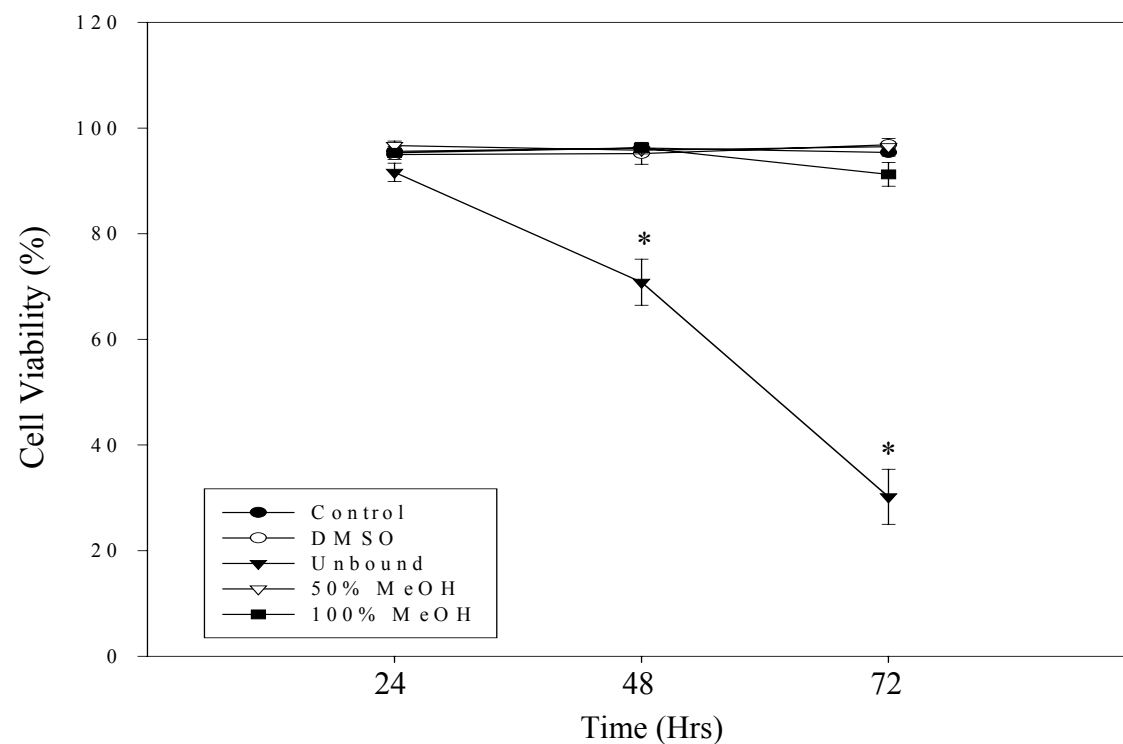
Figure 8. Cell Viability (% live cells /total cells) of passion fruit juice treated MOLT-4 cells over time. The unbound fraction contained compounds not bound to the C_{18} column. The 50% MeOH fraction were compounds from C_{18} column eluted with 1:1 ratio of methanol: deionized water and 100% MeOH fraction were compounds eluted from the C_{18} column with 1:0 ratio of methanol: deionized water. DMSO was control for 100% MeOH fraction only. Fractions added as 2% concentration, equivalent to 2% of stock PFJ. Significant difference from the control ($p<0.05$) designated by *.

Figure 9. Cell cycle analysis of passion fruit juice treated MOLT-4 cells with carotenoid (0.185 mg/mL) and aqueous polyphenolic (8.7 mg/mL) fractions. Fractionated passion fruit juice was added as a 2% percent of total volume of cells in µL. DMSO was the control for carotenoid only. Fractions added as 2% concentration, equivalent to 2% of stock PFJ.Significant difference from control (p<0.05) designated by *. ■ :G$_1$ Phase, ▨ :G$_2$ Phase, ▨ :S Phase
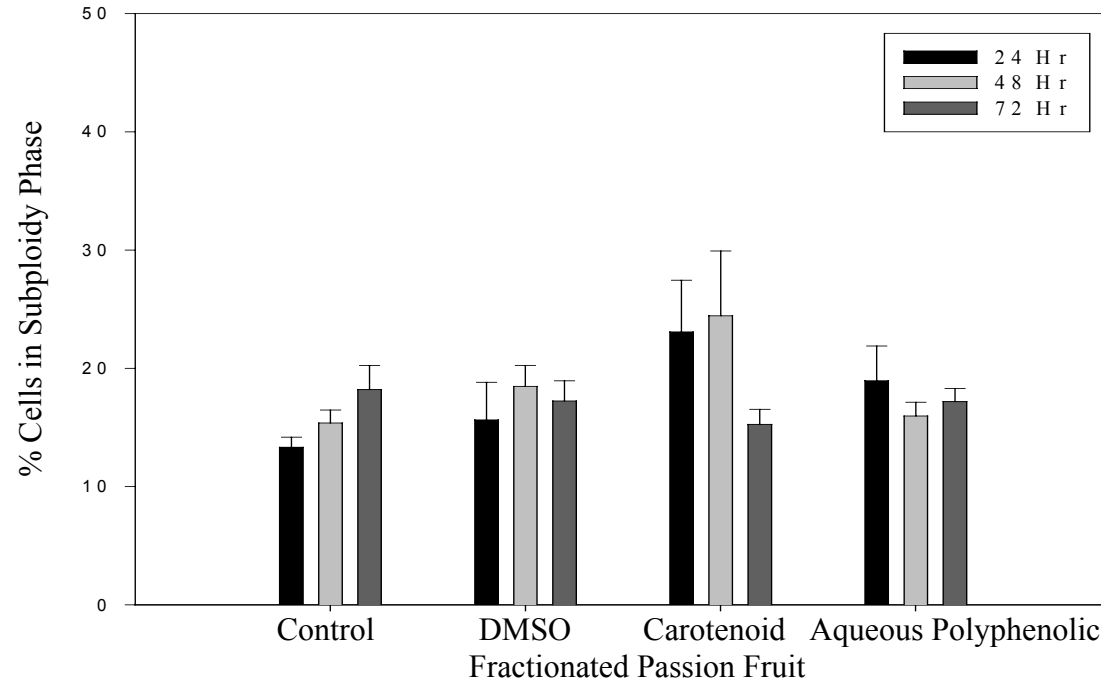
Figure 10. Percentage of cells in subploidy phase of carotenoid and aqueous polyphenolic fractionated passion fruit juice treated MOLT-4 cells. Carotenoid (0.185 mg/mL) and aqueous polyphenolic (8.7 mg/mL) fractionated passion fruit juice was added at a 2% percent of total volume of cells in μL. DMSO was the control for carotenoid fraction only. Fractions added as 2% concentration, equivalent to 2% of stock PFJ. Significant difference from control (p<0.05) designated by *.
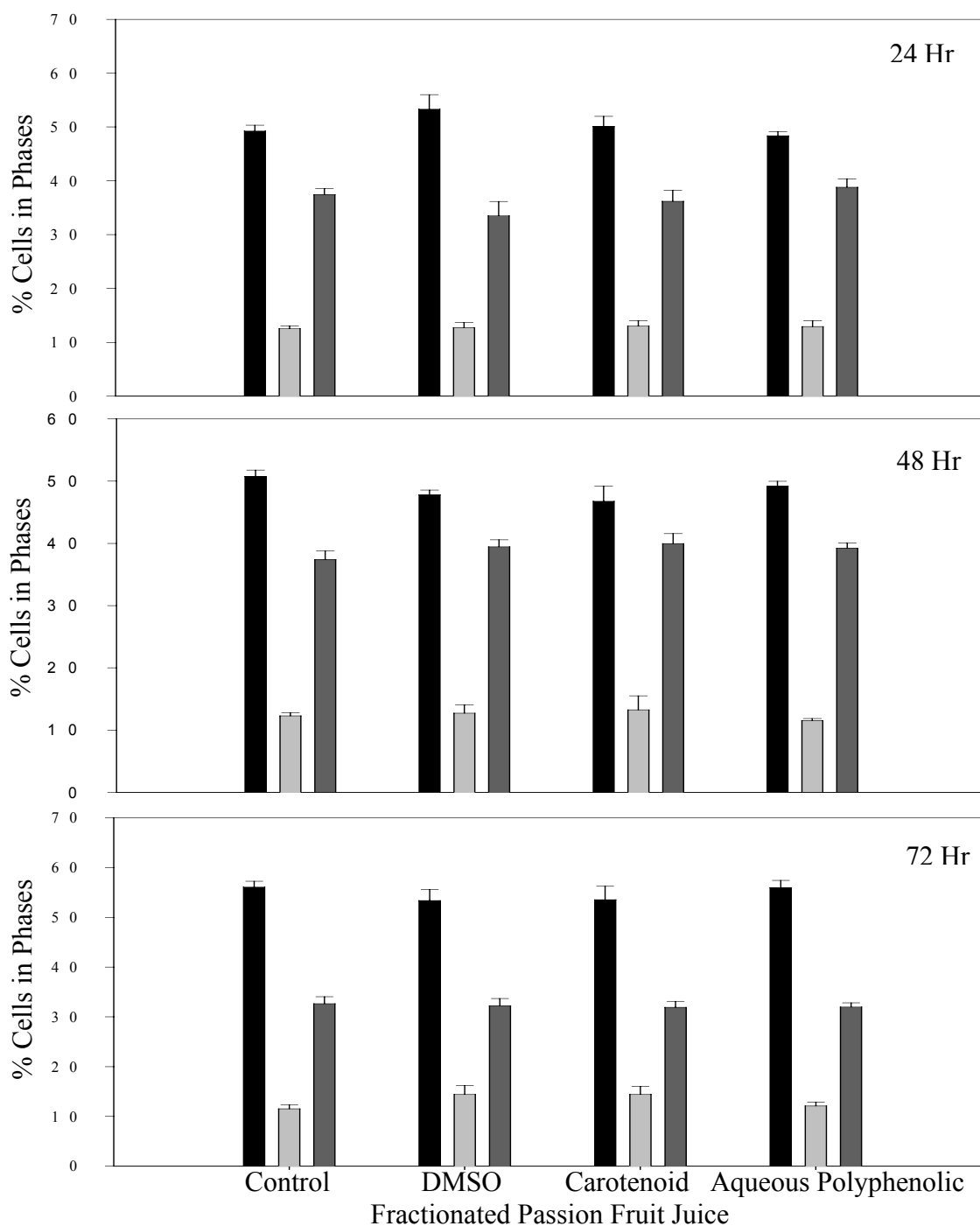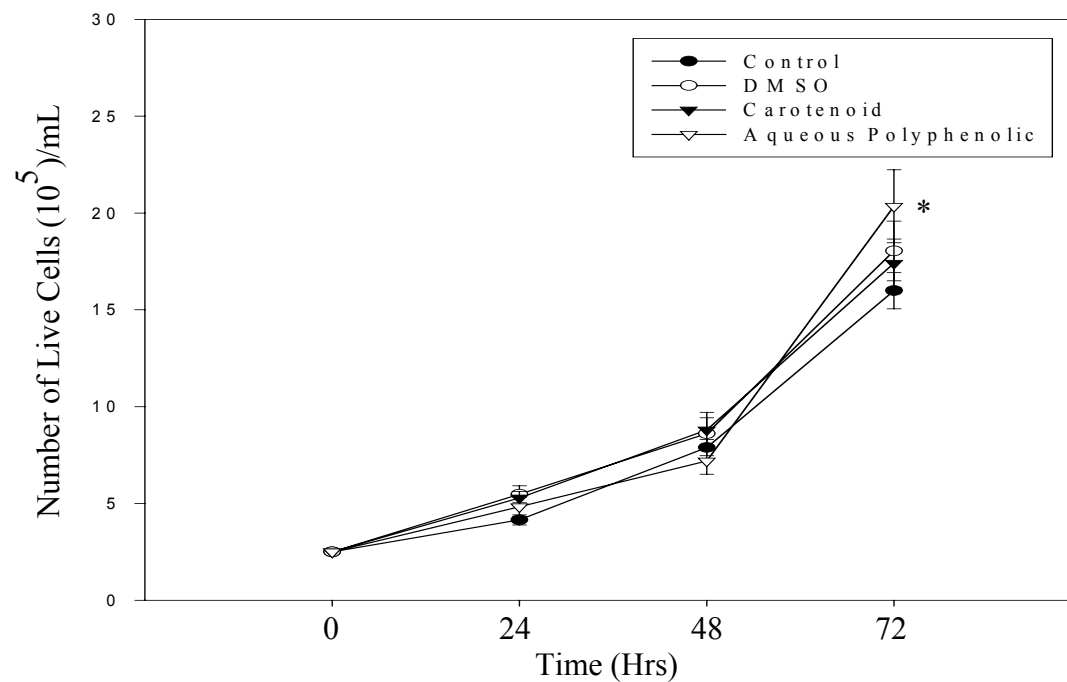
Figure 11. Cell Growth (10^5 cells/mL) of passion fruit juice treated MOLT-4 cells over time. Carotenoid (0.185 mg/L) and aqueous polyphenolic (8.7 mg/L) fractionated passion fruit juice was added at a 2% percent of total volume of cells in µL. DMSO was the control for carotenoid fraction only. Fractions added as 2% concentration, equivalent to 2% of stock PFJ. Significant difference from control (p<0.05) designated by *.

Figure 12. Cell Viability (% live cells/ total # cells) of carotenoid and polyphenolic fractionated passion fruit juice (PFJ) treated MOLT-4 cells over time. Carotenoid (0.185 mg/mL) and aqueous polyphenolic (8.7 mg/mL) fractionated PFJ was added at a 2% percent of total volume of cells in μL. DMSO was the control for carotenoid fraction only. Fractions added as 2% concentration, equivalent to 2% of stock PFJ. Significant difference from control ($p<0.05$) designated by *.
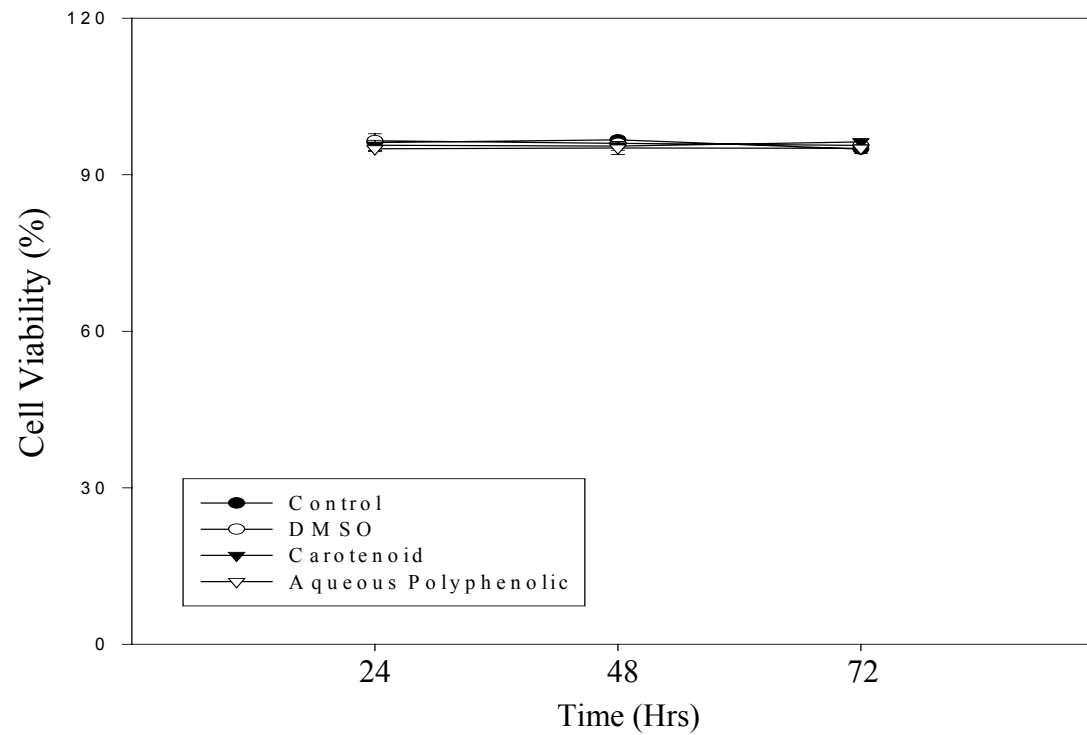
Figure 13. Cell cycle analysis of passion fruit juice treated MOLT-4 cells with 2%, 4%, 8% partitioned carotenoid/polyphenolics PFJ. ■: G_1 Phase, ▨: G_2 Phase, ▨: S Phase 2%= 240 μL (185 mg/L carotenoid/ 8700.0 mg/L aqueous polyphenolic) 4%= 480 μL (370 mg/L carotenoid/ 17400.0 mg/L aqueous polyphenolic) 8%= 960 μL (740 mg/L carotenoid/ 34800.0 mg/L aqueous polyphenolic) DMSO/EtOH was the control for the 2-8% PF samples. Fractions added as 2% concentration, equivalent to 2% of stock PFJ. Significant difference from control ($p<0.05$) designated by *.

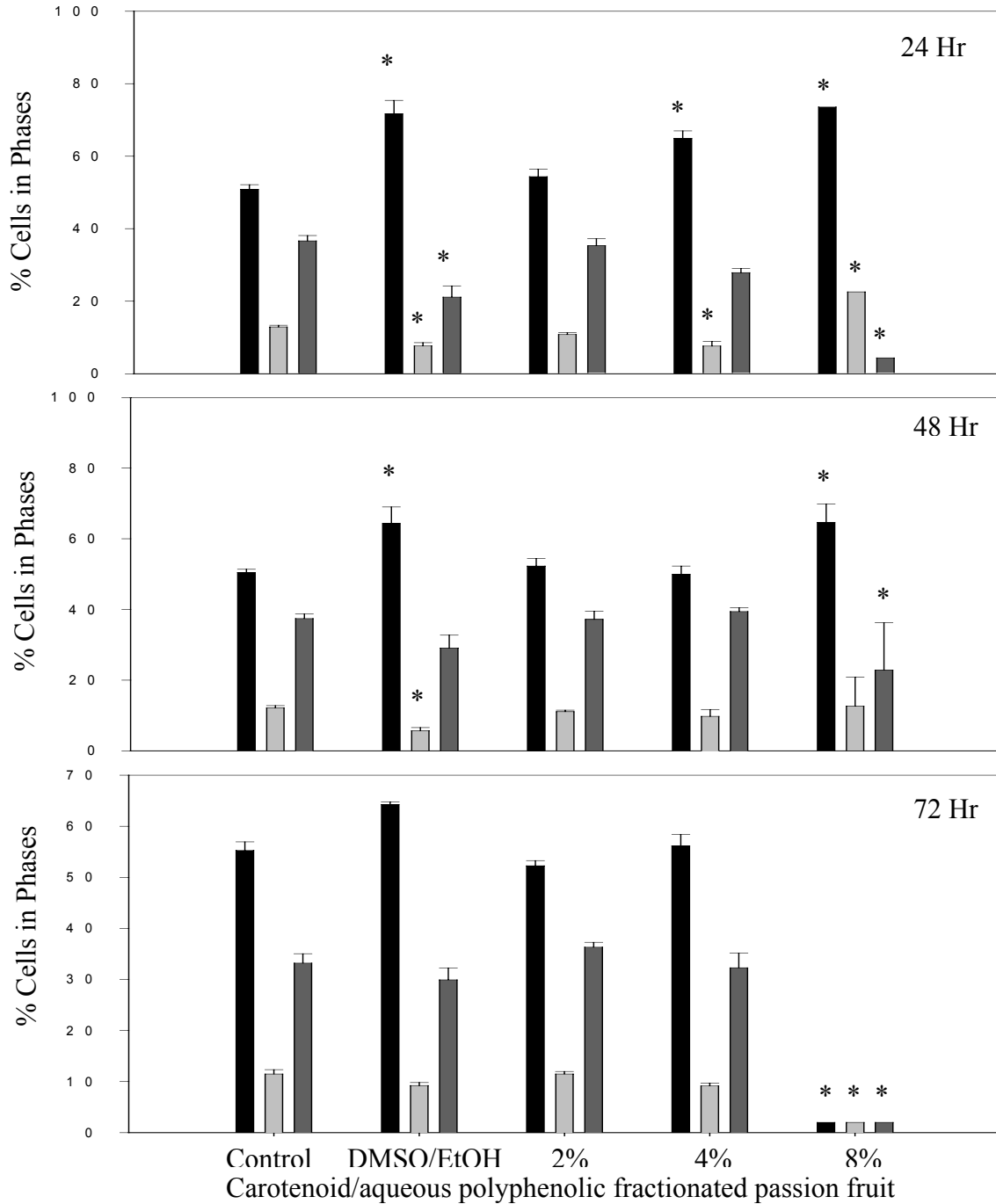Figure 14. Percentage of cells in subploidy region of carotenoid/aqueous polyphenolic fractionated passion fruit juice treated MOLT-4 cells. Carotenoid/aqueous polyphenolic fractionated passion fruit juice was added at a percent of total volume of cells in μL. 2%= 240 μL (185 mg/L carotenoid/ 8700.0 mg/L aqueous polyphenolic) 4%= 480 μL (370 mg/L carotenoid/ 17400.0 mg/L aqueous polyphenolic) 8%= 960 μL (740 mg/L carotenoid/ 34800.0 mg/L aqueous polyphenolic) DMSO/EtOH was the control for the 2-8% fraction. Significant difference from control (p<0.05) designated by *.
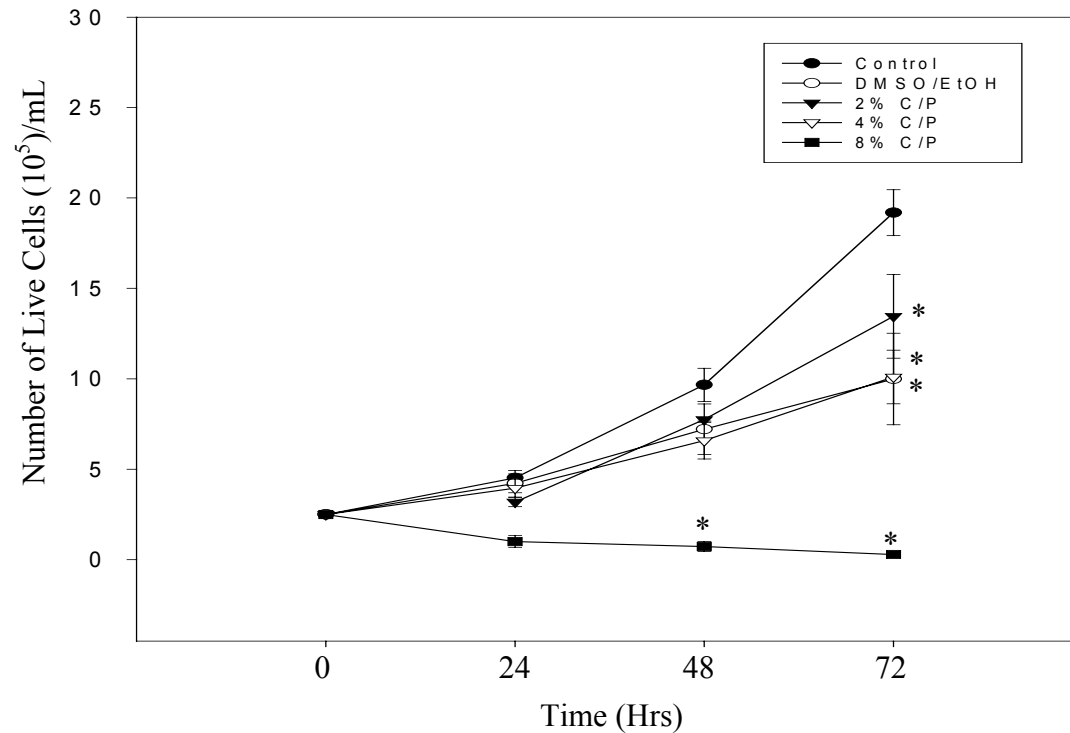
Figure 15. Cell Growth (10^5 cells/mL) of fractionated passion fruit juice (PFJ) treated MOLT-4 cells over time. PFJ fractionated carotenoid/aqueous polyphenolic was administered as percent of total volume of cell culture in μL. 2%= 240 μL (185 mg/L carotenoid/ 8700.0 mg/L aqueous polyphenolic) 4%= 480 μL (370 mg/L carotenoid/ 17400.0 mg/L aqueous polyphenolic) 8%= 960 μL (740 mg/L carotenoid/ 34800.0 mg/L aqueous polyphenolic) DMSO/EtOH was the control for 2-8% PFJ samples. Fractions added as 2% concentration, equivalent to 2% of stock PFJ. Significant difference from control (p<0.05) designated by *.
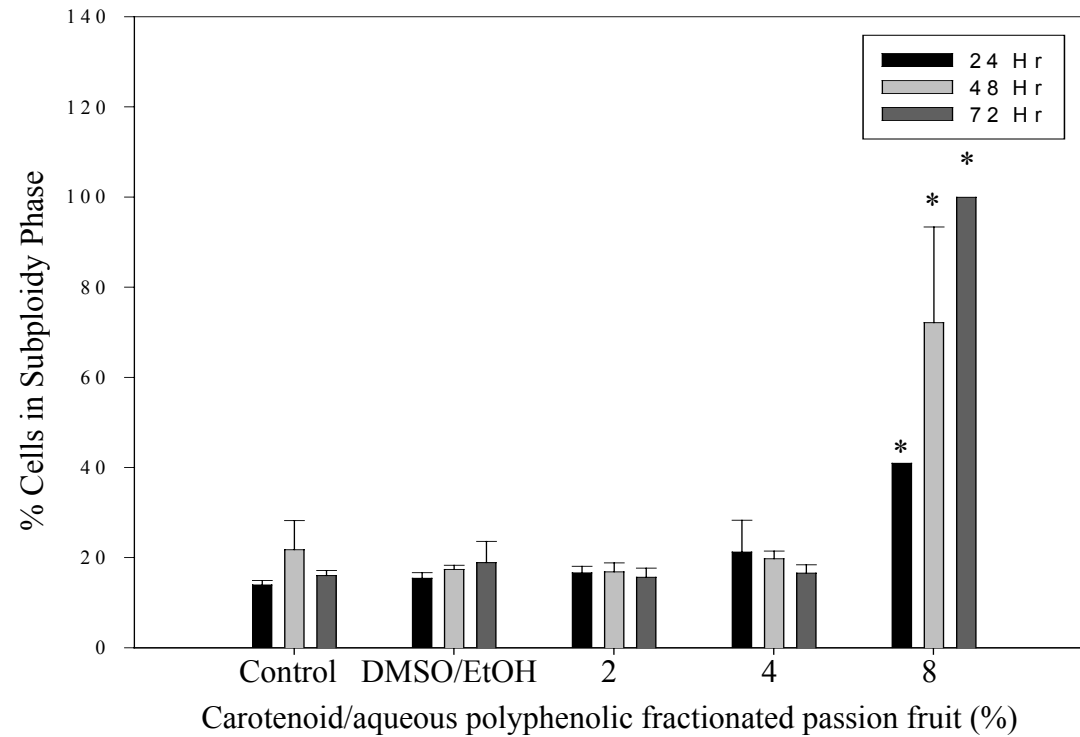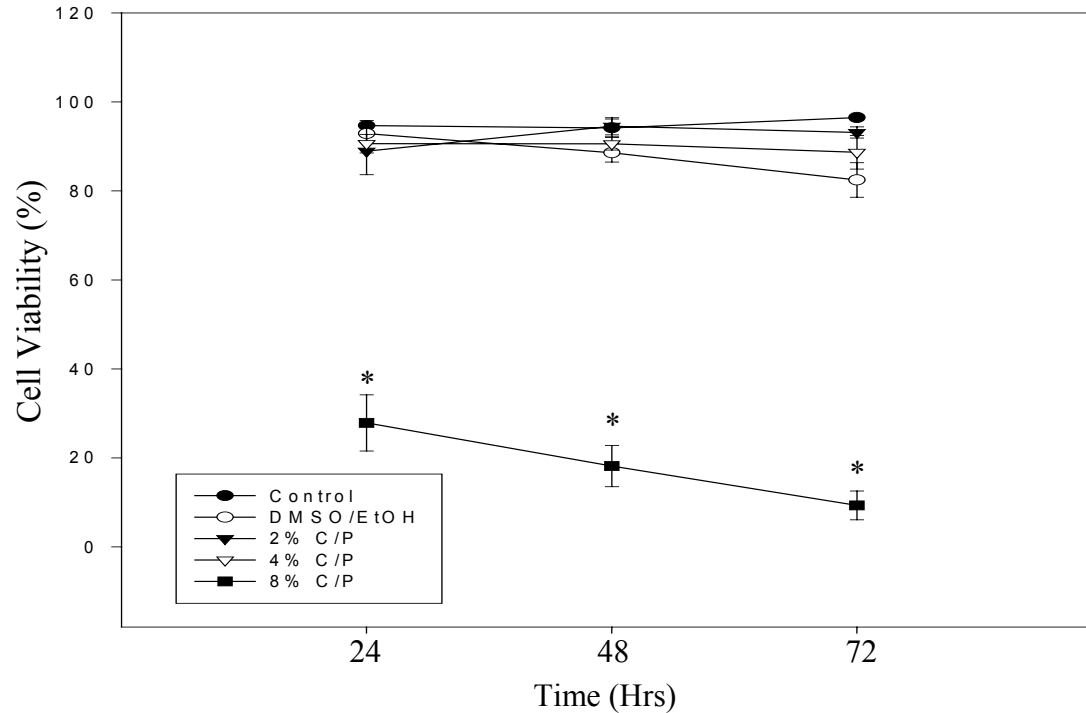
Figure 16. Cell Viability (% live cells/ total # cells) of fractionated passion fruit juice treated MOLT-4 cells over time. Passion fruit juice fractionated carotenoid/aqueous polyphenolic was administered as percent of total volume of cell culture in μL. 2%= 240 μL (185 mg/L carotenoid/ 8700.0 mg/L aqueous polyphenolic) 4%= 480 μL (370 mg/L carotenoid/ 17400.0 mg/L aqueous polyphenolic) 8%= 960 μL (740 mg/L carotenoid/ 34800.0 mg/L aqueous polyphenolic) DMSO/EtOH was the control for 2-8% PF samples. Fractions added as 2% concentration, equivalent to 2% of stock PFJ. Significant difference from control ($p < 0.05$) designated by *.
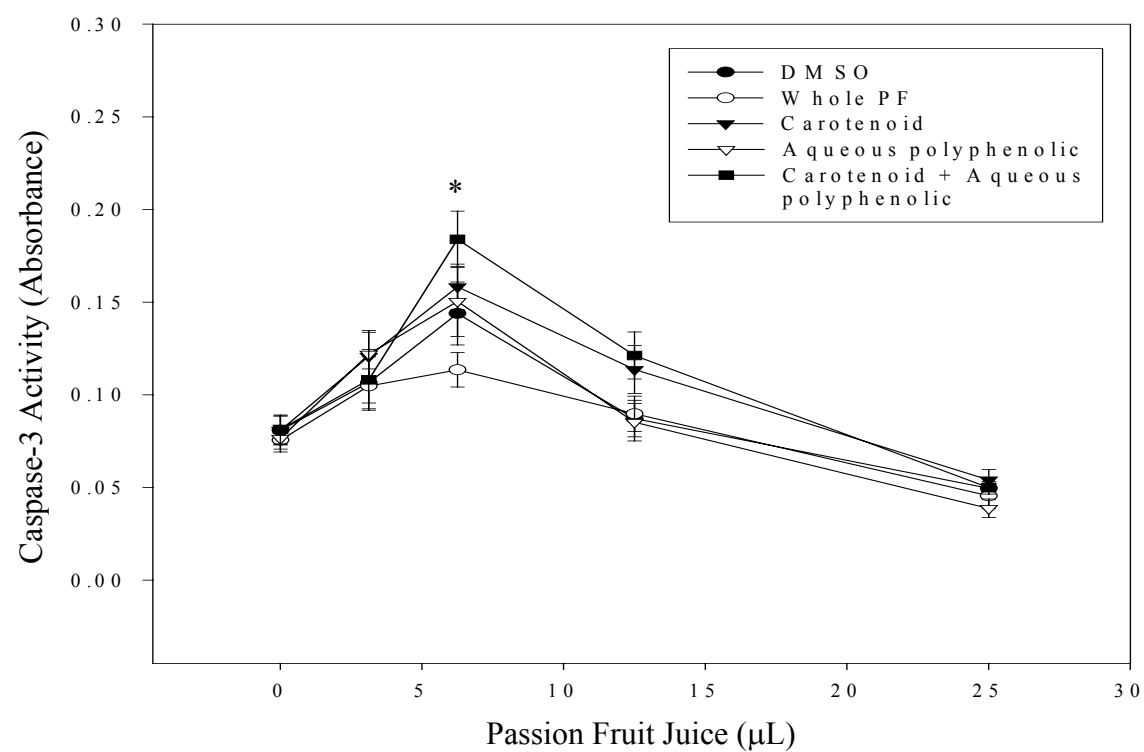
Figure 17: Absorbance from Caspase-3 activity of passion fruit juice treated MOLT-4 cells (μL). Carotenoid concentration: 25μL= 1.156 mg/L; 12.5 μL= 0.578 mg/L; 6.25 μL= 0.289 mg/L; 3.12 μL= 0.144 mg/L Aqueous polyphenolic concentration: 25μL= 54.375 mg/L; 12.5 μL= 27.1875 mg/L; 6.25 μL= 13.594 mg/L; 3.12 μL= 6.797 mg/L. DMSO was the control for all samples. Significant difference from control ($p<0.05$) designated by *.

Figure 18. Absorbance from MTT activity of passion fruit juice treated MOLT-4 cells when incubated for 4 hours. Carotenoid concentration: 25μL= 1.156 mg/L; 12.5 μL= 0.578 mg/L; 6.25 μL= 0.289 mg/L; 3.12 μL= 0.144 mg/L Aqueous polyphenolic concentration: 25μL= 54.375 mg/L; 12.5 μL= 27.1875 mg/L; 6.25 μL= 13.594 mg/L; 3.12 μL= 6.797 mg/L. DMSO was the control for all samples. Significant difference from control ($p < 0.05$) designated by *.
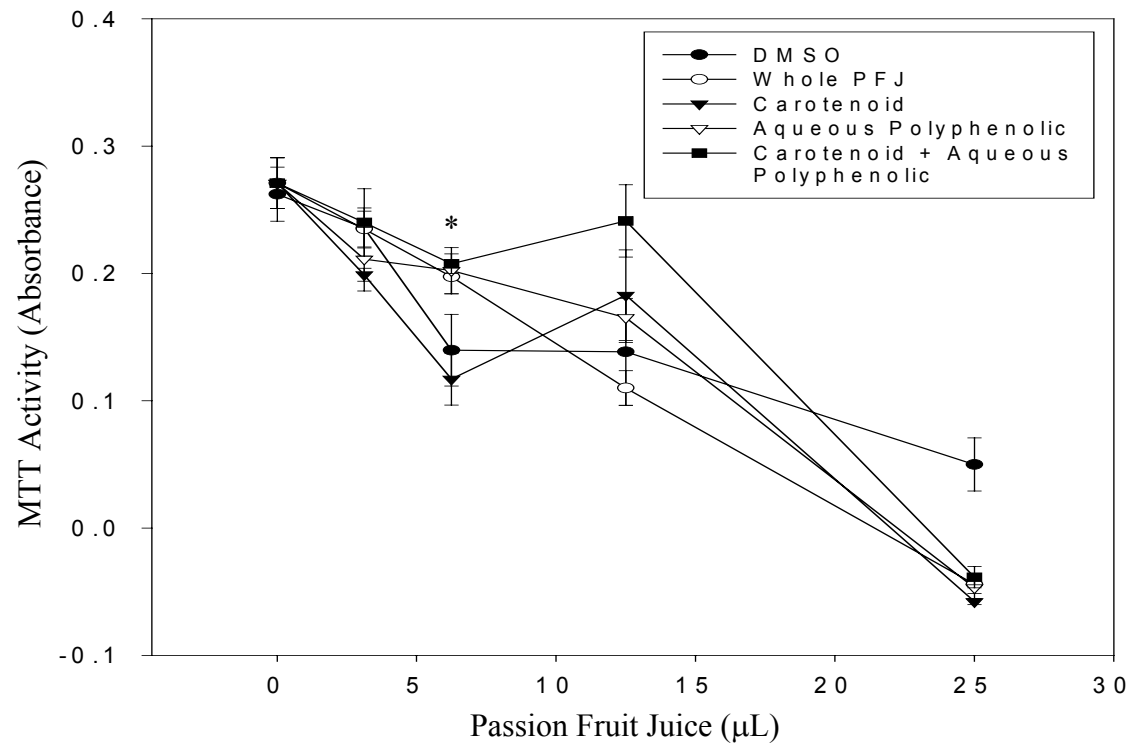
CHAPTER 5
DISCUSSION AND CONCLUSIONS

This study examined the relationship between the passion fruit juice phytochemicals, carotenoids and aqueous polyphenolics, and its effects on cell cycle kinetics, apoptosis activity and cell viability of leukemia lymphoma MOLT-4 cells. Previous studies have not investigated passion fruit juice phytochemicals on MOLT-4 cells, however, individual carotenoids and polyphenolic have been evaluated in other leukemia cell lines such as HL-60 myeloid leukemia, CEM leukemia T-cells, and K562 human leukemia cells. Stock passion fruit juice as well as fractioned whole carotenoids and polyphenolics were used to determine which fraction was more beneficial in the inhibition of cell cycle and induction of apoptosis.

Whole passion fruit had demonstrated a dose response on leukemia. At 2% stock passion fruit juice, a significant increase of G_0/G_1 phase and decrease in the G_2/M phase was indicative of G_0/G_1 arrest in leukemia ($p<0.05$) at 48 hours. This was consistent with the results that both beta-carotene and lycopene arrested the HL-60 cells at the G_0/G_1 phase. It seems that since HL-60 myeloid B cells and MOLT-4 lymphoid T cells are similar because they are leukemia but differ by their morphology, seem to have similar effects of phytochemicals. Since the cell cycle effect of whole passion fruit cannot infer which phytochemicals are responsible for the arrest, several fractions at 2% were compared to stock passion fruit juice to determine what was responsible for the cell cycle arrest.

The unbound fraction had an accumulation of cells in the G_0/G_1 phase with a decrease G_2/M indicating a G_0/G_1 arrest. This arrest was due to the organic acids, amino acids, and proteins present in the fraction. Since the 100% MeOH fraction, consisting of non-polar carotenoids and aqueous polyphenols, or the 50% MeOH fraction had no significant effect, it can be inferred that at 2% the significant G_0/G_1 phase for whole passion fruit juice was probably due to the organic acids, amino acids and proteins, and not the carotenoids and aqueous polyphenols as we originally thought. To confirm this, the third set of fractions, the carotenoid and polyphenolic individual fractions, was used to determine whether individual phytochemicals could be responsible for the cell cycle arrest. Since there was no significance detected, the cell cycle was not affected by the individual carotenoid and aqueous polyphenolic fractions at 2% passion fruit juice.

The collective carotenoid and aqueous polyphenolic fraction effect on the cell cycle was consistent with the results from the previous fractions; at 2% there was not a significant effect on the cell cycle. However, after 24 hours, at 4% there was a significant increase in the G_0/G_1 phase and a decrease in the G_2/M, indicative of a G_0/G_1 arrest in a dose dependent manner. In addition, at 8% there was a significant increase in the G_0/G_1 phase, an increase in the G_2/M and a decrease in the S phase, indicative of a G_0/G_1 and a G_2/M arrest. This indicated that 2% concentration was not a high enough dose to induce a cell cycle arrest. Only at a dose greater or equal to 4% was large enough to promote a G_0/G_1 arrest, and a dose of 8% was needed for a G_2/M arrest. These results were similar to a study with β-carotene, where at low concentrations induced a G_0/G_1 phase and at high concentrations, induced a G_2/M arrest (Palozza 2002). The effect of DMSO/EtOH should be taken into consideration, it had a greater effect on the cell cycle

than was anticipated. It seemed to have more of an influence on G_0/G_1 phase than the G_2/M or S phase. The DMSO/EtOH might have an effect on the cell cycle arrest, but to what extent cannot be determined.

When MOLT-4 cells were treated with several fractions of passion fruit, whole passion fruit juice, unbound and combined carotenoid and aqueous polyphenolic fractions, had a decreased cell counts at 2 and 8% concentrations, and increased subploidy regions after 72 hours. We believed that passion fruit that this decreased in cell viability was due to induced apoptosis in MOLT-4 cells, a mechanism which we investigated using a caspase-3 assay. At 4 hours, all passion fruit fractions stimulated caspase-3 with a peak at 6.25 uL, which indicates an induction of apoptosis. We also demonstrated that passion fruit fractions had a 3-fold decrease in cell viability in a dose dependent manner. These results show that passion fruit induced apoptosis with a decrease in cell viability of MOLT-4 leukemia cells.

In summary, we have demonstrated that passion fruit at 2% concentrations has an effect on the cell cycle due to the organic acids, amino acids and proteins. In order to show a G_0/G_1 arrest a $\geq$ 4% concentration of carotenoids and polyphenolics is needed, however more studies about the effect of DMSO/EtOH must be further investigated. Likewise in order to establish if carotenoids or polyphenols are more beneficial, more studies at higher concentrations are needed. We also demonstrated that the most crucial time points to evaluate passion fruit was at 24 and 48 hours, by 72 hours all cells have recovered. For concentrations at 8%, it was interesting to show a G_0/G_1 and G_2/M arrest, which might suggest that even higher concentrations, there might be a greater induction of G_2/M arrest.

Overall, we have demonstrated that passion fruit juice might not be as beneficial by inhibiting the cancer cell cycle because of the large solvent effect noticed. The results did however, show that fractions of passion fruit juice was capable of inducing apoptosis and decreasing cell viability of MOLT-4 leukemia lymphoma. The cell cycle might not be a good mechanism to study because of the solvent effects, so other methods of study such as antioxidant activity as well as continued research of apoptosis activity might be further studied to evaluate the beneficial effects of passion fruit juice on cancer.

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BIOGRAPHICAL SKETCH

Cindy De Neira was born in Long Island, New York on January 27, 1979, and lived in Boca Raton, Florida, from 1985 until she graduated from high school in 1997. While at the University of Florida, she received a bachelor's degree in chemistry in 2001 and then a master's degree in nutrition from the Food Science and Human Nutrition Department in 2003. In the fall of 2003, she will be attending Des Moines University Osteopathic Medical Center, where she will be obtaining a Doctor of Osteopathic Medicine degree.