Characterization of Phytochemicals and Antioxidant Activities of Specialty Tomatoes

by

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ABSTRACT

CHARACTERIZATION OF PHYTOCHEMICALS AND ANTIOXIDANT ACTIVITIES OF SPECIALTY TOMATOES

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Twenty-eight specialty tomato cultivars were studied for the phenolic compositions, antioxidant activities in both hydrophilic and hydrophobic fractions of the samples, and the potential effect of simulated in vitro digestion on phenolic composition. Antioxidant activities were evaluated by both chemical-based assays (DPPH, FRAP and ORAC-L/ORAC-H) and cell-based antioxidant assay (CAA). Phenolic compositions were analyzed by HPLC-DAD, which showed that in addition to the phytochemicals commonly known for tomatoes, cultivars such as Lemon Boy Hybrid had a unique composition of phenolics. Cultivar Cuban Yellow Grape showed the highest total phenolic content at 7.12 mg GAE/g DW, and exhibited the highest antioxidant activities from all three chemical-based antioxidant assays. Cultivar Brand Sweet Plum had the highest total carotenoid content (541.13 μg/g DW), which did not show corresponding antioxidant activities. Results indicated that phenolic compounds were bioavailable to the cells as demonstrated by the cell-based antioxidant assay. Phenolic compounds, such as rutin and naringenin were reduced by approximately 70% after simulated gastrointestinal digestion, which may be in part due to the interactions between polyphenols and food matrix such as proteins and dietary polysaccharides. This study showed that specialty tomatoes such as Cuban Yellow Grape, with a unique phytochemical composition, possess higher phenolic content and stronger antioxidant activity, therefore can potentially have added health benefits to humans.
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# TABLE OF CONTENT

Acknowledgements ........................................................................................................ iii

List of Tables .................................................................................................................... vii

List of Figures .................................................................................................................. viii

List of Abbreviations ...................................................................................................... viii

Chapter 1 : Literature Review ......................................................................................... 1

1.1 Tomatoes ..................................................................................................................... 1

1.1.1 Overview ................................................................................................................ 1

1.1.2 Phenolic Compounds ............................................................................................. 9

1.1.3 Carotenoid Compounds in Tomatoes ..................................................................... 17

1.2 Tomato Bioactive Compounds and Disease Prevention ........................................... 22

1.2.1 Dietary Phenolics as Therapeutic Agents ........................................................... 22

1.2.2 Dietary Carotenoids and their Roles in Disease Prevention ............................... 23

1.2.3 Tomato Consumption and Chronic Diseases ....................................................... 24

1.3 Antioxidant and Anti-Inflammation Properties of Tomato Bioactive Compounds .......... 28

1.3.1 Oxidative Stress .................................................................................................. 28

1.3.2 Mechanism of Antioxidant Action ...................................................................... 29

1.3.3 Potential Synergy of Phytochemicals in Tomato .................................................. 31

1.3.4 Chemical-Based Antioxidant Activity Assay ....................................................... 33

Chapter 2 : Rationale, Hypotheses, and Objectives ....................................................... 35

2.1 Rationale ................................................................................................................... 35

2.2 Hypotheses .............................................................................................................. 36

2.3 Objectives ............................................................................................................... 36

Chapter 3 : Composition and Antioxidant Activities of Phenolic compounds of Specialty Tomatoes ........................................................................................................... 37

3.1 Introduction ............................................................................................................. 37

3.2 Materials and Methods .......................................................................................... 38
### Chapter 3: Phenolic Compounds

3.2.1 Tomato Varieties and Preparation of Tomato Samples ........................................... 38
3.2.2 Phenolic Extraction .................................................................................................. 38
3.2.3 Determination of Total Phenolic Content (TPC) ..................................................... 39
3.2.4 Antioxidant Activities of Phenolic Extracts by Chemical-based Assays .................. 39
3.2.5 HPLC–DAD Analysis of Phenolic Compounds ..................................................... 41
3.2.6 Mass Spectrometry Analysis of Phenolic Compounds ....................................... 42
3.2.7 Statistical Analysis ................................................................................................. 43

### Chapter 4: Antioxidant Activities of Carotenoid Bioactive Compounds of Specialty Tomatoes

4.1 Introduction .................................................................................................................. 51
4.2 Materials and Methods ............................................................................................... 52
4.2.1 Tomato Varieties and Preparation of Tomato Samples ....................................... 52
4.2.2 Carotenoid Extraction ............................................................................................ 52
4.2.3 Determination of Total Carotenoid Content (TCC) ................................................. 52
4.2.4 Carotenoid Antioxidant Activities by Chemical-Based Assays ............................. 53
4.2.5 Statistical Analysis ................................................................................................. 53

### Chapter 5: In vitro Digestion Model and Cell-Based Antioxidant Activities of Phenolic Extracts

5.1 Introduction .................................................................................................................. 60
5.2 Materials and Methods ............................................................................................... 61
5.2.1 Sample Preparation and Extraction .......................................................... 61
5.2.2 Simulation of in vitro Gastric and Gastrointestinal Digestion Model .......... 62
5.2.3 Cell-based Antioxidant Assay .................................................................. 63
5.2.4 Statistical Analysis .................................................................................. 64
5.3 Results and Discussion .............................................................................. 64
  5.3.1 Phenolic Contents and Profiles Before and After Digestion .................. 64
  5.3.2 Cell-based antioxidant assay (CAA) ..................................................... 69

Chapter 6: Conclusions and Future Directions .................................................... 72
  5.1 Conclusions ............................................................................................... 72
  5.2 Future Directions ....................................................................................... 74

Chapter 7: References ...................................................................................... 79

LIST OF TABLES

Table 1. List of flavonoids, phenolic acids and other phenolics analyzed in tomato fruits. .......... 16
Table 2. Total phenolic content (TPC) and antioxidant activities measured by DPPH, ORAC and FRAP. .......................................................... 45
Table 3. Total carotenoid content (TCC) and antioxidant activities measured by DPPH and ORAC ....56
Table 4. Degradation (%) of major phenolic compounds in cultivar Lemon Sugar Hybrid, Cuban Yellow Grape and Sun Sugar Hybrid after gastro and gastrointestinal digestions................................. 67
LIST OF FIGURES

Figure 1. Chemical structures of commonly found phenolic acids ........................................ 11
Figure 2. General structure of flavonoids (left) and anthocyanidin (right).............................. 14
Figure 3. Chemical structures of commonly found flavonoids............................................. 14
Figure 4. Chemical structure of beta-carotene.................................................................... 18
Figure 5. Chemical structures of commonly found carotenoids in tomato............................. 21
Figure 6. Total phenolic content of the hydrophilic extracts of 28 specialty tomato cultivars...... 44
Figure 7. Antioxidant activities of the lipophilic extracts of 28 specialty tomato cultivars....... 48
Figure 8. Chromatographic profiles of phenolics in cultivar Lemon Boy Hybrid (A), Cuban Yellow Grape (B), Sun Sugar Hybrid(C) and mix standard (D) at 360 nm. ............................ 50
Figure 9. Total carotenoid content in hydrophobic extracts of 28 specialty tomato cultivars.... 55
Figure 10. Antioxidant activities of the lipophilic extracts of 28 specialty tomato cultivars..... 59
Figure 11. Chromatographic profiles of phenolics before and after in vitro digestion at 360 nm.66
Figure 12. Cell-based Antioxidant Activity (CAA) Assay for Assessing Antioxidants.......... 70
Figure 13. Cell-based antioxidant activity of phenolic extracts in H_2O_2-stimulated Caco-2 cells. ........................................................................................................................................ 71

LIST OF ABBREVIATIONS

AAE: ascorbic acid equivalent
AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride
ANOVA: one-way analysis of variance
ATP: adenosine triphosphate
CAA: cell-based antioxidant assay
CAT: catalase
CP: chronic pancreatitis
CP: chronic pancreatitis
CVD: cardiovascular disease

DCFH-DA: Dichloro-dihydro-fluorescein diacetate

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DW: dry weight

FRAP: ferric ion reducing antioxidant power

FW: fresh weight

GAE: gallic acid equivalent

GPx: glutathione peroxidase

GRx: glutathione reductase

GSH: glutathione

HPLC: high performance liquid chromatography

HPLC-DAD: HPLC with diode array detection

LC-MS: Liquid chromatography–mass spectrometry

LDL: low-density lipoproteins

MS: mass spectroscopy

NMR: nuclear magnetic resonance

ORAC-H: Oxygen radical absorbance capacity for hydrophilic

ORAC-L: Oxygen radical absorbance capacity for lipophilic

PBS: phosphate buffer saline

PCL: photochemiluminescence

PMSF: phenylmethylsulfonyl fluoride

PPO: polyphenoloxidase

PSA: prostate-specific antigen
RNS: reactive nitrogen species

ROS: reactive oxygen species

SD: standard deviation

SOD: superoxide dismutase

SPE: solid phase extraction

TCC: total carotenoid content

TE: trolox equivalent

TPC: total phenolic content

TPTZ: 2, 4, 6-tripyridyl-s-triazines
CHAPTER 1 : LITERATURE REVIEW

1.1 Tomatoes

1.1.1 Overview

1.1.1.1 Geographic Origin; botanic origin/classification (types of different tomatoes)

Tomato belongs to the Solanaceae family and there are more than 3,000 species that have been identified. In particular, the genus Solanum consists of 13 species: the cultivated tomato, Solanum lycopersicum, which is the only domesticated species, and 12 wild species (such as S. chmielewskii, S. habrochaites, S. pennellii, and S. pimpinellifolium). Although botanically tomato is characterized as a vegetable, it is commonly recognized by people as a fruit. The genus Lycopersicon of the Solanaceae family is believed to have originated in a narrow costal area of western South America, from Ecuador south to northern Chile, and the Galapagos Islands. In contrast, some writers supported an origin in Mexico, and believed that tomato was first domesticated in Mexico as seeds were taken to Europe from Mexico after Cortez conquered Mexico City in 1519 [1, 2]. In the mid-16th century, tomato was first recorded in Europe, and was cultivated as ornamental or curiosity plants but was not often eaten, except in Italy and Spain. Notably, the fruit was thought by many to be poisonous due to the close resemblance to deadly nightshade. Tomato was not accepted as a vegetable crop until the late sixteenth century in Europe, and it was not introduced as an important vegetable back into America until the 18th century.

During the seventeenth century, tomato was first being placed in the genus Solanum under the Solanaceae family, a Latin word for “the nightshade plant”, and tomato was commonly
referred to as *S. pomiferum*. In 1694, Tournefort placed tomatoes in a new grouping of plants within *Solanaceae*, and first considered cultivated tomatoes as a distinct genus called *Lycopersicon*, meaning “wolf peach” in Greek. This Greek term appears to follow an old German word for tomato, *wolfpfirsich*, which also translates into English as “wolf peach.” This name was not rejected until 1753 by a well-known botanist Carl Linnaeus in his work *Species Plantarum*. Carl Linnaeus, is often called the Father of Taxonomy. His system for naming, ranking, and classifying organisms (kingdom, phylum, order, family, genus, and species) is still in wide use today. He rejected Tournefort’s separate genus *Lycopersicon* and placed tomatoes back in *Solanum*, naming the tomato the familiar *S. Lycopersicon* — both poison and wolves. After that tomato was recognized as *Solanum lycopersicon* by many modern authors [2, 3].

It is well known that tomato plants can vary from white to, pink, yellow, orange, red, purple, mottled, or striped. Tomatoes can be classified according to three of the most basic distinctions: determinate vs. indeterminate, heirloom vs. hybrid, and shape/size. Heirloom tomatoes, or “open pollinated” tomatoes, are varieties that have been reproduced for generations by the traditional method of growing tomatoes from seeds with favorable qualities [4, 5]. As a result, the seeds possessing desired qualities would be retained and those seeds with aberrant and undesirable qualities would be gradually discarded. How old a commercial tomato variety needs to be considered an heirloom is a controversy. In general, a variety is recognized as an heirloom if it was introduced before 1940, or it has been bred for more than 50 years in circulation. Hybrid tomatoes are obtained from cross-breeding of two genetically different tomato varieties. With a hybrid, you get the best traits of each of the parents. The cross-pollination is intentionally controlled by breeders to ensure the desired combination of characteristics, such as bigger size or better disease resistance [4, 6].
There are two types of growing habits for tomatoes—Determinate and Indeterminate [7, 8]. Determinate tomatoes, often called “bush” tomatoes, bloom and set fruit all at once, usually within one to two weeks, and then decline. Their blossoms grow at the ends of the shoots, so the plants stop growing when the fruit sets on the terminal bud. These tomato plants in nature are genetically programmed to produce a certain number of stems, leaves and flowers, so no pruning is required. Determinate varieties are usually compact plants that can grow to about 3 or 4 feet high with some level of staking or caging, and they are generally favored by tomato industry or mass production growers aiming a massive harvest of tomatoes all at once. Indeterminate tomatoes, are often called “vine” tomatoes. They grow and produce stems, leaves, flowers and tomatoes consistently throughout the summer until the plant dies. Because the flowers grow along the vines rather than at the ends, strong staking or caging support systems are needed. The taller they get the heavier they are and the more support they need. Indeterminate tomatoes are preferred by growers who want to harvest tomatoes all season long.

Along with fruit color and plant growth habit, the shape and size of tomato fruit are defining features that distinguish one variety from another. Globally tomatoes are the most heavily commercially-cultivated vegetables. On average a tomato fruit ranges from 70 to 150 g and generally measures about 50 to 70 mm in diameter [9]. Cherry tomatoes are the smallest fruit of tomatoes which are spherical to slightly oval in shape. Generally, a cherry tomato is about 12 to 25 g in weight and 25 to 35 mm in diameter. The Super Sweet 100 is such a hybrid cultivar developed in the United States which not only has an outstanding flavor but is also resistant to certain commonly found fungal diseases. Similar to cherry tomatoes, small plum tomatoes are in oval shape that elongate and bulge in the middle, and are often eaten fresh on their own or in salads. Larger plum tomato varieties, measured up to 600 mm, are usually preferred for tomato
sauce making, but not for eating fresh. The last distinct variety needs to be mentioned is the beefsteak. Beefsteak tomato is the largest variety among those of the classic type, having a meaty flesh and numerous seeds. It can weigh about 200 g to well over 1 kg and great for eating fresh [10, 11].

1.1.1.2 Production /historic consumption

Tomato is a crop consumed worldwide. According to USDA (United States Department of Agriculture), its world production is estimated to be around 159 million tons per annum [12]. Particularly, 91% of the world production of tomato is found in North America (Canada & California) and South America, China, Australia, and Mediterranean area (France, Greece, Italy, Spain, and Portugal (WPTC - The World Processing Tomato Council) [13]. The United States, as one of the world's leading producers of tomatoes, second only to China, generates more than $2 billion profit annually from the fresh and processed tomatoes industry. In the past decade, tomato consumption has further increased since tomato fruits supply both fresh market and processed products such as soups, juices, purees and sauces. Among fresh vegetables, only onions, head lettuce, and potatoes are consumed more frequently than raw tomatoes, but tomatoes are by far the most commonly consumed processed vegetable. Fresh tomato is consumed in large quantities (17.9 pounds per year) and processed tomato is the most commonly consumed processed vegetable (68.7 pounds per year) by an average American [14]. According to the Economic Research Service of the USDA, 35% of raw tomatoes are processed into sauces, the rest are into either tomato paste (18%), canned tomatoes (17%), ketchup (15%) and tomato juice (15%).
1.1.1.3 Health benefits (Mediterranean diet)

Traditional Mediterranean diet is characterized by: (i) diet rich in olive oil, (ii) high consumption of nuts, beans, fruits, vegetables, (iii) moderate consumption of fish, poultry, and even wine, and (iv) low consumption of eggs and red meat [15]. Hundreds of studies over the past few decades have linked the Mediterranean diet to increased longevity, maintenance of a healthy weight, and reduced risk of diabetes, cardiovascular and coronary heart disease [16-18]. Among the vegetables, tomatoes are a main component of the traditional Mediterranean diet. The high content of the antioxidant compounds of the fruit, particularly carotenoids (lycopene and \( \alpha \)-carotene), ascorbic acid, and polyphenols [19], may help to explain the beneficial effects of the Mediterranean diet in the prevention of certain diseases.

Tomatoes contain significant quantities of carotenoids, phenolic, potassium, and vitamin E. Numerous animal studies have shown that consumption of tomatoes is linked to the reduced risk of prostate cancer. Campbell et al. supported that freeze-dried whole-tomato powder or lycopene alone reduced the growth of prostate tumors in rats [20]. Bosland et al. applied N-methyl-N-nitrosourea (NMU) and testosterone-induced prostate cancer model to examine the effects of tomato consumption on prostate cancer development. The results supported that rats fed with a diet containing 10% tomato powder had significantly longer survival from prostate cancer [21].

1.1.1.4 Recent clinic studies

Tomatoes and tomato products are one of the most familiar vegetables in the world diet. Quantitatively, they are the most consumed non-starchy vegetable and are the most significant
source of dietary antioxidants which could contribute tremendously to human health. Many studies have reported that eating tomato fruits has beneficial effects on inflammation, hypertension, cardiovascular disease, and diabetes. In recent decades, much attention has been given to improve the levels of health-promoting phytochemicals in tomato.

Tomato, a low glycemic index food with a rich source of antioxidants, is believed to have protecting roles in diabetics and cardiovascular diseases. In a randomized control trial, a supplementation of tomato juice to diabetic patients was accounted for a nearly 3-fold increase of plasma lycopene and an elevated intrinsic resistance of low-density lipoproteins (LDLs) to oxidation by 42%, suggesting tomato to be a valuable component of a cardioprotective diet [22]. Similarly, Farzad et al. concluded that consumption of 200 g raw tomato for 8 weeks produced a significant decrease in serum glucose and blood pressure in type 2 diabetic patients [23].

Since tomatoes are the most prevalent vegetable source of lycopene which has been associated with reduced risk of prostate cancer, this food item was of particular interest. A number of case-control studies and prospective studies have been conducted in the past decades in order to evaluate the anti-cancer properties of tomatoes. Mills et al. suggested higher consumption of tomatoes was statistically significantly related to lower risk of prostate cancer [24] In contrast, no significant association was found in other studies [25, 26].

Pancreatic cancer is the 4th leading cause of cancer-related deaths in both men and women in Canada. Substantial evidence exists that high consumption of vegetables, particularly tomatoes that are rich in carotenoids, may be associated with reduced pancreatic cancer risk [24, 27, 28]. Chronic pancreatitis (CP) is at a greater risk of developing micronutrient deficiencies due to malabsorption. Insufficient absorption of carotenoids in CP patients could contribute to an increased risk of developing pancreatic cancer compared with the general population [29]. An intervention study by Quilliot et al. demonstrated that consumption of 40 g tomato paste
(approximately 24 mg lycopene) was associated with a significant increase in plasma lycopene levels after daily supplements of tomato products in CP patients [30]. However, no clinical benefits were investigated in this study.

Research in cancer and cardiovascular disease, particularly those related to the antioxidant effects of tomatoes, has led to research in other areas including skin protection and bone health. Although these emerging areas are not yet thoroughly investigated in literature, promising results have been reported. Consumption of dietary tomato paste (40 g per day), providing approximately 16 mg/d of lycopene, was found to protect against ultraviolet light-induced erythema (skin redness or rash) in humans [31]. A similar result was observed, suggesting for the photoprotective effects of dietary carotenoids after 10-12 weeks of intervention in volunteers [32]. Epidemiological studies also suggest a positive correlation between dietary lycopene and bone mass. A 17-year follow-up from the Framingham Osteoporosis Study reported a lower risk of hip fracture ($p =0.01$) and non-vertebral fracture ($p = 0.02$) with higher lycopene intake [33].

1.1.1.5 Major bioactive components (phenolics and carotenoids)

Tomatoes contain a variety of antioxidant phytochemicals that have health-beneficial effects on humans. Two of the major components are carotenoids and polyphenols (or phenolics; these two are used interchangeably in this thesis), which by nature are lipophilic and hydrophilic antioxidants, respectively. Carotenoids are tetraterpenes belonging to fat-soluble pigments. They include provitamin A carotenoids, such as β-carotene and β-cryptoxanthin, and non-provitamin A carotenoids, such as lutein and lycopene. More than 600 carotenoids have been identified in nature, but only about 40 are found in the human diet [34]. Tomato is mostly the sole source of dietary lycopene for humans. It is well known that carotenoids contribute to human health owing
to their antioxidant power. Studies have demonstrated that carotenoids are powerful free radical scavengers particularly due to their capability in quenching singlet oxygen \( ^1\text{O}_2 \), neutralizing sulfenyl radicals and stabilizing peroxy radicals through the hydrophobic chain of polyene units [35].

Phenolic compounds are widely distributed phytochemicals composed of an aromatic ring and one or more hydroxyl substituents. Most common phenolics in tomato fruit include flavonoids, phenolic acids and tannins, which are effective free radical scavengers. In recent years, the antioxidant activity of phenolics has been found to play important roles in human health. Phenolic compounds have been ascribed to their antioxidant activity in protecting humans from cancer and coronary heart disease, which are mainly induced by oxidative stress[36].

Along with carotenoids and phenolics, vitamins such as vitamins E also contribute to the beneficial properties of tomatoes. Vitamin E is a fat-soluble antioxidant, and its nutrient content is defined by \( \alpha \)-tocopherol activity. The molecules that contribute to \( \alpha \)-tocopherol activity are four tocopherols and four tocotrienols which differ in the number and position of the methyl groups on the chromane ring, identified by the prefixes alpha- (\( \alpha \)-), beta- (\( \beta \)-), gamma- (\( \gamma \)-), and delta- (\( \delta \)-). Tocopherols contain a phytyl chain while tocotrienols contain a geranylgeranyl chain. Tocopherols are the most abundant vitamins in tomato plant and have been suggested to act either independently or synergistically with other bioactive compounds in their health-promoting effects. Studies have revealed a synergistic effect of vitamin E and lycopene in terms of increased efficacy in prostate cancer prevention [37]. A study by Böhm et al. also suggested that interactions between vitamin C, carotenoids, and \( \alpha \)-tocopherol led to enhanced efficiency in free-radical scavenging activities [38].
Vitamin C, a water-soluble compound including both ascorbic and dehydroascorbic acid, plays a critical role in preventing tomato itself against autoxidative damage. Ascorbic acid in tomatoes is susceptible to oxidation, light, and high temperatures. Therefore, significant losses of ascorbic acid can occur during the post-harvest storage period [39]. Noticeably, ascorbic acid has been reported to act synergistically with other vitamins and polyphenols in its antioxidant activity. For instance, caffeic acid and ferulic acid have been found to have an indirect synergistic interaction with vitamins C and E in the antioxidant process [40].

1.1.2 Phenolic Compounds

1.1.2.1 Phenolics Classification, Chemical Structure and Occurrence (factors affecting biosynthesis of phenolics, genetics vs. environment vs. processing)

Phenolics are phytochemicals abundantly found in fruits vegetables and grains. The biological roles of phenolics in plants include attracting pollinators, protecting plants against infections caused by microorganisms and injuries by insects [41]. Tomato fruit has been known for its abundant content of phenolic compounds, including phenolic acids and flavonoids. Structurally speaking, phenolics compounds are a group of phytochemicals composed of an aromatic ring and one or more hydroxyl substitutes. The basic structure of a phenolic compound is known as an aglycone, which can associate with various carbohydrates and organic acids to form glycosides. Many phenolic compounds can also join together to form polymers. So far at least 10 classes of phenolics have been identified based on the number of phenolic rings and the structural elements linking these rings [42].

The most common phenolics found in tomato fruit include flavonoids, phenolic acids and tannins which are effective free radical scavengers. Recent studies have pointed to the antioxidant functionality of phenolics in human health benefits. Phenolic compounds have been
ascribed to their antioxidant activity in protecting humans from cancer and coronary heart disease, which are mainly induced by oxidative stress [36].

**Phenolic acids**

Molecules that do not possess a polyphenol structure and have a carboxyl group attached to the benzene ring are known as phenolic acids. Phenolic acids, responsible for the astringent taste of vegetables and fruits [43] may be distinguished depending on their structure: hydroxybenzoic acids and cinnamic/hydroxycinnamic acids [44]. Hydroxybenzoic acids possess a common C6-C1 structure, whereas hydroxycinnamic acids are aromatic compounds with a three-carbon side chain (C6-C3). These two classes of phenolic acids have been found to exist widely in fruits and vegetables at different concentrations. The main hydroxybenzoic acids are gallic, ellagic, vanillic acid, protocatechuic and 4-hydrobenzoic acids (Figure 1). Compare to the hydroxycinnamic acids, the hydroxybenzoic acid concentration in fruits and vegetables is generally lower except for certain pigmented vegetables, such as black radish, onion, and potatoes [45, 46], and Japanese alder, oriental ginseng [47]. Hydroxycinnamic acids are mainly represented by caffeic, ferulic, sinapic and p-coumaric acids (Figure1). Caffeic acid, found in coffee, wine, and beans, is the most common and accounts for up to 70% of total hydroxycinnamic acids in fruits [48, 49]. Ferulic acid is mainly found in legumes, pumpkin, and cereal grains which constitute its main dietary source [50]. Chlorogenic acid refers to the ester of caffeic acid and quinic acid and it commonly occurs in coffee, apples, pears, and berries. p-Coumaric acid can be found in beans, wheat, oats, apples and grapefruits [44]. A single cup of coffee may contain 70–350 mg of chlorogenic acid. In western countries, intake of phenolic acids is generally high (100 mg to 2 g/d) due mainly to the high rate of coffee
Phenolic acids are generally known for their antioxidant activity, but studies also have shown that they may play a role in the anti-cancer activity. Plant-derived caffeic acids were found to induce DNA fragmentation and apoptotic cell death in cancer cells, thus exhibits a potent anticancer effect in a study using the HT-1080 human fibrosarcoma cell line [52].

![Chemical structures of commonly found phenolic acids](image)

**Figure 1. Chemical structures of commonly found phenolic acids**

**Flavonoids**

Flavonoids are the largest family of phenolic compounds in tomatoes, and found to be present mainly in the peel of the fruit and account for the aroma, fragrance, and color of the tomato. Flavonoids are found ubiquitously in plant foods, however, they are not uniformly distributed throughout the plant kingdom. Studies found that broccoli, cranberry, and orange
juices contained a relatively lower amount of flavonoids and/or tannins as compared to red wine, teas, fruits (apples, blueberries) and dark chocolate [53-55]. Black and oolong teas, particularly, contain high to moderate level of tannins [56], which are considered to be responsible for the health-promoting benefits of these beverages.

Flavonoids are built upon a basic flavone skeleton of diphenylpropanes (C6–C3–C6) with different oxidation levels of the central pyran ring (Figure 2). To date, more than 4000 flavonoids have been profiled, including flavanols, flavonols, flavanones, isoflavones and anthocyanidins [57]. Derivatives of flavonoids are present in each family according to the number and nature of substituent groups attached to the flavonoid nucleus [58]. Additionally, many flavonoids in foods can be polymerized into large molecules to form condensed tannins. Some common flavonoids found in tomatoes include quercetin, kaempferol, myricetin, naringenin, naringin and rutin (Figure 3).

A great variety of studies has been carried out to determine the factors affecting the level of phenolic compounds in fruits and other plant foods. However, only a few have been conducted particularly for tomatoes. Environmental factors are known to affect the total phenolic content in tomato fruits. Light, temperature, fertilization, irrigation, origins of the cultivars and ripening stage at harvest may all contribute to the variations of phenolic contents in tomatoes. For example, plants effectively synthesize flavonoids and other phenolics under stimulation by ultraviolet (UV-B) light. These phenolic compounds act as protectors against further solar ultraviolet B radiation (280–320 nm). In a study with two Roma tomato varieties, tomato fruits had two-fold increase in flavonoid and other phenolic content due to the effect of UV-B light exposure [59].
Phenolic compounds are also susceptible to the food processing procedures and food storage conditions. In order to prolong the shelf life of food products, thermal processing such as hot water immersion is required [60]. The level of flavonoids in vegetables has been found to decrease when cooked in hot water. This may be explained by the polarity of flavonoids which make them readily dissolved in water (polar solvent). In foods having a high surface area or ruptured cell walls, flavonoids can particularly easily escape the cellular compartments [61]. Tomato purees were boiled in water (70 °C) for 21 – 26s and were analyzed for their phenolic contents and antioxidant capacities. The results indicated that thermal treatment had insignificant impact on phenolic contents. In addition, a slight but non-significant reduction in antioxidant activity was observed for thermally processed tomato purée as compared to the un-processed purée [62]. However, a recent study indicated that the total phenolics concentration and the water-soluble antioxidant capacity increased during the thermal processing of tomatoes [63]. Although studies have been conducted to evaluate the influence of storage conditions on polyphenol micronutrients in tomatoes, varied results have been found. After storing the bottled tomato pulp at room temperature (20.0±1.8 °C) for 180 days, a significant increase in total phenolic concentration was reported in a study by Ordóñez-Santos [64]. A more recent study analyzed the impact of storage (3, 6, and 9 months) on phytochemicals in ketchup and tomato. Notable reductions of the total polyphenol content and antioxidant capacity of the water soluble fraction were observed in ketchup and tomato juices. Specifically, glycosylated polyphenols showed greater stability during storage, in comparison to the aglycones quercetin, caffeic and ferulic acid [65].
Figure 2. General structure of flavonoids (left) and anthocyanidin (right).

Figure 3. Chemical structures of commonly found flavonoids.
1.1.2.2 Soluble vs. Insoluble-Bound Phenolics

It is well known that phenolics exist in soluble free, soluble conjugated, and insoluble bound forms [66]. Free and some conjugated phenolics are soluble in water or polar organic solvents such as methanol and ethanol. Soluble phenolic conjugates are referred to as phenolics which are conjugated with small peptides or oligosaccharides [67, 68]. Conjugated phenolics are often hydrolysed under acidic or alkalinic conditions to release the free forms of phenolic compounds for the purpose of identification and quantification [69, 70]. The non-extractible fraction of phenolics is mainly composed of bound phenolics, in which carboxylic and hydroxyl groups of insoluble phenolics are bound to cell wall polymers through ester and ether linkages, respectively [71]. Contribution of bound phenolics to the total phenolic content can be higher than that of free and conjugated phenolics in certain foods [72], and consequently making significant contribution to antioxidant capacity [71-73].

1.1.2.3 Tomato Phenolic Content and Profile

Tomato contains a number of flavonoids and phenolic acids that make it a healthy diet. Currently found polyphenol compounds in tomatoes are summarized in Table1. Many hydroxycinnamic acids together with their conjugates have been found in tomato skin and flesh. Chlorogenic acid, an ester formed between a certain hydroxycinnamic acid and quinic acid has been reported as the main phenolic acid in tomatoes. In addition to chlorogenic acid and its isomers, ferulic, caffeic, p-coumaric acid [74] and trace amounts of vanillic and sinapic acids have been detected in tomato fruits [75].

The history of flavonoid reported to occur in tomatoes can be traced back to 1947 when rutin was first isolated from leaves and stem epidermis of the tomato plant [76]. In 1958, two flavonoids naringenin and quercitrin (quercetin 3-rhamnoside) were first time isolated from the...
fruit skins and leaves of three tomato cultivars [77]. In 1981, Davies and Hobson published a literature review with respect to the previously reported polyphenols in tomatoes. They stated that rutin was the most common flavonoid in tomatoes, and they characterized kaempferol 3-rutinoside, and quercetin 3-glucoside (isoquercitrin) [78, 79]. Nevertheless, the presence of these flavonoids was reported in trace amount. Today, increasing numbers of flavonoids have been identified due to the advances in HPLC instrumentation and also in combination with mass spectrometry (LC-MS). According to the USDA flavonoid database, commercial red tomatoes (fresh weight), on average contain 15 mg of flavonoids (reported as aglycones) per kg. Particularly, Naringenin (45%) is determined to be the most common flavonoid, followed by quercetin (39%), myricetin (10%) and kaempferol (5%) [56].

Table 1. List of flavonoids, phenolic acids and other phenolics analyzed in tomato fruits. The compounds have been characterized by use of high-performance liquid chromatography (HPLC), mass spectrometry (MS), nuclear magnetic resonance (NMR), or a combination of these methods.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Method of Characterization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chalconaringenin</td>
<td>LC-MS, NMR</td>
<td>[80-82]</td>
</tr>
<tr>
<td>Naringenin</td>
<td>LC-MS</td>
<td>[79, 83]</td>
</tr>
<tr>
<td>Naringenin 7-rutinoside</td>
<td>LC-MS</td>
<td>[84]</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>LC-MS</td>
<td>[61, 65, 85, 86]</td>
</tr>
<tr>
<td>Kaempferol 3-glucoside</td>
<td>LC-MS</td>
<td>[80, 86]</td>
</tr>
<tr>
<td>Kaempferol 3-O-rutinoside</td>
<td>LC-MS, NMR</td>
<td>[87]</td>
</tr>
<tr>
<td>Quercetin</td>
<td>LC-MS</td>
<td>[57, 85, 87-90]</td>
</tr>
<tr>
<td>Quercetin 3-rhamnoside (Quercitrin)</td>
<td>LC-MS</td>
<td>[80, 91]</td>
</tr>
<tr>
<td>Quercetin 3-glucoside (Isoquercitrin)</td>
<td>LC-MS</td>
<td>[91, 92]</td>
</tr>
<tr>
<td>Quercetin 3-O-rutinoside</td>
<td>MS, NMR</td>
<td>[61, 75, 77, 79, 84, 85, 88]</td>
</tr>
<tr>
<td>Compound</td>
<td>Method</td>
<td>References</td>
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<td>--------------------------------</td>
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</tr>
<tr>
<td>Rutin</td>
<td>LC-MS</td>
<td>[93-95]</td>
</tr>
<tr>
<td>Myricetin</td>
<td>LC-MS</td>
<td>[93-95]</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>HPLC</td>
<td>[96]</td>
</tr>
<tr>
<td>Hydroxybenzoic acid hexose</td>
<td>LC-MS</td>
<td>[82]</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>HPLC</td>
<td>[97, 98]</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>HPLC</td>
<td>[98]</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>HPLC</td>
<td>[57, 77, 86, 89, 99]</td>
</tr>
<tr>
<td>m-Coumaric acid</td>
<td>HPLC</td>
<td>[100]</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>HPLC</td>
<td>[90, 101, 102]</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>HPLC</td>
<td>[96, 97]</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>HPLC</td>
<td>[43, 88, 89]</td>
</tr>
<tr>
<td>Chlorogenic acid (3-O-caffeoylquinic acid)</td>
<td>LC-MS</td>
<td>[20, 56, 77, 78, 81, 94]</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>HPLC</td>
<td>[50, 61, 75, 89]</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>LC-MS</td>
<td>[91, 97, 103]</td>
</tr>
</tbody>
</table>

1.1.3 Carotenoid Compounds in Tomatoes

1.1.3.1 Carotenoids Classification, Chemical Structure and Occurrence (factors affecting biosynthesis of carotenoids, genetics vs. environment vs. processing)

Carotenoids are lipid-soluble pigments naturally synthesized by all photosynthetic organisms, such as phytoplankton, algae, and higher plants; they can also be produced by some bacteria, fungi, and yeast. Carotenoids are composed of two major classes. The first group contains highly unsaturated hydrocarbons, including lycopene and α-, β-, and γ-carotenes that contain only carbon- and hydrogen-atoms but lack oxygen. The other group of carotenoids are xanthophylls, are those that have at least one oxygenated group on their terminal rings, such as in β-cryptoxanthin, lutein, and zeaxanthin [104]. The basic structure of carotenoids is a 40 carbon tetraterpene which is composed of 8 isoprene units, such as found in β-carotene, a symmetrical
The unique chemical structure of carotenoids, a conjugated polyene chain, is responsible for the distinct colors, going from colorless to yellow, orange, red, pink, and blue with an increasing number of conjugated double bonds. The polyene chain not only provides a base for cis-trans isomerization but also accounts for the sensitivity of carotenoids towards oxidizing reagents, strong acids, heat and light [105]. Thus, extraction of carotenoids from plants must be performed in an environment with dim light and also free of strong acids and peroxides. More than 600 carotenoids have so far been identified in the plant kingdom, but only about 40 can be found in the human diet [34]. It is well known that carotenoids contribute to human health due to their provitamin A activity and antioxidant power. Particularly, studies have demonstrated that carotenoids are powerful free radical scavengers due to their capability in quenching singlet oxygen \( ^1\text{O}_2 \), neutralizing sulfenyl radicals and stabilizing peroxyl radicals through the hydrophobic chain of polyene units [35].

**Figure 4. Chemical structure of beta-carotene.**

Factors affecting the total carotenoid content and its relationship with the total antioxidant activity of tomato have been studied widely: genetics, geographic location, environmental conditions, maturity, processing techniques and post-harvest storage conditions have been found to have the most impact [106]. Several studies have shown the instability of carotenoids. For instance, a 6-month storage of a conventionally thermal processed tomato juice at 25 °C, led to a significant decrease in total carotenoids and lycopene by 16% and 12%,
respectively [107]. Another study which investigated the effect of industrial processing on the antioxidant profiles of tomato, and found distinctly reduced levels of lycopene (32%) during the production of tomato paste [108]. Contrasting results have been observed in recent studies. During storage of bottled tomato pulp up to 180 days, no significant reduction of lycopene level was reported by Ordóñez-Santos et al. [64]. Moreover, studies have shown an increase in carotenoid content in processed tomato products compared to raw tomatoes. A study found a significant increase in lycopene content (up to 30%) during the process of producing tomato sauce [109]. Abushita et al. reported the stability of β-carotene and an increase for lycopene (36%) under thermal processing [110].

Lycopene, with a molecular formula \( \text{C}_{40}\text{H}_{56} \), is a key member of the hydrocarbon carotenoids family. It is synthesized by many plants but not by animals or humans [111]. The conjugated polyene structure which gives lycopene deep-red color and the antioxidant properties is represented by an acyclic open-chain consisting of 13 double-bonds. Eleven of them are conjugated, whereas two are non-conjugated double bonds, which enables lycopene to exist in both the \textit{cis} and \textit{trans} isomeric configurations [104]. However, in fresh tomatoes, lycopene is mainly found in the all-\textit{trans} isomeric form, which gives it thermodynamically most stable configuration [112]. Unlike \( \alpha\)- and \( \beta\)-carotene, lycopene is a non-provitamin A carotenoid due to the absence of a \( \beta\)-ionone ring in its acyclic structure [113]. Nevertheless, with a lipophilic characteristic, lycopene is easily soluble in chloroform, benzene, and ether and nearly insoluble in methanol, ethanol and water [114].

1.1.3.2 Carotenoid Content and Profile of Tomato

The most abundant carotenoid in tomato is lycopene, followed by phytoene, phytofluene, \( \zeta\)-carotene, \( \gamma\)-carotene, \( \beta\)-carotene, neurosporene, and lutein [115] (Figure 5). The lycopene
concentration in tomato fruits depends on a variety of factors including environmental conditions, geographic location, climatic situation, species and maturity, but with an average of about 5 to 10 mg lycopene per 100 g fresh tomato [12, 28]. As the most abundant carotenoid in tomato, up to 15 mg lycopene in 100 g fruit has been reported for deep-red tomato varieties, whereas yellow species are less rich in lycopene, with a content of only about 0.5 mg per 100 g [28]. In addition, the tomato skin was recorded for containing five times more lycopene than the tomato pulp [116], suggesting for the nutritional significance of consuming the entire tomato fruit.

β-Carotene, which gives carrots, sweet potatoes, and squashes their orange color, is considered the major dietary precursor of vitamin A. β-Carotene is converted into retinol which is most well-known for its role in vision. Tomato is known as a good source of β-carotene. It is reported that the average amount of β-carotene in regularly grown tomato is in the range of 0.28 to 1 mg/100 g fresh weight (FW, and this value can reach up to 1.2 mg/100 g FW in cherry tomato varieties [93].

Lutein is one of the most prevalent carotenoid synthesized by plants, and like other xanthophylls, it is found in high quantities in green leafy vegetables such as spinach as well as corn, sweet potatoes and tomatoes. It is reported that the average concentration of lutein in regular tomato is 32 µg/100 g FW [117], but can be as high as 800 µg/100 g FW in cherry tomato varieties [118]. Lutein is responsible for the preservation of visual function, particularly in protecting the macular of the human eyes [119], protection against cardiovascular disease via. inhibition of NF-KB signaling [120]. In addition, a study by Herrero-Barbudo et al. showed that lutein consumption is associated with increased DNA resistance to endogenous damage and
repair capacity [121]. However, the role of regular lutein consumption in terms of protection of DNA, protein and lipids from oxidative damage was not supported by other studies [117].

Figure 5. Chemical structures of commonly found carotenoids in tomato
1.2 Tomato Bioactive Compounds and Disease Prevention

1.2.1 Dietary Phenolics as Therapeutic Agents

Recent studies have shown that phenolic compounds are responsible for reduced risk of a number of human diseases including inflammation, cancer, cardiovascular disease, ulcer, diabetes and liver diseases.

As one of the leading causes of the death worldwide, cancer has been mainly treated with synthetic chemotherapeutic methods which are often accompanied by severe side effects. This has led to the search for natural therapeutic agents. Phenolic acids, particularly, exert their anticancer functions by providing anti-oxidant properties to vital cellular components, such as lipids, proteins, and DNA. Moreover, phenolic compounds can inhibit proliferation and stimulate apoptosis of cancer cells. The study found anti-colon cancer potential of ellagic acid as a result of increased the production of ROS, decreased cell proliferation, and induced apoptosis in HCT-15 colon adenocarcinoma cells [122]. Prostate cancer is the second cause of cancer deaths in men in the USA. Ellagic acid, on the other hand, has been shown to increased cell adhesion and exhibit beneficial effect on metastasis of prostate cancer in SCID mouse tumor model [123].

Diabetes mellitus is a metabolic disease characterized by hyperglycemia resulting from impairment in glucose metabolism for a prolonged period. Chronic hyperglycemia of diabetes may cause dysfunction and failure of multiple organs, including the nerves, eyes, kidneys, heart and blood vessels [124]. Natural polyphenol compounds having anti-diabetic properties have been widely studied. Chlorogenic acid has been found to induce glucose uptake via increased expression of GLUT4 and PPAR-\( \gamma \) transcript [125]. \( p \)-Coumaric acid also exerts beneficial effects on metabolic disorders via stimulation of fatty acid \( \beta \)-oxidation and inhibition of oleic acid-induced triglyceride accumulation [126].
Heart and blood vessel disease, also called cardiac disease, is often associated with the formation of diabetes mellitus. Hyperlipidemia, hyperglycemia, and oxidative stress can promote a process called atherosclerosis [127]. Caffeic acid and ellagic acid were also found to have cardiac protective effect demonstrating anti-dyslipidemia, anti-coagulatory, anti-oxidative, and anti-inflammatory properties in cardiac tissues of diabetic mice [128]. Atherosclerosis is a chronic inflammatory disease characterized by a build-up of plaque inside arteries. Gallic acid and was reported to have anti-atherosclerotic function via inhibition of platelet activation and platelet-leukocyte aggregation, whereas protocatechuic aldehyde attenuated atherosclerosis by inhibiting migration and proliferation of vascular smooth muscle cells [129, 130].

Health-promoting effects of polyphenol compounds are mainly due to its significant radical scavenging capacity. In particular, polyphenol resveratrol, and flavonoids such as rutin and quercetin, have been reported to exert intestinal anti-inflammatory activity [131] as well as to inhibit LDL oxidation in human after supplementation [132]. Not only so, anthocyanins, the most abundant group of flavonoid pigments commonly found in highly pigmented fruits and vegetables including blueberry, blackberry, and eggplant, have been recently discovered to be the most potent antioxidants in purple tomato[88]. Studies have revealed that anthocyanins play important roles in protecting against certain cancers, cardiovascular disease and age-related degenerative diseases [133, 134].

1.2.2 Dietary Carotenoids and their Roles in Disease Prevention

Lycopene, the most prevalent carotenoid in tomato, is known for many beneficial health effects. Studies have supported the role of lycopene in preventing cancer [135], osteoporosis [136] through its antioxidant action and down-regulation of pro-inflammatory cytokines [137].
Indeed, lycopene has also been suggested to protect human against cardiovascular disease (CVD) [138] as well. It has been reported that lycopene, a strong radical scavenging antioxidant, synergized with other lipophilic fraction of tomato, such as vitamin E, in its antioxidant actions such as inhibition of LDL oxidation [139]. In addition, lycopene is suggested to have an inhibitory effect on inflammation through down-regulation of pro-inflammatory cytokines such as IL-6 and TNF-alpha. A study by Markovits et al. demonstrated that consumption of tomato-derived lycopene down-regulated the markers of inflammation and oxidative stress in obese patients [137].

β-Carotene is a strong antioxidant known to protect humans from photo-oxidative damages. Studies have shown that consumption of β-carotene may contribute to the inhibition of atherosclerosis and prevention of myocardial infarction [113]. However, the protecting role of β-carotene has been challenged by other studies [140]. A study by Kataja-Tuomola et al. showed that supplements with β-carotene at doses of 20 mg daily for 5-8 years did not have a protective effect on type 2 diabetes in male smokers [141]. In order to fully understand the beneficial effect associated with β-carotene consumption, further trials will be required.

1.2.3 Tomato Consumption and Chronic Diseases

1.2.3.1 Tomato Fruit Consumption and Inflammation, Diabetes, Cardiovascular Disease and Prostate Cancer

Several studies suggest that components of tomato varieties have anti-inflammatory properties to different extents. A purple tomato breeding line, V118 with an accumulation of a small amount of anthocyanins in the skin was bred by introgression of a trait from a wild purple tomato, was found to have stronger antioxidant activities than commercial red tomatoes[88, 101].
In an *in vivo* carrageenan-induced paw oedema rat study, both the purple and red tomato extracts significantly reduced the paw oedema by 7.48–13.08%. Moreover, the purple tomato containing anthocyanins was found to exert higher anti-inflammatory and antioxidant activity in the tissue and, therefore, can potentially provide better protection against oxidative stress [89].

Diabetic patients whose plasma levels of some antioxidants are relatively low, are at increased risk of developing coronary heart disease. High glucose levels induce oxidation of low-density lipoproteins (LDLs) therefore contribute to increased risk of arterial disease. Tomato, a low glycemic index food with a rich source of antioxidants, has been believed to have a protective role in diabetics and cardiovascular diseases. In a randomized control trial, 57 patients with well-controlled type 2 diabetes were randomized to receive supplementation with tomato juice [22]. After a 4-week treatment, plasma lycopene levels were elevated nearly 3-fold and intrinsic resistance of LDL to oxidation was increase by 42%, suggesting that tomato can be a valuable component of a cardioprotective diet. Similarly, Farzad *et al.* concluded that consumption of 200 g raw tomato for 8 weeks produced a significant decrease in serum glucose and blood pressure in type 2 diabetic patients, supporting that tomato might be beneficial for reducing cardiovascular risk associated with type 2 diabetes [23]. However, inconsistent results have been reported in some animal studies which showed a decrease in serum glucose [142, 143]. Not only so, Wang *et al.* found little evidence for a significant association between the risk of type 2 diabetes in women and consumption of tomato-based foods [142].

Prostate cancer is the most common cancer in American men. Because tomatoes are the only vegetable source of lycopene which has been associated with reduced risk of prostate cancer, this food item was of particular interest. A number of case-control studies and prospective studies have been conducted in the past decades in order to evaluate the anti-cancer
properties of tomatoes. However, contradicting results have been obtained. A case-control study involving a total of 932 men (449 black and 483 white) with histologically confirmed prostate cancer showed no statistically significant associations between consumption of tomato products and prostate cancer risk [26]. In a multiethnic case-control study of male subjects who had been diagnosed with prostate cancer, results indicated an inverse but not statistically significant association for cooked tomatoes in African-American and Japanese men but not in the white or Chinese men. Overall, the study did not find an association between raw or cooked tomato intakes and total or advanced prostate cancer risk [25].

A cohort study was conducted based on follow-up questionnaires sent to 47,894 eligible subjects initially free of diagnosed cancer through 1986 to 1992. They reported that tomato sauce and tomatoes have significantly inverse association with prostate cancer risk [144]. After a 6-year follow-up cohort study among 14,000 Seventh-day Adventist men, Mills et al. suggested higher consumption of tomatoes was statistically significantly related to lower risk of prostate cancer [24]. In a randomized placebo-controlled study, thirty-two patients with localized prostate adenocarcinoma were treated with tomato sauce-based entrees for 3 weeks, and their prostate tissue was collected for inspection [145]. The result suggested for increased serum (1.97-fold) and prostate lycopene (2.92-fold) concentrations and a reduction in serum prostate-specific antigen (PSA) concentration (a biomarker of prostate carcinogenesis). The available data suggest that increased consumption of tomatoes and tomato-based products should be prudent when treating cancer patients.

Pancreatic cancer is the 4th leading cause of cancer-related deaths in both men and women in Canada. Survival is not promising, with a 5-year survival rate of 8% in Canada and 5% worldwide. It was estimated that in 2015, 4,800 Canadians would be diagnosed with pancreatic cancer [146]. Substantial evidence suggested that high consumption of vegetables, particularly
tomatoes with high levels of carotenoids, may be associated with reduced pancreatic cancer risk. Potential mechanisms of beneficial effects of carotenoids on pancreatic cancer were suggested, including antioxidant activity, enhancement of immune function, inhibition of cellular proliferation and enhancement of gap junctional intercellular communication which plays an important role in cell growth control and carcinogenesis [147, 148]. A case-control study of 462 histologically diagnosed pancreatic cancer subjects and 4721 population-based controls was carried out across eight Canadian provinces for 5 years via. food frequency questionnaires. The study demonstrated an inverse dose-response relation between the consumption of tomato and tomato-based products and pancreatic cancer in men, suggesting that tomato consumptions may play a role in the prevention of pancreatic cancer [149].

Chronic pancreatitis (CP) is a progressive fibroinflammatory disease that impairs body’s ability to digest food and regulate blood sugar. Patients with exocrine pancreatic deficit are at a greater risk of developing micronutrients deficiencies because of malabsorption [150, 151]. Insufficient absorption of carotenoids in CP patients could contribute to an increased risk of developing pancreatic cancer compared with the general population [29]. A study of serum collected from 25802 volunteers was conducted to evaluate lycopene level in the serum of patients before they develop pancreatic cancer and compare their prediagnostic levels with matched control subjects who had not developed cancer. The result demonstrated a strong association between deficient levels of serum lycopene with pancreatic cancer [30]. An intervention study by Quilliot et al. asked 22 volunteer CP patients who had low serum lycopene concentration to consume 40 g tomato paste (approximately 24 mg lycopene) daily [30]. As a result, despite pancreatic malabsorption syndrome, they observed a significant increase in plasma lycopene levels after daily supplements of tomato products in CP patients. However, no effects
on clinical symptoms were reported by this study due to the nature of the short length of follow-up.

1.3 Antioxidant and Anti-Inflammation Properties of Tomato Bioactive Compounds

1.3.1 Oxidative Stress

The oxidative property of oxygen plays an essential role in various biological process. However, oxygen can also aggravate the damage within the cell by oxidative events. When cells use oxygen to generate energy, and free radicals are formed naturally as by-products of ATP (adenosine triphosphate) generation by the mitochondria. These oxidative forms of molecules are reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox process. A free radical is a molecule possessing one or more unpaired electrons that are alone in an atomic or molecular orbital. Free radicals are formed from molecules via the breakage of a chemical bond such that each fragment keeps one electron, by cleavage of a radical to give another radical and, finally via redox reactions [152]. Free radicals include hydroxyl (OH•), superoxide (O2• −), nitric oxide (NO•), nitrogen dioxide (NO2•), peroxyl (ROO•) and lipid peroxyl (LOO•). Peroxynitrite (ONOO−), hypochlorous acid (HOCl), hydrogen peroxide (H2O2), singlet oxygen (1 O2), ozone (O3), nitrous acid (HNO2) and dinitrogen trioxide (N2O3) are not free radicals and generally called oxidants, but can easily lead to free radical reactions in living organisms [152, 153].

These reactive species play a dual role in human as both toxic and beneficial compounds since they can be either harmful or helpful to the body. At low or moderate levels, reactive species acts beneficially on cellular redox signaling and immune function, but high accumulation of these reactive species could lead to oxidative stress, a harmful process that can damage cell
function and structures. For humans, oxidative stress is a result of disequilibrium between reactive oxygen/nitrogen species generation and the cellular capacity to detoxify these species with antioxidant nutrients and enzymes. Oxidative stress is an important causative factor in the development and progression of chronic and degenerative diseases, including cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases.

ROS and RNS are generated from either endogenous normal cell metabolisms \textit{in situ} or from external sources (pollution, cigarette smoke, radiation, medication). These biological free radicals are highly unstable but also highly reactive due to the presence of an unpaired electron to react with various organic substrates such as lipids, proteins, DNA. For example, hydroxyl radical (HO•) and hydroperoxyl (HO•₂) in excess can inflict direct damage to lipids by a process called lipid peroxidation that generates cytotoxic and mutagenic products [154]. In addition to the damage to cell membranes by way of lipid peroxidation, oxidative stress can lead to structural changes and loss of enzyme activity in proteins, and also the formation of different oxidative DNA lesions which can cause mutations. Fortunately, the body has several mechanisms to counteract these attacks by using DNA repair enzymes and/or antioxidants [152, 154].

1.3.2 Mechanism of Antioxidant Action

The human body has several mechanisms to prevent free radical induced diseases by producing antioxidants, which are either naturally produced \textit{in situ} (endogenous antioxidants), or externally take them from food /or supplements (exogenous antioxidants). Endogenous and exogenous antioxidants act as “free radical scavengers” neutralizing the excess of free radicals,
preventing and repairing damages caused by ROS and RNS, and, therefore, can enhance the immune defense and lower the risk of cancer and degenerative diseases [155-157].

Endogenous compounds in cells are composed of enzymatic antioxidants and non-enzymatic antioxidants. The major antioxidant enzymes directly involved in the neutralization of ROS and RNS are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx). The non-enzymatic antioxidants are divided into metabolic antioxidants and dietary antioxidants. Metabolic antioxidants, which is defined as endogenous antioxidants, are produced by metabolism in the body, including reduced glutathione (GSH), lipid acid, L-arginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, and transferrin. On the other hand, exogenous dietary antioxidants are referred to as the compounds available through dietary intake or supplements. Popular dietary and nutritive antioxidants are vitamin E, vitamin C, carotenoids, flavonoids, trace metals (selenium, manganese, zinc), omega-3 and omega-6 fatty acids [155].

It has been well established that phytochemicals, such as polyphenols and carotenoids, play significant roles in total antioxidant capacity of fruits and vegetables. These antioxidative phytochemicals can be classified based on their mode of action: 1) Free radical scavengers by inhibiting free radical formations; 2) Singlet oxygen quenchers by donating an electron or hydrogen atom; 3) Transition metal chelator by converting reactive metal ions to stable forms [155, 158]. Antioxidant molecules are not all equally powerful in these varied mechanisms. For example, phenolic acids are effective in trapping free radicals but not as good at chelating metals, while flavonoids can do both efficiently i.e. to scavenge free radicals and to chelate metals [158].
1.3.3 Potential Synergy of Phytochemicals in Tomato

1.3.3.1 Definition of Synergistic Interaction

The total antioxidant capacity of a food is the ultimate outcome of antioxidant activities from all the above-discussed exogenous antioxidant compounds potentially found in a particular food. These structurally diverse phytochemicals may possess similar or different, but complementary antioxidant activities, interacting with each other when they are consumed together as a whole food. The interactions among the different components in plant-based food can be synergistic, additive and antagonistic, which may, in turn, alter their physiological impacts.

A synergistic antioxidant interaction refers to the antioxidant effect of two or more components when applied in combination is greater the sum of individual antioxidant effects applied separately. An additive antioxidant effect occurs when the antioxidant effect of combination provides the sum of the individual antioxidant effects. Antagonistic antioxidant interaction, on the other hand, is defined when the total antioxidant effect is less than the mathematical sum which is predicted from individual components [159].

1.3.3.2 Synergistic effect on anti-oxidant and anti-inflammatory activities

In recent years, the synergism between bioactive constituents in food has been increasingly documented because the bioactive compounds in edible plants are not consumed individually, but instead, in the form of natural mixtures. Therefore, approaches from single antioxidant are no longer adequate to assess the pharmacological benefits contributed by the mixture of natural phytochemicals [160]. When ingested as a mixture, phytochemicals in food undergo multifaceted interactions, promoting health benefits and protecting human against disease threatens. Due to
the nature of diverse mechanisms of action that work simultaneously, it is difficult but critical to identify these natural interactions and to elucidate some of the most powerful naturally derived mixtures.

Consumption of tomato powder, but not lycopene alone was reported to inhibit prostate carcinogenesis in a study using rat model, suggesting that the health promoting effects associated with lycopene consumption for modifying prostate carcinogenesis are enhanced through both additive and synergistic interactions with various other tomato carotenoids and phenolics [27]. Furthermore, significant synergistic anti-proliferative effects were discovered when different flavonoids were administrated in combination manner in a mouse liver cancer cell-line study, suggesting that combinations of flavonoids exert a more efficient inhibitory effect against cancer cell growth than individual flavonoids [28]. Therefore, the importance of choosing the best combination of antioxidants should be taken into consideration by food scientists or industry when designing functional foods.

1.3.3.3 Reported Synergistic Interactions between Phenolics, Carotenoids, and Vitamins

The health benefits of tomato are commonly attributed to the antioxidant phytochemicals thereof. However, tomato is also a good source of vitamins A and E, which possess significant antioxidant effects on human health as well. Recently, the roles of phytochemicals, particularly polyphenols and carotenoids in the vitamin antioxidant regeneration network have been discovered [161]. Noticeably, polyphenols have been reported to act synergistically with vitamin C and E due to the unique amphiphilic property. Phenolic compounds are soluble in both aqueous and lipid phases, and thus, are capable of being in contact with both vitamin C and vitamin E, which are water soluble and lipid soluble, respectively. Flavonoids has been reported
to exert synergistic interaction with the chain-breaking antioxidant alpha-tocopherol (vitamin E) via the action of regeneration of alpha-tocopherol [162]. In addition, phenolic acids, such as caffeic acid and ferulic acid have been found to have an indirect synergistic interaction with vitamins C and E in the antioxidant process [40]. While many polyphenols including phenolic acids and flavonoids have been found to be synergists to the vitamin antioxidants, the antioxidant interactions among individual polyphenolic compounds themselves are not well documented.

Phenolic acids, which existed in a mixture of fruit and vegetables, have been reported for their synergistic interactions. It is shown that the major phenolic acids present in 'Ataulfo' mango pulp including gallic, protocatechuic and chlorogenic acids exert synergistic effects on total antioxidant activity, although antagonism was also observed between vanillic acids and other phenolics in the fruit [163].

Carotenoids, on the other hand, have been suggested to act synergistically with vitamins as well. A study by Jacob et al. reported that the consumption of tomato juice (500 ml for 2 weeks) reduced inflammation more effectively when a high dose of vitamin C was administrated. This result suggested that the synergy between lycopene and vitamin C might be the key to release oxidative stress and inflammation [164]. In a cell-based study, the lipophilic extracts obtained from different tomato varieties were examined for their antioxidant activities. Among the two-compound mixtures the highest synergistic effect was detected in α-tocopherol-lycopene mixtures, and the synergism also occurred for lycopene-β-carotene, lycopene-lutein, and lutein-β-carotene mixtures [165].

1.3.4 Chemical-Based Antioxidant Activity Assay

Because antioxidants involve in several mechanisms, a single assay cannot account for all
the different modes of action. As a consequence, over the years, researchers have developed many antioxidant assays which are often used in conjunction to assess the overall antioxidant potency. *In vitro* chemical models are commonly used to assess antioxidant activities of phytochemicals. The ferric reducing antioxidant power assay (FRAP) and oxygen radical absorbance capacity (ORAC) method are the two most frequently used chemical assays for determining the total antioxidant activities of phenolic compounds. The FRAP assay measures the reducing power of antioxidants to reduce ferric-tripryridyl-triazine (Fe3+-TPTZ) complex to the blue colored ferrous form (Fe2+). On the other hand, the ORAC method has been used to assess antioxidant activity of phytochemicals by way of inhibiting free radical oxidation and degradation of a fluorescent compound. Particularly, ORAC for Lipophilic Extracts (ORAC-L) is used for detecting the antioxidant activity of lipophilic fractions in tomato (mainly the carotenoids) and ORAC Assay for Hydrophilic Extracts (ORAC-H), on the other hand, is used for measuring the antioxidant capacity of hydrophilic fraction in tomato (mainly the phenolics).

Another colorimetric method has been recommended for its fast and accurate evaluation of the total antioxidant activity in food and beverage. An organic nitrogen radical known as 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a dark purple-colored, stable free-radical molecule used to measure the reducing ability of antioxidants toward the DPPH radical. The scavenging capacity is measured by monitoring the absorbance decrease at 515-528 nm until the absorbance remains constant.
CHAPTER 2: RATIONALE, HYPOTHESES, AND OBJECTIVES

2.1 Rationale

Oxidative stress, a result of disequilibrium between reactive oxygen/nitrogen species generation and the cellular capacity to detoxify these species with antioxidant nutrients and enzymes, is an important causative factor in the development and progression of chronic and degenerative diseases, including cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases. The human body has several mechanisms to prevent free radical induced diseases by counteracting with antioxidants, which are either naturally produced in situ (endogenous antioxidants), or externally take them from food /or supplements (exogenous antioxidants). In recent decade, a large number of studies has investigated the potential therapeutic activity of dietary phenolic compounds against human diseases, including anti-inflammation, anti-cancer, anti-cardiovascular disease, anti-ulcer, anti-diabetes, anti-liver diseases [123, 124, 133, 134]. Studies have also supported the role of carotenoids, especially lycopene, in preventing cancer [135], osteoporosis [136], and also protecting humans against cardiovascular disease (CVD) [138].

Tomato, a rich source of bioactive antioxidants including polyphenols, carotenoids, vitamin A, E, and C, has been well known for its health-promoting effect due to the significant antioxidant activities contributed by these phytochemicals. Emerging evidence suggests that natural interventions, such as consumption of lycopene/flavonoids alone or in the form of tomato fruit/ tomato products, can reduce the risk of certain cancers, epidermal diseases and cardiovascular diseases [14, 20, 28, 31, 32, 142, 144, 145, 166, 167]. Therefore, evaluations of
phytochemical content and antioxidant activities in commercial tomato and purple tomato varieties have been the focus of several recent studies [14, 86, 88, 101]. Nevertheless, no particular research has been conducted to investigate the phytochemical profiles and antioxidant activities of selected specialty tomatoes which are not widely available in the market and commonly consumed. Information on the bioaccessibility of and bioavailability of phenolics or carotenoids of specialty tomato varieties are also not available. The unique color, size and flavor of these specialty tomatoes have made them highly demanded in recent years, and there is a niche market for increased production. The health benefits of these tomatoes as compared to the conventionally tomato cultivars are not known, therefore there is an urgent need to characterize the bioactive components in these tomatoes.

2.2 Hypotheses

The overall hypothesis of this thesis is that specialty tomatoes may possess different phytochemical compositions in comparison with commercial red tomato varieties, as well as the purple tomatoes that have been previously studied by our group. The antioxidant activity, bioaccessibility and bioavailability of the phytochemicals may also be different in these specialty tomatoes. These differences can potentially lead to distinct health benefits in humans when such tomatoes are consumed.

2.3 Objectives

1. To characterise and identify the phenolic and carotenoid compounds in extracts of specialty tomato cultivars and to assess their contribution the potential health benefits by chemical and cellular antioxidant assays.

2. To assess the bioaccessibility of phenolic compounds in selected tomato cultivars using \textit{in vitro} gastrointestinal digestion model
CHAPTER 3: COMPOSITION AND ANTIOXIDANT ACTIVITIES OF PHENOLIC COMPOUNDS OF SPECIALTY TOMATOES

3.1 Introduction

Tomatoes contain a variety of antioxidant phytochemicals that have potential health-beneficial effects on humans. Although commonly consumed tomatoes are best known for their rich content of lycopene, a carotenoid known for many beneficial health effects, increasing number of studies has reported a significant antioxidant role of phenolics in protecting humans from cancer and coronary heart disease through their antioxidant potential [36]. In the past decade, increasing efforts have been made for the characterization of polyphenols in commercial tomato fruits. [78, 104, 110, 113, 168, 169]. Nevertheless, for certain uncommon tomato varieties, identities of some phenolic compounds and their contribution to the total antioxidant activity are not clearly elucidated. Our previous study assessed phytochemical compositions and antioxidant activities of a newly developed nongenetically modified purple tomato V118, which was found to possess significantly higher antioxidant and anti-inflammatory potentials compared to commercial tomatoes [89, 170]. Eight phenolic compounds commonly found in other tomatoes were identified in V118, including protocatechuic acid, chlorogenic acid, gentistic acid, caffeic acid, \( p \)-coumaric acid, ferulic acid, rutin and naringenin. Noticeably, an unknown peak which was found in other varieties of tomato, was detected in the purple tomato V118 as well [171]. These findings have led to our interest in investigating the phenolic composition and antioxidant activities of the specialty tomato cultivars. The objective of this chapter is to measure the total phenolic content of the hydrophilic fractions of 28 specialty tomato cultivars using the Folin-Ciocalteu method, assess their antioxidant activity by antioxidant assays: DPPH, ORAC-H
and FRAP assay, and analyze their phenolic composition by using HPLC-DAD. Our hypothesis is that these specialty tomatoes may contain higher content of total phenolic compounds as well as a unique profile of phenolics in comparison with that of commercial tomato varieties, or even the purple tomato variety. Such unique phenolic composition may lead to different antioxidant activities as well.

3.2 Materials and Methods

3.2.1 Tomato Varieties and Preparation of Tomato Samples

The specialty tomato varieties used in this study were grown at the Greenhouse and Processing Crops Research Centre, Agriculture and Agri-Food (AAFC), Harrow, Ontario. Nutrient feedings and climate settings were the same as commercial practice. Cultivars were planted in October, 2014 and were harvested from December 2 to 19, 2014 at commercial maturity. The whole tomatoes were washed, cut into pieces and ground with a commercial blender. The pulp was immediately freeze-dried and ground into fine powder, and stored in polyethylene tubes at -80°C before analysis.

3.2.2 Phenolic Extraction

Hydrophilic phytochemicals, mainly phenolic compounds, were extracted from the dried powder of the whole fruit of specialty tomatoes with acidified aqueous methanol. In brief, the freeze-dried tomato powder (0.3g) was accurately weighed and transferred into a 15 mL polypropylene tube and mixed with 15 mL 80% methanol containing 0.1% HCl (v/v). The extraction was carried out on a rotary shaker (Scientific Industries Inc., Bohemia, NY, USA) at 400 rpm for 4 hs, at room temperature followed by 15 min ultra-sonication (VWR, Mississauga, Ontario, Canada). The mixture was centrifuged at 6000 rpm for 5 min (Eppendorf centrifuge
The supernatant was collected, and the extraction was repeated three times. The supernatants were combined, topped up to 15 mL and used as the crude extract for further studies on the different bioactive component. All procedures were performed in dim lighting to avoid light-induced changes. Samples were extracted in triplicate.

3.2.3 Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) of the extract was estimated using the Folin-Ciocalteu assay with modifications [172]. Briefly, 25 μL of gallic acid standard and tomato extracts were transferred into the 96-well microplates, mixed with 125 μL of Folin-Ciocalteu reagent (diluted 10x) and allowed to react for 10 min at room temperature. Then 125 μL of 7.5%(w/v) sodium carbonate (Na$_2$CO$_3$) solution was added and allowed to incubate for 30 min at room temperature before the absorbance of the reaction mixture was read at 765 nm using a visible-UV microplate kinetic reader (EL 340, Bio-Tek Instruments Inc., Winooski, VT, USA) The results were expressed in milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g DW). All samples were tested in triplicate.

3.2.4 Antioxidant Activities of Phenolic Extracts by Chemical-based Assays

3.2.4.1 DPPH Assay of Hydrophilic Extracts

The radical scavenging activity of the extracts was assessed spectrophotometrically in a UV–Vis plate reader by monitoring the disappearance of DPPH at 517 nm, according to a described procedure of Li et al. [170]. Briefly, 25 μL of appropriately diluted samples or trolox
solutions (62.5, 125, 250, 500, 750, and 1000 μM) were added to 200 μL of DPPH solution (350 μM, dissolved in methanol) in a well of 96-well plate. The mixture was allowed to react at room temperature in the dark for 4 h at which time the absorbance was recorded at 517 nm. The DPPH radical scavenging activity was expressed as micromoles of trolox equivalents (TE) per gram of sample (μmol TE/g).

3.2.4.2 ORAC-H Assay

The ORAC assay for hydrophilic compounds (ORAC-H) of tomato was conducted according to reported protocols with slight modifications [101]. Trolox was used as the standard and fluorescein as the fluorescent probe. A stock solution of fluorescein (Sigma Aldrich, St. Louis, MO, USA) (144.65 x 10^{-3} mM) was prepared by dissolving 4.8 mg of fluorescein in 100 mL phosphate buffer (75 mM, pH7.4). The working fluorescein solution (8.68 x 10^{-6} mM) was prepared daily by diluting 9 μL of the stock solution to 14.991 mL of phosphate buffer. The AAPH (2,2’-Azobis(2-amidinopropane) dihydrochloride) (Sigma-Aldrich) radical solution used as a peroxyl radical generator (153 mM) was prepared daily by taking 414 mg of AAPH and making it up to 10 mL with 75 mM phosphate buffer. As the ORAC assay is extremely sensitive, the samples must be diluted appropriately before analysis to avoid interference. In this study, the crude phenolic extracts were diluted X50 with phosphate buffer.

To each well of a polystyrene 96-well microplate (Greiner Bio-One GmbH, Frickenhausen, Germany), 25 μL of an appropriately diluted sample, blank or a series of standards of trolox solutions (6.25, 12.5, 25, 50 and 100 μmol/L) were added and mixed with 150 μL of working fluorescein solution (8.68x10^{-5} mM in phosphate buffer, pH 7.4), and incubated for 30 min at 37 °C. Subsequently, 25 μL of AAPH (153 mM in phosphate buffer) was
added to each well to initiate the reaction. The fluorescence (excitation/emission wavelength = 485/528 nm) was read every minute for 120 min or until it reached zero in a fluorescence plate reader equipped with an automatic thermostatic holder (PLX 800, Bio-Tek Instruments, Inc.). A calibration curve was constructed daily by plotting the calculated differences of the area under the fluorescein decay curve between the blank and the sample. The result values were expressed as μmol of Trolox equivalent per gram of dry weight tomato sample (μmol TE/g).

3.2.4.3 FRAP Assay of Hydrophilic Extracts

The ferric reducing antioxidant power (FRAP) assay was determined according to Li at el. [173]. The FRAP assay measures the ability of the antioxidants in tomato extracts to reduce the ferric-tripyrindyl-triazine (Fe³⁺-TPTZ) complex to the blue colored ferrous form (Fe²⁺) that absorbs light at 593 nm. Briefly, 10 μL of standards (62.5, 125, 250, 500, 750, and 1000 μM) or sample extract were added and mixed with 300 μL of ferric-TPTZ reagent (prepared by mixing 300 mM acetate buffer, pH 3.6; 10 mM TPTZ in 40 mM HCl; and 20 mM FeCl₃•6H₂O at a ratio of 10:1:1 (v/v/v)). The plate was allowed to incubate at room temperature for 120 min and the absorbance readings were taken at 593 nm against the reagent blank using the aforementioned microplate kinetic reader. The antioxidant activity was expressed as micromole ascorbic acid equivalents (AAE) per gram dry weight tomato (μmol AAE/g DW).

3.2.5 HPLC–DAD Analysis of Phenolic Compounds

HPLC analysis was performed using an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) system consisted of a degasser, a quaternary gradient pump, a thermostatted autosampler and a diode array detector (DAD), and the ChemStation software. Separation was in a Kinetex XB-C18 column (100 mm x 4.6 mm, 2.6 μm) (Phenomenex Inc., Torrance, CA). The
binary mobile phase consisted of 5% formic acid in water (v/v) (solvent A) and 95% methanol mixed with 5% acetonitrile (v/v) (solvent B). Injection volume was 7 μL and flow rate was kept at 0.7 mL/min for a total run time of 50 min. The solvent gradient was as follows: 0–40 min, 0–80% B; 40–42 min, 80–100% B; 42–44 min, 100% B; 44–44.5 min, 100–0%B. Peaks were monitored at 280 nm for phenolic acids and 360 nm for flavonols. Phenolic compounds were identified by comparing retention time and UV absorption spectra with available external standards and confirmed by LC–MS. Compounds with no standard reference materials were tentatively identified by UV spectrum, MS data and by matching with published data.

3.2.6 Mass Spectrometry Analysis of Phenolic Compounds

Mass spectra were obtained using a Dionex UHPLC UltiMate 3000 LC interfaced to a 42rotoc SL ion trap mass spectrometer (Bruker Daltonics, Billerica, MA, USA), following separation on a C18 column (Agilent Poroshell 120, 2.7 μm particle size, 150 x 4.6 mm, Santa Clara, CA, USA). The initial mobile phase condition was 2% acetonitrile in 0.1% formic acid. The gradient went to 98% acetonitrile in 0.1% formic acid in 30 min. The flow rate was maintained at 0.4 mL/min. The mass spectrometer electrospray capillary voltage was maintained at 4.5 kV and the drying temperature at 220 °C. The drying gas flow rate was set to 10 L/min with nebulizer pressure at 40 psi. Nitrogen was used as both nebulising and drying gas; helium was used as collision gas at 60 psi. For the monitoring of phenolic compounds, the mass-to-charge ratio was scanned across the m/z range 100–1500 in enhanced resolution negative-ion auto MS/MS mode. The smart parameter setting (SPS) was used to automatically optimize the trap drive level for precursor ions. The instrument was externally calibrated with the ESI (TuneMix, Agilent).
3.2.7 Statistical Analysis

All assays or tests were conducted in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad software (San Diego, CA, USA) and SPSS (Version 18.0, Chicago, IL). The statistical significance of the data was determined using one-way ANOVA followed by Duncan’s multiple-comparison test with a p<0.05 taken as value of significance.

3.3 Results and Discussion

3.3.1 Total Phenolic Content

The phenolic contents of the twenty-eight specialty tomato varieties are summarized in Table 2. TPC varied significantly among tested tomatoes, ranging from 3.05 to 7.12 mg GAE/g DW (Figure 6). Cultivar Cuban Yellow Grape had the highest TPC at 7.12 mg GAE/g DW followed by Kellog’s Breakfast and Snow White at 6.17 and 5.82 mg GAE/g DW, respectively. The phenolic content of these three cultivars: Cuban Yellow Grape, Kellog’s Breakfast and Snow White are slightly higher than that of common tomato varieties (from 2.90 to 5.00 mg GAE/g DW) [99], but similar to what has been previously reported for purple cultivars (6.59 mg GAE/g DW) [89]. The difference in TPC could be attributed to mainly the genotype variations of the tested tomatoes, as all of these were grown under the same environmental conditions.
Figure 6. Total phenolic content of the hydrophilic extracts of 28 specialty tomato cultivars. TPC, total phenolic contents, values are expressed as mg GAE/g DW tomato; values are means ± SD, n = 3, values followed by the same letter in the same assay are not significantly different (p < 0.05).
Table 2. Total phenolic content (TPC) and antioxidant activities measured by DPPH, ORAC and FRAP.

<table>
<thead>
<tr>
<th>Tomato Varieties</th>
<th>TPC (mg GAE/g DW)</th>
<th>DPPH (μmol TE/g DW)</th>
<th>ORAC (μmol TE/g DW)</th>
<th>FRAP (μmol AAE/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chenkee Chocohlate</td>
<td>4.68±0.15jkl</td>
<td>30.34±0.57defg</td>
<td>86.41±3.00hi</td>
<td>22.97±0.82h</td>
</tr>
<tr>
<td>Grighthires Pride</td>
<td>4.11±0.10fg</td>
<td>26.79±0.55bcde</td>
<td>80.34±4.56efghi</td>
<td>18.60±0.64bc</td>
</tr>
<tr>
<td>Kellog's Breakfast</td>
<td>6.17±0.08q</td>
<td>31.48±1.08fgghi</td>
<td>88.35±7.20i</td>
<td>25.76±0.25jk</td>
</tr>
<tr>
<td>Straberry Organic</td>
<td>3.57±0.11c</td>
<td>32.23±0.64gh</td>
<td>78.63±1.85defghi</td>
<td>21.11±0.67efg</td>
</tr>
<tr>
<td>Black</td>
<td>5.07±0.08n</td>
<td>29.39±0.94cdefg</td>
<td>77.02±2.89defghi</td>
<td>22.45±0.24gh</td>
</tr>
<tr>
<td>Lemon Boy Hybrid</td>
<td>3.93±0.07ef</td>
<td>129.11±5.17</td>
<td>359.47±15.82k</td>
<td>52.60±1.64l</td>
</tr>
<tr>
<td>Cuban Yellow Grape</td>
<td>7.12±0.17r</td>
<td>129.47±9.15</td>
<td>375.71±15.33l</td>
<td>59.67±3.57m</td>
</tr>
<tr>
<td>Black Zebra</td>
<td>4.46±0.26ij</td>
<td>35.64±5.67i</td>
<td>102.98±6.25j</td>
<td>20.05±0.69cde</td>
</tr>
<tr>
<td>Bull's Heart</td>
<td>4.47±0.05ij</td>
<td>28.43±1.22cdefg</td>
<td>104.29±4.09j</td>
<td>19.19±0.59bcd</td>
</tr>
<tr>
<td>Japanese Trifele Black</td>
<td>4.18±0.05fgh</td>
<td>28.46±0.42cdefg</td>
<td>63.80±0.85bc</td>
<td>19.98±0.59bcd</td>
</tr>
<tr>
<td>Black Cherry</td>
<td>4.51±0.08ijk</td>
<td>29.27±0.78cdefg</td>
<td>68.67±6.02bcdefg</td>
<td>20.45±0.83def</td>
</tr>
<tr>
<td>Orange Russian</td>
<td>4.73±0.07klm</td>
<td>30.56±0.68efgh</td>
<td>58.33±7.83b</td>
<td>22.07±0.44fgh</td>
</tr>
<tr>
<td>Cherokee Purple Organic</td>
<td>4.15±0.05fgh</td>
<td>27.27±0.35bcde</td>
<td>70.87±2.50bcdefg</td>
<td>19.46±0.06bcd</td>
</tr>
<tr>
<td>White Beauty</td>
<td>4.83±0.11lmn</td>
<td>34.24±0.41hi</td>
<td>65.75±4.23bcde</td>
<td>25.44±0.17jk</td>
</tr>
<tr>
<td>Sun Sugar Hybrid</td>
<td>5.32±0.23o</td>
<td>43.47±2.47</td>
<td>81.05±9.83fghi</td>
<td>26.51±1.20k</td>
</tr>
<tr>
<td>Mint Jalep</td>
<td>3.83±0.07de</td>
<td>25.87±0.54bcde</td>
<td>41.26±14.31a</td>
<td>14.56±0.05a</td>
</tr>
<tr>
<td>Black Pear</td>
<td>4.21±0.10gh</td>
<td>31.46±0.44fghi</td>
<td>60.46±13.53b</td>
<td>19.20±0.26bcd</td>
</tr>
<tr>
<td>Sweet baby girl hybrid</td>
<td>4.61±0.24ijkl</td>
<td>32.64±1.86gh</td>
<td>105.03±9.65j</td>
<td>25.25±0.33jk</td>
</tr>
<tr>
<td>Super Sweet 100 hybrid</td>
<td>3.83±0.23de</td>
<td>28.49±0.61cdefg</td>
<td>112.83±3.84j</td>
<td>23.10±0.18hi</td>
</tr>
<tr>
<td>Yellow Pear Organic</td>
<td>3.38±0.14bc</td>
<td>22.97±0.69a</td>
<td>84.62±2.86ghi</td>
<td>18.26±0.35b</td>
</tr>
<tr>
<td>Copia</td>
<td>4.95±0.08mn</td>
<td>22.81±0.57a</td>
<td>77.49±3.00cdefghi</td>
<td>19.98±0.51bcd</td>
</tr>
<tr>
<td>Green Zebra</td>
<td>4.96±0.10mn</td>
<td>24.82±0.45ab</td>
<td>76.86±3.90cdefghi</td>
<td>19.92±0.16bcd</td>
</tr>
<tr>
<td>TT80154</td>
<td>3.05±0.25a</td>
<td>32.70±1.24gh</td>
<td>64.07±10.40bcdf</td>
<td>22.41±0.03gh</td>
</tr>
<tr>
<td>Italian Red Pear</td>
<td>3.61±0.09cd</td>
<td>21.12±0.46</td>
<td>72.40±2.52bcdefg</td>
<td>18.61±0.43bc</td>
</tr>
<tr>
<td>Juliet hybrid</td>
<td>4.38±0.07hi</td>
<td>25.73±0.89bcde</td>
<td>71.24±2.68bcdefg</td>
<td>20.15±0.43cde</td>
</tr>
<tr>
<td>Esteria hybrid Organic</td>
<td>3.06±0.13a</td>
<td>38.14±0.68</td>
<td>77.50±2.88cdefghi</td>
<td>23.15±0.89hi</td>
</tr>
<tr>
<td>Snow white</td>
<td>5.82±0.13p</td>
<td>44.27±1.12</td>
<td>88.87±3.35i</td>
<td>24.63±0.22ij</td>
</tr>
<tr>
<td>Brand sweet plum</td>
<td>3.16±0.16ab</td>
<td>28.71±1.30cdefg</td>
<td>79.06±4.17efghi</td>
<td>22.06±0.76fgh</td>
</tr>
</tbody>
</table>
3.3.2 Antioxidant Activities of the Hydrophilic Extracts

Natural phenolic compounds widely distributed in food plants are considered to exert their beneficial health effects mainly through their antioxidant activities. Different methods were used to evaluate the antioxidant activity of the specialty tomatoes, as no single chemical assay can accurately evaluate the contribution of the hydrophilic components to the total antioxidant action of the plant food. In this study, the antioxidant activities of the phenolic fractions were evaluated using three common chemical antioxidant assays, i.e. DPPH, FRAP, ORAC assays, and were found to vary widely and significantly among 28 tested tomatoes (Table 2). As shown, the antioxidant activity as assessed by the DPPH assay ranged from 21.12 to 129.47 μmol TE/g DW, the FRAP value which is a measurement of the reducing power ranged from 14.56 to 59.67 AAE/g DW. The ORAC values of the tomato extracts varied most significantly, from 41.26 to 375.71 μmol TE/g DW (Figure 7).

Distinctively high antioxidant activities were found in cultivar Cuban Yellow Grape followed by Lemon Boy Hybrid for all three antioxidant assays. The ORAC-H value for the Cuban Yellow Grape was 375.71 μmol TE/g DW, which is distinctively higher than that of the ORAC-H values of the purple tomato (323.23 μmol TE/g DW) and 2.5-folds higher than that of the traditional tomato cultivar San Marzano (140 μmol TE/g DW) [174]. The FRAP value of Cuban Yellow Grape was 59.67 μmol AAE/g DW, which is significantly higher than what has been reported for other tomatoes (48.6 μmol AAE/g DW) [175] as well as for the purple tomato V118 previously reported by our group (54.95 μmol AAE/g DW) [101]. Attempts were made to compare the DPPH value of our samples with other commercial tomato and V118 purple tomatoes [89, 176, 177]. However, in our work, DPPH was
calculated as micromoles of trolox equivalents (TE) per gram of sample ($\mu$molTE/g), whereas percent scavenging (%) was used in other studies.
Figure 7. Antioxidant activities of the lipophilic extracts of 28 specialty tomato cultivars. (A) DPPH assay, values are expressed as μ mol TE/g DW tomato; (B) ORAC assay, values are expressed as μ mol TE/g DW tomato; (C) FRAP assay, values are expressed as μ mol TE/g DW tomato; values are means ± SD, n = 3, values followed by the same letter in the same assay are not significantly different (p < 0.05).

3.3.3 Correlation Coefficient Between Total Phenolic Contents and Antioxidant Activities

In order to assess the contribution of total phenolic content to the antioxidant activity, attempts were made to analyse the correlation between the antioxidant activities (DPPH, FRAP and ORAC) and phenolic contents (TPC) using the Pearson’s correlation coefficient (r). The antioxidant activities of the phenolic fractions showed poorly positive but significant correlation with TPC, with the correlation coefficient r values 0.3779, 0.4665 and 0.3671, respectively (p < 0.05). However, the Cuban Yellow Grape was the only one consistently showed both highest TPC and antioxidant activities.
3.3.4 HPLC and Mass Spectrometry Profiles of Phytochemical Antioxidants

Some major phenolic compounds commonly found in tomatoes were identified in the selected specialty tomato cultivars with high TPC by matching the retention times, UV-visible spectra, and mass spectrometric data with those of the corresponding standards. For example, in cultivar Lemon Boy Hybrid which had previously exhibited high antioxidant activities, gentisic acid (peak1), chlorogenic acid (peak 2), ferulic acid (peak4), rutin (peak6) and naringenin (peak8) were identified comparing their retention time and UV spectra (Figure 8) with those of the corresponding commercial standards and the results were confirmed by MS.

In contrast, phenolic acids that have been commonly found in tomato cultivars, such as protocatechuic acid, caffeic acid, p-coumaric acid, quercetin and kaempherol [101, 178] were not detected in any of the selected specialty tomatoes. Many peaks were not able to be identified in this study due to limited time and resources. Particularly, unknown peaks uk1 (at 13.072 min), uk2 (at 19.180 min) and uk3 (at 23.188 min) which were found in all three selected cultivars, but did not match with any known phenolics commonly found in tomato. Identification of these compounds and their contribution to the antioxidant activities are recommended for future studies.
Figure 8. Chromatographic profiles of phenolics in cultivar Lemon Boy Hybrid (A), Cuban Yellow Grape (B), Sun Sugar Hybrid (C) and mix standard (D) at 360 nm. Peaks: 1=gentisic acid; 2=chlorogenic acid; 3=p-coumaric acid; 4=ferulic acid; 5=3,5-dimethoxy-4-hydroxycinnamic acid; 6=rutin; 7=quercetin; 8=naringenin; 9=kaempferol
4.1 Introduction

Like in many other plants, carotenoids are the most vital colored phytochemicals that accumulate as secondary metabolites in the chromoplasts, providing distinct red, pink, orange and yellow color of tomato[32, 110]. The most abundant carotenoid in tomato is lycopene, followed by phytoene, phytofluene, ζ-carotene, γ-carotene, β-carotene, neurosporene, and lutein [115]. The lycopene concentration in tomato fruits shows a great variability, depending on environmental conditions, geographic location, climatic situation, species, and maturity, but with an average of about 5 to 10 mg lycopene per 100 g fresh tomato [12, 28]. For humans, oxidative stress due to the imbalance between reactive oxygen/nitrogen species generation and the cellular capacity to detoxify these species with antioxidant compounds and enzymes, plays a critical role in the development and progression of several human chronic diseases, including cardiovascular disease and cancer [164, 179]. Carotenoids are good antioxidant candidates that reduce oxidative DNA damage, lipid peroxidation and cholesterol synthesis [180], and also modulate inflammation responses through down-regulation of pro-inflammatory cytokines[137]. In this chapter, we have selected specialty tomatoes, and analyzed the contents of total carotenoids, and how they contribute to the antioxidant activities using chemical-based models including 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorption capacity(ORAC) assays.
4.2 Materials and Methods

4.2.1 Tomato Varieties and Preparation of Tomato Samples

Specialty tomatoes studied were the same 28 cultivars as stated above in Section 3.2.1.

4.2.2 Carotenoid Extraction

The extraction of lipophilic phytochemicals was conducted under the same protocol as those described above (Section 3.2.2) except a different solvent acetone/ethanol (1:1, v/v) was used to extract the samples.

4.2.3 Determination of Total Carotenoid Content (TCC)

The total carotenoid contents (TCC) in the tomato samples were determined according to the method of Ndolo et al. with some modification [181]. The absorbance was measured at 450 nm using a UV–Vis plate reader (EL 340; Bio-Tek Instruments Inc., Winooski, VT). Total carotenoid content (TCC) was calculated using the following equation and expressed as μg β-carotene equivalent/g sample:

\[
C = \frac{V \times A}{S \times W} [\mu g/g]
\]

Where A is the absorbance reading at 452 nm, S is the regression coefficient (the number that expresses the relationship which is created based on the concentration of β-carotene standard solutions in μg/mL and the absorbance), V is the total volume of extract, W is the sample dry weight. All procedures were performed in dim lighting to avoid light-induced changes.
4.2.4 Carotenoid Antioxidant Activities by Chemical-Based Assays

4.2.4.1 DPPH Assay of Hydrophobic Extracts

The protocol of DPPH assay of hydrophobic extracts followed as same as the protocol for hydrophilic extracts (Section 3.2.5).

4.2.4.2 ORAC-L Assay

The ORAC assay for the lipophilic extract of tomato (ORAC-L) was conducted according to Li et al. with slight modifications [101]. The protocols were the same as those described above for the ORAC-H assay except that a different solvent i.e. 7% randomly methylated β-cyclodextrin (RMCD) instead of 75 mM phosphate buffer was used to dissolve the Trolox and samples, to ensure the solubility of the lipophilic antioxidants in the reaction mixture. The 7% RMCD solvent was made in a 50% acetone-water mixture (v/v) and was shaken for 1 h at room temperature on an orbital shaker at 200 rpm. The sample solution was ready for analysis after further dilution with 7% RMCD.

4.2.5 Statistical Analysis

All assays or tests were conducted in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical analysis was carried out using GraphPad software (San Diego, CA, USA) and SPSS (Version 18.0, Chicago, IL). The statistical significance of the data was determined using one-way ANOVA followed by Duncan’s multiple-comparison test with a $p<0.05$ taken as value of significance.
4.3 Results and Discussion

4.3.1 Total Carotenoid Content

Carotenoids are the predominant antioxidant phytochemicals of the lipophilic extract which may also contain other bioactive compounds such as vitamin E [20]. Carotenoids commonly found in tomato are reported as lycopene (most abundant), followed by phytoene, phytofluene, ζ-carotene, γ-carotene, β-carotene, neurosporene, and lutein [115], which act synergistically in exerting the antioxidant potential. Carotenoids were, therefore, the focus of the present study as tomato cultivars with different carotenoid compositions were found to affect the antioxidant potential differently. The total carotenoid contents of the 28 specialty tomatoes were analyzed and the results are summarized in Table 3. TCC varied from 36.68 to 541.13 μg/g DW (Figure 2), which showed significantly wider range than what have been reported by other research groups (from 120.50 to 278.00 μg/g DW) [20, 168]. Particularly, Cultivar Brand Sweet Plum had the highest total carotenoid content (541.13 μg/g DW) which was 2-fold higher than the commercial red tomato variety and a purple tomato V118 [168], followed by cultivar Snow White (529.23 μg/g DW) and Esteria Hybrid Organic (389.65 μg/g DW) (Figure 9).
Figure 9. Total carotenoid content in hydrophobic extracts of 28 specialty tomato cultivars. TCC, total carotenoid contents, values are expressed as µg/g DW tomato; values are means ± SD, n = 3, values followed by the same letter in the same assay are not significantly different (p < 0.05).
Table 3. Total carotenoid content (TCC) and antioxidant activities measured by DPPH and ORAC.

<table>
<thead>
<tr>
<th>Tomato Varieties</th>
<th>TCC µg/g DW</th>
<th>DPPH (µmol TE/g DW)</th>
<th>ORAC (µmol TE/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chenkee Chocoholate</td>
<td>389.65±11.05o</td>
<td>6.28±0.08bcd</td>
<td>54.20±5.67hij</td>
</tr>
<tr>
<td>Grighthires Pride</td>
<td>529.23±12.13p</td>
<td>7.99±0.24hi</td>
<td>58.62±3.04ijk</td>
</tr>
<tr>
<td>Kellog’s Breakfast</td>
<td>128.13±1.68efg</td>
<td>7.28±0.22fg</td>
<td>53.27±2.92hij</td>
</tr>
<tr>
<td>Straberry Organic</td>
<td>85.69±3.06c</td>
<td>6.81±0.26def</td>
<td>45.35±1.47defgh</td>
</tr>
<tr>
<td>Black</td>
<td>541.13±21.49q</td>
<td>9.08±0.63j</td>
<td>49.38±3.77efghi</td>
</tr>
<tr>
<td>Lemon Boy Hybrid</td>
<td>81.67±1.19c</td>
<td>11.01±1.00m</td>
<td>66.91±2.34k</td>
</tr>
<tr>
<td>Cuban Yellow Grape</td>
<td>141.93±2.73hi</td>
<td>14.31±0.12o</td>
<td>49.33±6.59efghi</td>
</tr>
<tr>
<td>Black Zebra</td>
<td>352.99±16.71n</td>
<td>9.78±0.27kl</td>
<td>48.63±1.85efghi</td>
</tr>
<tr>
<td>Bull’s Heart</td>
<td>121.58±2.24de</td>
<td>5.86±0.16b</td>
<td>50.06±1.42efghi</td>
</tr>
<tr>
<td>Japanese Trifile Black</td>
<td>239.62±4.79l</td>
<td>7.08±0.31efg</td>
<td>51.92±3.84ghi</td>
</tr>
<tr>
<td>Black Cherry</td>
<td>285.29±0.61m</td>
<td>6.62±0.52cde</td>
<td>49.58±3.38efghi</td>
</tr>
<tr>
<td>Orange Russian</td>
<td>150.99±1.52i</td>
<td>4.66±0.19a</td>
<td>68.78±9.10k</td>
</tr>
<tr>
<td>Cherokee Purple Organic</td>
<td>139.08±2.85gh</td>
<td>4.53±0.20a</td>
<td>29.69±1.79ab</td>
</tr>
<tr>
<td>White Beauty</td>
<td>36.68±0.31a</td>
<td>5.07±0.03a</td>
<td>60.17±0.55jk</td>
</tr>
<tr>
<td>Sun Sugar Hybrid</td>
<td>144.47±1.06hi</td>
<td>6.03±0.21bc</td>
<td>63.06±6.64jk</td>
</tr>
<tr>
<td>Mint Jalep</td>
<td>171.74±4.97j</td>
<td>6.23±0.30bcd</td>
<td>54.14±3.80hij</td>
</tr>
<tr>
<td>Black Pear</td>
<td>186.20±7.05k</td>
<td>9.31±0.41jkl</td>
<td>28.95±1.18ab</td>
</tr>
<tr>
<td>Sweet baby girl hybrid</td>
<td>126.92±5.09ef</td>
<td>12.11±0.59n</td>
<td>52.57±5.74hij</td>
</tr>
<tr>
<td>Super Sweet 100 hybrid</td>
<td>116.32±1.00de</td>
<td>9.56±0.39jkl</td>
<td>42.36±5.45cdefghi</td>
</tr>
<tr>
<td>Yellow Pear Organic</td>
<td>53.74±0.28b</td>
<td>4.48±0.19a</td>
<td>40.41±3.78bcdefg</td>
</tr>
<tr>
<td>Copia</td>
<td>135.08±0.99fgh</td>
<td>7.52±0.13gh</td>
<td>44.02±1.97cdefgh</td>
</tr>
<tr>
<td>Green Zebra</td>
<td>110.92±0.55d</td>
<td>9.04±0.91j</td>
<td>36.60±5.42abcd</td>
</tr>
<tr>
<td>TT80154</td>
<td>231.28±1.81l</td>
<td>12.06±0.41n</td>
<td>39.81±3.68bcdef</td>
</tr>
<tr>
<td>Italian Red Pear</td>
<td>118.40±1.89de</td>
<td>8.29±0.21i</td>
<td>40.57±1.10a</td>
</tr>
<tr>
<td>Juliet hybrid</td>
<td>151.51±5.16i</td>
<td>9.10±0.10j</td>
<td>32.79±4.40abc</td>
</tr>
<tr>
<td>Esteria hybrid Organic</td>
<td>64.04±0.45b</td>
<td>10.19±0.11l</td>
<td>35.72±1.19abcd</td>
</tr>
<tr>
<td>Snow white</td>
<td>63.16±0.47b</td>
<td>13.86±0.24o</td>
<td>38.25±0.29abcde</td>
</tr>
<tr>
<td>Brand sweet plum</td>
<td>184.56±3.59k</td>
<td>10.06±0.08l</td>
<td>35.22±1.57abcd</td>
</tr>
</tbody>
</table>

4.3.2 Antioxidant Activities of Lipophilic Extracts

The antioxidant activities of the lipophilic extracts of specialty tomatoes were examined using the DPPH and ORAC-L methods. The DPPH assay measures the scavenging ability of antioxidants towards DPPH radical while the ORAC assay assesses antioxidant activity of
phytochemicals by way of inhibiting free radical oxidation and degradation of a fluorescent compound. The antioxidant activities of tomato lipophilic extracts varied significantly (p < 0.05) among the lipophilic extracts of tested varieties (Figure 10). The antioxidant activities of the carotenoid extracts as measured by the DPPH assay ranged from 4.48 to 14.31 μmol TE/g DW and the ORAC values were ranged from 28.95 to 68.78 μmol TE/g DW, respectively. Cuban Yellow Grape showed highest DPPH value at 14.31 μmol TE/g DW, followed by Snow White (13.86 μmol TE/g DW) (Table 3). Although DPPH assay was calculated differently thus difficult to compare with previously reported work, the ORAC values of the lipophilic fraction of our specialty tomatoes (28.95 to 68.78 μmol TE/g DW) were significantly higher than what have been reported for other tomatoes which ranged from 10.47 to 13.76 μmol TE/g DW [165, 168]. Moreover, cultivar Orange Russian showed the highest ORAC value 68.78 μmol TE/g DW, which is almost 4-fold higher than the orange tomato Jaune Flammee (13.76 μmol TE/g DW).
4.3.3 Correlation Coefficient Cetween Total Carotenoid Contents and Antioxidant Activities

The total carotenoid content as discussed above may be the general descriptor for the total antioxidant activities. The amount and composition of individual carotenoids in a particular tomato are believed as the determining factors affecting the antioxidant activities of the lipophilic fractions. Attempts were made to analyse the correlation between the antioxidant activities (DPPH and ORAC-L) and TCC using the Pearson’s correlation coefficient (r). However, no significant correlations were found in this study, suggesting other contents e.g. tocopherols, might have contributed to the antioxidant activity of the lipophilic extracts of these specialty tomatoes. This identifies need for further studies.
CHAPTER 5: IN VITRO DIGESTION MODEL AND CELL-BASED ANTIOXIDANT ACTIVITIES OF PHENOLIC EXTRACTS

5.1 Introduction

Antioxidants have been known for their role in reducing oxidative stress, which is the result of an imbalance between the production of reactive oxygen species (ROS) and antioxidant levels in cells and is believed to contribute to the development of many chronic diseases. Different chemical methods have been applied (DPPH, ORAC, and FRAP) to evaluate the antioxidant potential of phenolic extracts. Chemical-based methods are useful for screening, and are low-cost, high-throughput and can yield an index value (expressed as equivalents of Trolox) that allows comparing and ordering different products. However, their ability to predict in vivo activity is lacking in that they do not account for the uptake, distribution, and metabolism of antioxidants in the cell.

In general, animal and human studies are more time consuming and costly. This makes cell culture based assays very attractive as intermediate testing methods. In comparison with chemical antioxidant assays, the cell-based antioxidant (CAA) assay is more physiologically relevant, which reveals the actual in situ activity, uptake, metabolism and distribution of antioxidants under physiological conditions. The in vitro digestion model mimics the physiological processes occurring in the gastrointestinal tract of the human digestive system and has been widely used to study the complex multistage process of human digestion. The model simulates digestion in the oral cavity, the stomach, and the small intestine, and it could
effectively predict phenolic compounds released from the food matrix, bioaccessibility, and assess changes in their profiles prior to absorption.

The phenolic extracts of three representative cultivars were selected for further assessment. Cultivar Cuban Yellow Grape, which showed the highest TPC at 7.12 mg GAE/g DW, and strongest antioxidant potential from the three chemical-based assays, is on the list of our top interest. Along with the variety Cuban Yellow Grape, Lemon Boy Hybrid was also evaluated because of its uniquely high antioxidant activity but a relatively low TPC value (3.93 mg GAE/g DW). The Sun Sugar Hybrid, which showed ordinary performance in total phenolic content and hydrophilic antioxidant activities, was picked for assessment for comparison purposes. The purpose of this chapter is to investigate the cellular antioxidant activities of the selected varieties and the effect of in vitro digestion process on their phenolic profiles.

5.2 Materials and Methods

5.2.1 Sample Preparation and Extraction

5.2.1.1 Crude Extraction

Selected tomato samples were extracted for hydrophilic compounds under the same protocol as previously described in section 3.2.3, except 80% methanol was used instead of 80% methanol containing 0.1% HCl (v/v). In addition, the crude extracts of this experiment were concentrated to remove methanol prior to further clean up by solid phase extraction (SPE).

5.2.1.2 Solid Phase Extraction (SPE)

SPE was used for further purifying the phenolic extracts. The purification was accomplished using Strata-X 200 μm Polymeric Reversed-Phase cartridges (Phenomenex,
Torrance, CA, USA) [101]. The cartridge was activated by passing 50 mL of MeOH solution and equilibrated with 50 mL of distilled water. The crude extract concentrated by a rotary evaporator was diluted with H$_2$O$_2$ (1:1 v/v). The solution was carefully loaded onto the cartridge, which was then washed with 50 mL of distilled water to remove soluble sugars, protein, and substances that could interfere with the analysis of phenolic compounds. The elution of the polyphenols was performed by the addition of 2x10 mL of methanol. The eluate was dried by rotary evaporator.

5.2.2 Simulation of in vitro Gastric and Gastrointestinal Digestion Model

An in vitro digestion model system was performed according to the method of Sessa et al., 2011 with slight modifications [182]. Briefly, to study the effect of gastric digestion, 40 µL of each purified extract solution (12.5 mg equivalent phenolic content/mL) was mixed with 1.2 mL saliva which was collected 5 min after volunteers consumed 250 mL milk and added to 2.8 mL HBSS. The mixtures were incubated for 15 min at 37 ºC in a water bath shaker at 200 rev/min. After that, 3 mL phosphate buffer saline (PBS) was added and the pH was adjusted to 2 with 1 M HCl. Porcine pepsin (Amresco) (final concentration: 1.3 mg/mL on the basis of earlier titrations) was mixed with the homogenates and incubated for 90 min under the same conditions. Half of the total volume was taken to a new tube and 1 mM PMSF (final concentration: 0.174 mg/mL) was added to stop the digestion. To study the effect of the gastrointestinal digestion (separate sets of experiment), the other half volume (left after the 90 min incubation) was continued by adjusting the pH of the mixture to 7-8 by drop-wise addition of 1 M NaOH. After estimating the volume, pancreatin(Sigma) (final concentration: 0.175 mg/mL) and porcine bile salt (final concentration: 1.1 mg/mL) were added and the pH was adjusted to 6.5 by adding 1 M NaHCO$_3$. Subsequently, the digestate was incubated in a shaking water bath at 37 ºC for another 2 h. PMSF (final concentration: 0.174 mg/mL) was added to stop the digestion process. In both
digestions, the resulting mixtures (the digestates) were centrifuged at 4000 rpm for 10 min (5810R, Brinkman Instruments Inc.) and the supernatants were collected for analyses and assays. A blank (without the added sample) was incubated under the same conditions to correct the interferences from the digestive enzymes and buffers. This system was used to simulate the human salivary, gastric and small intestinal digestive process. HPLC-DAD was used to analyze the digested bioactive compound (dissolved in 80% MeOH).

5.2.3 Cell-based Antioxidant Assay

The antioxidant activity at the cellular level was evaluated by the method described by Li et al. with slight modifications [89]. Caco-2 cells (ATCC) used in this study with passages between 30 to 40 times were seeded at a density of 4 x 10⁴ cells/ml in a 96-well black/clear flat bottom Costar cell culture plate (Corning®, Fisher Scientific Co., Ottawa, CANADA) and grown with 100 µL of growth medium, the cells were incubated for 5-7 days at 37 °C with fresh media replacements every 2-3 days. The cells were rinsed with 100 µL HBSS+10% FBS at the end of the incubation. Cells were treated with 100 µL of purified tomato extract (0.25 mg equivalent phenolic content/mL) at different concentrations and 100 µM 2',7'-dichlorofluorescin diacetate (DCFH-DA) at the same time, and then incubated for 30 min at 37 °C. After washing twice with PBS, cell media were removed and replaced with 100 µL 50 µM H₂O₂ for each well. The fluorescence intensity was measured using a fluorescence spectrophotometer PLX800 at an excitation wavelength of 485 nm and an emission wavelength of 528 nm for 1 h. The results were calculated according to the following equation: Eq. CAA unit=(100-(∫ SA - ∫ BA)/∫ CA) × 100, where ∫ SA is the integrated area under the sample fluorescence vs. time curve, ∫ BA and ∫ CA are the integrated area from the blank and control curves, respectively[183].
5.2.4 Statistical Analysis

All assays or tests were conducted in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad software (San Diego, CA, USA) and SPSS (Version 18.0, Chicago, IL). The statistical significance of the data was determined using one-way ANOVA followed by Duncan’s multiple-comparison test with a $p<0.05$ taken as value of significance.

5.3 Results and Discussion

5.3.1 Phenolic Contents and Profiles Before and After Digestion

When consuming a tomato, the total amount of an ingested phenolic compound does not always reflect the amount that is available to the body. Only a certain amount of the phenolic compounds will be bioavailable after an extensive metabolism by intestinal, hepatic enzymes and by the intestinal microflora. Bioaccessibility, therefore, describes the fraction of a compound potentially available for further uptake and absorption. During consumption of the tomato fruits, phenolic compounds need to undergo enzymatic hydrolysis in the digestive tract or by the gut microflora before becoming absorbed. Many methods are used to assess the rates and the degrees to which these phenolic compounds are digested and absorbed [182]. In this study, *in vitro* digestion model was applied to mimic the physiological release of food bioactives occurring in the gastrointestinal tract of the human digestive system, in an attempt to explore the bioaccessibility of the phenolic compounds of selected specialty tomatoes [184].
Figure 11. Chromatographic profiles of phenolics before and after in vitro digestion at 360 nm. (a’), before digestion; (b’), after gastric digestion; (c’) after intestinal digestion. Panel A: phenolics extracted from Lemon Boy Hybrid; Panel B: phenolics extracted from Cuban Yellow Grape; Panel C: phenolics extracted from Sun Sugar Hybrid.

The in vitro digestion model mimics the physiological processes occurring in the gastrointestinal tract of the human digestive system and has been widely used to study the complex multistage process of human digestion. In this study, all of the phenolic compounds decreased significantly during the gastric digestion and after the gastrointestinal digestion (p < 0.01) (Figure11). For instance, in Lemon Boy Hybrid, phenolics such as rutin and naringenin were reduced by approximately 70% and 72%, respectively after gastrointestinal digestions. Moreover, gentistic acid and chlorogenic acid were completely degraded (Table 4). In addition to the possible degradation, reduction of the major phenolic compounds may be in part due to the high affinity of polyphenols for proteins and dietary fibers. The formation of such complexes may lead to their loss to the solid pellet removed by centrifugation.
Table 4. Degradation (%) of major phenolic compounds in cultivar Lemon Sugar Hybrid, Cuban Yellow Grape and Sun Sugar Hybrid after gastro and gastrointestinal digestions.

<table>
<thead>
<tr>
<th>Phenolic Compound</th>
<th>Crude Extract (%)</th>
<th>Reduction after Gastric Digestion (%)</th>
<th>Reduction after Gastrointestinal Digestion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lemon Boy Hybrid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentistic Acid</td>
<td>100</td>
<td>68</td>
<td>100</td>
</tr>
<tr>
<td>Chlorogenic Acid</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Rutin</td>
<td>100</td>
<td>56</td>
<td>70</td>
</tr>
<tr>
<td>Narigenin</td>
<td>100</td>
<td>40</td>
<td>72</td>
</tr>
<tr>
<td>uk1</td>
<td>100</td>
<td>74</td>
<td>89</td>
</tr>
<tr>
<td>uk2</td>
<td>100</td>
<td>62</td>
<td>70</td>
</tr>
<tr>
<td>uk3</td>
<td>100</td>
<td>57</td>
<td>78</td>
</tr>
<tr>
<td><strong>Cuban Yellow Grape</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>100</td>
<td>37</td>
<td>76</td>
</tr>
<tr>
<td>Rutin</td>
<td>100</td>
<td>5</td>
<td>90</td>
</tr>
<tr>
<td>uk1</td>
<td>100</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>uk2</td>
<td>100</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td><strong>Sun Sugar Hybrid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rutin</td>
<td>100</td>
<td>48</td>
<td>74</td>
</tr>
<tr>
<td>uk1</td>
<td>100</td>
<td>77</td>
<td>84</td>
</tr>
<tr>
<td>uk2</td>
<td>100</td>
<td>55</td>
<td>77</td>
</tr>
<tr>
<td>uk3</td>
<td>100</td>
<td>46</td>
<td>72</td>
</tr>
</tbody>
</table>

Many factors may affect the bioaccessibility of polyphenols when using the *in vitro* model, pH-dependent chemical reactions including degradation, epimerization, hydrolysis, and oxidation within the gastrointestinal tract, and also interactions between polyphenols with other proteins and digestive enzymes can all occur during the digestion. It is generally accepted that high-molecular weight polyphenols (such as tannins) tend to form insoluble complexes with proteins in solution [185]. Phenolic compounds may interact with proteins both reversibly and
irreversibly. Reversible interactions often occur via hydrophobic interaction, hydrogen bonding or van der Waals forces, whereas ionic and covalent bonds are generally formed between the polyphenols and proteins in irreversible interactions [186].

Human salivary enzymes including amylase (mainly), albumin, mucins, and proline- and histidine-rich proteins have been found to affect the digestibility and absorption of specific polyphenols. Phenolic compounds can have strong affinities with salivary proline- and histidine rich proteins and are known to effectively precipitate proteins through hydrogen bonding and hydrophobic interactions [185]. The carbonyl oxygen next to the secondary amine in proline residues acts as a strong hydrogen bond acceptor, forming a hydrogen bond of proline-rich proteins with tannins. Histidine-rich proteins have been suggested to be more potent than proline-rich proteins in precipitating condensed tannins as well as tannic acid in certain studies [187, 188].

Several structural properties of polyphenols can influence their non-covalent binding capacity to protein molecules. First, the higher molecular weight and higher degree of polymerization of phenolics (an increase of the hydrophobicity of the molecule) precipitate or interact with proteins more effectively [189, 190]. Second, the greater the conformational mobility and flexibility of the phenolics, the higher capacity to interact with proteins, by allowing accessibility to more sites of complexation [191]. Last, the strength of polyphenol-protein interaction is positively correlated with polyphenol hydrophobicity. When a polyphenol molecule is water soluble, it has a weaker affinity for proteins and, therefore, less likely to form complexation with proteins [190, 192, 193].

The formation of covalent bonds between proteins and polyphenol is generally characterized as the irreversible interactions. The biochemical mechanisms for forming this
irreversible phenolic-protein complexation involves enzymatic/non-enzymatic oxidation of phenolic compounds, with the formation of o-quinones or o-semi-quinones [194]. Enzymatic oxidation of polyphenols occurs when the enzyme polyphenoloxidase (PPO) catalyzes the oxidation of phenolic compounds in the presence of molecular oxygen. Non-enzymatic oxidation of polyphenol involves various mechanisms including auto-oxidation, metallic cations catalyzed or thermal-induced oxidation. Consequently, the electrophilic o-quinones or the o-semi-quinones can easily form covalent bonds with nucleophiles available from functional groups of proteins, such as thiols, thioether, and primary and secondary amines [194, 195].

By contrast, binding of polyphenols with polysaccharides may also occur and influence the bioaccessibility of polyphenols. This has long been neglected. Although little has been published in the literature about the mechanism of complexation of polyphenols with polysaccharides, increasing interest has been shown by researchers in recent years. The suggestive mechanisms of polyphenol-polysaccharide interactions would be similar to the polyphenol-protein association, which is mediated by van der Waals interactions, hydrogen bonds and hydrophobic interactions [196, 197]. The high affinity of polyphenols for proteins and dietary fibers may therefore at least partially explain the loss of phenolics during the solid removal step by centrifugation [186].

5.3.2 Cell-based antioxidant assay (CAA)

Currently, various chemistry assays are used to assess the antioxidant activity, but their ability to predict in vivo activity is lacking in that they do not account for the uptake, distribution, and metabolism of antioxidants in cells, tissues or organs. In comparison with chemical antioxidant assays, the cell-based antioxidant (CAA) assay is more physiologically relevant, which reveals the actual in situ activity, uptake, metabolism and distribution of antioxidants.
under actual biochemical conditions of the living cells [183]. Dichlorofluorescin is a probe that is trapped within cells and is easily oxidized to fluorescent dichlorofluorescein (DCF). The CAA approach measures the ability of phenolic compounds to prevent the formation of DCF by 2, 2′-azobis (2-amidinopropane) dihydrochloride (ABAP)-generated peroxyl radicals in Caco-2 cells. The decrease in cellular fluorescence, when compared to the control cells, indicates the antioxidant capacity of the compounds [183] (Figure 12). Only the phenolic compounds that are uptaken by the cells will have the antioxidant activity in CAA.

**Figure 12. Cell-based Antioxidant Activity (CAA) Assay for Assessing Antioxidants.**

In this study, Caco-2 cells grown for 5-7 days with fresh media replacements every 2-3 days were first treated with different dosages (at final concentrations of 0.025, 0.25, 2.5 and 25 μg/mL cell culture medium) of SPE-purified tomato extracts for 30 min, and then exposed to H$_2$O$_2$ (50 μM) to induce cellular responses. Our results showed that the Sun Sugar Hybrid had strong cellular antioxidant activity potential even at the lower dose (0.025μg/mL); all three
extracts (Lemon Boy Hybrid, Cuban Yellow Grape, and Sun Sugar Hybrid) showed significant CAA values and the activities were dose-dependent for all selected cultivars (Figure 13). There was significant difference found among the three extracts (p<0.05).

Figure 13 Cell-based antioxidant activity of phenolic extracts in H\textsubscript{2}O\textsubscript{2}-stimulated Caco-2 cells. Values are means ± SD, n = 4. Concentrations are expressed in μg phenolic content equivalent/mL medium. The 100% of the control represents 4x10\textsuperscript{4} cells/ml.
CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

Tomato has been well known for containing a variety of antioxidant phytochemicals as well as vitamins, which exert health-beneficial effects on humans. Carotenoid and phenolic compounds, as two major categories of phytochemicals in tomato, have been reported for their high antioxidant activities in numerous studies. Nevertheless, the phytochemical profiles of specialty tomato cultivars have not been reported. Total phytochemical contents and compositions before and after digestion were determined by using spectrophotometric methods and HPLC. Antioxidant activities of phytochemicals (hydrophilic extracts and hydrophobic extracts) were evaluated by both chemical-based assays, including DPPH, ORAC, FRAP assays, as well as CAA. In vitro digestion model was studied to examine the accessibility of phenolic compounds in selected cultivars.

This study consisted of two parts: 1) chemical analysis of phenolic/carotenoid content and antioxidant activity of 28 specialty tomatoes; and 2) assessment of the effects of in vitro digestion process on the phenolic profile.

In Chapter 3, the objective was to quantify the total phenolic content and antioxidant activity in the hydrophilic extracts of 28 tomato varieties and, to determine the individual phenolic composition of selected cultivars: Cuban Yellow Grape, Lemon Boy Hybrid, and Sun Sugar Hybrid. In this study, we found that in all tomato varieties analyzed, cultivar Cuban Yellow Grape showed the highest TPC. Antioxidant activities assessed by DPPH, ORAC-H and
FRAP assays were also the highest for cultivar Cuban Yellow Grape, followed by Lemon Boy Hybrid, among all the 28 specialty tomatoes. Moreover, their antioxidant activities were significantly higher than what had been reported previously by other researchers for the commercial tomato varieties, as well as the purple tomato V118.

Believing that Cuban Yellow Grape and Lemon Boy Hybrid may have a unique phenolic composition which contributes to the distinctively high phenolic content and antioxidant activity, next step, we applied HPLC-DAD which helped us to elucidate the phenolic profiles of these two candidate cultivars. Sun Sugar Hybrid, which showed ordinary performance in total phenolic content and hydrophilic antioxidant activities, was also selected for composition analysis for comparison purposes. Commonly found phenolics such as gentistic acid, chlorogenic acid, ferulic acid, rutin, and naringenin were identified in the Lemon Boy Hybrid by matching the retention times, UV-visible spectra, and mass spectrometric data with those of the corresponding standards. However, several peaks were not identified in this study. Particularly, unknown peaks uk1 (at 13.072 min), uk2 (at 19.180 min) and uk3 (at 23.188 min), which also existed in other two cultivars did not match with any known phenolics commonly found in commercial tomatoes. Future works for identification of these unknown peaks are needed.

In Chapter 4, the total carotenoid content and antioxidant activities in the lipophilic fractions of 28 specialty tomato varieties were examined. Cultivar Brand Sweet Plum had the highest total carotenoid content (541.13μg/g DW), which was 2-fold higher than red tomato variety and purple tomato V118. Orange Russian was found to have the highest ORAC-L value while Cuban Yellow Grape possessed the highest antioxidant activity in DPPH assay. According to results of the present study, the total carotenoid content did not show as a general descriptor for the total antioxidant activities of the lipophilic fraction.
In Chapter 5, cultivar Cuban Yellow Grape, Lemon Boy Hybrid, and Sun Sugar Hybrid were selected for further analysis. The bioaccessibility of phenolic compounds was investigated via an *in vitro* digestion model which mimicked the physiological release of food bioactives occurring in the gastrointestinal tract of the human digestive system. In conclusion, bioaccessibility of the phytochemical antioxidants: phenolics of the selected specialty tomatoes were not stable during the simulated gastrointestinal digestion. For instance, phenolics such as rutin and naringenin from Lemon Boy Hybrid, were reduced by approximately 70% and 72%, respectively after gastrointestinal digestions. Moreover, gentistic acid and chlorogenic acid were completely undetected. Reduced detection of the major phenolics may be in part due to the high affinity of polyphenols for proteins and polysaccharides, resulting in the formation of complexation that may lead to their loss during the solid removal step by centrifugation. The CAA assay was applied in this study to investigate the actual *in situ* activity under near physiological conditions. Our results indicated that phenolic compounds were bioavailable to the cells as demonstrated by the cell based antioxidant activity. The Sun Sugar Hybrid, particularly, had stronger cellular antioxidant potential even at the lower dose (0.025μg/mL).

### 5.2 Future Directions

Among the 28 specialty tomato cultivars, the hydrophilic extract of Cuban Yellow Grape was found to have the highest total phenolic content, and antioxidant activities in DPPH, ORAC-H and FRAP assays. This led to our strong interest to investigate the phenolic composition of high antioxidant potential cultivars, such as Cuban Yellow Grape and Lemon Boy Hybrid. Although several phenolic compounds were identified via HPLC-DAD methods with further confirmation by mass spectrometry, a number of unknown phenolic compounds represented by
uk1, uk2 and uk3 still remained uncharacterized in this study, which need to be studied in future research.

Previous studies on the phenolic profiles of plant foods based their analysis on extracts obtained from 80% methanol. This procedure is not sufficient to completely extract phenolic compounds, including those conjugated to small peptides or oligosaccharides [186]. The extractable phenolics, both free and conjugated. Conjugated phenolics can be acid hydrolysed to release the aglycones or other monomeric phenolic compounds which simplifies the phenolic profile and helps further identification and quantification [69, 70]. Complete characterization of individual phenolics of the specialty tomatoes using HPLC-MS, particularly accurate mass spectrometry is necessary in future studies in order to fully understand how individual hydrophilic phytochemicals contribute to the total phenolic content and total antioxidant activities in these specialty tomatoes.

The NMR or the nuclear magnetic resonance spectroscopy identifies the carbon-hydrogen framework of an organic compound. Using this method and other instrumental methods including mass spectrometry, will help elucidating molecular structures and, therefore, identify the unknown phenolic compounds in the selected specialty tomatoes.

The lipophilic extracts of 28 specialty tomatoes were analyzed for their total carotenoid content and antioxidant capability. However, according to our result, the total carotenoid content did not correlate to the total antioxidant activities of the lipophilic extracts. Composition of individual carotenoids was not examined in this study due to the limit of time. Existing carotenoids in tomatoes have been well identified in studies, including lycopene, followed by phytoene, phytofluene, ζ-carotene, γ-carotene, β-carotene, neurosporene, and lutein [115].
However, identification and quantification of carotenoid compounds will help to elucidate how individual component of the lipophilic extracts contribute to the total carotenoid content and total antioxidant activities in these specialty tomatoes. A chemical-based model photochemiluminescence (PCL) assay which measures the superoxide radical-scavenging capability of antioxidants was commonly used in the analysis of total and individual antioxidant activities of lipophilic tomato extracts [168]. It was reported by Zhang et al. that PCL value exhibited higher correlation with total carotenoid content in comparison with DPPH [173]. PCL assay could provide additional information on the antioxidant capacity of carotenoids and other lipophilic components, thus should be considered in future research.

In the present study, bioaccessibility of the phenolics in selected specialty tomatoes was not great as found in the simulated gastrointestinal digestion experiment. The reduced detection of the major phenolic compounds may be caused by factors other than the degradation of phenolic compounds, such as binding to proteins and polysaccharides. To validate this hypothesis, the future work may focus on finding an organic solvent or other means that can dissociate this complexation without interference with individual components, and compare the effect of in vitro digestion on dissociated phenolics with the crude extracts. If the level of the phenolic compounds after the simulated gastrointestinal digestion can be recovered by such means, we can conclude a formation of insoluble complexation of polyphenols with proteins or polysaccharides during the in vitro gastrointestinal digestion.

In recent years, the synergism between bioactive constituents in food has been increasingly documented because the bioactive compounds in edible plants are not consumed individually, but instead, in the form of natural mixtures. Therefore, approaches to assess the pharmacological
benefits contributed by the mixture of natural phytochemicals become crucial [172]. To date, very scarce analysis of antioxidant synergy between phenolics and carotenoids in tomato has been reported [89]. Although additive or synergistic interactions have been found among different phytochemicals and vitamins in tomato [163, 164], no particular studies have been conducted on the synergy of antioxidant activity between carotenoids and polyphenols in specialty tomato varieties. Therefore, this could be a leading topic for further studies on interaction

Although the antioxidant activity of phytochemicals is well recognized, it should be pointed out that they can also display prooxidant activities under certain conditions, such as at high doses or in the presence of metal ions [198]. For instance, phenolic compound may become a radical once it loses an electron or when it acts as a reducing agent. Its oxidized intermediates such as semiquinones and quinones may become prooxidants and have adverse effects to human health when present at high concentrations. Interaction between polyphenols and transition metal ions can result in formation of prooxidant in human GI tract due to high doses of phenolics ingestion [199]. Therefore, polyphenols therefore can be a double-edged sword. On the one hand, physiologic doses of exogenous antioxidants are required to protect against excess oxidative stress such as ROS, thus beneficial to health. However, high doses of exogenous antioxidants such as by taking supplements may disrupt redox balance and therefore exert deleterious effects on human health.

In closing word, by comparing the outcomes of previous research done by our group (same protocols and methodologies), we found selected specialty tomato cultivars (especially the Cuban Yellow Grape) may potentially have greater health benefits due their high phenolic content/carotenoid content, and superior antioxidant capabilities. The unique phenolic profiles of
these samples and the quantification of individual phenolic compounds and carotenoids need to be clearly elucidated in the future for fully understanding how individual phytochemicals contribute to the total antioxidant activities in these specialty tomatoes. Information from this study can help tomato breeders for developing unique tomato varieties with high concentrations of both carotenoids and phenolics, which potentially have stronger antioxidant activities and thus better help to reduce the risk of cancer, heart disorder, and other chronic diseases.
CHAPTER 7: REFERENCES

Primary Sources

Secondary Sources

Uncategorized References


