

Health-promoting phytochemicals: (1) In response to environmental factors in lettuce, spinach  
and tomato; (2) Development of 3D cell culture model for potential anticancer role

by

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B.S., Harbin University of Commerce, 2013  
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## **Abstract**

As health-promoting agents, phytochemicals are biosynthesized in the plants that typically respond to environmental stresses. This study focused on the analysis of phytochemical contents in vegetables in response to environmental changes of high tunnel and light spectra. A potential anticancer activity was further studied by developing a novel 3D cell culture model. Three specific studies were conducted as follows.

Study 1: High tunnel production has been applied in mid-west for many years due to the advantages of extending growing season and increasing crop yield. Previous studies, however, showed high tunnel resulted in reduction of phenolic contents in vegetables. Therefore, the first study was to confirm the effect of high tunnel on phenolic contents in two varieties of lettuce ('Two Star' and 'Red Fire') and carotenoid contents in two varieties of tomatoes ('Mountain Fresh' and 'Celebrity'). Phenolics in lettuce and carotenoids in tomato were isolated and quantitated, respectively, by HPLC. High tunnel resulted in a significant reduction of phenolic contents in 'Two Star' but not in 'Red Fire' lettuce when compared with open field. A significant decrease of carotenoid contents in 'Celebrity' but not in 'Mountain Fresh' tomato was also observed. Therefore, this study confirmed that high tunnel application reduced phenolic or carotenoid contents in one of the two lettuce or tomato varieties, suggesting the effect of high tunnel production is variable and genotype specific.

Study 2: Light is an important environmental factor influenced not only photosynthesis but also phenolic biosynthesis in plants. The objective of this study was to investigate the effect of supplemental light spectra including red, far-red, and blue light on phenolic contents in two varieties of lettuce (green-leaf variety 'Two Star' and red-leaf variety 'Red Fire') and two varieties of spinach ('Avon' and 'Bloomsdale'). The phenolics were extracted and quantitated by

HPLC. Far-red and blue light but not red light resulted in an increase of phenolic contents in 'Two Star' lettuce. In 'Red Fire' lettuce, a significant increase in phenolic contents were observed when exposed to red light, while far-red and blue light reduced phenolic contents. Supplemental lighting did not alter flavonoid contents in two varieties of spinach. Taking together, the results showed that supplemental lighting and its spectral quality had significant effect on the phytochemical contents of lettuce but not spinach, and the impact varies depending upon the genotype.

Study 3: Traditionally, cancer research is primarily relied on *in vitro* 2D monolayer cell culture and *in vivo* animal model studies. Given a flat 2D cell culture that usually lacks 3D microenvironmental cell-cell interaction and considering an animal model that is typically expensive and time-consuming, an alternative 3D cell culture has been promising. This pilot study was to develop a novel 3D hydrogel cell culture model of human hepatocarcinoma HepG2 cells or colorectal adenocarcinoma SW480 cells by treating with chlorogenic acid (CGA) at 0-40  $\mu\text{M}$ . The results showed both HepG2 and SW480 cells grew much better in 3D hydrogel culture system than 2D by extended exponential phase and high proliferation. CGA treatment resulted in a dose- and time-response inhibition of HepG2 and SW480 growth in exponential phase, while HepG2 cells were more susceptible than SW480 cells. Establishment of this novel 3D hydrogel culture model for future phytochemical function may bridge the gap between *in vitro* 2D cell culture and *in vivo* animal model studies.

Taken together, this dissertation of three studies focused on phytochemicals from quantitation analysis in vegetables in response to environmental factors of high tunnel and light spectra to a novel 3D hydrogel cell culture development for potential phytochemical anti-cancer function. The conclusions, i.e., (1). high tunnel application reduced phenolic or carotenoid contents in

special genotype of lettuce or tomato varieties; (2). lighting and its spectral quality had significant effect on the phytochemical contents of lettuce but not spinach; (3). establishment of a novel 3D hydrogel culture model for phytochemical treatment may bridge the gap between 2D cell culture and *in vivo* animal model studies, could be of particular significance in health-promoting phytochemical research and functional food application.

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Taken together, this dissertation of three studies focused on phytochemicals from quantitation analysis in vegetables in response to environmental factors of high tunnel and light spectra to a novel 3D hydrogel cell culture development for potential phytochemical anti-cancer function. The conclusions, i.e., (1). high tunnel application reduced phenolic or carotenoid contents in

special genotype of lettuce or tomato varieties; (2). lighting and its spectral quality had significant effect on the phytochemical contents of lettuce but not spinach; (3). establishment of a novel 3D hydrogel culture model for phytochemical treatment may bridge the gap between 2D cell culture and *in vivo* animal model studies, could be of particular significance in health-promoting phytochemical research and functional food application.

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## **Dedication**

To my dear parents and husband

I dedicate my dissertation to my parents, Ziping Xu and Dongzhi Wang

and my husband Zhengjun Wang

for their everlasting love, support, and encouragement.

## **Preface**

This dissertation is the original work completed by Jingwen Xu. All chapters were formatted according to the standards of Journal of Food Chemistry.

# Chapter 1 - Literature review

## Summary

Essential nutrients like carbohydrate, protein (amino acid), and nucleic acid synthesized by plants for growth and development are defined as primary metabolites (Crozier, Jaganath, & Clifford, 2006). Other bioactives in plants like small molecular-weight non-nutrient compounds are categorized as secondary metabolites (Crozier, Jaganath, & Clifford, 2006). Secondary metabolites, known as phytochemicals, comprise the families of polyketides, terpenoids, phenolics, alkaloids, and other compounds containing sulfur and nitrogen (Dillard and German, 2000). These phytochemicals are widely distributed in nature among vegetables, fruits, cereals, and herbs. Phytochemicals contribute to the sensory attributes of vegetables and fruits (Ignat, Volf, & Popa, 2011). The phytochemical profile of a plant is determined by genotype factors, but the contents are influenced by environmental factors like cultivation methods, UV light intensity and quality, postharvest conditions, changes in temperature, and changes in fertilizer and irrigation (Oh, Carey, & Rajashekar, 2011).

Eating vegetables and fruits rich in phytochemicals is associated with reducing the risk of cancer and age-related chronic diseases (Liu 2013; Birt, Hendrich & Wang, 2001). Antioxidant phytochemicals like vitamin C, vitamin E, and provitamin A in vegetables and fruits can protect against overproduction of oxygen in the human body (Garcia-Salas, Morales-Soto, Segura-Carretero, & Fernández-Gutiérrez, 2010; Wang & Goodman, 1999). Overproduction of reactive oxygen species (ROS) in the human body results in aging-related chronic diseases. Antioxidants from dietary polyphenols can scavenge free radicals in the human body and help prevent these diseases. Antioxidant polyphenols have anti-cancer attributes, as shown in primarily *in vitro* studies; they modulate initiation of carcinogenesis by protecting against DNA damage, inhibit

cell proliferation, induce cancer cell death, and downregulate the expression of cancer-related genes (Zhang et al., 2015).

Currently, more than 70% of cancer research relies on 2D monolayer culture before correlating to *in vivo* studies (Hutmacher, 2010). However, cells in 2D monolayer culture lack a complex 3D microenvironment as well as cell-cell interaction resulting in poor experimental results and even failures. The development of this novel 3D culture is a promising alternative that can mimic the tumor microenvironment more closely. Cancer cells growing in a 3D culture can closely reflect human physiology. However, the lack of standardized experimental protocols, high costs, and small experimental scales mean our understanding of cancer cells in 3D culture incomplete.

**Part I. Biosynthesis and classification of phenolics and carotenoids in vegetables; identifying, isolating, and quantifying phenolics and carotenoids; environmental effects of high tunnel production and light on phenolics and carotenoids in vegetables.**

To date, more than 8000 phytochemicals have been identified (Tsao, 2010). Depending on the different precursors from primary metabolites, secondary metabolites are classified into three primary groups: polyketides derived from acetal coA, terpenoids originating from fatty acids, and phenolic compounds biosynthesized from amino acids (Crozier et al., 2006). This review will focus on biosynthesis, extraction, identification, separation of phenolics and carotenoids, as well as changes in phenolics and carotenoids in plants in response to the environment.

Traditionally, primary three extraction methods are used experimentally: liquid-liquid extraction; solid-liquid extraction, also known as solid-phase extraction; and supercritical fluid extraction (SFE). To date, several novel extraction systems have been developed: pressurized-liquid extraction (PLE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) (Wang & Weller, 2006; Ignat et al., 2011). Compound separation primarily relies on chromatographic techniques and depends on affinities that are not miscible in two phases. The common chromatographic techniques involve paper chromatography, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), high speed counter current chromatography (HSCCC), and ultra-high performance liquid chromatography (UPLC). Quantifying chemical compounds is done primarily using spectrophotometric and chromatographic methods (Tsao & Yang, 2003; Naczk & Shahidi, 2006; Davis et al 2018). Mass spectrometry (MS) is a powerful technique for qualifying analytes. MS is also sensitive enough to identify compounds. The commonly used approaches for analyzing

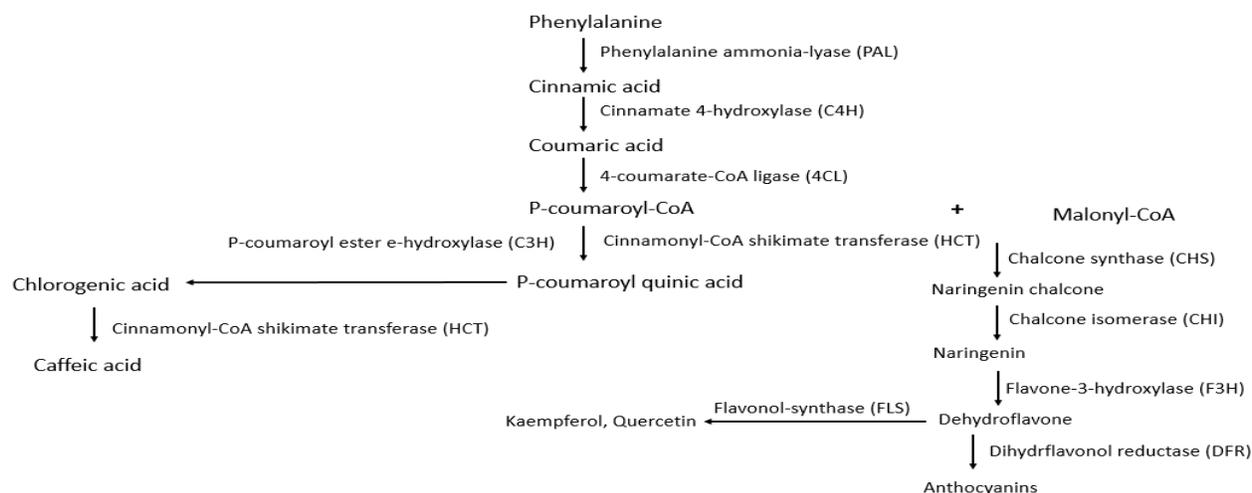
phenolic compounds are electrospray ionization (ESI) and atmospheric pressure ionization (API). Liquid chromatography/ Mass spectrometry (LC/MS) and mass spectrometry/mass spectrometry (MS/MS) are good analytical techniques to identify, qualify, and quantify compounds. Specific extraction methods, identification, characterization, and quantification of phenolics and carotenoids will be discussed separately.

### Biosynthesis and classification of phenolics in plants

Phenolic compounds are synthesized via the shikimic pathway or phenylpropanoid pathway.

Phenylpropanoids originate from cinnamic acid formed from phenylalanine via the enzyme phenylalanine ammonia-lyase (PAL) (Dixon & Paiva, 1995). This enzyme is the branch point enzyme between the primary metabolites and secondary metabolites (Dixon & Paiva, 1995).

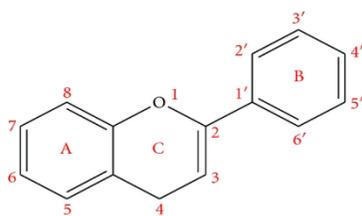
With the help of a series of enzymes, cinnamic acid will turn into other phenolic acids and flavonoids. Through the shikimic pathway, three important aromatic amino acids are produced: phenylalanine, tryptophan, and tyrosine (Dixon & Paiva, 1995). Figure 1.1 shows the biosynthesis of phenolic compounds.



**Figure 1.1. Biosynthesis of phenolic compounds via the shikimic pathway.**

Adapted from Dixon & Paiva, 1995.

Chemically, phenolics consist of more than one phenol ring with more than one hydroxyl group. Phenolic compounds are further subdivided into simple phenols, phenolic acids, flavonoids, tannins, and stilbenes, and lignans (Ignat et al., 2011; Ayella, Trick & Wang, 2007). Approximately one-third of dietary phenols are phenolic acids (Robbins, 2003). Phenolic acids have two subgroups: the hydroxybenzoic acids like gallic acid, vanillic acid, and syringic acid and hydroxycinnamic acids like caffeic acid and ferulic acids. Tannins are high molecular compounds consisting of groups of hydrolysable and condensed tannins (proanthocyanidins) (Ignat et al., 2011). Stilbenes are usually glycosylated with sugars in plants and lignans. Flavonoids are the largest subgroup of phenolic compounds and fall into more than 4000 categories (Ignat et al., 2011). The flavonoid skeleton consists of fifteen carbons with two aromatic rings linked by a three-carbon bridge. It is a C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> configuration as shown in figure 1.2. The alteration of the degree of oxidation of the three-carbon bridge will sort the flavonoid into subclasses like flavanone, flavone, isoflavone, and flavonols. Substitutions as in oxygenation, alkylation, glycosylation, and acylation to rings A and B will create other subgroups of flavonoids (Balasundram, Sundram, & Samman, 2006)



**Figure 1.2. Flavonoid skeleton.**

### **Extracting, isolating, and separating phenolics**

Phenolics are naturally conjugated with glycosides, acylglycosides, and aglycones in vegetables. Thus, isolating phenolics requires diminishing and purifying the interference and conjugation. However, because various phenolics are present in plants and because phenolics have different

polarities, no single method can extract, isolate, and separate phenolics from vegetables.

Therefore, the extraction and isolation of phenolics depends on research interest.

Earlier research interest has focused primarily on phenolic acids like gallic acid, caffeic acid, ferulic acid, chicoric acid, and chlorogenic acid and flavonoids like luteolin, quercetin, rutin, apigenin, and kaempferol. The hydroxyl groups in phenolic acids and flavonoids cause polar attributes, so the extraction solvent is usually a high polarity solvent like methanol and water.

Reverse phase column C18 has been used extensively for phenolic acid and flavonoid isolation and separation in our lab (Su et al, 2017; Su et al, 2016; Xu et al, 2015). Table 1.1 summarizes extraction solvents and separation columns used in extracting and separating phenolics and flavonoids.

**Table 1.1. Extraction solvents and separation columns used in phenolic and flavonoid extraction and separation.**

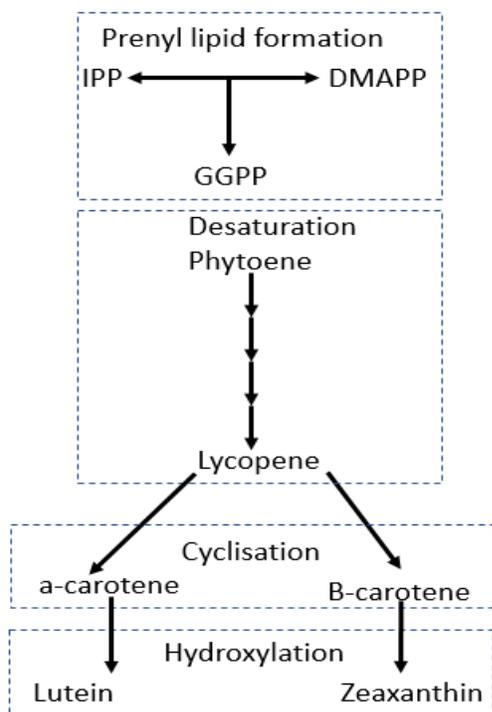
Analytes	Extraction solvents	Separation column	Reference
Caffeoyltartaric acid, chlorogenic acid, dicaffeoyltartaric acid, quercetin-3-glucoside, rutin, etc	Methanol and acetonitril	C18	Ferreres et al., 1997
Quercetin, quercetin 3-glucuronide, luteolin, chlorogenic acid, chicoric acid	chloroform	C18	Hohl et al., 2001
Chlorogenic acid, quercetin-3- <i>O</i> -rutinoside, quercetin-3- <i>O</i> -glycoside, luteolin-7- <i>O</i> -glucoside	80% ethanol	C18	Zhao, Carey, Young, Wang, & Iwamoto, 2007
Anthocyanins, polyphenols, and flavonoids	methanol	C18	Boo et al., 2011
Anthocyanins and flavonols	Methanol/water/ acetic acid (85:14.5:0.5)	C18	Cheng et al., 2014
Caffeic acid, chlorogenic acid, cyanidin, quercetin-3- <i>O</i> -glycoside, luteolin-7- <i>O</i> -glucoside, chicoric acid	70% methanol	C18	Santos et al., 2016

### **Biosynthesis and classification of carotenoids in plants**

Carotenoids are composed of either oxygenated or non-oxygenated hydrocarbons containing at least 40 carbons and including double carbon bond systems (Abuajah, Ogbonna, & Osuji, 2015). Carotenoids are further divided into two subgroups: carotenes, which lack oxygen functions

(e.g.,  $\beta$ -carotene and lycopene) and xanthophylls, which contain oxygen functions (e.g., lutein and zeaxanthin) (Johnson, 2002).

Carotenoids are biosynthesized in plants from the five carbon precursor isopentenyl pyrophosphate (IPP, C<sub>5</sub>), then change into another precursor, geranylgeranyl pyrophosphate (GGPP, C<sub>20</sub>), before becoming carotenes (Enfissi, Nogueira, Bramley, & Fraser, 2017). The biosynthesis of carotenoids in plants are shown in figure 1.3.



**Figure 1.3. Biosynthesis of carotenoids in vegetables.**

Adapted from Enfissi, Nogueira, Bramley, & Fraser, 2017. IPP: isopentenyl pyrophosphate. GGPP: geranylgeranyl pyrophosphate. DMAPP: dimethylallyl diphosphate.

### **Extracting, isolating, and separating carotenoids**

Carotenoids are abundant in colorful plants among carrot, pepper, spinach, sweet potato, tomato, and watermelon (Johnson, 2002). Carotenoids are low polarity and soluble in lipids, so they were first successfully extracted via solutions like isooctane, chloroform, acetonitrile, diethyl ether, and methanol (Zakaria, 1979). The extraction solution is then altered using acetone and diethyl

ether (Wills, Nurdin, & Wootton, 1988; Guil-Guerrero, Reboloso-Fuentes, & Isasa, 2003; Guil-Guerrero & Reboloso-Fuentes, 2009; Shen et al, 2017). Lin and Chen (2003) developed five solvent systems: ethanol/hexane (4:3, v/v), acetone/ hexane (3:5, v/v), ethanol/acetone/hexane (2:1:3, v/v/v), ethyl acetate/hexane (1:1, v/v), and ethyl acetate to compare extraction efficiency (Lin & Chen, 2003). The mixture of ethanol/hexane (4:3, v/v) resulted in the most extraction (Lin & Chen, 2003). Barba, Hurtado, Mata, Ruiz, & Tejada (2006) extracted and isolated carotenoids in plants by comparing six extraction solvents: methanol, ethyl ether, tetrahydrofuran (THF), methanol/THF (98:2, v/v), methanol/THF (50:50, v/v), and hexane/acetone/ethanol (50:25:25, v/v/v) (Barba, Hurtado, Mata, Ruiz, & Tejada, 2006). Among these six solvents, hexane/acetone/ethanol (50:25:25, v/v/v) was the most efficient extraction solvent. Table 1.2 provides a summary of the separation columns and mobile phases used in carotenoid separation in previous studies.

**Table 1.2. Separation columns and applied mobile phases in carotenoid separation.**

Column	Mobile phase	Reference
C18	Methanol/acetonitrile/water	Wills, Nurdin, & Wootton, 1988 (1988)
C18	Acetonitrile-1-butanol/methylene chloride	Lin and Chen, 2013
C18	Methanol/acetone	Barba et al., 2014
C18	Acetone/water	Sérino, Gomez, Costagliola, & Gautier, 2009
C18	Acetonitrile/methanol/ethyl acetate	GAMA, TADIOTTI, & Sylos, 2009
C30	Methanol/ MTBE	Seybold, Fröhlich, Bitsch, Otto, & Böhm, 2004
C30	Methanol/ MTBE	Guil-Guerrero & Reboloso-Fuentes, 2009
C30	Methanol/water/ MTBE	Gupta, Sreelakshmi, & Sharma, 2015
C30	Methanol/ MTBE	Hernández, Hellín, Fenoll, & Flores, 2015
ODS-1 reverse phase column	Acetonitrile/methanol/ tetrahydrofuran	Varzakas & Kiokias, 2016

## **Effect of the environment (high tunnel production and light spectra) on phenolic and carotenoid content in plants**

Phenolic and carotenoid profiles in plants are determined by genotypes, but those profiles are influenced by environmental factors like cultivation method (high tunnel, green house), light intensity and quality, temperature, water, oxygen, pest infection, and postharvest processing (Oh et al., 2011).

### **High tunnel cultivation**

High tunnel production is newly developed, primarily used in America's Mid-west and in Asia. Polyethylene films cover the tunnel, so high tunnels can maintain a mild temperature and retain humidity, protecting crops from insects and fluctuations of temperature. The primary advantage of high tunnel is to extend the growing season into the winter season and increase crop yield (Zhao, Rajashekar, Carey, & Wang, 2006), allowing the growing season to begin 1 to 4 weeks earlier in spring and extend it by 2 to 8 weeks in autumn (Wells & Loy, 1993). Production system environment can be better controlled in high tunnel production than in open field production (Wallace et al., 2012). However, studies showed cultivation in a high tunnel reduced the phenolic compound contents in lettuce (Zhao, Carey, Young, Wang, & Iwamoto, 2007; Zhao, Iwamoto, & Carey, 2007; Oh et al., 2011), and in pac choi (Zhao, Iwamoto, et al., 2007). Hence, the first objective of this study was to confirm the effect of high tunnel production on the phenolic content of two varieties of lettuce. Tomato is also a high-value vegetable, consumed daily by many people. However, the effect of high tunnel production on carotenoid contents in tomato remains incompletely understood. Therefore, we would study the effect of high tunnel cultivation on the carotenoid content in two varieties of tomato as well.

## **Light spectra**

Phenolic and carotenoid biosynthesis in plants is affected by light, an important abiotic factor. UV light is critical to the synthesis of phenolics and flavonoids (Gruda, 2005; Garcia-Macias et al., 2007). Visible light is also important in the biosynthesis of phenolics and carotenoids in vegetables. For example, red light improves plant growth (Stutte, Edney, & Skerritt, 2009; Chory, 2010), increases phenolics and flavonoids in lettuce (Li & Kubota, 2009), and increases carotenoids in Chinese cabbage (Fan et al., 2013). Blue light increases chlorophyll content in Chinese cabbage (Fan et al., 2013), increases carotenoids in green oak lettuce (Chen, Xue, Guo, Wang, & Qiao, 2016), and increases vitamin C in plants (Amoozgar, Mohammadi, & Sabzalian, 2017). However, little is known about how supplemental light spectra impact phenolics and flavonoids in leafy greens like lettuce and spinach. Thus, the second objective of this research was to identify how supplemental light, especially in the red, far-red, and blue spectra, impacts phenolics in two varieties of lettuce and flavonoids in two varieties of spinach.

## **Conclusion**

Isolating and separating phenolics and carotenoids requires chromatographic techniques like HPLC. The most commonly used separation column used to isolate and separate phenolics is the C18 reverse phased column while carotenoids require the C30 reverse phased column. In vegetables, genes determine the profiles of phenolics and carotenoids, but profiles vary because of the environment, especially cultivation method and light spectra. High tunnel production has resulted in decreased phenolics in vegetables. To our best knowledge, the effect of light spectra on phenolics in leafy greens has not been studied. Therefore, we will conduct research to study the effects of supplemental light on phenolics in leafy greens.

## **Part II. Novel 3D cell culture and cancer research in 2D and 3D cell culture systems**

To date, three primary 2D cell culture systems have been used in cancer research: tissue-culture plates, Petri-dishes, and cover slips (Hutmacher, 2010). However, cells in 2D monolayer culture lacked the complex 3D microenvironment and cell-cell interactions that exist *in vivo* (DesRochers, Palma, & Kaplan, 2014), so 2D culture cannot completely and accurately reflect cancer cell and tumor growth in a 3D microenvironment. Such inadequate experimental tools cannot accurately reflect either tumor growth or the effect of phenolics on cancer prevention. This inadequacy will result in experimental failures. Therefore, there is an increasing tendency of altering 3D culture by 2D culture because 3D culture can more closely mimic complex tumor microenvironment and reflect cell behaviors. 3D cell culture has shown metabolic and proliferative gradients across spherical geometry, a more accurate representation than 2D cell culture (Verjans, Doijen, Luyten, Landuyt, & Schoofs, 2018).

### **3D cell culture platforms**

Materials used in 3D culture influence cell behavior (Yamada & Cukierman, 2007). Thus, using 3D culture platforms is crucial for cell growth and for the success of the whole experiment. 3D culture platforms fall into two categories: scaffolds-free and scaffold models.

#### **Scaffold-free models, spheroids, and microfluidics**

Spheroids are the aggregates of single tumor cells or co-cultures with other types of cells, either suspended or embedded in 3D microenvironment (Friedrich, Ebner, & Kunz-Schughart, 2007). Spheroids are spherical aggregates of cells formed in cultures that retain a 3D shape with tissue-specific functions (Fitzgerald, Malhotra, Curtin, O' Brien, & O' Driscoll, 2015). Spheroids have advantages, including retaining tumor characteristics and morphologies from human bodies, are

sustainable for 2 weeks, mimic cell-cell interaction in co-culture spheroids, and are convenient (Huang & Gao, 2018). Multicellular tumor spheroids (MCTS) are quite promising because of their 3D structure and their penetration barrier, for treating tumors (Huang & Gao, 2018). One well known example of a scaffold-free model is the hanging drop method, referring to seeding single cells using a hanging drop culture and then incubating until the cells form 3D spheroids (Verjans et al., 2018). The scaffold-free magnetic method binds cells with nanoparticles to make them magnetic and resuspends them in a culture supplied with a magnetic field to form aggregates (Verjans et al., 2018). However, scaffold-free methods have some common drawbacks. Their wide application is limited because of their high cost, the difficulty of developing spheroid co-cultures, and a lack of standardized experimental conditions for specific cell lines.

### **Scaffolds**

Scaffolds are synthetic 3D cultures that support cells as they proliferate and differentiate. Hydrogels, decellularized tissues/organs, and cell-derived matrices are the three types of divided scaffolds (Fitzgerald et al., 2015). Natural materials like collagen, gelatin, and laminin can be synthesized to porous scaffolds (Ravi, Paramesh, Kaviya, Anuradha, & Solomon, 2015) and to synthetic polymers like poly-ethylene glycol (PEG) and poly-ethylene oxide (PEO) (Place, George, Williams, & Stevens, 2009). Natural polymers, however, are difficult to control because of their biochemistry and material properties (Cushing, & Anseth, 2007). Synthetic polymers, on the other hand, are made of polylactic and polyglycolide with large fiber diameters and pores that provide poor structure and mechanical properties and, consequently, affect cell growth in 3D microenvironment (Cushing, & Anseth, 2007).

## *Hydrogels*

Peptide-based biological polymer materials, hydrogels made of charged and/or noncharged amino acids, or blocks of hydrophilic and/or hydrophobic copolymers do not have these drawbacks and thus perform properly. Hydrogels composed of amino acid are structurally and mechanically similar to natural extra cellular matrices (ECMs) (Salick, Pochan, & Schneider, 2009). Hydrogels are networks of crosslinked hydrophilic polymers that swell in water but do not dissolve; they facilitate nutrient, oxygen, and waste transport (Verjans et al., 2018).

Hydrogels allow the diffusion of nutrients and oxygen and hence help cell growth within the scaffold matrix. The main advantage of hydrogel is that hydrogel can be fractured into small pieces, performing like liquid gel under high shear. Moreover, hydrogels can cross-link the network and perform like gel again when shear ceases (Dong, Paramonov, Aulisa, Bakota, & Hartgerink, 2007; Yan et al., 2010; Bakota, Wang, Danesh, & Hartgerink, 2011). In 3D hydrogel cell culture, cells can easily grow within the scaffold matrix when mildly encapsulated.

However, hydrogel swelling is affected by temperature, ionic strength, and pH (Sri, Ashok, & Arkendu, 2012). One promising hydrogel model called PepGel overcomes these drawbacks of environmentally sensitive hydrogels. The composition of amino acids in hydrogels means peptide monomers can fold into stable structures like  $\alpha$ -helical or  $\beta$ -sheet, which helps associate structures and cross-link the network (Huang, Herrera, Luo, Prakash, & Sun, 2012). Cells can be seeded at room temperature and cells cultured in PepGel in a 37°C incubator to allow gel formation. PepGel does not need ice to cool down and avoids acidic conditions or UV light. PepGel can be shipped and stored at 4°C, which is also convenient. PepGel in cell culture can be easily broken down via gentle pipetting. It has been demonstrated PepGel has no significant

effect on viability in 3D hydrogel cell culture in contrast to 2D monolayer culture (Huang, Ding, Sun, & Nguyen, 2013; Liang, Susan Sun, Yang, & Cao, 2017).

### ***Decellularized tissues/organs, and cell-derived matrices***

Decellularized tissues/organs is another type of scaffold that has been successfully applied in tissue engineering. However, the ability to remove cells from the tissues depends on origin of the tissue and the removal method (Gilbert, Sellaro, & Badylak, 2006). The last subgroup, the cell-derived matrix, refers to a coating from deposition of extra cellular matrix (ECM) obtained from culturing cells on a biomaterial surface (Fitzgerald et al., 2015).

### **Phenolics effect on liver cancer and colon cancer**

#### **Liver and colon cancer**

Liver cancer is one of the frequent death-leading cancer globally (Sener & Grey, 2005).

Colorectal cancer (CRC) is the third most common cancer in males and second in females worldwide (Favoriti et al., 2016). Liver cancer research based on using HepG2 cell line has been extensively studied (Musonda & Chipman, 1998; Kuo & Lin, 2003; Chun, Chang, Choi, Kim, & Ku, 2005; Rezaei et al., 2012), indicating HepG2 cell line is a good model for liver cancer research (Knasmüller et al., 1998; Mersch-Sundermann, Knasmüller, Wu, Darroudi, & Kassie, 2004; Ramos, Alía, Bravo, & Goya, 2005). Several colorectal cancer cells were used as models to study including cell line HT-29 and Caco-2 (Yi, Fischer, Krewer, & Akoh, 2005; Shimizu et al., 2005), and SW480, HT-29, and Cacao-2 (Wang et al., 2000). Among these three cell lines, SW480 is a good colorectal adenocarcinoma model for colorectal cancer research.

Altering diet and lifestyle is an important part of reducing the risk of CRC (Willett, 1995).

Phenolics have health benefits: antioxidative, antimicrobial, anticarcinogenic, anti-inflammatory, and cholesterol reduction (Leitzmann, 2016). Phenolic antioxidants have anti-inflammatory, anti-

cancer, and immune-modulating action and help prevent chronic diseases (Abuajah et al., 2015; Zhang et al., 2015). This review will briefly cover the antioxidative and anticancer effects of phenolics.

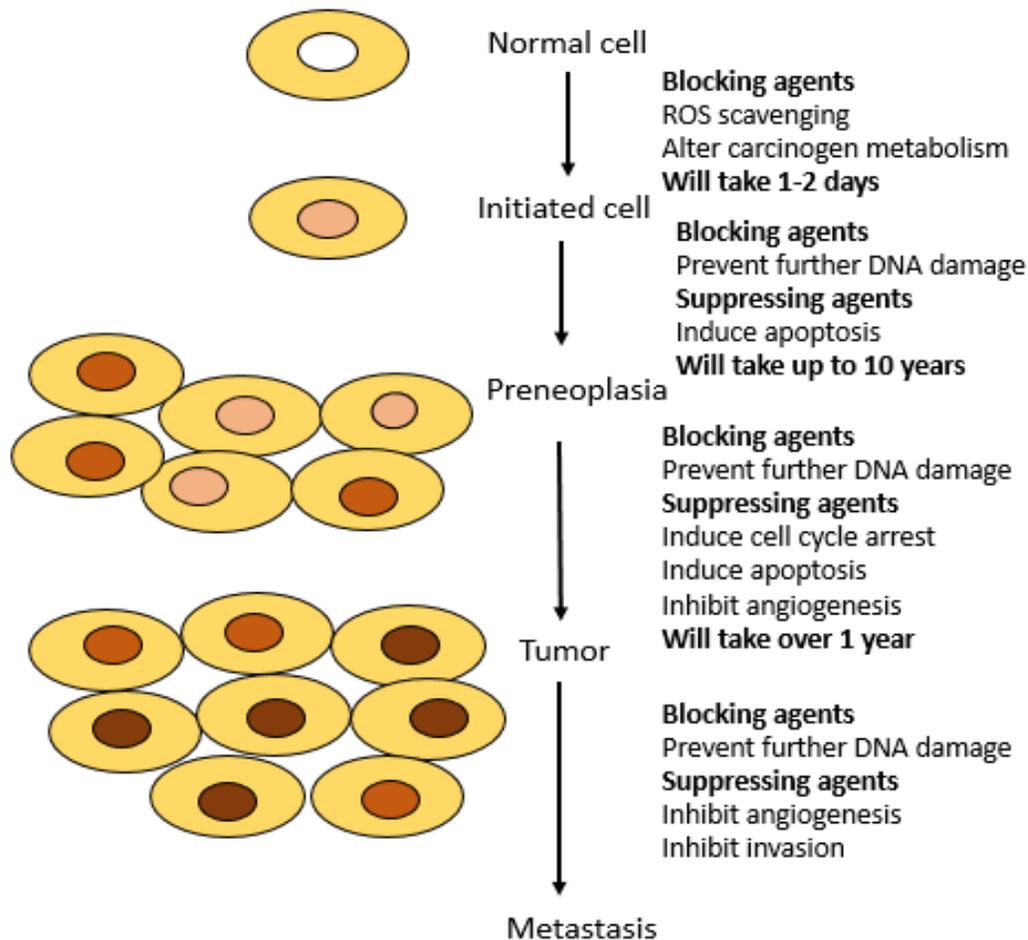
### **Antioxidant effect**

Age-related chronic diseases are caused by overproduction of reactive and nitrogen oxidants in human body. Oxidants like nitric oxide (NO), hydroxyl radical (OH), and superoxide anion ( $O_2^-$ ), all of which are physiologically produced reactive oxygen species (ROS), if overproduced in the human body causes imbalance and oxidative damage to large biomolecules like DNA, proteins, and lipids, leading further to aging related diseases. The hydroxyl groups on phenolics can act as an electron or hydrogen atom donor, neutralizing free radicals and ROS. Hydroxyl groups on polyphenols help in scavenging free radicals because they function as substitutes for scavengers (Rokayva et al., 2013). More hydroxylic groups have a stronger anticancer effect than polyphenols without hydroxylic groups or containing  $-OCH_3$  moieties (Anantharaju, Gowda, Vimalambike, & Madhunapantula, 2016). In dietary polyphenols, flavonoids account for 60% and phenolic acid account for 30% (Ramos, 2007). Phenolics often conjugate with other compounds in plants like glycosides, acylglycosides, and aglycones (Zhang & Tsao, 2016). Free or simple conjugates of polyphenols are absorbed in the upper gastrointestinal (GI) tract. Absorbed phenolics can be further transported to other tissues and organs via blood vessels, but those unabsorbed phenolics will be further metabolized or released by gut microbiota in the colon.

### **Anti-cancer effect**

Cancer is a complex disease with functioning oncogenes, loss of functioning tumor suppressor genes, and tumor mutations caused by the environment (Thoma, Zimmermann, Agarkova, Kelm,

& Krek, 2014). Tumor initiation starts with DNA in cells exposed to carcinogens (smoking, infection/inflammation, and nutrition/diet) and causes further damage (Doll and Peto, 1981). Three primary phases in the process of carcinogenesis are tumor initiation, promotion, and progression (Ramos, 2007). Initiation is a rapid and irreversible process in intracellular and extracellular chain events. Tumor promotion is a long and reversible process in which active cells proliferate and accumulate, and progression refers to neoplasm transformation or the growth of tumors and metastasis (Surh, 2003). Chemoprevention of cancer varies depending on the phase, by blocking (preventing carcinogens from reaching the target site) or suppressing (inhibiting malignant transformation of initiated cells, in the promotion or progression stage). The underlying anti-cancer effects of phenolics involve regulating growth factors or receptors; regulating cell signaling involving kinase and transcription factors, important genes that control the cell cycle and involving cell cycle arrest, cell survival, and apoptosis (Wahle, Brown, Rotondo, & Heys, 2010); and inhibiting oncogene transformation to normal cells, suppressing tumor proteins like p53, angiogenesis, and metastasis (Anantharaju et al., 2016). Several possible mechanisms for preventing cancer depend on controlling or modulating key molecules in cell proliferation and survival. This may involve altering the signaling pathways through mitogen-activated protein kinase (MAPKs), phosphoinositide-3-kinase (PI3K), and protein kinase B (PKB); modifying transcription factors like activator protein-1 (AP-1) and nuclear factor  $\kappa$ B (NF- $\kappa$ B); and alternating and modulating key molecules controlling cell cycles like cyclin-dependent kinases (CDKs), inhibitors, or apoptosis (Bcl-2 family) (Manson, 2003). If these key molecules are suppressed or inhibited, cancer cells will undergo cell-cycle arrest or apoptosis. Figure 1.4 shows the potential intervention in carcinogenesis and is adapted from (Manson, 2003; Surh, 2003).



**Figure 1.4. Potential intervention in carcinogenesis.**

ROS: reactive oxygen species. Adapted from Manson, 2003 and Surh, 2003.

### **Current cancer research in 2D and 3D cell culture**

As mentioned, so far, more than 70% of cancer research relies on 2D monolayer culture before correlating to *in vivo* studies (Hutmacher, 2010). To date, few researchers have studied the toxicity of drugs on liver cancer cells in 3D cultures, including hepatocytes in microfluidic systems (Chao, Maguire, Novik, Cheng, & Yarmush, 2009), Caco-2 cells in microfluidic chips (Imura, Asano, Sato, & Yoshimura, 2009), hepatic co-culture platforms (Novik, Maguire, Chao, Cheng, & Yarmush, 2010), HepG2/G3a cells in microfluidic biochip models (Prot et al., 2011), and collagen scaffolds containing villi microchip (Yu, Peng, Luo, & March, 2012).

Previous research has extensively considered the effect of dietary phenolics on cancer, primarily in 2D cultures. However, to the best of our knowledge, no reports have been published on the effect of phenolics on human hepatomacarcinoma HepG2 cells in 3D hydrogel culture. Similarly, we have found no studies of the effect of phenolics on colorectal cancer cells SW480 in 3D hydrogel culture. Thus, the first objective of this study was to develop a protocol for seeding and isolating HepG2 and SW480 cells in 3D cultures. The second objective was to study the effect of chlorogenic acid on inhibiting HepG2 cell growth and SW480 cell growth.

### **Conclusion**

In a summary, while cancer research in 2D cultures *in vitro* provides prerequisite knowledge of the underlying mechanisms and suggested phenolics concentration in experiments preparing for *in vivo* studies, 2D cell culture is not ideal. 3D cell models, on the other hand, can mimic microenvironments and cell-cell interactions more accurately. However, the high cost and small scale of experiments limit wider application of 3D culture, and standardized experimental protocols for 3D models are lacking because cell seeding density is cell-, organ-, and model-dependent. Therefore, experiments involving different cell lines and diverse anti-cancer phenolics should be conducted to fill this gap in the research.

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## **Chapter 2 - Determination of phenolic contents in lettuce and carotenoid contents in tomato grown in high tunnel and open field**

### **Abstract**

Phytochemicals such as phenolics and carotenoids in plants are health-promoting factors associated with preventing cancer and chronic diseases. While many crops are increasingly grown in high tunnel to increase their yield and extend growing season, however, previous studies showed high tunnel production resulted in decrease in phenolic contents in vegetables. Therefore, the aim of this research was to confirm the effect of high tunnel production on phenolic contents in lettuce and carotenoid contents in tomato. Two varieties of lettuce ('Two Star' and 'New Red Fire') and two varieties of tomato ('Mountain Fresh' and 'Celebrity') were grown in high tunnel in contrast to traditional cultivation of open field. Phenolics in lettuce and carotenoids in tomato were characterized by using High Performance Liquid Chromatography (HPLC). Four phenolic acids (gallic acid, chlorogenic acid, caffeic acid and chicoric acid) and four flavonoids (luteolin-7-glucoside, rutin, apigenin-7-glucoside and kaempferol) in lettuce were identified and quantified. Three carotenoids including lutein,  $\beta$ -carotene, and lycopene in tomato were identified and quantified. The results showed phenolic contents of 'Two Star' (a green leaf lettuce) grown in high tunnel was significantly lower in contrast to those grown in open field. High tunnel production resulted in reduction of predominant phenolic acids (chlorogenic acid and chicoric acid) and flavonoid (luteolin-7-glucoside) in 'Two Star' lettuce. However, high tunnel production did not affect phenolic contents in 'New Red Fire' (red leaf lettuce). Carotenoid contents in 'Celebrity' grown in high tunnel were significantly lower than

those grown in open field. However, there was no significant difference of carotenoid contents within 'Mountain Fresh' tomato grown in high tunnel in comparison to open field. This study confirmed that high tunnel production decreased phenolic contents in 'Two Star' lettuce and reduced carotenoid contents in 'Celebrity' tomato. The effect of high tunnel production on phenolic and carotenoid contents in vegetables is genotype dependent.

Key words: lettuce, tomato, phenolic, carotenoid, high tunnel, open field

## 2.1. Introduction

Epidemiological studies suggest dietary intake of vegetables and fruits rich in phytochemicals associated with reducing risk of cancer and chronic diseases such as cardiovascular disease, diabetes, obesity, and other age-related diseases (Slavin & Lloyd, 2012; Liu, 2013; Abuajah, Ogbonna, & Osuji, 2015; Zhang et al., 2015; Leitzmann, 2016). Phytochemicals, also known as secondary metabolites, are bioactives uniquely to plants. So far, over 8000 phytochemicals have been identified and primarily categorized into four groups including alkaloids, phenolics, terpenoids, sulfur containing compounds and nitrogen containing compounds (Garcia-Salas et al., 2010).

Lettuce is a commonly consumed leafy green primarily via salad. Lettuce is a good source of macronutrients (eg., carbohydrate and fiber) and micronutrients (eg., iron, folate, and vitamin C) and meantime low in calories, fat and sodium (Kim, Moon, Tou, Mou, & Waterland, 2016).

Lettuce is rich in health-promoting phenolic acids such as chlorogenic acid, chicoric acid, caffeic acid, and flavonoids such as luteolin (Santos et al., 2016). Extensive studies demonstrate health benefits of these phenolics such as effect of luteonlin-7-glucoside on inflammation of skin diseases (Palombo et al., 2016); chlorogenic acid against colorectal cancer (Hou, Liu, Han, Yan, & Li, 2017), kaempferol against cervical cancer (Kashafi, Moradzadeh, Mohamadkhani, & Erfanian, 2017), and effect of chicoric acid on inhibiting oxidized low-density lipoprotein (LDL) thus to prevent atherosclerotic cardiovascular disease (Tsai et al., 2017).

Tomato is a warm season crop consumed as vegetable and fruit via fresh or processed, and contains carbohydrates, vitamin C, vitamin E and folic acid, phenolics and carotenoids (eg., lutein,  $\beta$ -carotene, and lycopene) (Kelebek et al., 2017). Previously, health benefits of carotenoids have been demonstrated for example, xanthophylls in tomato such as lutein and

zeaxanthin absorbed damaging blue light in macular region of retina therefore protected vision (Johnson, 2002). Carotenoids can improve visual performance, sleep quality and adverse physical symptoms (Stringham, Stringham, & O'Brien, 2017). Dietary intake of food rich in  $\beta$ -carotene and  $\alpha$ -carotene is correlated with reducing risk of type 2 diabetes in healthy humans (Slujis et al., 2015). Carotenoids can prevent prostate cancer and breast cancer (Linnewiel-Hermoni et al., 2015).

It is well-known that profiles of phenolic and carotenoid in plants are determined by the genotype factors, but can be influenced by environmental factors. Phenolics in plants are synthesized via 'shikimic pathway' through the enzyme phenylalanine ammonia-lyase (PAL) (Dixon & Paiva, 1995; Oh, Carey, & Rajashekar, 2011). Phenylalanine ammonia-lyase (PAL) enzyme is sensitive and responsive to diverse biotic and abiotic factors including cultivation methods, light intensity and spectra, insect/pathogen attack, wounding, change of temperature and nutrient status (Dixon & Paiva, 1995). Conventionally, plants are grown in open field. However, plant yield is quite often affected by fluctuation of natural environment such as harsh temperature, drought or waterlogging conditions. Un heated polyethylene films covered on the tunnel, known as high tunnel, has been developed as a protective measure. High tunnel production has been widely applied in Asia and mid-west America. High tunnel has many advantages such as protecting plants from natural climatic variation, extending growing season, reducing the pest infection, and increasing crop yield (Lamont et al., 2003; Rader and Karlsson, 2006; Luthria, Mukhopadhyay, & Krizek, 2006). Wells and Loy (1993) report high tunnel production extends growing season from 1 to 4 weeks in spring and 2 to 8 weeks in autumn. High-value crops are primarily grown in high tunnel such as leafy greens, tomato, pepper, small fruit, and ornamentals (Carey et al., 2009).

Previously, some research focused on the effect of high tunnel production on phenolic contents in lettuce and carotenoid contents in tomato. For example, Zhao et al (2007) reported the effect of high tunnel on phenolics in two varieties of lettuce ('Red Sails' and 'Kaulara') in contrast to conventional cultivation open field, and showed high tunnel reduced phenolic contents in both two varieties of lettuce. Oh et al (2011) investigated the impact of high tunnel on phenolic contents in two varieties of lettuce 'Red Sails' and 'Baronet' in contrast to open field, and demonstrated high tunnel decreases in phenolic contents in both two varieties. A two-year study compared the lycopene and phenolic contents in tomato grown in high tunnel in contrast to open field and results showed high tunnel reduced phenolic contents but improved lycopene content in the first year of production, however, high tunnel reduced lycopene contents but did not affect phenolic contents in tomato in the second year of cultivation (Cowan, Miles, Andrews, & Inglis, 2014). The results on the effect of high tunnel on phenolic contents and carotenoid contents in plants are controversial. Limited studies cannot completely reveal the overall trend of phenolic contents in lettuce grown in high tunnel. Therefore, driven by this impetus, the objective of this research was to study the influence of high tunnel on phenolic contents in two varieties of lettuce including 'Two Star' and 'New Red Fire' and carotenoid contents in two varieties of tomato including 'Mountain Fresh' and 'Celebrity' in contrast to cultivation of open field.

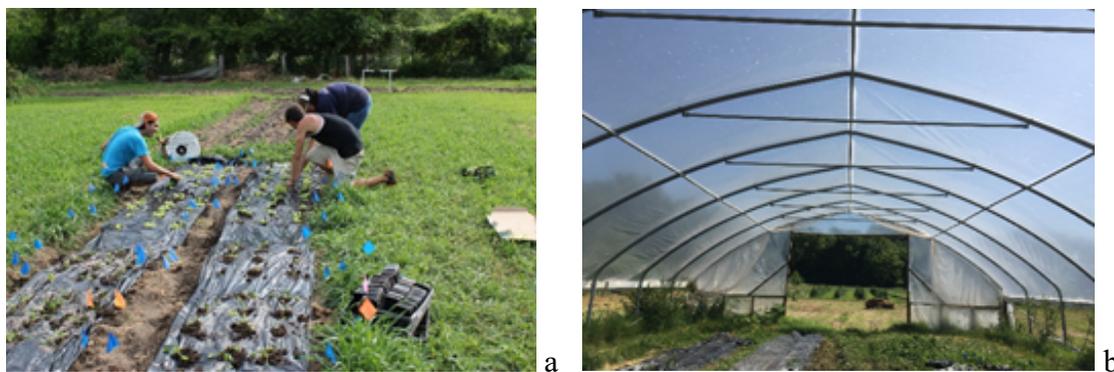
## **2.2. Materials and Methods**

### **2.2.1. Lettuce and tomato samples**

Leaf lettuce ('New Red Fire' and 'Two Star') and tomato ('Mountain Fresh' and 'Celebrity') were harvested during the summer of 2016 at Willow Lake Student Farm, Manhattan, KS.

Lettuce and tomato samples were freeze dried after harvesting and kept in -20 °C until analysis.

Treatment for lettuce and tomato had 3 replications and 1-3 replications, respectively.



**Figure 2.1. Open field (a) and high tunnel (b) at Willow Lake Student Farm, Manhattan, KS.**

### **2.2.2. Chemical reagent and solvent**

3,4,5-trihydroxybenzoic acid (gallic acid) was purchased from ACROS organics (Marris, NJ). 1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate) (chlorogenic acid), 3,4-hydroxycinnamic acid (caffeic acid), chicoric acid, and kaempferol were purchased from Sigma Aldrich (St. Louis, Mo). Luteolin-7-glucoside and apigenin-7-glucoside were purchased from Indofine Chemical (Hillsborough, NJ). Quercetin-3-rutinoside (rutin) was purchased from ACROS organics (Marris, NJ). Flavone was used as internal standard and purchased from BioChemika (San Angelo, TX). Lutein standard was purchased from Iodofine Chemical Company (Hillsborough, NJ).  $\beta$ -carotene was purchased from Alfa Aesar (Ward Hill, MA). Lycopene standard was purchased from MP Biomedicals (Solon, OH). Trans-beta-8-apo'-carotenal was used as internal standard (I.S.) purchased from Sigma Aldrich (St.Louis, MO). HPLC grade solvents were purchased from Fischerscientific (Fair Lawn, NJ) including methanol, and methyl tert-butyl ether (MTBE). Formic acid was purchased from Sigma Aldrich (St. Louis, MO). 0.45  $\mu$ m and 0.2  $\mu$ m syringe filter were purchased from Conring Incorporated (Corning, NY). Sodium Chloride was purchased from Fischerscientific (Fair Lawn, NJ).

Extraction solvent ethanol was purchased from Alfa Aesar (Ward Hill, MA) and hexane was purchased from Fischerscientific (Fair Lawn, NJ).

### **2.2.3. Extraction of phenolic compounds in lettuce**

Dry leaf sample (0.15 g) was extracted with 15 ml of 70% aqueous methanol containing flavone as internal standard (I.S.) and mixed overnight via orbital shaking (Benchmark, Edison, NJ). Each sample was centrifuged by Eppendorf centrifuge (5810R) at 20 °C, 3950 rpm for 30 min and filtered. The collected supernatant was brought to 25 ml in a volumetric flask by adding 70% aqueous methanol. Two ml solution was evaporated under gentle nitrogen gas. Then re-dissolved in 200  $\mu$ L 70% aqueous methanol and filtered using 0.45  $\mu$ m Whatman syringe filter for HPLC analysis.

### **2.2.4. HPLC separation and quantification of phenolic compounds in lettuce**

A Shimadzu HPLC system (Kyoto, Japan) was used for chromatographic separation and analysis. This system was composed of a DGU-20A3 degasser, an LC-20AB liquid deliver pump, a SIL-20A auto-sampler, a CBM-20A communication bus module, a SPD-20A diode array detector, and a CTO-20AC column oven. A Waters (Milford, MA) C18 reversed phase column (250 mm length, 4.6 mm diameter) was used for phenolics separation. Data was analyzed using LC solution software (Kyoto, Japan). Diode array detector was used and UV/vis scans ranged from 190 to 800 nm.

For separating phenolics in lettuce, the elution was performed with mobile phase A (5% formic acid in de-ionized water) and mobile phase B (5% formic acid in 95% methanol). An optimum column oven was 31 °C. The flow rate is 0.8 ml/min. The gradient conditions were set up as follows: solvent B contents at 0-10% for 5 min, 10-40% for 25 min, 40-70% for 10 min, and held at 70% for 16 min before returning to 0% at 64 min. Phenolic acids were expressed as equivalent

of vanillic acid and flavonoids were expressed as equivalent of quercetin. Retention time and specific wavelength of each analyte was identified with commercial authentic standard. Analytes were quantified by its peak area and calibration curves. Retention time and specific wavelength of each phenolic is shown in table 2.1.

**Table 2.1. Retention time and specific wavelength of phenolics in lettuce.**

Phenolic acid/ flavonoid	Wavelength (nm)	Retention time (min)
Gallic acid	271	6.74
Chlorogenic acid	272	19.28
Caffeic acid	325	21.10
Chicoric acid	327	30.25
Luteolin-7-glucoside	350	32.89
Rutin	335	33.35
Apigenin-7-glucoside	354	35.83
Kaempferol	347	36.36

### **2.2.5. Carotenoids extraction in tomato**

The extraction method was adapted from Li and others (2012). Tomato sample (0.3 g) was added in 6 ml extraction solution (ethanol: hexane, 4:3, v/v) and mixed via orbital shaking (Benchmark, Edison, NJ) for 3 hr. The mixture was centrifuged by Eppendorf centrifuge (5810R) at 3950 rpm at 20 °C for 30 min. Supernatant was collected and added 8 ml hexane and centrifuged again.

This step was repeated one more time. All the supernatant was collected in a separatory funnel and washed by 50 ml distilled water then 50 ml 10% sodium chloride (NaCl) solution. The lipid layer was collected and evaporated to dryness under gentle Nitrogen gas. Then re-dissolved in 1 ml methanol/ MTBE (1:1, v/v). Solution was filtered through 0.2 µm membrane filter waited for HPLC analysis. The tubes were covered by aluminium foil and all procedures were performed in the dark with the amber light for the purpose of avoiding carotenoid degradation.

### 2.2.6. HPLC separation and quantification of carotenoids in tomato

YMC C30 reversed phase column (3  $\mu$ m, 4.6 x 250 mm) was used for separating carotenoids. Mobile phase was assayed as followings: A: methanol/ MTBE (7:3, v/v), B: MTBE. Mobile phase flow rate was 1 ml/min. Column temperature was 25 °C. Carotenoids were detected at 450 nm. The gradient elution method was described as followings: t=0 min, 10%B; t=2 min, 20%B; t=10 min, 20%B; t=20 min, 70%B; t=25 min, 10%B; t=30 min, 10%B. Retention time of individual carotenoid is shown in table 2.2.

**Table 2.2. Retention time of carotenoids detected at 450 nm.**

Carotenoid	Retention time (min)
lutein	4.51
$\beta$ -carotene	8.92
Lycopene	24.5

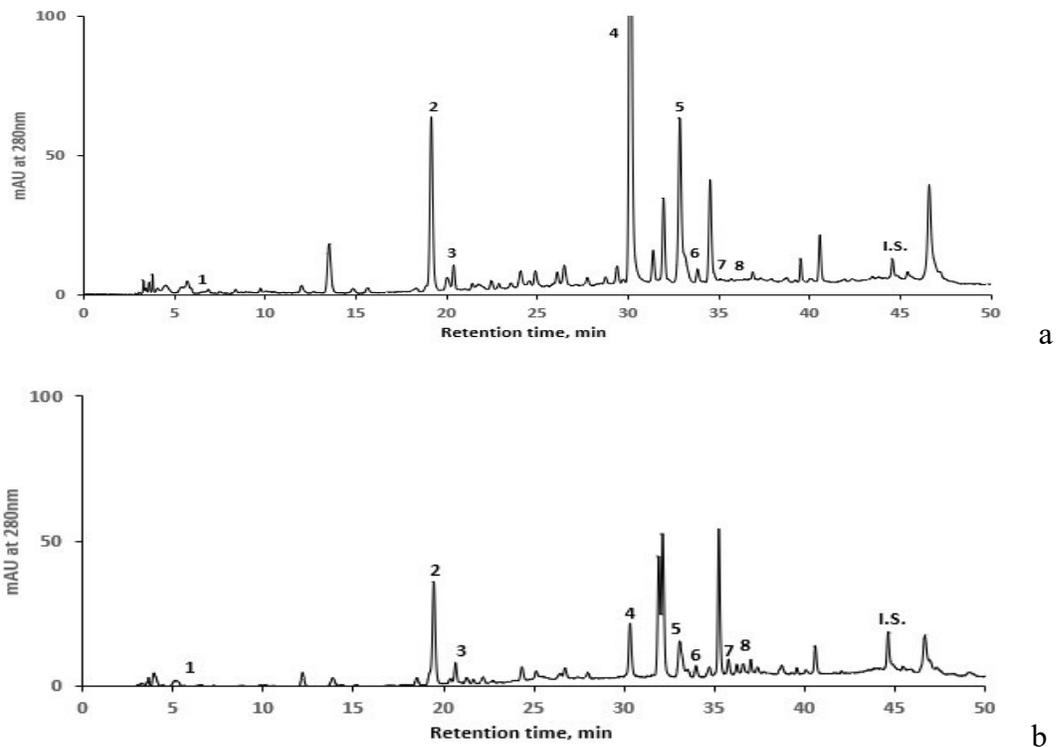
### 2.2.7. Statistical analysis

Analysis of variance (ANOVA) was performed using Statistical Analysis System (SAS 9.4) version. Data was exhibited as mean  $\pm$  standard deviation. Tukey's test was applied to compare the means ( $\alpha=0.05$ ).

## 2.3. Results and Discussion

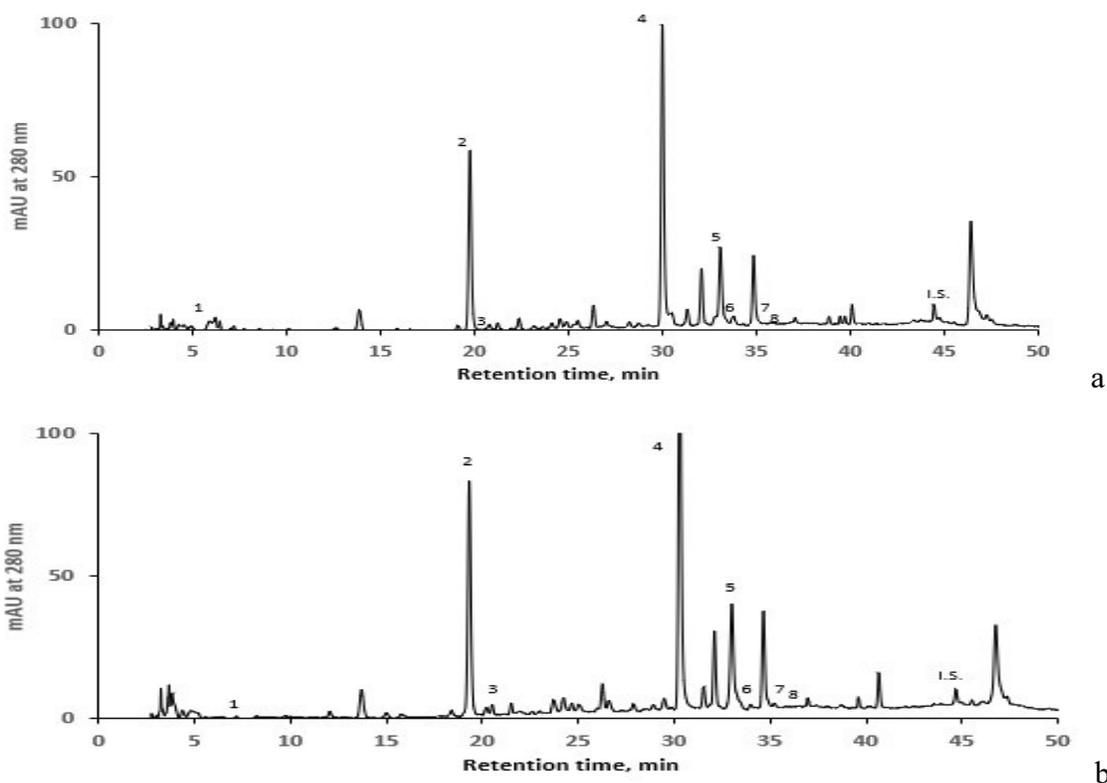
### 2.3.1. Effect of high tunnel production on phenolic contents in lettuce

Representative chromatograms of 'Two Star' and 'New Red Fire' grown in open field and high tunnel are shown in figure 2.2 and 2.3 respectively. The total identified phenolic acid and flavonoid contents are shown in figure 2.4. The individual phenolic acid and flavonoid content of two varieties grown in high tunnel and open field is shown in table 2.3.



**Figure 2.2. Representative HPLC chromatograms of ‘Two Star’ lettuce grown in open field (a) and high tunnel (b).**

Peak 1: gallic acid; peak 2: chlorogenic acid; peak 3: caffeic acid; peak 4: chicoric acid; peak 5: luteolin-7-glucoside; peak 6: rutin; peak 7: apigenin-7-glucoside; peak 8: kaempferol; I.S.: flavone.



**Figure 2.3. Representative HPLC chromatograms of ‘New Red Fire’ lettuce grown in open field (a) and high tunnel (b).**

Peak 1: gallic acid; peak 2: chlorogenic acid; peak 3: caffeic acid; peak 4: chicoric acid; peak 5: luteolin-7-glucoside; peak 6: rutin; peak 7: apigenin-7-glucoside; peak 8: kaempferol. I.S.: flavone.

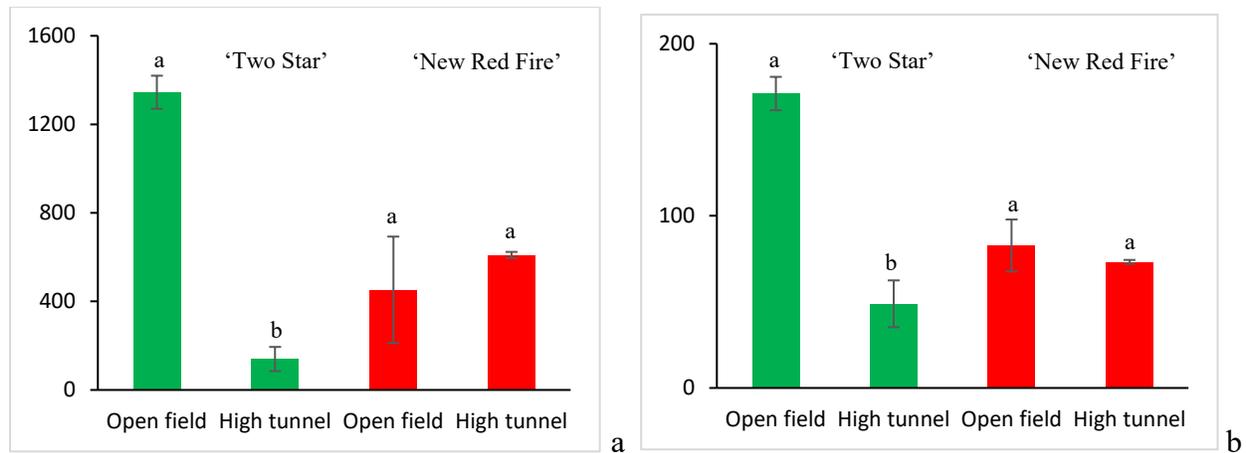
Chlorogenic acid and chicoric acid were the two predominant phenolic acids in lettuce and the combination of these two phenolic acids accounted for over 90% of total identified phenolic acid contents. High tunnel production resulted in significant reduction of chlorogenic acid and chicoric acid contents in ‘Two Star’ in contrast to open field, thus resulted in lower total identified phenolic acid contents of ‘Two Star’ lettuce grown in high tunnel in contrast to open field. Luteolin-7-glucoside accounted for the highest amount of identified flavonoids in lettuce. High tunnel production significantly reduced the luteolin-7-glucoside contents in ‘Two Star’ lettuce, therefore leading to decline the total identified flavonoid contents in comparison with open field. Although the high tunnel improved the contents of rutin, apigenin-7-glucoside and

kaempferol, the combination of these three flavonoid contents accounted for small amount of contents of total identified flavonoid. Overall, total identified flavonoid contents of 'Two Star' grown in high tunnel were decreased. Our results were in accordance with previous studies that high tunnel production reduced phenolic contents in lettuce (Zhao, Carey, Young, Wang, & Iwamoto, 2007; Oh et al., 2011). Polyethylene films covered on the tunnel resulted in blocking light intensity and reducing UV radiation (Antignus et al., 1996). High light intensity and solar UV radiation played important roles in phytochemical accumulation in plants (Rajashekar et al., 2009). Exposure to UV light improved the phenolic contents in plants (Gruda, 2005; Luthria, Mukhopadhyay, & Krizek, 2006; Taber, Havlovic, & Howell, 2010). Shading on tunnel resulted in significant decrease in anthocyanin contents in lettuce (Kleinhenz, Gazula, & Scheerens, 2003), and UV-transparent films increased flavonoid contents in lettuce in contrast to UV block films (García-Macías et al., 2007). Thus, reducing light intensity and blocking UV light in high tunnel resulted in reducing phenolic contents in 'Two Star' lettuce. However, high tunnel did not affect phenolic acid and flavonoid contents in 'New Red Fire' lettuce. Therefore, the effect of high tunnel on phenolic contents in lettuce was genotype dependent.

**Table 2.3. Individual phenolic acid and flavonoid content of ‘Two Star’ and ‘New Red Fire’ lettuce grown in open field and high tunnel.**

	‘Two Star’		‘New Red Fire’	
	Open field	High tunnel	Open field	High tunnel
gallic acid	2.4±0.7 <sup>a</sup>	6.6±6.6 <sup>a</sup>	10.7±1.0 <sup>a</sup>	2.5±1.4 <sup>b</sup>
chlorogenic acid	102.9±9.4 <sup>a</sup>	31.6±18.9 <sup>b</sup>	36.3±33.4 <sup>a</sup>	60.3±1.2 <sup>a</sup>
caffeic acid	2.1±0.2 <sup>a</sup>	7.1±2.9 <sup>a</sup>	2.2±1.5 <sup>a</sup>	2.6±0.6 <sup>a</sup>
chicoric acid	1236.4±96.8 <sup>a</sup>	94.1±39.0 <sup>b</sup>	544.3±14.2 <sup>a</sup>	410.1±316.6 <sup>a</sup>
luteolin-7-glucoside	171.4±11.7 <sup>a</sup>	35.2±5.9 <sup>b</sup>	75.0±8.2 <sup>a</sup>	64.6±1.3 <sup>a</sup>
rutin	1.2±0.2 <sup>b</sup>	7.8±6.0 <sup>a</sup>	5.8±8.2 <sup>a</sup>	11.7±5.7 <sup>a</sup>
apigenin-7-glucoside	1.3±0.4 <sup>a</sup>	4.8±2.7 <sup>a</sup>	1.0±0.8 <sup>a</sup>	1.2±0.3 <sup>a</sup>
kaempferol	1.5±0.5 <sup>b</sup>	9.0±0.6 <sup>a</sup>	1.1±0.8 <sup>a</sup>	2.1±0.6 <sup>a</sup>

Values are means ± SD, n=3. Means without a common letter within the same row within the same variety differ ( $p \leq 0.05$ ). Phenolic acid content was expressed as equivalent of vanillic acid ( $\mu\text{g/g}$  dry weight), and flavonoid content was expressed as equivalent of quercetin ( $\mu\text{g/g}$  dry weight).



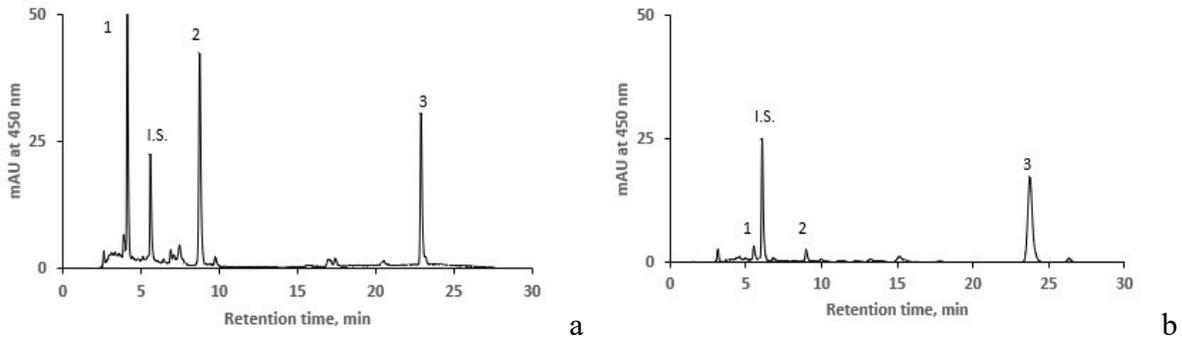
**Figure 2.4. The total identified phenolic acid contents (left) and total identified flavonoid contents (right) in lettuce grown in open field and high tunnel.**

Total identified phenolic acid contents were expressed as equivalent of vanillic acid ( $\mu\text{g/g}$  dry weight), total identified flavonoid contents were expressed as equivalent of quercetin ( $\mu\text{g/g}$  dry weight).

### 2.3.2. Effect of high tunnel production on carotenoid contents in tomato

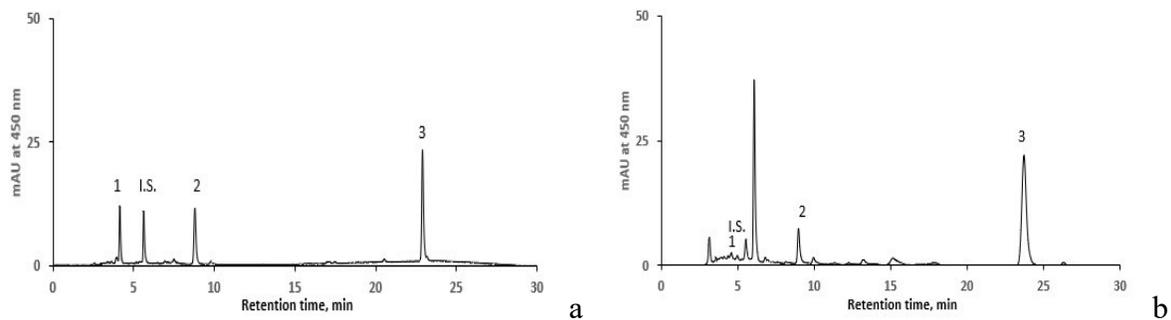
Representative chromatograms of carotenoids in ‘Celebrity’ and ‘Mountain Fresh’ grown in high tunnel and open field are shown in figure 2.5 and 2.6, respectively. Total identified carotenoid contents in ‘Celebrity’ and ‘Mountain Fresh’ grown in high tunnel and open field are shown in

figure 2.7. The individual carotenoid content of ‘Celebrity’ and ‘Mountain Fresh’ grown in high tunnel and open field is shown in table 2.4.



**Figure 2.5. Representative HPLC chromatograms of ‘Celebrity’ tomato grown in open field (a) and high tunnel (b).**

Peak 1: lutein; peak 2:  $\beta$ -carotene; peak 3: lycopene.



**Figure 2.6. Representative HPLC chromatograms of 'Mountain Fresh' tomato grown in open field (a) and high tunnel (b).**

Peak 1: lutein; peak 2:  $\beta$ -carotene; peak 3: lycopene.

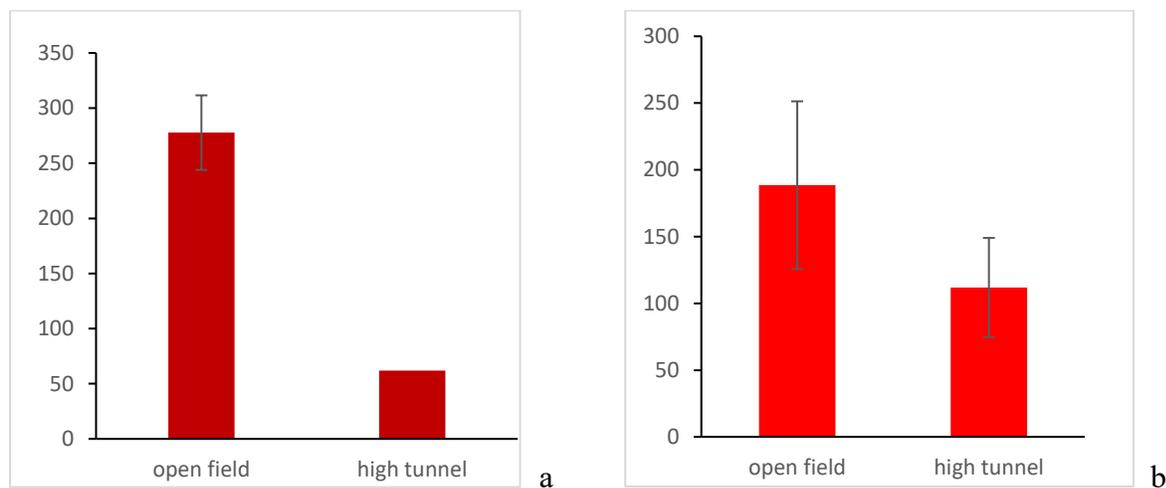
High tunnel decreased lutein,  $\beta$ -carotene and lycopene contents of ‘Celebrity’ tomato in contrast to open field. Our result was in the agreement with previous study that shaded tunnels reduced carotenoid contents in tomato in contrast to those grown under direct sunlight (Marais, Van Wyk, & Rapp, 1991). Infrared and short-wave radiation played important roles in coloring of tomato (Lipton, 1970) and lycopene biosynthesis was influenced by exposure to solar radiation (Dumas, Dadomo, Di Lucca, & Grolier, 2003). However, polyethylene films blocked UV light and reduced light intensity, therefore resulted in reducing lutein,  $\beta$ -carotene and lycopene in tomato

grown in high tunnel in contrast to open field. However, there was no significant difference of carotenoid contents in ‘Mountain Fresh’ grown in high tunnel compared with open field. Hence, the impact of high tunnel on carotenoid contents in tomato was determined by genotype factors.

**Table 2.4. Individual carotenoid content of ‘Celebrity’ and ‘Mountain Fresh’ grown in open field and high tunnel.**

Individual carotenoid	‘Celebrity’		‘Mountain Fresh’	
	Open field	High tunnel	Open field	High tunnel
lutein	83.8±14.6 <sup>a</sup>	6.5 <sup>b</sup>	58.4±36.3 <sup>a</sup>	7.8±2.9 <sup>a</sup>
β-carotene	123.9±1.0 <sup>a</sup>	8.9 <sup>b</sup>	78.3±38.7 <sup>a</sup>	21.7±7.9 <sup>a</sup>
lycopene	96.5±33.8 <sup>a</sup>	57.3 <sup>b</sup>	109.0±45.4 <sup>a</sup>	96.5±31.4 <sup>a</sup>

Values are means ± SD, n=2-3. Data of carotenoid in ‘Celebrity’ grown in high tunnel has 1 replicate. Means without a common letter within the same row within the same variety differ ( $p \leq 0.05$ ). Carotenoid content was expressed as equivalent of β-carotene (μg/g dry weight).



**Figure 2.7. Comparison of total identified carotenoid contents in 'Celebrity' (a) and 'Mountain Fresh' (b) grown in open field and high tunnel.**

Carotenoid content was expressed as equivalent of β-carotene (μg/g dry weight).

## **2.4. Conclusion**

High tunnel reduced phenolic contents in certain variety of lettuce ('Two Star') and carotenoid contents in certain variety of tomato ('Celebrity') in contrast to open field. The effect of high tunnel on phenolics in lettuce and carotenoids in tomato was genotype dependent. The negative effects of high tunnel on phenolics in lettuce and carotenoids in tomato could be overcome by variety selection and genetic engineering.

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## **Chapter 3 - Effect of supplemental light on phenolic contents in leafy greens (lettuce and spinach)**

### **Abstract**

Light is an important abiotic factor influenced plant growth and phenolic biosynthesis. However, little is known about effect of visible light on accumulation of phenolic compounds in leafy greens. Two varieties of lettuce ‘Two Star’ and ‘New Red Fire’ and two varieties of spinach ‘Bloomsdale’ and ‘Avon’ were grown in growth chamber under fluorescent lighting (control) and red (658 nm), far-red (734 nm), and blue (476 nm) spectra. The phenolics extracted from the edible leaves of lettuce and spinach and were identified and quantified by using High Performance Liquid Chromatography (HPLC). Far-red and blue light significantly increased phenolic acid contents and flavonoid contents in ‘Two Star’ lettuce, while red light did not. In ‘New Red Fire’ lettuce, a significant increase in phenolic acid and flavonoid content was observed by exposure to red light. However, far-red and blue light reduced phenolic acid and flavonoid contents in ‘New Red Fire’ lettuce compared to the control. Light quality did not alter flavonoid contents in two varieties of spinach (‘Bloomsdale’ and ‘Avon’). Hence, the results suggest that the effect of visible light on phenolics in leafy greens are genotype dependent.

Key words: lettuce, spinach, phenolics, light spectra

### 3.1. Introduction

The dietary intake rich in vegetables and fruits is associated with reducing risk of cancer and chronic diseases such as cardiovascular disease and diabetes (Liu, 2013). Although the mechanisms of dietary intake of vegetables and fruits associated with lowering risk of chronic diseases are not well known. Phytochemicals present in the vegetables and fruits are considered to play important roles in preventing chronic diseases. Spinach is a good source of macronutrients such as carbohydrate, fiber, fat and micronutrients such as vitamin, pro-vitamin, as well as non-nutrient phytochemicals like  $\alpha$ -carotene and  $\beta$ -carotene, and flavonoids (Roberts & Moreau, 2016). Common flavonoids such as luteolin, apigenin, quercetin, and kaempferol are not present or occurred very low levels in spinach (Bergquist et al., 2005). Alternatively, flavonoids conjugated with four primary types of aglycones including patuletin, spinacetin, spinatoside, and jaceidin exist in spinach (Koh & Ng, 2009). Consumption of spinach showed increasing antioxidant capacity in serum in human which could help prevent chronic diseases (Cao, Russell, Lischner, & Prior, 1998). Another study demonstrated dietary intake of spinach inhibited human gastric cancer cell growth (Murakami et al., 2003). Maeda et al (2009) reported 20 mg/kg spinach glycolipid intake delayed the colon cancer cell growth. Except spinach, lettuce is another high value vegetable consumed daily which is rich in carbohydrate and micronutrients such as iron, folate, and vitamin C, and phenolics (Kim et al., 2016).

It is well-known that light is an important abiotic factor influenced plant growth and phenolics biosynthesis. For example, red and blue light improved plant growth due to absorbance of chlorophyll a and chlorophyll b (Chory, 2010). Previously, several studies have been conducted to understand the effect of visible light on bioactives in vegetables. Stutte et al (2009) demonstrated red light (640 nm) improved chlorophyll contents, blue light (460 nm) enhanced

anthocyanin contents, and far-red (730 nm) reduced anthocyanin contents in red leaf lettuce (Stutte et al., 2009). Green light (505/535 nm) improved total phenolic contents in lettuce (Samuolien, Sirtautas, Brazaityt, & Duchovskis, 2012). Red light (658 nm), blue light (460 nm) and combination of red and blue light (6:1) improved chlorophyll and carotenoid contents in Chinese cabbage (Fan et al., 2013). Another study reported red light (660 nm) and blue light (450 nm) increased chlorophyll and carotenoid contents in green oak lettuce (Chen et al., 2016). Phenolic acid and flavonoid contents in red leaf lettuce increased in response to exposure to blue light (Taulavuori, Hyöky, Oksanen, Taulavuori, & Julkunen-Tiitto, 2016). Chlorophyll and carotenoid contents of lettuce increased by 70% red light (650-665 nm) and 30% blue light (460-475 nm) and 100% blue light (460-475 nm) (Amoozgar et al., 2017). Another study showed that red light (638 nm) enhanced flavonoid contents in spinach (Bliznikas et al., 2012). To date, the effect of visible light on phenolics in lettuce and spinach is not clear. Therefore, the aim of this research was to study the effect of light spectra including fluorescent (control group), red light (658 nm), far-red light (734 nm), and blue light (476 nm) on phenolic contents in two varieties of lettuce ('Two Star' and 'New Red Fire') and flavonoid contents in two varieties of spinach ('Bloomsdale' and 'Avon').

## **3.2. Materials and Methods**

### **3.2.1. Plant growth**

Lettuce and spinach were grown in growth chamber at 22/18°C (day/night) and 10-hour photoperiod under various light treatments until harvest. Only LEDs were used as supplemental lighting source and contained red light (658 nm), far red light (734 nm), and blue light (476 nm). The light intensity was maintained at 250  $\mu\text{mol}/\text{m}^2/\text{s}$  by using movable banks of LEDs. Each treatment has 3 replications.

### **3.2.2. Chemical reagent and solvent**

3,4,5-trihydroxybenzoic acid (gallic acid) was purchased from ACROS organics (Morris, NJ). 1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate) (chlorogenic acid), 3,4-hydroxycinnamic acid (caffeic acid), chicoric acid, and kaempferol were purchased from Sigma Aldrich (St. Louis, Mo). Luteolin-7-glucoside and apigenin-7-glucoside were purchased from Indofine Chemical (Hillsborough, NJ). Quercetin-3-rutinoside (rutin) was purchased from ACROS organics (Morris, NJ). Flavone was used as internal standard and purchased from BioChemika (San Angelo, TX).

Methanol was purchased from Fischer Scientific (Fair Lawn, NJ). Formic acid was purchased from Sigma Aldrich (St. Louis, MO). 0.45  $\mu\text{m}$  Whatman syringe filter was purchased from Corning Incorporated (Corning, NY).

### **3.2.3. Extraction of phenolics in lettuce and flavonoids in spinach**

Dry sample (0.15 g) was extracted with 15 ml of 70% aqueous methanol containing flavone as internal standard in 50 ml tube and mixed via orbit shaking (Benchmark, Edison, NJ) overnight. Each treatment was centrifuged by Eppendorf centrifuge (5810R) at 4  $^{\circ}\text{C}$ , 3950 rpm for 30 min and filtered. The collected supernatant was brought to 25 ml volumetric flask and 2 ml sample was evaporated under gentle Nitrogen gas. Then re-dissolved in 200  $\mu\text{L}$  70% aqueous methanol and was filtered by 0.45  $\mu\text{m}$  syringe then used for HPLC analysis.

### **3.2.4. HPLC separation and quantification of phenolics in lettuce**

A Shimadzu HPLC system (Kyoto, Japan) was used for chromatographic analysis and separation. This system consisted of a DGU-20A3 degasser, an LC-20AB liquid deliver pump, a SIL-20A auto-sample, a CBM-20A communication bus module, a SPD-20A diode array detector, and a CTO-20AC column oven. A Waters (Milford, MA) C18 reverse phase column

(250 mm length, 4.6 mm diameter) was used for phenolic compounds separation. Data were analyzed using LC solution software (Kyoto, Japan). The phenolics were detected by diode array detector and UV/vis scans were between 190 and 800 nm.

For lettuce characterization, the elution was performed with mobile phase A (5% formic acid in de-ionized water) and mobile phase B (5% formic acid in 95% methanol). An optimum column oven temperature was 31 °C. The flow rate is 0.8 ml/min. The gradient conditions were set up as follows: solvent B volume at 0-10% for 5 min, 10-40% for 25 min, 40-70% for 10 min, and held at 70% for 16 min before returning to 0% at 64 min. Flavone was used as the internal standard for quantification and phenolic acids were expressed as equivalent of vanillic acid and flavonoids were expressed as equivalent of quercetin. Phenolics in lettuce were quantified by retention time at unique wavelength shown in table 3.1.

**Table 3.1. Retention time and wavelength of phenolics in lettuce.**

Phenolic acid/ flavonoid	Wavelength (nm)	Retention time (min)
Gallic acid	271	6.74
Chlorogenic acid	272	19.28
Caffeic acid	325	21.10
Chicoric acid	327	30.25
Luteolin-7-glucoside	350	32.89
Rutin	335	33.35
Apigenin-7-glucoside	354	35.83

### **3.2.5. HPLC separation and quantification of flavonoids in spinach**

For spinach flavonoids characterization, the elution was performed with mobile phase A (water/methanol/formic acid, 69:30:1, v/v/v) and mobile phase B (methanol). An optimum column oven was 31 °C. The flow rate is 0.7 ml/min. The gradient conditions were set up as follows: solvent B volume at 15-45% for 18 min, 45-80% for 9 min, and held at 80% for 3 min

before returning to 15% at 40 min. Spinach flavonoids were expressed as equivalent of quercetin. Identification and quantification of each compound was compared with the literature (Bergquist, Gertsson, Knuthsen, & Olsson, 2005).

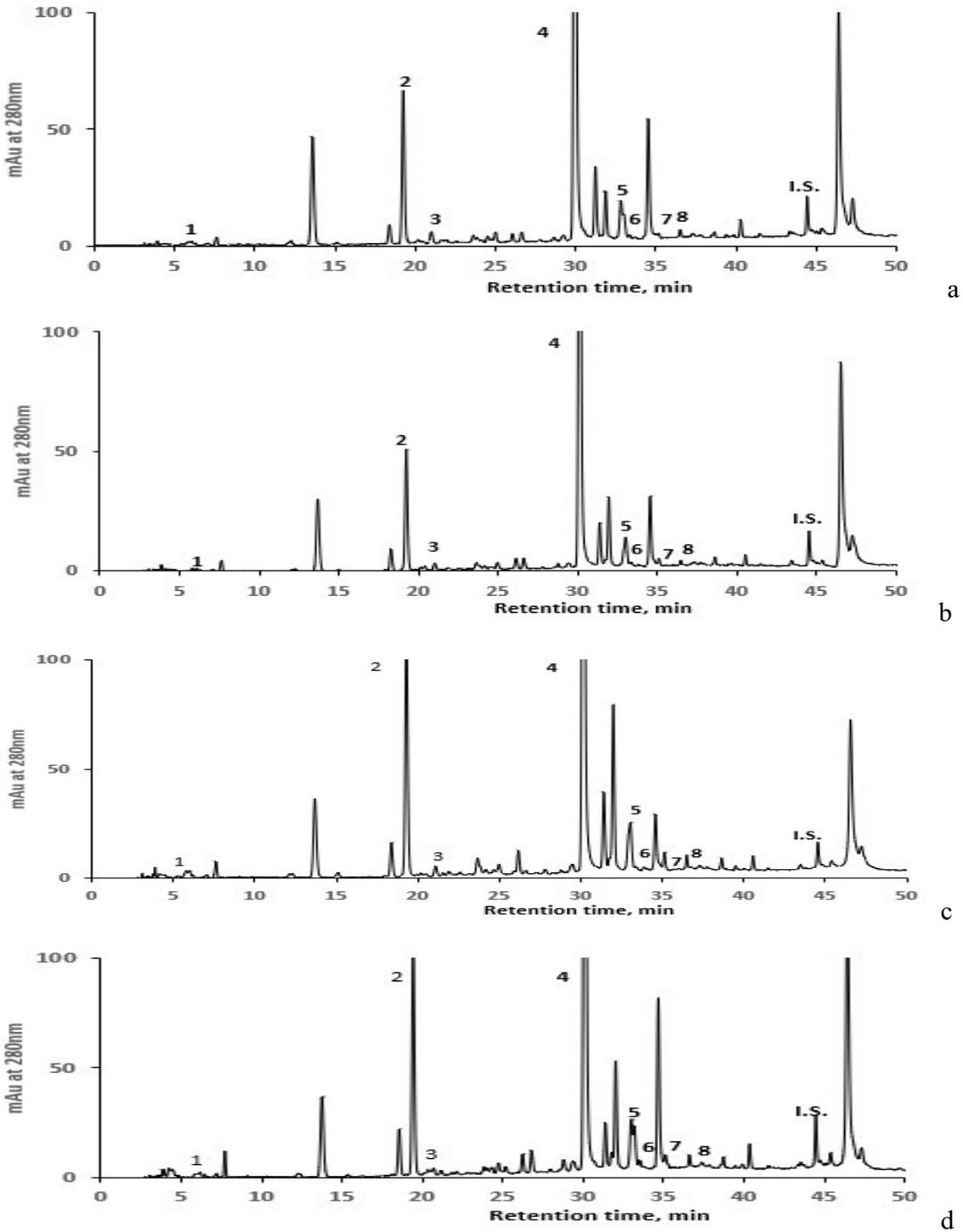
### **3.2.6. Statistical analysis**

Analysis of variance (ANOVA) was performed using Statistical Analysis System (SAS 9.4) version. Data were shown as mean  $\pm$  standard deviation. Tukey's test was applied to compare the means ( $\alpha=0.05$ ).

## **3.3. Results and Discussion**

### **3.3.1. Effect of supplemental light spectra on phenolic contents in lettuce**

Representative HPLC chromatograms of 'Two Star' treated with different light spectra are shown in figure 3.1 and total identified phenolic contents of 'Two Star' were shown in figure 3.2. Representative HPLC chromatograms of 'New Red Fire' treated with various light spectra are shown in figure 3.3 and total identified phenolic contents were shown in figure 3.4. Individual phenolic acid and flavonoid content in 'Two Star' and 'New Red Fire' lettuce treated with light spectra is shown in table 3.2 and 3.3 respectively.



**Figure 3.1. HPLC chromatograms of 'Two Star' lettuce grown under different light wavelength, fluorescent (a), red (b), and far-red (c), and blue (d).**

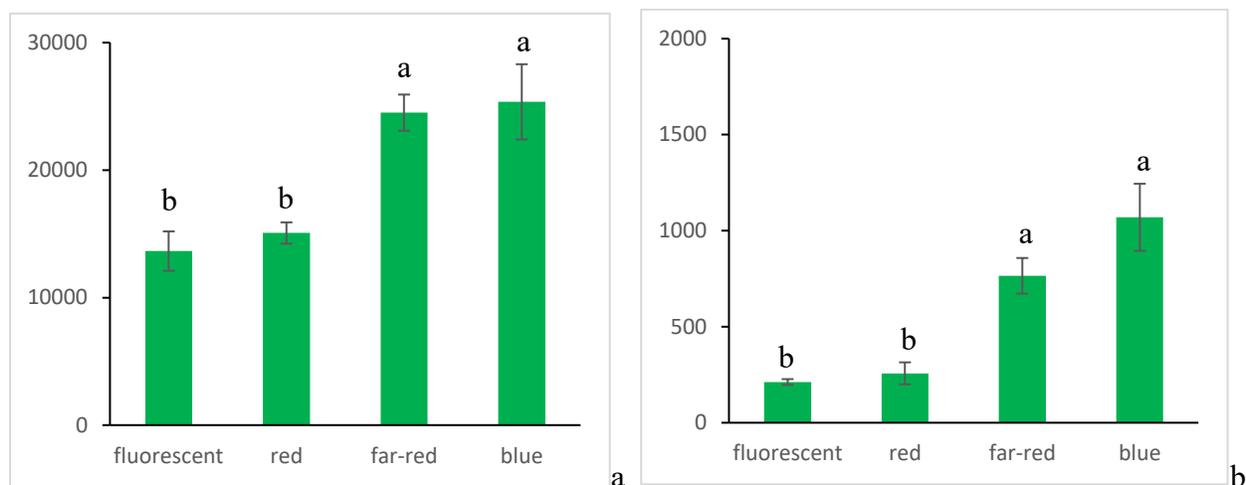
Peak 1: gallic acid; peak 2: chlorogenic acid; peak 3: caffeic acid; peak 4: chicoric acid; peak 5: luteolin-7-glucoside; peak 6: rutin; peak 7: apigenin-7-glucoside; peak 8: kaempferol. I.S.: flavone.

Chlorogenic acid and chicoric acid were the two predominant phenolic acids present in lettuce, and chicoric acid content accounted for over 95% of total identified phenolic acid contents. Far-red (734 nm) and blue light (476 nm) significantly increased chlorogenic acid and chicoric acid contents in ‘Two Star’ lettuce, thus resulted in significant increase in total identified phenolic acid contents in ‘Two Star’. However, red light (658 nm) did not alter phenolic acid contents in ‘Two Star’ lettuce. Luteolin-7-glucoside was the predominant flavonoid in lettuce and accounted for 80% of flavonoid contents in ‘Two Star’ lettuce. A significant increase in luteon-7-glucoside was achieved by exposure to far-red (734 nm) and blue (476 nm) light spectra. However, red light (658 nm) did not influence flavonoid contents in ‘Two Star’ lettuce. Far-red (734 nm) improved phenolic contents in green leaf lettuce (Lee, Son, & Oh, 2016) and this was in accordance with our results that far-red (734nm) increased phenolics in green leaf lettuce (‘Two Star’). It has been reported that blue light can regulate metabolic pathways thus help accumulate bioactives in plants (Stutte et al., 2009). This was in an agreement with our findings that blue light (476 nm) improved phenolics in ‘Two Star’ lettuce.

**Table 3.2. Individual phenolic content in 'Two Star' lettuce grown under different light spectra.**

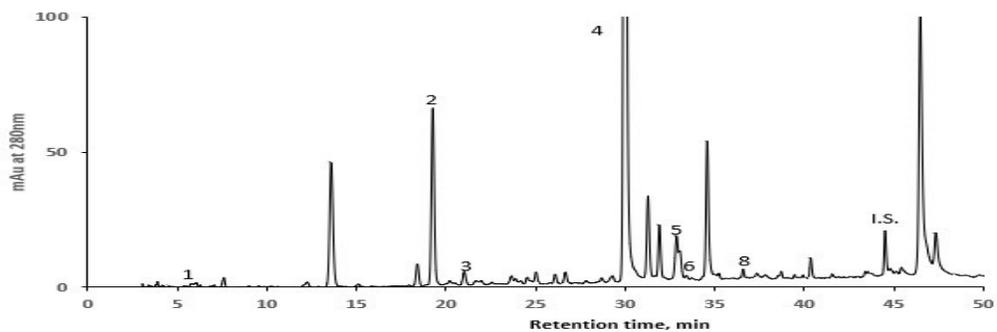
Individual phenolic	fluorescent	red	far red	blue
gallic acid	18.8±11.1 <sup>ab</sup>	6.3±4.6 <sup>b</sup>	54.5±21.2 <sup>a</sup>	27.7±16.9 <sup>b</sup>
chlorogenic acid	500.8±35.6 <sup>b</sup>	577.0±38.3 <sup>b</sup>	1405.7±103.1 <sup>a</sup>	1307.8±255.5 <sup>a</sup>
caffeic acid	100.9±13.7 <sup>ab</sup>	59.1±35.0 <sup>b</sup>	178.4±36.9 <sup>a</sup>	117.0±45.2 <sup>ab</sup>
chicoric acid	13026.5±1487.9 <sup>b</sup>	14418.0±816.9 <sup>b</sup>	22863.0±1299.0 <sup>a</sup>	23901.0±2731.3 <sup>a</sup>
luteolin-7-glucoside	172.9±14.9 <sup>c</sup>	228.4±56.9 <sup>c</sup>	686.4±70.7 <sup>a</sup>	442.3±28.1 <sup>b</sup>
rutin	26.8±1.4 <sup>b</sup>	23.3±4.1 <sup>b</sup>	31.4±6.2 <sup>b</sup>	551.9±172.3 <sup>a</sup>
apigenin-7-glucoside	3.9±0.0 <sup>b</sup>	1.9±0.1 <sup>b</sup>	17.9±7.6 <sup>a</sup>	19.5±4.0 <sup>a</sup>
kaempferol	6.9±2.2 <sup>b</sup>	3.7±2.2 <sup>b</sup>	19.8±10.1 <sup>ab</sup>	31.4±13.2 <sup>a</sup>

Values are means ± SD, n=3. Means without a common letter within the same row between different light spectra differ ( $p \leq 0.05$ ). Phenolic acid content expressed as vanillic acid equivalent ( $\mu\text{g/g}$  dry weight). Flavonoid content expressed as quercetin equivalent ( $\mu\text{g/g}$  dry weight).

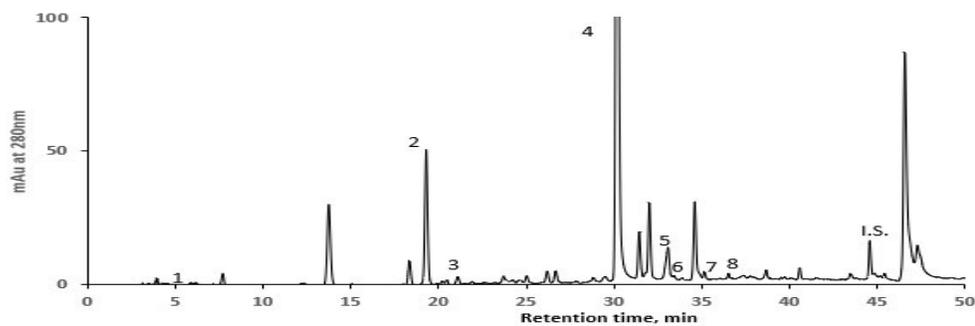


**Figure 3.2. Effect of light spectra on total identified phenolic acid contents (a) and total identified flavonoid content (b) in 'Two Star' lettuce.**

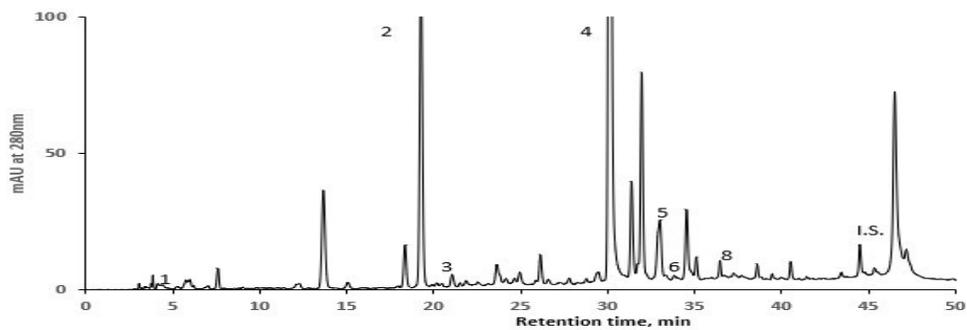
Values are means  $\pm$  SD,  $n=3$ . Means without a common letter differ ( $p \leq 0.05$ ). Phenolic acid contents were expressed as vanillic acid equivalent ( $\mu\text{g/g}$  dry weight). Flavonoid contents were expressed as quercetin equivalent ( $\mu\text{g/g}$  dry weight).



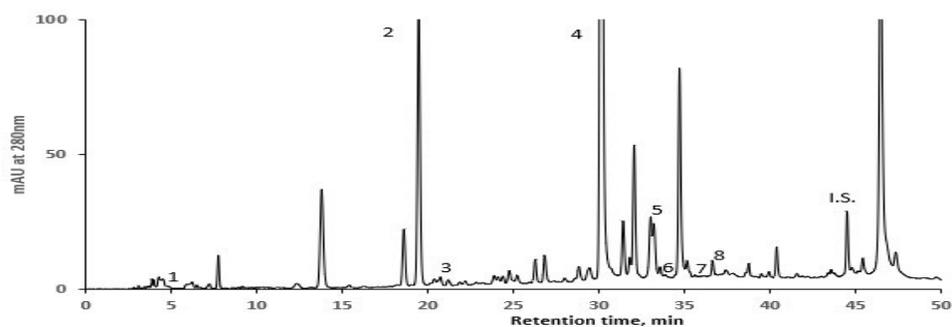
a



b



c



d

**Figure 3.3. Representative HPLC chromatograms of 'New Red Fire' lettuce grown under light wavelength, fluorescent (a), red (b), far-red (c), and blue (d).**

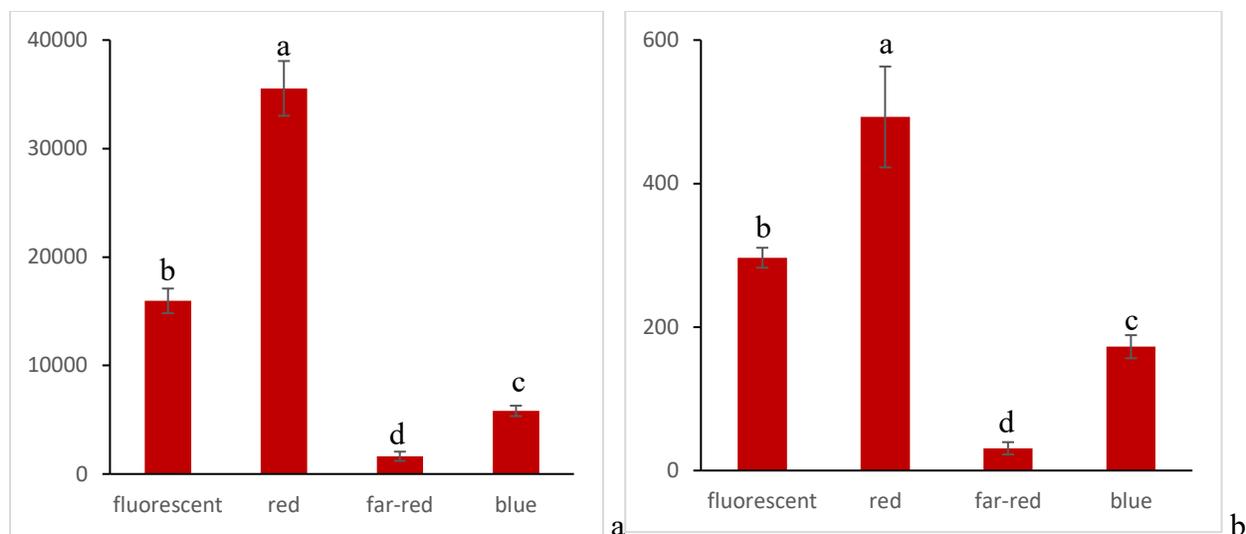
Peak 1: gallic acid; peak 2: chlorogenic acid; peak 3: caffeic acid; peak 4: chicoric acid; peak 5: luteolin-7-glucoside; peak 6: rutin; peak 7: apigenin-7-glucoside; peak 8: kaempferol. I.S.: flavone.

In ‘New Red Fire’ lettuce, red light (658 nm) significantly increased phenolic contents in contrast to fluorescent (control), while both far-red (734 nm) and blue light (476 nm) significantly reduced phenolic contents. Similar observation was reported by Li and Kubota (2009) that red light improved phenolic contents in red leaf lettuce ‘Red Cross’ but blue light did not (Li & Kubota, 2009). Similarly, red light increased phenolic contents in red lettuce (Samuolien, Sirtautas, Brazaityt, & Duchovskis, 2012). However, the mechanisms of the influence of light spectra on phenolics is not clear. The underlying mechanisms of how light spectra influenced phenolics biosynthesis in plants should be further studied.

**Table 3.3. Individual phenolic content in 'New Red Fire' lettuce grown under different light spectra.**

Individual phenolic	fluorescent	red	far red	blue
gallic acid	85.1±8.3 <sup>ab</sup>	101.5±64.0 <sup>a</sup>	5.1±1.8 <sup>b</sup>	4.6±0.5 <sup>b</sup>
chlorogenic acid	1147.9±50.1 <sup>b</sup>	2070.3±181.5 <sup>a</sup>	109.0±26.6 <sup>d</sup>	414.9±43.1 <sup>c</sup>
caffeic acid	48.7±2.7 <sup>b</sup>	218.7±15.5 <sup>a</sup>	4.4±1.4 <sup>d</sup>	13.4±9.0 <sup>c</sup>
chicoric acid	14670.9±1079.1 <sup>b</sup>	33142.7±2264.9 <sup>a</sup>	1513.0±398.0 <sup>d</sup>	5575.41±444.5 <sup>c</sup>
luteolin-7-glucoside	259.7±11.5 <sup>b</sup>	405.1±74.1 <sup>a</sup>	24.9±6.4 <sup>d</sup>	145.6±14.2 <sup>c</sup>
rutin	30.3±2.2 <sup>b</sup>	67.4±5.9 <sup>a</sup>	4.8±1.8 <sup>c</sup>	21.9±2.0 <sup>b</sup>
apigenin-7-glucoside	undetectable	4.8±0.7 <sup>a</sup>	undetectable	1.5±0.2 <sup>b</sup>
kaempferol	8.4±0.8 <sup>b</sup>	17.4±4.2 <sup>a</sup>	2.1±0.4 <sup>c</sup>	3.5±0.2 <sup>b</sup>

Values are means ± SD, n=3. Means without a common letter within the same row between different light spectra differ ( $p \leq 0.05$ ). Phenolic acid content was expressed as vanillic acid equivalent ( $\mu\text{g/g}$  dry weight). Flavonoid content was expressed as quercetin equivalent ( $\mu\text{g/g}$  dry weight).

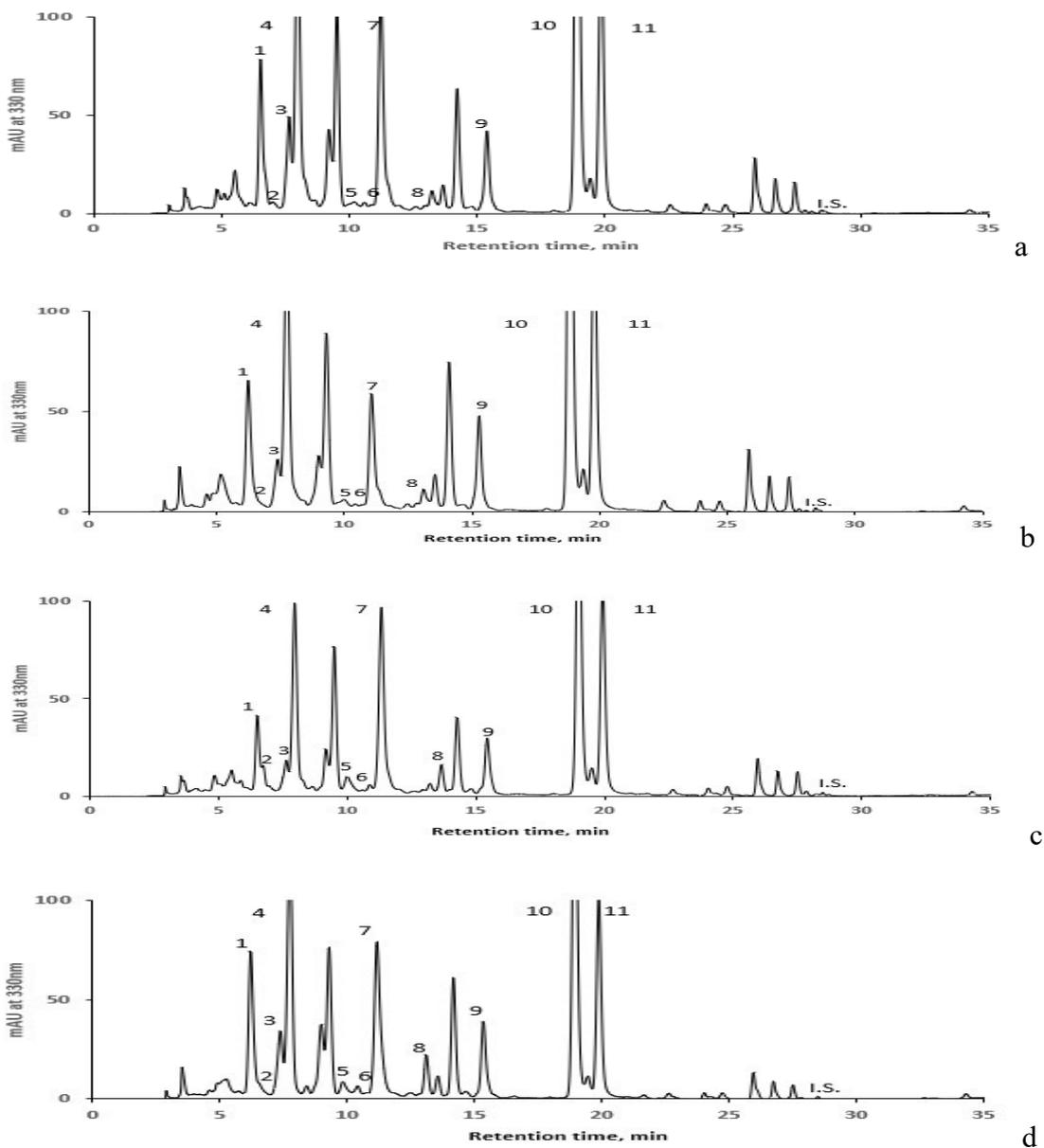


**Figure 3.4. Effect of light spectra on total identified phenolic acid contents (a) and total identified flavonoid contents (b) in 'New Red Fire' lettuce.**

Phenolic acid contents were expressed as vanillic acid equivalent ( $\mu\text{g/g}$  dry weight). Flavonoid contents were expressed as quercetin equivalent ( $\mu\text{g/g}$  dry weight).

### 3.3.2. Effect of supplemental light spectra on flavonoid contents in spinach

Representative HPLC chromatograms of 'Bloomsdale' and 'Avon' spinach treated with different light spectra are shown in figure 3.5 and 3.6 respectively. Comparison of total identified flavonoid contents of 'Bloomsdale' and 'Avon' spinach treated with different light spectra are shown in figure 3.7. Individual flavonoid content in 'Bloomsdale' and 'Avon' spinach is shown in table 3.4 and 3.5.



**Figure 3.5. Representative HPLC chromatograms of 'Bloomsdale' spinach grown under light wavelength, fluorescent (a), red (b), far-red (c), and blue (d).**

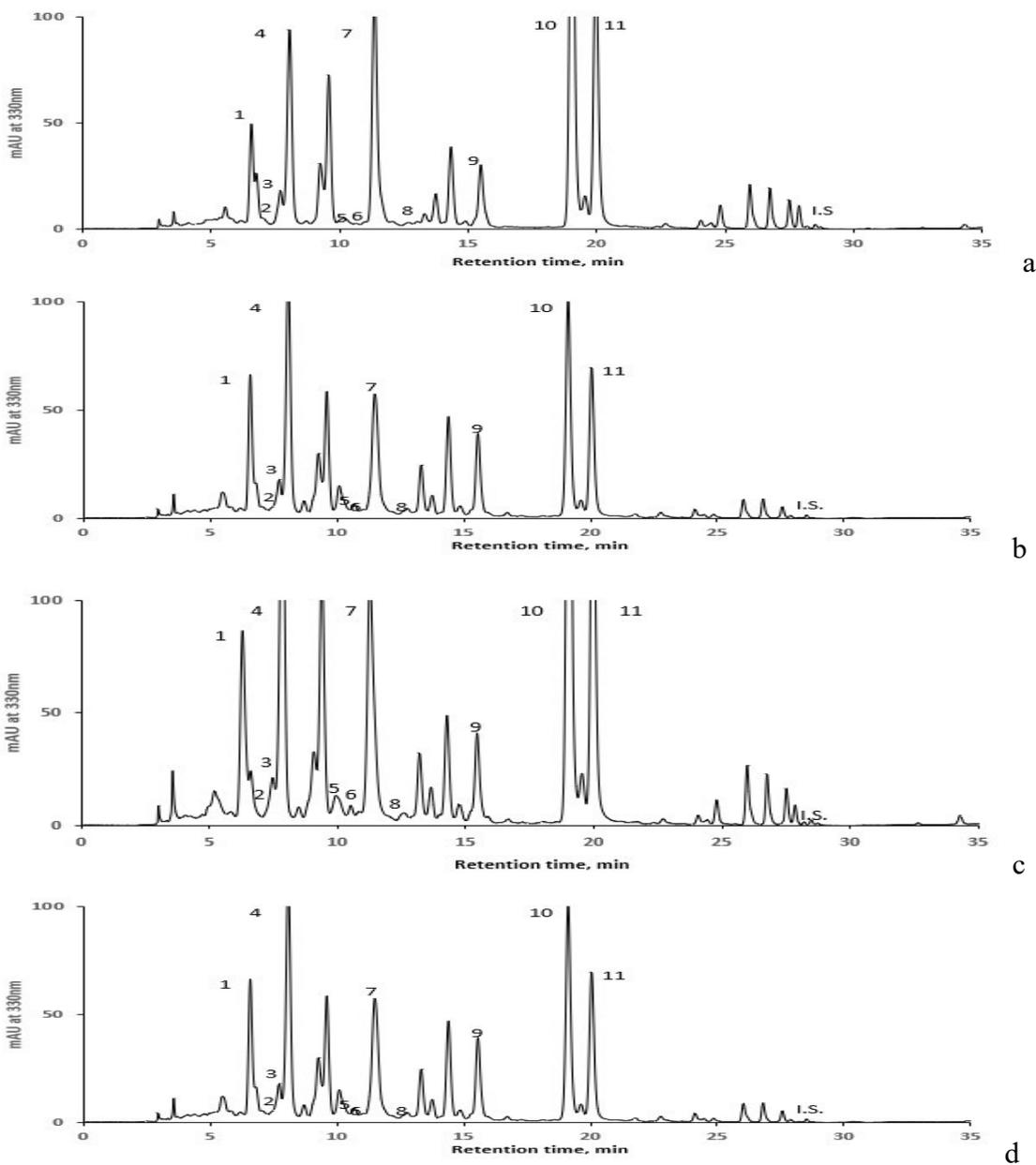
Peak 1: patuletin-3-glucosyl-(1-6)[apiosyl(1-2)]-glucoside; peak 2: spinacetin-3-glucosyl-(1-6)[apiosyl(1-2)]-glucoside including a tentatively identified patuletin glycoside; peak 3: patuletin-3-gentiobioside including a tentatively identified patuletin glycoside; peak 4: patuletin-3-(2''-feroylglucosyl)(1-6)[apiosyl(1-2)]-glucoside; peak 5: spinacetin-3-(2''-feroylglucosyl)(1-6)[apiosyl(1-2)]-glucoside; peak 6: spinacetin-3-gentiobioside including a compound probably of patuletin with gentiobioside and rhamnoside; peak 7: a patuletin-3-gentiobioside substituted with feroyl; peak 8: probably a compound of patuletin with gentiobioside and rhamnoside; peak 9: spinacetin-3-(2''-feroylglucosyl)(1-6)-glucoside; peak 10: spinatoside-4'-glucuronide; peak 11: 5,3',4'-trihydroxy-3-methoxy-6:7-methylene-dioxflavone-4'-glucuronide.

Compounds 1, 10 and 11 were the three predominant flavonoid compounds in ‘Bloomsdale’ spinach. Blue light (476 nm), red (658 nm) and far-red (476nm) significantly altered the contents of compound 2, 3, and 7 in contrast to control. However, these three compounds accounted for small amount of in total identified flavonoids contents. Thus, various light spectra did not affect total flavonoid contents in ‘Bloomsdale’ spinach.

**Table 3.4. Individual flavonoid content in 'Bloomsdale' spinach grown under light wavelength.**

compounds	fluorescent	red	far red	blue
Peak 1	1304.7±187.7 <sup>a</sup>	1345.4±105.2 <sup>a</sup>	1220.5±318.2 <sup>a</sup>	1537.1±76.3 <sup>a</sup>
Peak 2	25.3±20.2 <sup>b</sup>	29.7±5.6 <sup>b</sup>	12.4±6.3 <sup>b</sup>	66.2±4.4 <sup>a</sup>
Peak 3	233.0±55.4 <sup>ab</sup>	176.6±17.9 <sup>b</sup>	170.8±41.7 <sup>b</sup>	315.4±23.8 <sup>a</sup>
Peak 4	745.4±70.3 <sup>a</sup>	722.8±87.7 <sup>a</sup>	694.3±127.3 <sup>a</sup>	747.6±101.4 <sup>a</sup>
Peak 5	29.0±7.0 <sup>a</sup>	29.8±4.4 <sup>a</sup>	19.4±10.1 <sup>a</sup>	14.6±10.6 <sup>a</sup>
Peak 6	69.2±11.4 <sup>ab</sup>	82.9±12.4 <sup>a</sup>	49.6±7.4 <sup>b</sup>	45.7±5.8 <sup>b</sup>
Peak 7	579.5±60.8 <sup>a</sup>	495.2±86.3 <sup>a</sup>	333.0±193.0 <sup>ab</sup>	174.3±42.7 <sup>b</sup>
Peak 8	20.6±2.5 <sup>a</sup>	21.0±5.2 <sup>a</sup>	25.5±9.9 <sup>a</sup>	31.6±12.5 <sup>a</sup>
Peak 9	437.51±46.4 <sup>a</sup>	382.35±90.6 <sup>a</sup>	376.19±43.4 <sup>a</sup>	457.43±166.8 <sup>a</sup>
Peak 10	2595.9±145.0 <sup>ab</sup>	2761.3±183.5 <sup>a</sup>	2135.0±198.9 <sup>b</sup>	2422.8±286.2 <sup>ab</sup>
Peak 11	1337.6±64.7 <sup>a</sup>	1274.3±148.6 <sup>a</sup>	1189.8±135.3 <sup>a</sup>	1178.3±100.7 <sup>a</sup>

Values are means ± SD, n=3. Means without a common letter within the same row between different light spectra differ (p≤0.05). Flavonoid contents were expressed as quercetin equivalent (µg/g dry weight). Individual name of peak 1 to peak 11 shown below the figure 3.5.



**Figure 3.6. Representative HPLC chromatograms of 'Avon' spinach grown under light wavelength, fluorescent (a), red (b), far-red (c), and blue (d).**

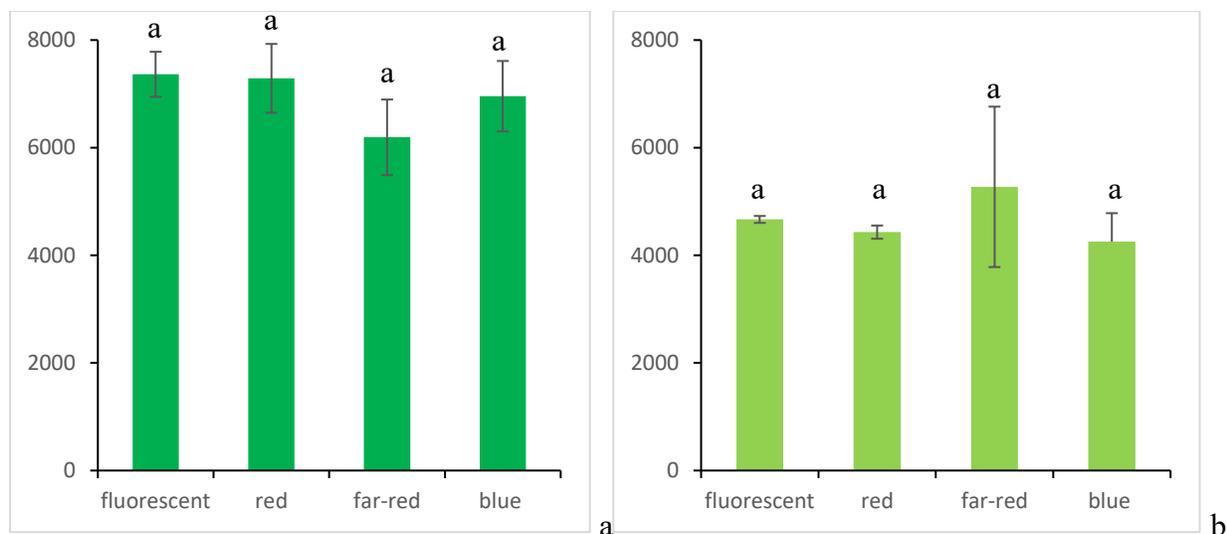
Peak 1: patuletin-3-glucosyl-(1-6)[apiosyl(1-2)]-glucoside; peak 2: spinacetin-3-glucosyl-(1-6)[apiosyl(1-2)]-glucoside including a tentatively identified patuletin glycoside; peak 3: patuletin-3-gentiobioside including a tentatively identified patuletin glycoside; peak 4: patuletin-3-(2''-feroylglucosyl)(1-6)[apiosyl(1-2)]-glucoside; peak 5: spinacetin-3-(2''-feroylglucosyl)(1-6)[apiosyl(1-2)]-glucoside; peak 6: spinacetin-3-gentiobioside including a compound probably of patuletin with gentiobioside and rhamnoside; peak 7: a patuletin-3-gentiobioside substituted with feroyl; peak 8: probably a compound of patuletin with gentiobioside and rhamnoside; peak 9: spinacetin-3-(2''-feroylglucosyl)(1-6)-glucoside; peak 10: spinatoside-4'-glucuronide; peak 11: 5,3',4'-trihydroxy-3-methoxy-6:7-methylene-dioxflavone-4'-glucuronide.

Compound 1, 10, and 11 were the three predominant flavonoids in ‘Avon’ spinach. And the combination of these three compounds accounted for around 80% of total identified flavonoids in ‘Avon’ spinach. Various light spectra did not influence individual flavonoid content in ‘Avon’ spinach. Consequently, different light spectra did not alter total identified flavonoid contents in ‘Avon’ spinach.

**Table 3.5. Individual flavonoid content in 'Avon' spinach grown under light spectra.**

compounds	fluorescent	red	far red	blue
Peak 1	812.3±70.2 <sup>a</sup>	837.4±122.3 <sup>a</sup>	1012.5±501.5 <sup>a</sup>	1022.7±93.9 <sup>a</sup>
Peak 2	21.4±0.7 <sup>a</sup>	28.9±3.3 <sup>a</sup>	27.2±24.5 <sup>a</sup>	41.4±17.9 <sup>a</sup>
Peak 3	150.2±15.7 <sup>a</sup>	153.5±16.4 <sup>a</sup>	132.2±52.7 <sup>a</sup>	200.3±42.7 <sup>a</sup>
Peak 4	408.0±38.5 <sup>a</sup>	459.9±15.8 <sup>a</sup>	463.7±234.5 <sup>a</sup>	434.3±127.2 <sup>a</sup>
Peak 5	15.3±5.9 <sup>a</sup>	1.6±0.3 <sup>a</sup>	18.9±19.7 <sup>a</sup>	5.98±7.41 <sup>a</sup>
Peak 6	39.6±0.5 <sup>a</sup>	33.7±29.7 <sup>a</sup>	50.9±28.0 <sup>a</sup>	34.3±7.0 <sup>a</sup>
Peak 7	269.7±2.7 <sup>a</sup>	121.0±69.0 <sup>a</sup>	232.8±105.1 <sup>a</sup>	124.9±33.9 <sup>a</sup>
Peak 8	13.2±0.1 <sup>a</sup>	15.8±2.4 <sup>a</sup>	19.7±8.0 <sup>a</sup>	13.0±2.3 <sup>a</sup>
Peak 9	216.4±40.9 <sup>a</sup>	221.77±28.6 <sup>a</sup>	295.98±211.6 <sup>a</sup>	309.3±61.2 <sup>a</sup>
Peak 10	1887.5±173.5 <sup>a</sup>	1641.6±148.2 <sup>a</sup>	1954.6±438.9 <sup>a</sup>	1356.4±489.3 <sup>a</sup>
Peak 11	835.8±178.5 <sup>a</sup>	921.3±153.7 <sup>a</sup>	1066.6±426.2 <sup>a</sup>	815.8±97.8 <sup>a</sup>

Values are means ± SD, n=3. Means without a common letter within the same row between different light spectra differ ( $p \leq 0.05$ ). Flavonoid content expressed as quercetin equivalent ( $\mu\text{g/g}$  dry weight). Individual name of peak 1 to peak 11 shown below the figure 3.6.



**Figure 3.7. Effect of light spectra on total identified flavonoid contents in 'Bloomsdale' (a) and in 'Avon' (b).**

Values are means  $\pm$  SD, n=3. Means without a common letter between different light spectra differ ( $p \leq 0.05$ ). Flavonoid contents were expressed as quercetin equivalent ( $\mu\text{g/g}$  dry weight).

### 3.4. Conclusion

Various light spectra significantly influenced phenolic contents in two varieties of lettuce while not in two varieties of spinach. Therefore, the effect of light spectra on phenolic contents in vegetables is genotype dependent. Positive effects of different light spectra on phenolics in lettuce could be used as effective strategies to enhance phenolics in lettuce for the purpose of providing health benefits and prevent chronic diseases.

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## **Chapter 4 - Application of 3D hydrogel Culture Model for Potential Anticancer Function**

### **Abstract**

Traditionally, cancer research has primarily relied on *in vitro* 2D monolayer culture and *in vivo* animal model studies. However, 2D monolayer culture lacks 3D microenvironment and cell-cell interactions, and an animal model is both expensive and time-consuming. A new alternative 3D culture should be investigated. A novel 3D h9e peptide hydrogel is a promising model for cancer research. However, there is no report studying liver cancer and colon cancer research by culturing in 3D peptide hydrogel. Thus, this study performed a 3D hydrogel culture by culturing human hepatocarcinoma HepG2 cells and colorectal adenocarcinoma SW480 cells and treated with chlorogenic acid (CGA) at 0-40  $\mu\text{M}$  up to 72 hrs. Results showed both HepG2 cells and SW480 cells were better adaptable to 3D hydrogel culture which reflects higher proliferation than that in 2D culture. An extended exponential phase of both HepG2 cells and SW480 cells was observed in 3D culture in contrast to 2D culture. CGA treatment resulted in morphological changes of HepG2 cells but not in SW480 cells. CGA treatment resulted in a dose- and time-response inhibition on HepG2 growth and SW480 cell growth. SW480 cells were more resistant to CGA treatment than HepG2 cells. When cells were treated with media without CGA, HepG2 cells and SW480 cells recovered to grow rapidly in 3D culture but not in 2D culture. Inhibitory effect of CGA on HepG2 cell growth and SW480 cell growth in 2D culture was lasting longer than that in 3D culture. In conclusion, different inhibitory effect of CGA on HepG2 cell growth and SW480 cell growth in 3D culture indicated an overestimation of CGA inhibitory effect of cell growth in 2D cell culture. Difference in stimuli of cell growth inhibition in 3D hydrogel culture in contrast to 2D culture suggested 3D hydrogel culture could potentially bridge the gap

of cell response to chemopreventive agents between *in vitro* in 2D culture and *in vivo* animal model.

Key words: 3D hydrogel culture, HepG2 cell, SW480 cell, chlorogenic acid, cell growth inhibition

## 4.1. Introduction

Flat 2D cell culture has been used *in vitro* research for many decades. In cancer research, *in vitro* 2D monolayer culture has provided prerequisite knowledge and a fundamental understanding of the underlying mechanisms before experiments conducted *in vivo* in animal model. However, 2D monolayer culture cannot provide three-dimensional physiological microenvironment, nor is 2D monolayer culture natural for all cell types, which cannot reflect the complexity *in vivo* animal model. Thus 2D monolayer culture will result in the overestimation of response to stimuli leading to experimental failure (Hutmacher, 2010; Kim et al., 2014; Verjans, Doijen, Luyten, Landuyt, & Schoofs, 2018). In a long term, this experimental failure leads to money and time loss in research.

3D culture is a breakthrough *in vitro* in cancer research, because it creates an artificial three-dimensional microenvironment that allows cell-cell interactions (Antoni, Burckel, Josset, & Noel, 2015). In contrast to cells in 2D culture, cells in 3D culture have shown more physiological relevance and improvements, including cell viability, morphology, proliferation, differentiation, stability, lifespan, etc (Antoni et al., 2015). Hence, 3D culture is a promising model which can bridge the huge gap of cell response to chemopreventive stimuli between *in vitro* 2D cell culture and *in vivo* animal study (Rodrigues et al., 2018).

3D culture platforms include hydrogels, scaffolds, decellularized tissues or organs, and cell-derived matrices (Fitzgerald et al., 2015). Hydrogels are the network of hydrophilic polymers composed of over 95% volume as water, but the materials display solid-like attributes and facilitate nutrient, oxygen, and waste transport (Sathaye et al., 2015; Verjans et al., 2018).

Currently, many natural and synthetic materials have been used in hydrogels such as collagen, Matrigel, and synthetic peptide hydrogels such as EAK16 and RADA16, Fmoc-FF and Fmoc-

RDG, and h9e (Worthington, Pochan, & Langhans, 2015). Studies have confirmed the success of applying h9e peptide hydrogel in seeding and isolating breast cancer MCF-7 cells without pH and temperature adjustment and that peptide hydrogel is suitable for analysis, such as confocal imaging and Western blot (Huang, Ding, Sun, & Nguyen, 2013). Another study demonstrated a comparable HeLa cell viability in 3D peptide hydrogel, as opposed to 2D culture and peptide-hydrogel-facilitated anti-cancer drug diffusion to cells (Liang et al., 2017).

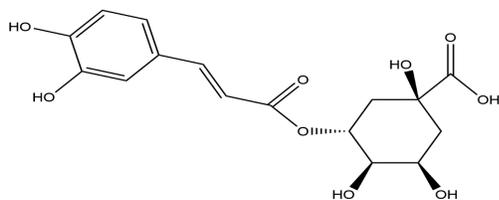
Liver cancer and colorectal cancer are frequent death-leading cancer globally (Sener & Grey, 2005). It has been reported colorectal cancer was a top leading cause of cancer related death among adults over the age of 50 in U.S. (Carini et al., 2017). SW480 cell is good colorectal adenocarcinoma model for colon cancer research. HepG2 cell, known as well-differentiated transformed cells, is a good model for liver cancer research (Mersch-Sundermann et al., 2004). Both cell lines, HepG2 cells and SW480 cells, have been used in *in vitro* in 2D monolayer cancer research for many decades.

Doll and Peto (1981) demonstrated that diet can alter 35% of human cancer-related mortality. Consumption of protective foods is estimated to prevent 66-75% of colon cancer and 33-66% of liver cancer (American Institute for Cancer Research & World Cancer Research Fund, 2007). Phytochemicals are health-promoting factors which are uniquely present in plants to provide health benefits like antioxidant, anti-cancer and anti-inflammatory property (Liu, 2013).

Phenolics, known as one classification of phytochemicals, have been reported many health benefits including the antioxidant, anti-cancer and anti-inflammatory property due to containing hydrogel groups on phenolics structure, which allow them to interact with proteins, causing changes of functions (Jafari, Saeidnia, & Abdollahi, 2014). The hydroxyl group of phenolics shares the proton in hydrogen bonds with proteins such as amide, guanidine, peptide amino, or

carboxyl groups. Therefore, phenolics can either prevent or cure cancer (Charlton et al., 2002; He, Lv, & Yao, 2007; Jafari et al., 2014).

Chlorogenic acid (CGA) is the most abundant hydroxycinnamic acid derived from vegetables, such as carrots, pears, apples, strawberries, berries, coffee, potatoes, etc (Clifford, 1999; Brown, 2005). The chemical structure of CGA is shown in figure 4.1. Previously, extensive studies reported that CGA inhibits human hepatocarcinoma HepG2 cell growth (Whiteman et al., 2005; Glei et al., 2006; Yip, Chan, Pang, Tam, & Wong, 2006; Kulisic-Bilusic, Schmöller, Schnäbele, Siracusa, & Ruberto, 2012; Yan, Liu, Hou, Dong, & Li, 2017), and it also inhibits colorectal adenocarcinoma SW480 cell growth (Zheng et al., 2002; García-Gutiérrez, Maldonado-Celis, Rojas-López, Loarca-Piña, & Campos-Vega, 2017; Sadeghi Ekbatan, Li, Ghorbani, Azadi, & Kubow, 2018). However, those studies are conducted in *in vitro* in 2D monolayer culture, so they risk the aforementioned overestimation and low correlation present in those studies.



**Figure 4.1. Chemical structure of chlorogenic acid.**

To date, to our best knowledge, there is no study of culturing human hepatocarcinoma HepG2 cells and colorectal adenocarcinoma SW480 cells in 3D peptide hydrogel culture. Additionally, little is known about the effect of CGA on inhibiting HepG2 cell growth and SW480 cell growth in 3D hydrogel culture in contrast to 2D culture. Hence, the first objective of this study is to culture HepG2 cells and SW480 cells in 3D peptide hydrogel culture. Secondly, this study will compare the effects of CGA's inhibition of HepG2 cell growth and SW480 cell growth between both 3D hydrogel culture and 2D monolayer culture.

## **4.2. Materials and Methods**

### **4.2.1. Materials**

HyClone Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Dulbecco's Phosphate Buffered Saline (DPBS), penicillin/streptomycin, and 0.5% trypsin-EDTA were purchased from Fisher Scientific Co. L.L.C (Pittsburgh, PA). Chlorogenic acid (CGA) was purchased from Sigma Aldrich (St. Louis, MO). HepG2 and SW480 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). PG works and PGmatrix-pure kit were provided by PepGel LLC (Manhattan, KS). Media was DMEM supplemented by 10% FBS, 100 µg/mL streptomycin, and 100 units/mL penicillin. AOPI staining solution was purchased from Nexcelom Bioscience (Lawrence, KS).

### **4.2.2. 2D Culture**

$1 \times 10^5$ /well HepG2 cell and SW480 cell were cultured in a 6-well plate respectively. Passage time was determined by cell confluency up to 80-90% via microscope by visual evaluation. To harvest HepG2 cell and SW480 cell from 2D culture, media was removed, and each well was rinsed with 1 ml DPBS and removed. Cells were enzymatically detached by using 0.05% trypsin-EDTA solution 1 ml/well and placed in 37°C incubator for 8 min and then added media 1 ml/well and cell suspension was collected in centrifuged tubes. Cells were separated by centrifuge at 0.6 rcf for 6 min (Eppendorf 5702, New York City). Upper supernatant was gently removed, and cell pellet was at the bottom.

### **4.2.3. 3D hydrogel culture**

To transfer HepG2 cell and SW480 cell from 2D to 3D hydrogel culture, cells were counted with a cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA) using AOPI staining method.

Cells were encapsulated in 3D hydrogel using PG works and PGmatrix-pure kit.  $5 \times 10^4$  /well HepG2 cell was cultured in 24-well plate.  $2 \times 10^4$  /well SW480 cell was cultured in 24-well plate. Cell suspension was mixed with PGworks and PGmatrix-pure kit by pipetting thoroughly without generating air bubbles. Cell culture was placed in a humidified 5% CO<sub>2</sub> atmosphere at 37°C for 0.5 hr to allow gel formation then added media 1 ml/well. Passage time was determined by cell confluency up to 80-90% via microscope by visual evaluation.

To isolate cells from 3D hydrogel culture, gel was disrupted by pipetting thoroughly, and mixture was collected in centrifuge tube. 1 ml/well DPBS was added to rinse the well and collected into tubes and mixed thoroughly with the mixture from the previous step. Cell suspension was centrifuged at 0.8 rcf for 10 min and then removed supernatant. 1 ml/well 0.05% trypsin-EDTA was added and gently pipetted to resuspend cells then placed in the incubator at 37°C for 8 mins, and media 1 ml/well was added and centrifuged again at 0.6 rcf for 6 min. Supernatant was removed, and cell pellets were at the bottom.

#### **4.2.4. Cell Morphology**

The morphological characters were determined by using a light microscope (Nicon Eclipse TE2000-u, Kanagawa, Japan). Morphologies of HepG2 cell and SW480 cell were cultured in 2D monolayer and 3D hydrogel culture respectively and observed every 24 hrs.

#### **4.2.5. Cell growth inhibition assay chlorogenic acid**

##### **4.2.5.1. Inhibitory assay in 3D cell culture**

HepG2 cells ( $2.5 \times 10^4$  / well) were cultured in 0.5% hydrogel in 48-well plate in a 37°C incubator. CGA treatment (0, 10, 20, and 40  $\mu$ M) was added in HepG2 cell in the exponential phase for continuous 24 hrs, 48 hrs, and 72 hrs. Then cells were treated with media without CGA

for another 24 hr, 48 hrs, and 27 hrs. HepG2 cells were harvested every 24 hrs. Each treatment had two replicates.

SW480 cells ( $1 \times 10^4$ /well) were cultured in 0.5% hydrogel in 48-well plate in a 37°C incubator. CGA treatment (0, 10, 20, and 40  $\mu$ M) was added in SW480 cells in the exponential phase for continuous 24 hrs, 48 hrs, and 72 hrs. Then cells were treated with media without CGA for another 24 hr, 48 hrs, and 27 hrs. SW480 cells were harvested every 24 hrs. Each treatment had two replicates.

#### **4.2.5.2. Inhibitory assay in 2D cell culture**

$2 \times 10^5$ /well of HepG2 cells or SW480 cells in exponential phase were seeded in a 6-well plate respectively and cultured in a humidified chamber for 1 day and allowed to be attached. CGA treatment (0, 10, 20, and 40  $\mu$ M) was added 24 hrs, 48 hrs, and 72 hrs continuously after cells attached. Then cells were treated with media without CGA for another 24 hr, 48 hr, and 27 hr. Cells were harvested every 24 hrs. Each treatment had two replicates.

#### **4.2.6. Cell counting**

After separation of cells, cells and cell viability were counted with a cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA) using AOPI dye.

#### **4.2.7. Statistical analysis**

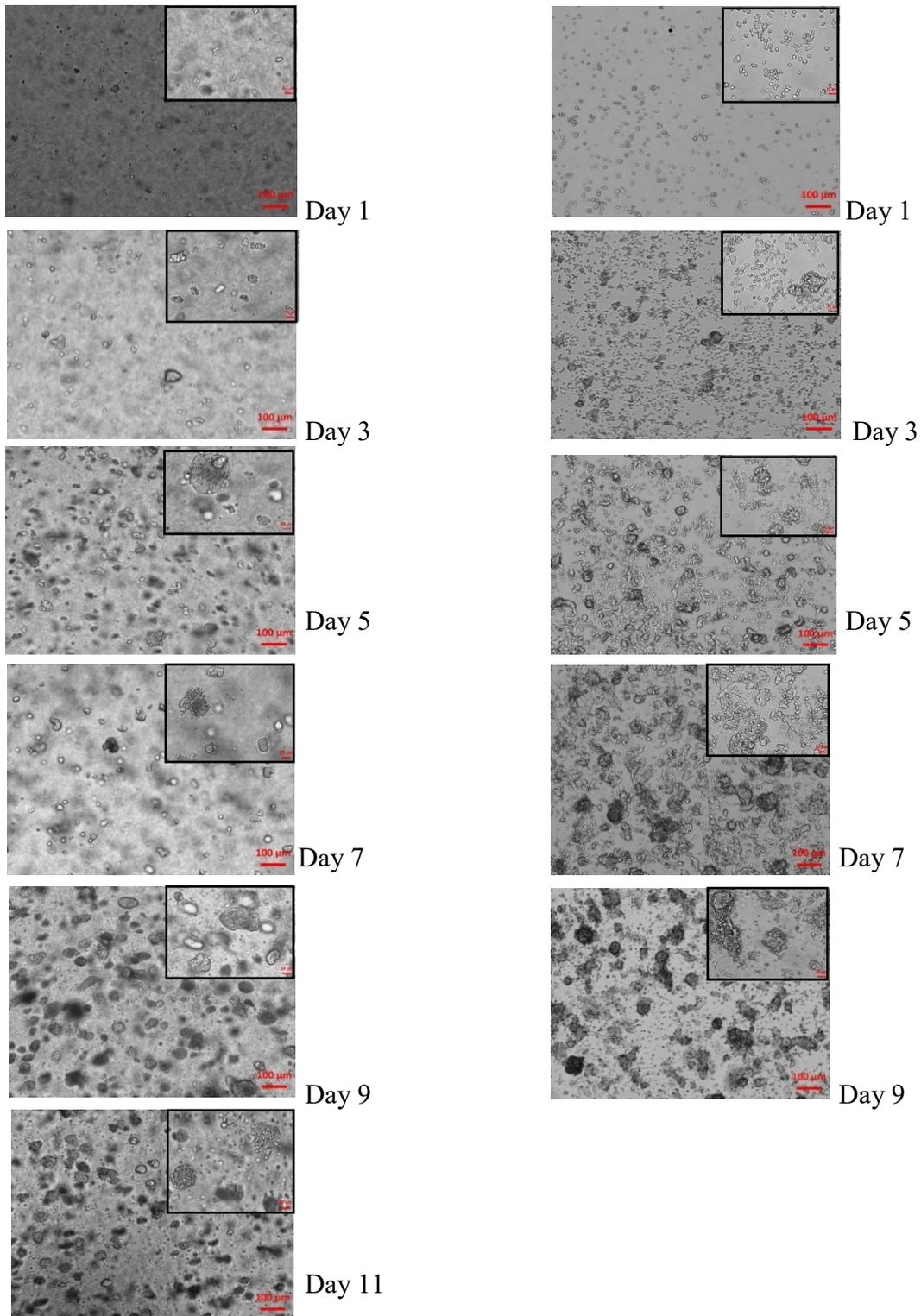
Analysis of variance (ANOVA) was performed using Statistical Analysis System (SAS 9.4) version. Data was exhibited as mean  $\pm$  standard deviation. Tukey's test was applied to compare the means ( $\alpha=0.05$ ).

### **4.3. Results and Discussion**

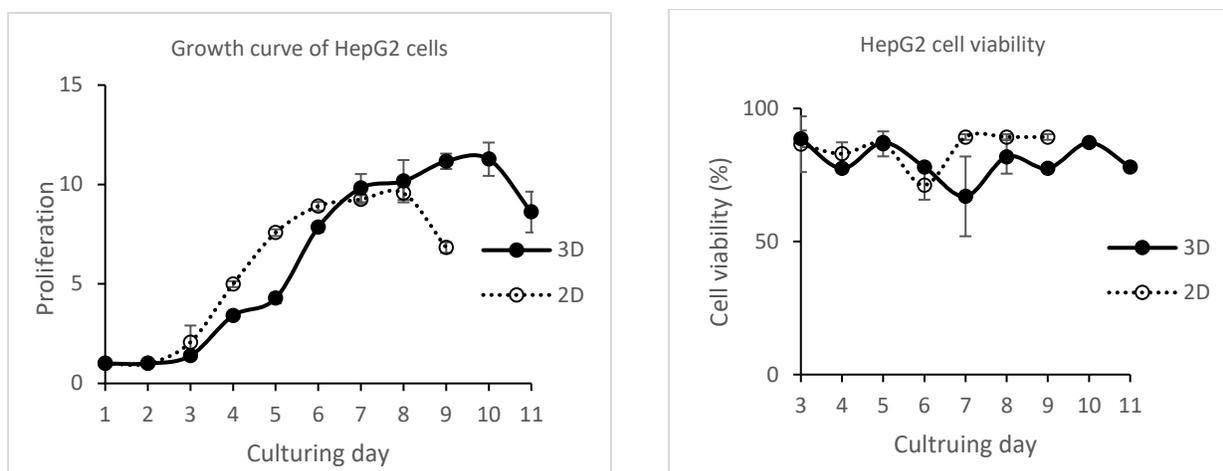
#### **4.3.1. Cell growth of HepG2 and SW480 cells in 3D and 2D culture**

##### **4.3.1.1 Cell growth of HepG2 cells in 3D and 2D culture**

HepG2 cells were floating in 3D hydrogel culture after incubation for one day. In 2D culture, HepG2 cells were attached to plate surface after being incubated for one day, which morphologies in day 1 are shown in figure 4.2. As incubation continued, HepG2 cells in 2D cell culture stretched and spread on the plate surface. On day 3, HepG2 cells started to form clusters on the surface. After day 8, HepG2 cell confluency was almost 80-90% on the plate surface, and some clusters were overlapped. In contrast to 2D culture, 3D hydrogel culture provided more room for HepG2 cells to grow at day 8. As incubation continued, HepG2 cells turned into large clusters. The growth continued even after day 9 of incubation in 3D culture. Combined with HepG2 cell proliferation data in 3D and 2D culture, HepG2 cell was found to grow slowly during the first three days in both 3D and 2D cell culture, which corresponded to morphological characters of HepG2 cells in 3D and 2D cell culture. According to the growth curve of HepG2 cells in 3D and 2D culture shown in figure 4.3, the exponential phase of HepG2 cells in 2D culture started from days 3 to 8, while the exponential phase of HepG2 cells in 3D culture took place from days 3 to 10, suggesting that 3D hydrogel culture extended the exponential phase of HepG2 cells. There was no significant difference of cell viability between HepG2 cells in 3D and 2D culture when compared within the same day.



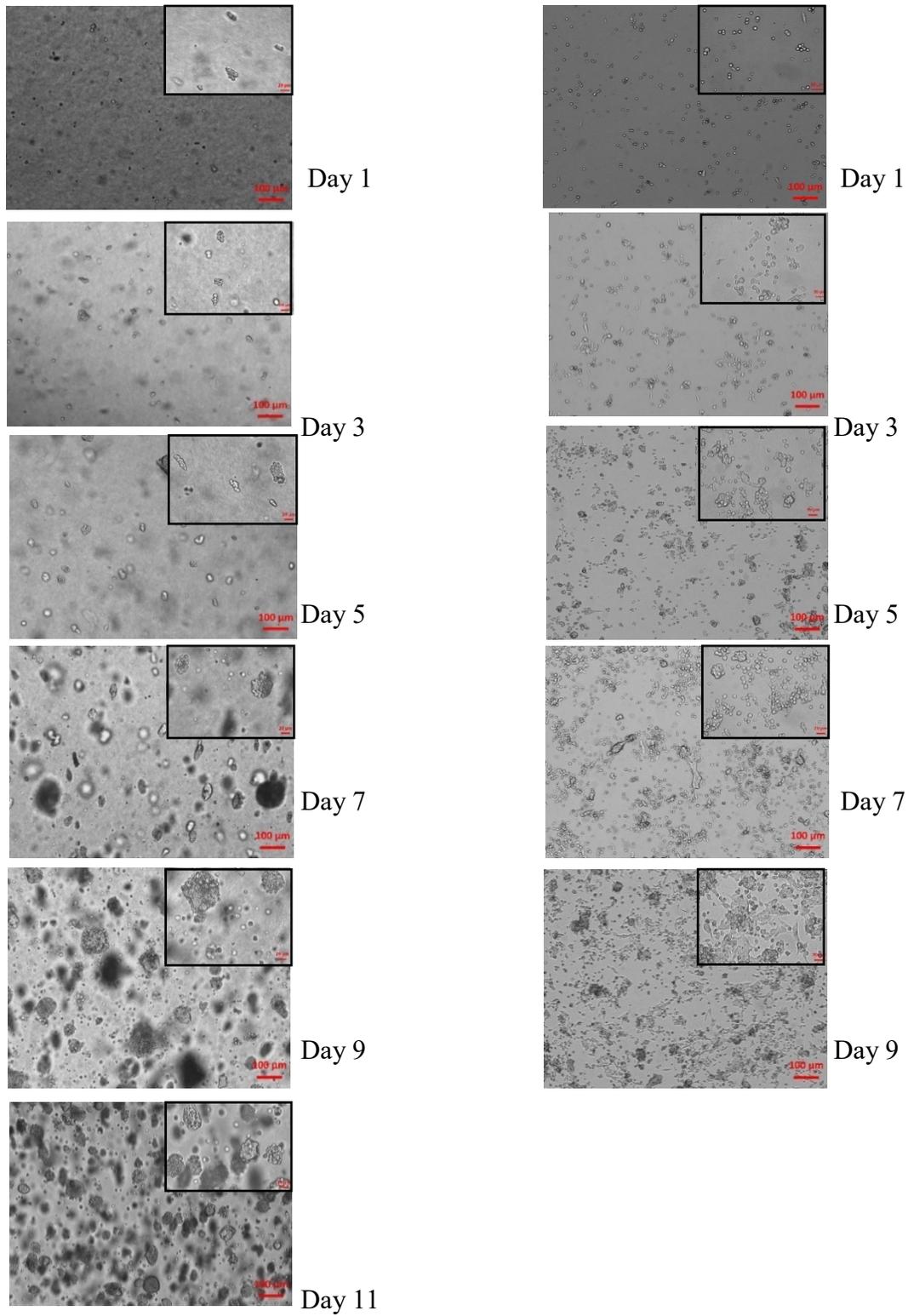
**Figure 4.2. HepG2 cell morphologies in 3D culture (left) and 2D culture (right).** Mag: 5x. Each image was shown 20x magnification on the right top.



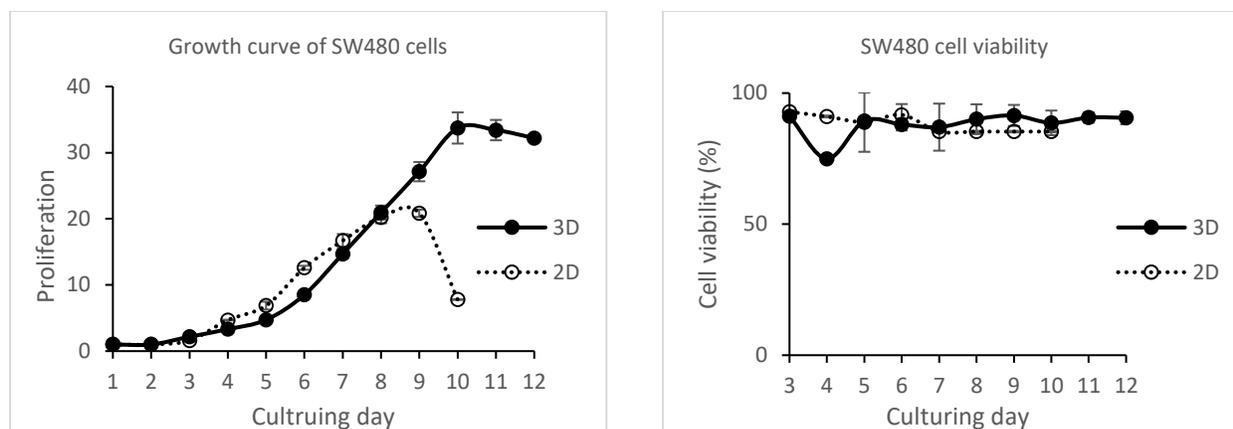
**Figure 4.3. Growth curve of HepG2 cells (left) and cell viability (right) in 3D and 2D culture.**

#### 4.3.1.2. Cell growth of SW480 cells in 3D and 2D culture

Morphological characters of SW480 cells in 3D culture and 2D culture are shown in figure 4.4. SW480 cells were floating in the 3D hydrogel culture after 1-day incubation, while SW480 cells attached to the surface of plate after 1-day incubation in 2D culture. As incubation time increased, SW480 cells stretched on the plate surface in 2D culture and turned to large clusters on day 5. After being incubated for 10 days, SW480 cell overlapped in 2D culture and cell confluency was around 80-90%. In contrast to 2D culture, 3D hydrogel culture provided more space for SW480 cell growth up to day 12. According to the growth curve shown in figure 4.5, the exponential phase of SW480 cells in 2D culture occurred from days 3 to 9, while the exponential phase of SW480 cells in 3D culture happened from days 3 to 10, suggesting 3D culture extended exponential phase of SW480 cells. SW480 cell proliferation in 3D hydrogel culture was significantly higher than that in 2D culture, indicating 3D culture was more favor of cell growth in contrast to 2D culture. There was no significant difference of cell viability between SW480 cells in 3D culture and SW480 cells in 2D culture when compared on the same incubation day.



**Figure 4.4. SW480 cell morphologies in 3D culture (left) and 2D culture (right).** Mag:5x. Each image was shown 20x magnification on the right top.



**Figure 4.5. Growth curve of SW480 cells culture (left) and SW480 cell viability (right) in 3D and 2D culture.**

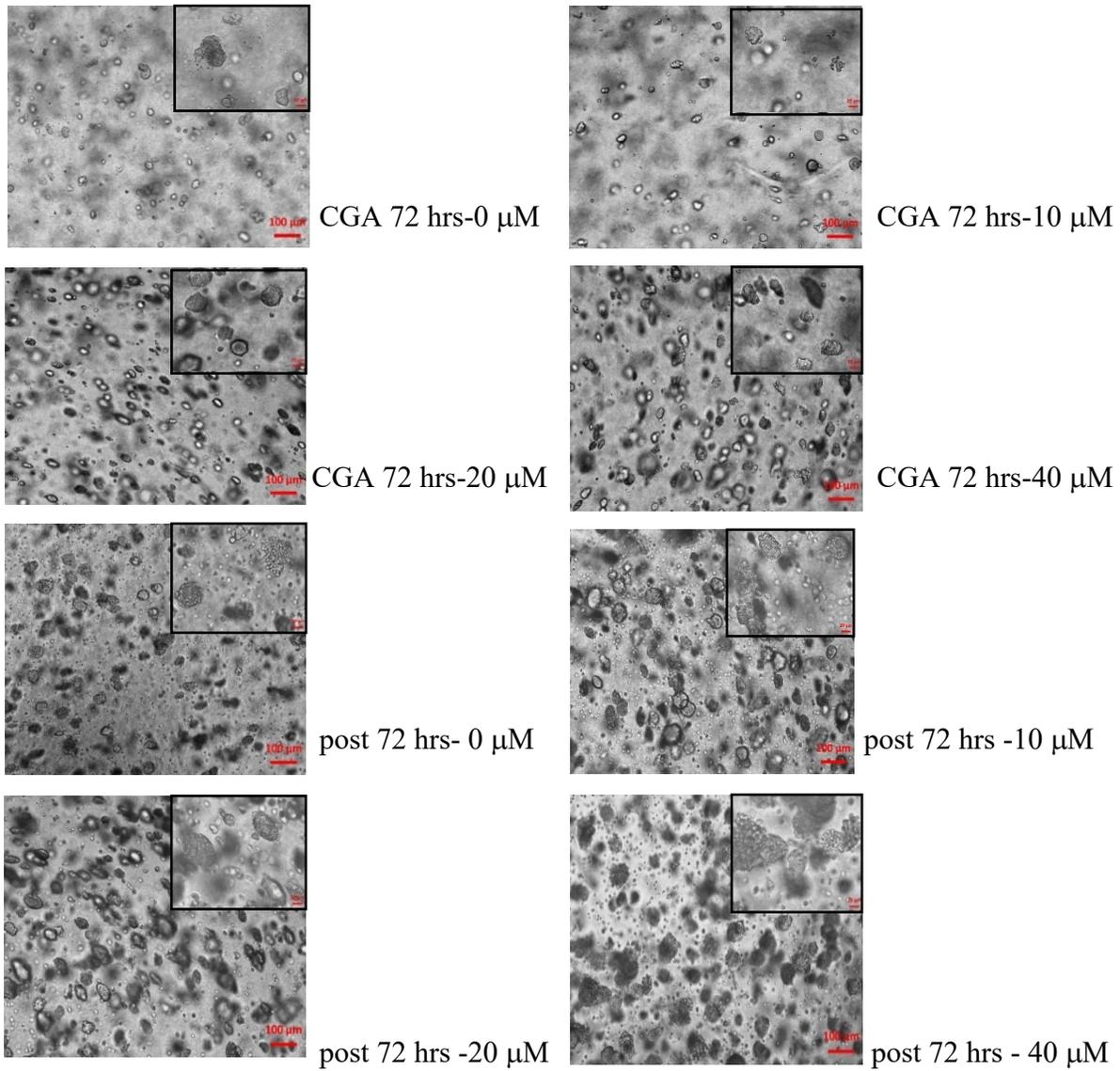
### **4.3.2. Effect of CGA on inhibiting HepG2 cell growth and SW480 cell growth in 3D and 2D cell culture**

#### **4.3.2.1. Effect of CGA on inhibiting HepG2 cell growth in 3D and 2D cell culture**

The effect of CGA treatment on HepG2 cell morphologies in 3D and 2D culture is shown in figure 4.6 and 4.7 respectively. CGA treatment did not affect HepG2 cell morphologies in 3D culture, but reduced HepG2 cell clusters in 2D culture. Effect of CGA on HepG2 cell growth and cell viability in both 3D and 2D culture is shown in figure 4.8.

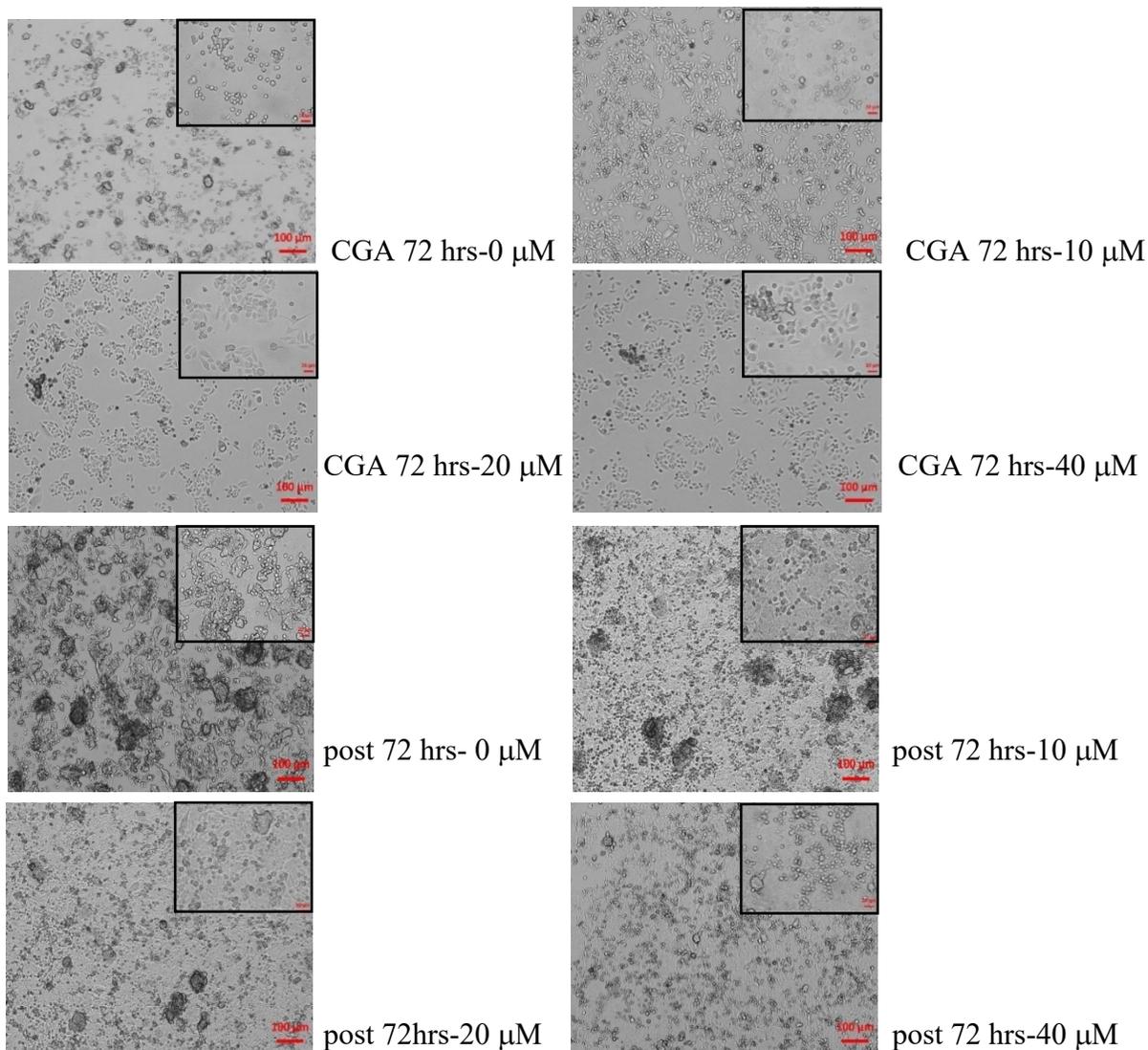
In 3D culture, CGA treatment (10, 20 and 40  $\mu$ M CGA) significantly reduced HepG2 cell growth at all concentrations and all tested time points up to 72 hrs. The impact of CGA on inhibiting HepG2 cell growth was dose- dependent. When cells treated with media without CGA, HepG2 cells were recovered for growth. However, cell growth of post CGA at 24 hrs and 48 hrs was lower than negative control, indicating effect of CGA on HepG2 cell growth was time- dependent. However, there was no significant difference of cell growth of post CGA at 72 hrs. HepG2 cell viability was not altered by CGA treatment at tested concentration and time point.

In 2D culture, CGA treatment (10, 20 and 40  $\mu$ M CGA) resulted in significant decrease in HepG2 cell growth at all concentrations and all tested time points up to 72 hrs, indicating that the impact of CGA on inhibiting HepG2 cell growth in 2D culture was dose- dependent. However, there was no significant difference of HepG2 cell growth among tested concentrations at the same tested time point. When cells treated with media without CGA at tested time point at 24 hrs, 48 hrs, and 72 hrs, HepG2 cells were recovered for growth. However, the cell growth was still lower than negative control indicating inhibitory effect of CGA on HepG2 cell growth was still lasting, and the effect of CGA on inhibiting HepG2 cell growth was time- dependent. CGA treatment did not affect HepG2 cell viability at all CGA concentration and all tested time points. Previously, studies showed that CGA could effectively induce HepG2 cell death in 2D culture, but the effective concentration of CGA varied from 60 to 1000  $\mu$ M (Yan, Liu, Hou, Dong, & Li, 2017), from 1.5 to 50  $\mu$ g/ml (Barahuie et al., 2017), or from 5 to 50  $\mu$ g/ml (Kan, Cheung, Zhou, & Ho, 2014). In this study, 10, 20, and 40  $\mu$ M CGA showed inhibitory effect on HepG2 cell growth in both 3D and 2D culture. When comparing HepG2 cell growth at the same concentration of CGA and time point, proliferation of HepG2 cell in 3D culture was higher than that in 2D culture, indicating that HepG2 cell in 3D culture was more resistant to CGA than that in 2D culture. After CGA removal, inhibitory effect of CGA on HepG2 cell growth in 3D culture was lasting longer than that in 2D culture. Contrary to cell viability, CGA treatment had greater effect on inhibiting HepG2 cell growth in 3D and 2D culture.



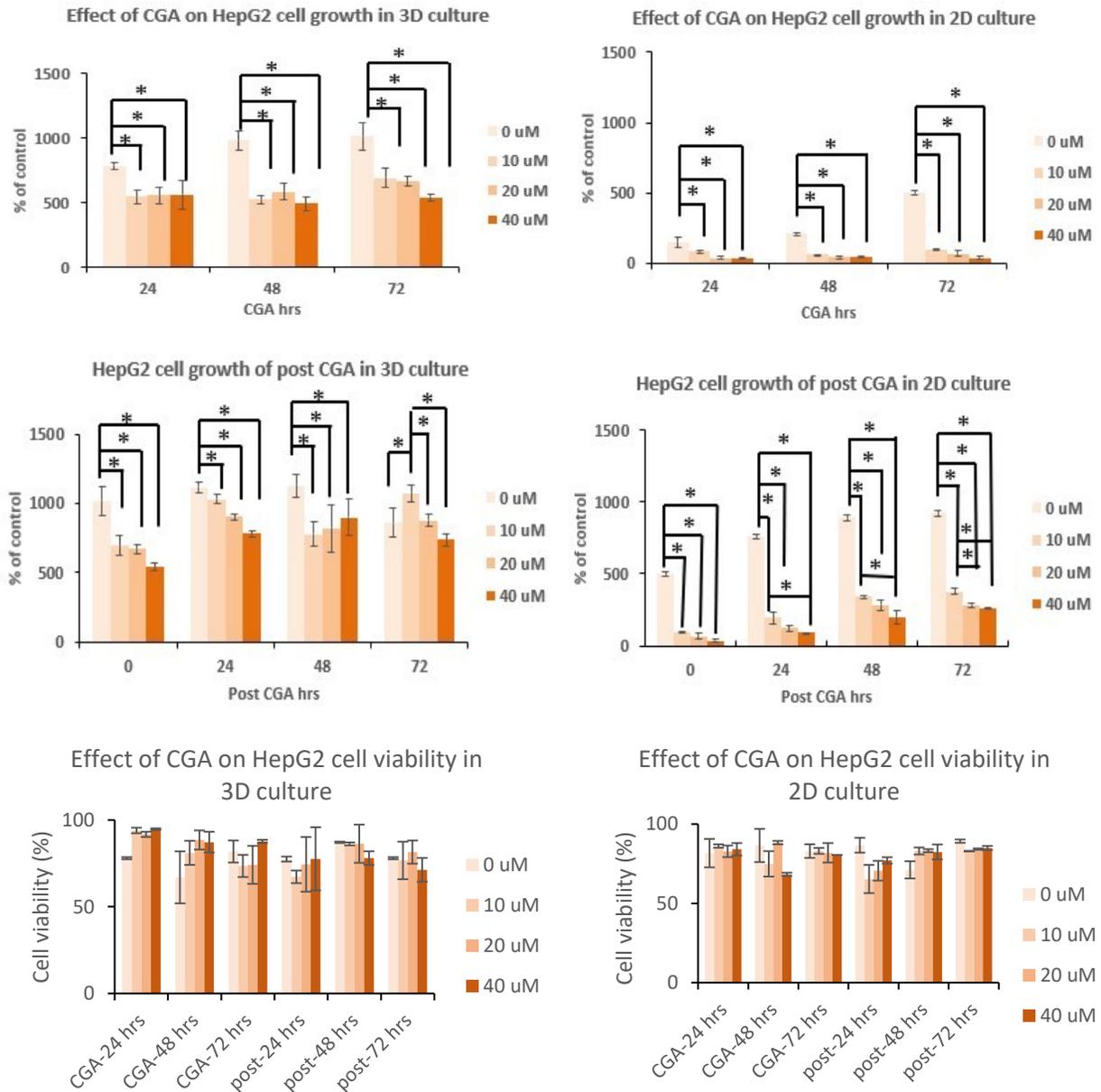
**Figure 4.6. Effect of CGA on HepG2 cell morphologies in 3D culture at 72 hrs and post CGA at 72 hrs.**

Mag: 5x. Each image was shown 20x magnification on the right top.



**Figure 4.7. Effect of CGA on HepG2 cell morphologies in 2D culture at 72 hrs and post CGA at 72 hrs.**

Mag: 5x. Each image was shown 20x magnification on the right top.



**Figure 4.8. Effect of CGA on inhibiting HepG2 cell growth in 3D and 2D culture, and HepG2 cell viability in 3D and 2D culture.**

#### 4.3.2.2. Effect of CGA on inhibiting SW480 cell growth in 3D and 2D cell culture

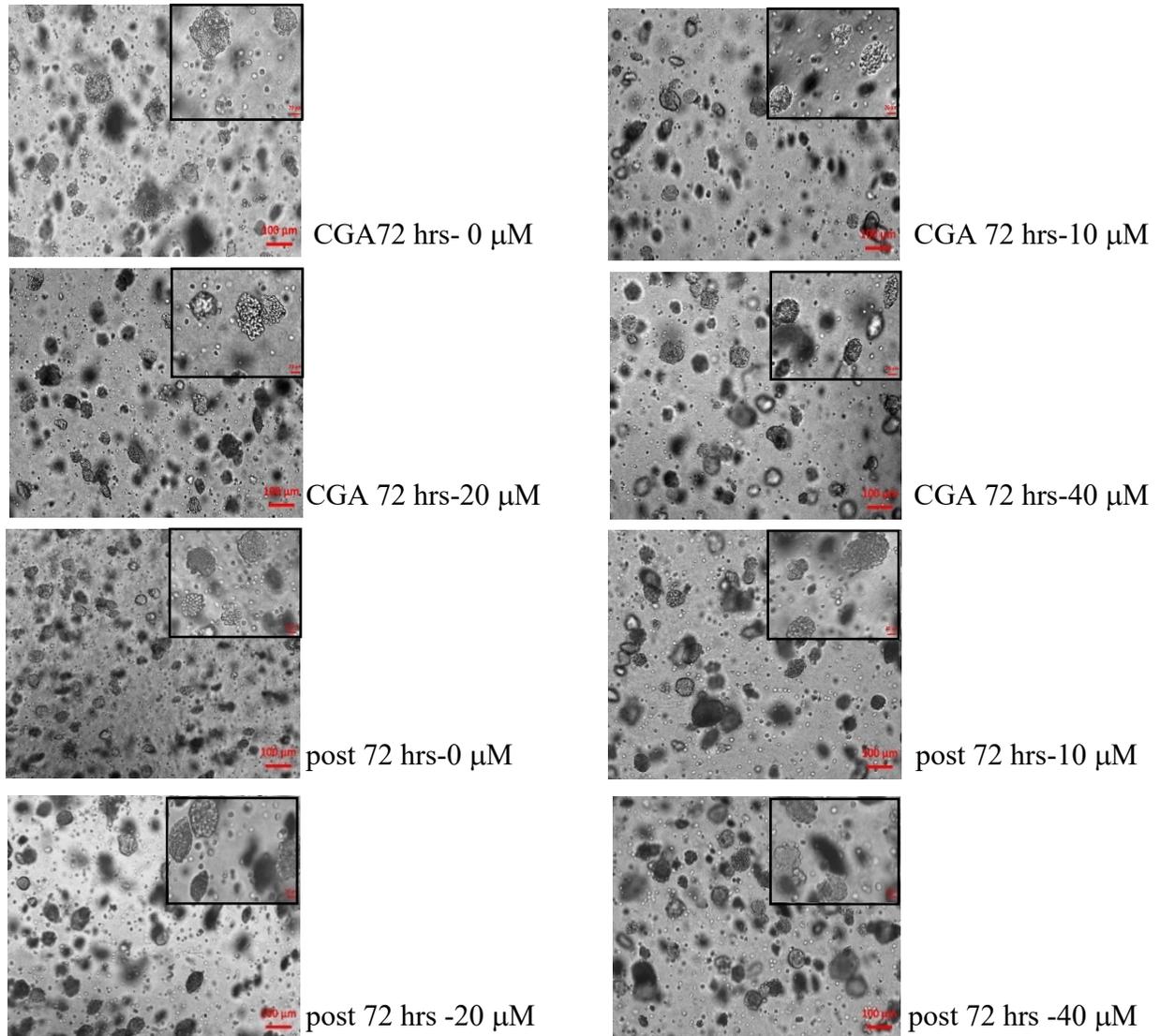
The CGA treatment did not affect the morphologies of SW480 cells in either 3D and 2D culture shown in figure 4.9 and figure 4.10. This finding was in accordance with the previous study that CGA did not affect morphologies of colon cancer colo32 cells (Zheng et al., 2002). Effect of CGA on SW480 cell growth and cell viability in both 3D and 2D culture is shown in figure 4.11.

In 3D culture, CGA treatment (10, 20, and 40  $\mu\text{M}$ ) did not affect SW480 cell growth at 24 and 48 hrs in contrast to negative control, but induced SW480 cell death at 72 hrs, indicating effect of CGA on SW480 cell growth was dose- dependent. CGA treatment did not change SW480 cell viability at tested time points. When cells treated with media without CGA, SW480 cells were recovered for growth. But the cell growth was lower than negative control of post CGA at 24 hrs, indicating effect of CGA on SW480 cell growth was time- dependent. Up to 48 hrs, cell growth of 40  $\mu\text{M}$  of post CGA was lower than negative control, cell growth of 10 and 20  $\mu\text{M}$  CGA of post has similar cell growth compared with negative control. There was no inhibitory effect of CGA on SW480 cell growth of post CGA at 72 hrs. CGA did not affect SW480 cell viability in 3D culture.

In 2D culture, CGA treatment (10, 20, and 40  $\mu\text{M}$ ) did not inhibit SW480 cell growth at 24hrs and 48 hrs. This was in accordance with the effect of CGA on cell growth in 3D culture. Up to 72 hrs, CGA treatment (10, 20, and 40  $\mu\text{M}$ ) induced SW480 cell death, but there was no significant difference of SW480 cell growth among tested CGA concentration. When cells treated with media without CGA, the inhibitory effect of CGA on SW480 cell growth was lasting at 24 hrs. Then cells were recovered for growth of post CGA at 48 hrs and 72 hrs, but cell growth was lower than negative control, indicating effect of CGA on SW480 cell growth was time- dependent.

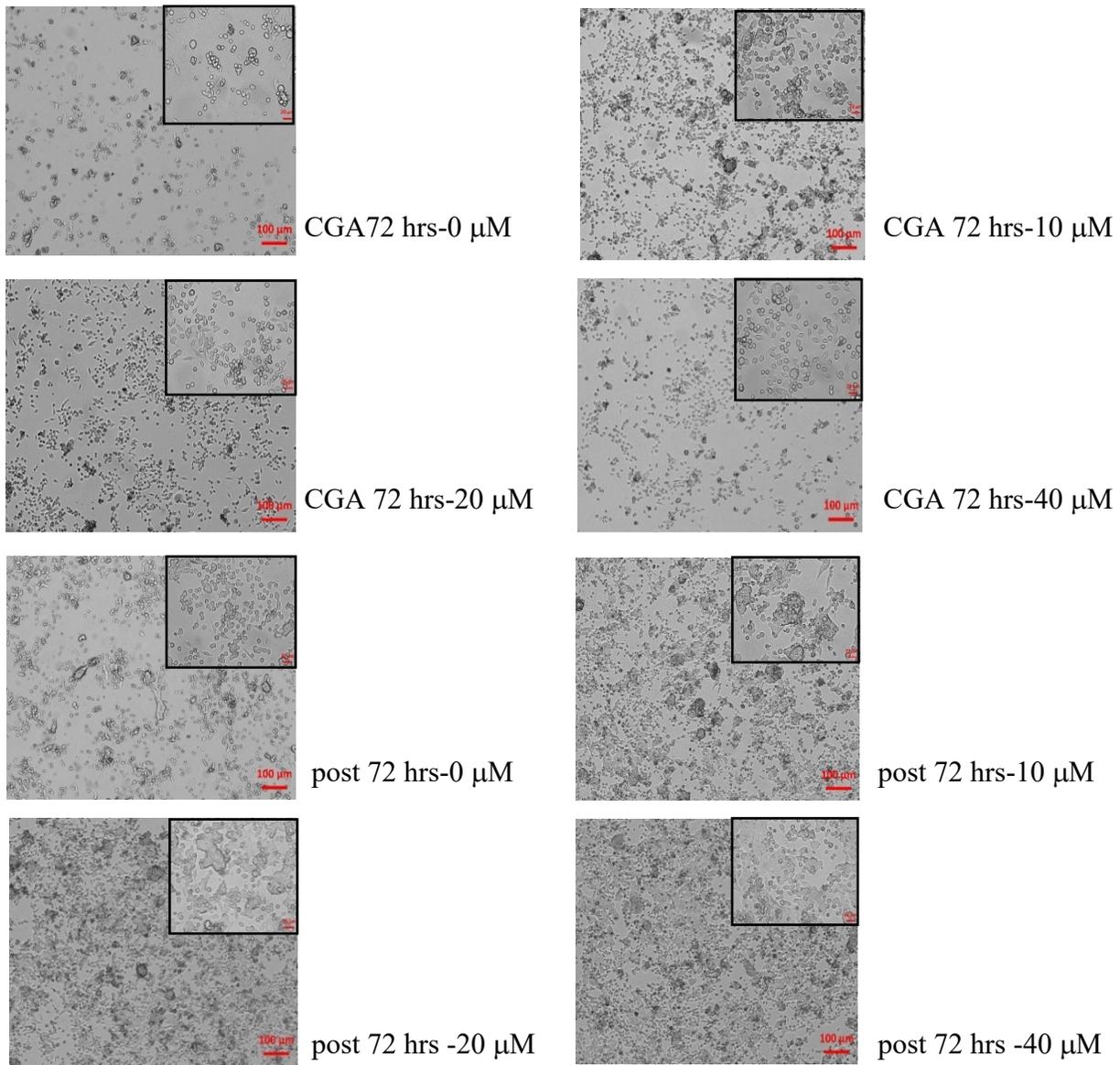
Previously, CGA effectively decreased proliferation of colon cancer cells (Caco-2 and SW480), but the concentration of effective CGA varied from 0.5 to 2 mmol/ L (Shin et al., 2015) or from 50 to 1000  $\mu\text{M}$  (Sadeghi Ekbatan, Li, Ghorbani, Azadi, & Kubow, 2018). In this study, CGA treatment up to 72 hrs resulted in inhibition of SW480 cell growth in both 3D and 2D culture, but the effective concentration was different. In 2D culture, all concentration resulted in significant

reduction of SW480 cell growth, while not in 3D culture, suggesting that SW480 cells were more resistant to CGA in 3D hydrogel culture than in 2D monolayer culture. After CGA removal, inhibitory effect of CGA on SW480 cell growth in 3D culture was lasting longer than that in 2D culture. In contrast to cell growth, CGA treatment has a minor effect of SW480 cell viability in both 3D and 2D culture.



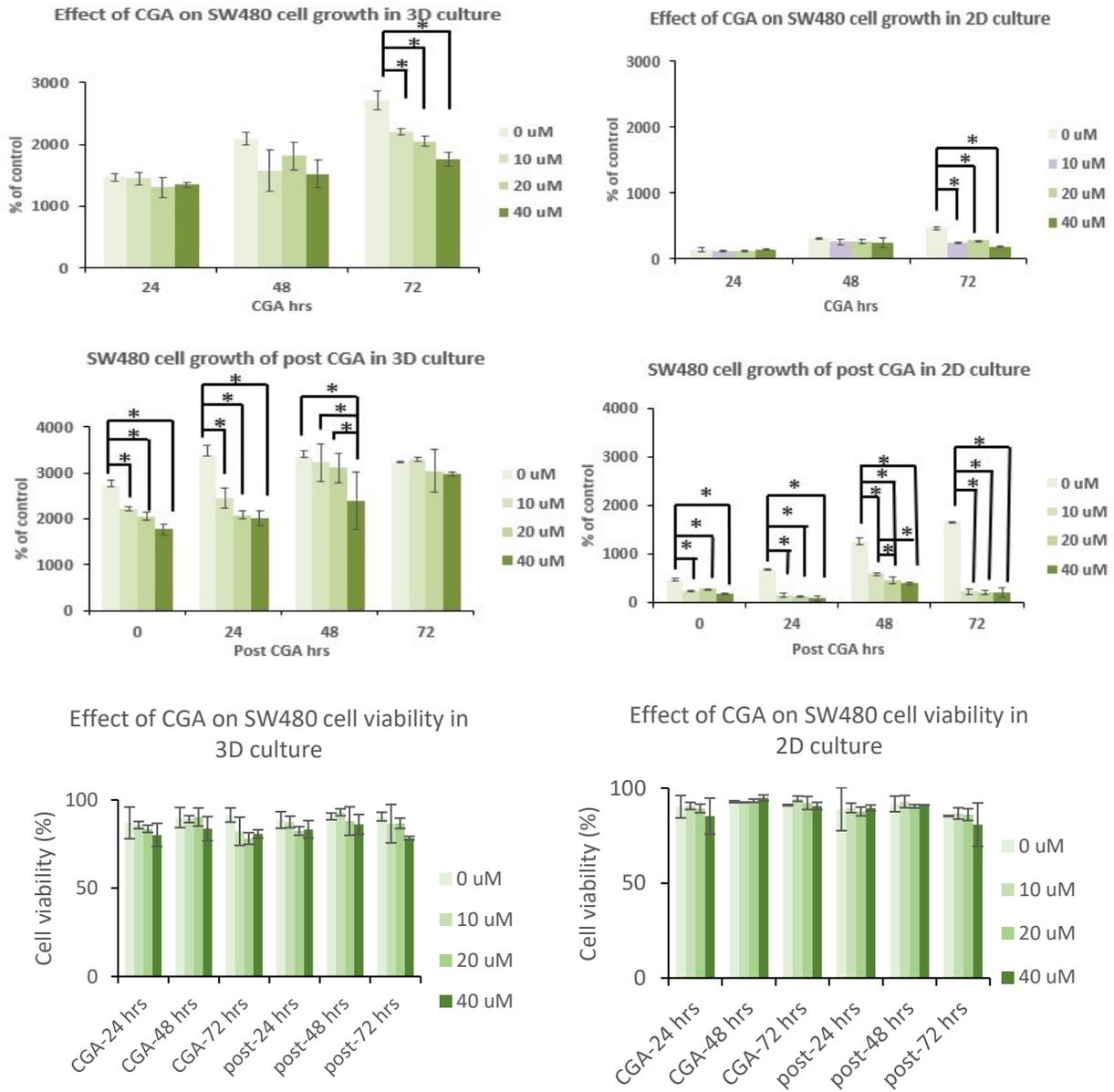
**Figure 4.9. Effect of CGA on SW480 cell morphologies in 3D culture at 72 hrs and post CGA at 72 hrs.**

Mag: 5x. 20x magnification of each image was shown on the right top.



**Figure 4.10. Effect of CGA on SW480 cell morphologies in 2D culture at 72 hrs and post CGA at 72 hrs.**

Mag: 5x. 20x magnification of each image was shown on the right top.



**Figure 4.11. Effect of CGA on inhibiting SW480 cell growth and cell viability in 3D and 2D culture.**

CGA treatment suppressed cell growth in a dose- and time- dependent manner in both HepG2 cell and SW480 cell lines, which indicates a chemopreventive effect of CGA on liver and colon cancer. Cells in 3D culture were more resistant to CGA in contrast to 2D culture. IC50 value is the half maximum inhibitory concentration to measure the potency of a treatment in inhibiting cell growth. In our study, IC50 values of HepG2 and SW480 cell treated with CGA at 72 hrs in

both 3D and 2D culture were calculated and shown in table 4.1. IC50 indicated the inhibition assay which induced half reduction of response. IC50 value of CGA on HepG2 cells in 3D culture was almost ten times higher than that in 2D culture. To SW480 cells, IC50 value in 3D culture was around fourteen times higher in contrast to 2D culture. To both HepG2 cell and SW480 cell, there was an increasing tendency of IC50 values from 2D monolayer culture to 3D hydrogel culture. This was in accordance with previous study that an increased IC50 value of flavone on colon cancer cell HT29 in 3D culture was observed in contrast to 2D monolayer culture (Silva et al., 2018). Another study investigated the effect of lycopene on inhibiting ovarian cancer cells in 2D monolayer culture and 3D spheroids culture, and it was found that lycopene reduced ovarian cancer cell growth in the first seven days, but increased cell viability after exposure up to 14 days (Holzapfel et al., 2016). Lower IC50 value of CGA on HepG2 cells was observed in contrast to SW480 cells, corresponding to SW480 cells were more resistant to CGA than HepG2 cells. That was in agreement with previous studies wherein CGA resulted in reductions of cell growth of human colon cells (HT290) and HepG2 cells, but HepG2 cells were more susceptible than HT290 cells (Glei et al., 2006), and Caco-2 cells were more resistant to CGA treatment than HepG2 cells (Wang et al., 2011).

**Table 4.1. IC50 values of CGA in HepG2 cell and SW480 cell in both 3D and 2D culture.**

Cells	IC50 ( $\mu\text{M}$ )	
	3D	2D
HepG2	56.3	5.2
SW480	251	17.4

#### 4.4. Conclusion

Using 3D hydrogel culture, which reflects natural tumor microenvironments, both HepG2 cells and SW480 cells extended the exponential phase and achieved more proliferation in contrast to 2D monolayer culture. HepG2 cells were more susceptible than SW480 cells to CGA treatment. CGA treatment suppressed HepG2 cell growth and SW480 cell growth and the effect was in a dose- and time-dependent manner in both 3D and 2D culture, while HepG2 cell and SW480 cell to CGA treatment in 3D culture was more resistant than 2D culture. The difference of cell growth obtained in 3D culture and 2D culture suggested 3D model was close to complex *in vivo* animal model and could potentially bridge the gap between *in vitro* 2D monolayer culture and *in vivo* animal model in future cancer research.

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## Chapter 5 - Conclusion

This dissertation focused on health promoting phytochemicals in response to environmental factors in lettuce, spinach and tomatoes and application of 3D hydrogel cell culture for anticancer function.

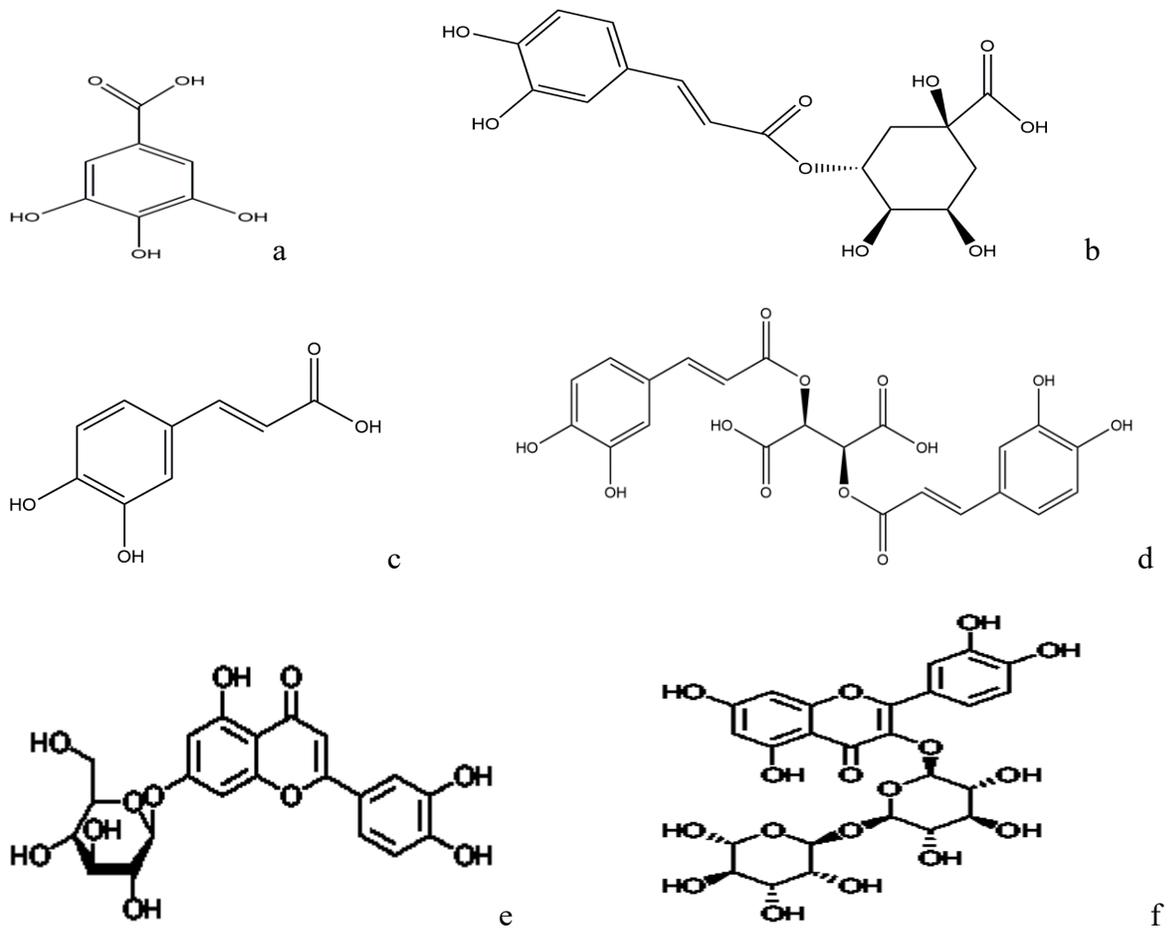
Study 1: This study selected two varieties of lettuce ('Two Star' and 'New Red Fire') and two varieties of tomato ('Celebrity' and 'Mountain Fresh') grown in high tunnel and open field and compared the phenolics in lettuce and carotenoids in tomato in high tunnel in contrast to open field. The results showed high tunnel decreased the phenolic contents in certain variety of lettuce and reduced carotenoid contents in certain variety of tomato. The variance of phenolics in lettuce and carotenoids in tomato indicated the effect of high tunnel production is genotype dependent. The negative effect of high tunnel on phenolics in lettuce and carotenoids in tomato can be improved by variety selection and genetic engineering.

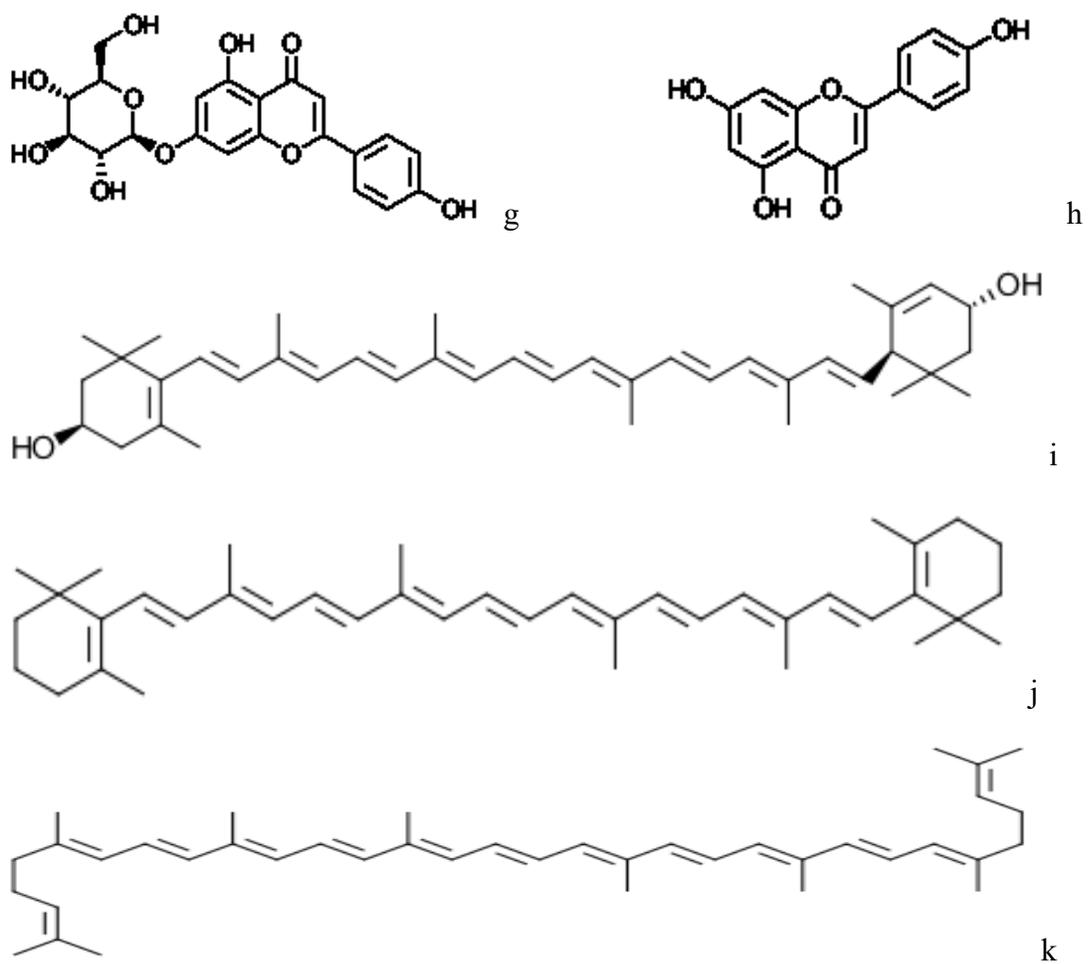
Study 2: Lettuce ('Two Star' and 'New Red Fire') and spinach ('Avon' and 'Bloomsdale') were grown under different light spectra such as red, far-red and blue light. Far-red and blue light significantly increased phenolic contents in 'Two Star' lettuce compared to the control, while red light did not. In 'Red Fire' lettuce, the phenolic contents were improved by red light in contrast to the control, however, far-red and blue light decreased the phenolic contents compared to the control. Light spectra did not alter flavonoid contents in two varieties of spinach. Different light spectra significantly influenced phenolics in lettuce, but not in spinach, indicating effect of light spectra on phenolics in plants is genotype dependent. The positive effect of light spectra on phenolics in lettuce could be used as effective strategies to increase phenolics for the purpose of improving health promoting factors for human health benefits. While negative effect of light spectra on phenolics in lettuce can be overcome by variety selection and genetic engineering.

Study 3: 3D hydrogel culture is a promising model for cancer research and showed favor of cell growth which was reflective as extended exponential phase of HepG2 cell and SW480 cell as well as more cell growth in contrast to 2D culture. Chlorogenic acid treatment suppressed HepG2 cell growth and SW480 cell growth in both 2D and 3D culture, and the effect of CGA on inhibiting HepG2 cell growth and SW480 cell growth is dose- and time- dependent. Chlorogenic acid treatment resulted in greater HepG2 cell growth inhibition and SW480 cell growth inhibition in 2D culture in contrast to 3D culture. SW480 cells were more resistant to chlorogenic acid than HepG2 cells. The difference of cell growth inhibition in 2D and 3D culture suggested 3D model was much closer to *in vivo* animal model and further could be potentially applied in bridging the gap of studies between *in vitro* 2D monolayer culture and *in vivo* animal model in future cancer research.

## Appendix A - Chemical structures of identified phenolics, flavonoids

### and carotenoids





**Figure A. 1. Chemical structure of each phenolic compounds, a: gallic acid, b: chlorogenic acid, c: caffeic acid, d: chicoric acid, e: luteolin-7-glucoside, f: rutin, g: apegenin-7-glusodie, h: kaempferol, i: lutein, j:  $\beta$ -carotene and k: lycopene.**

## Appendix B - Linear regression of standard curve

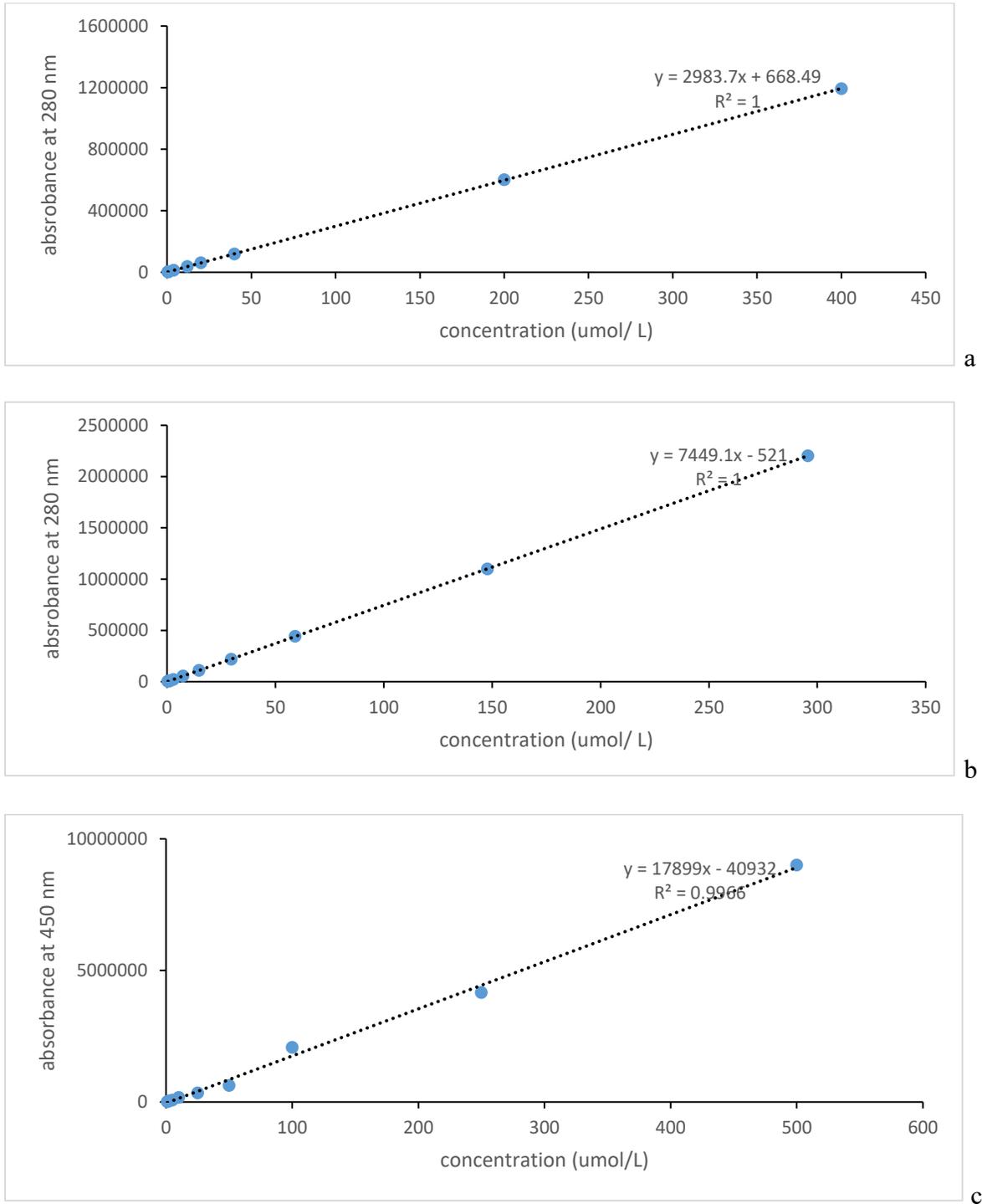
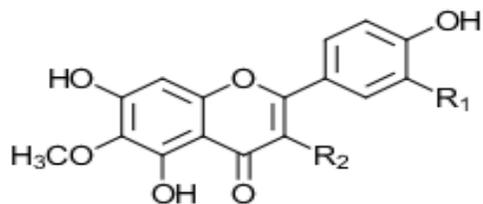


Figure B. 1. Linear regression curves of vanillic acid (a), quercetin (b), and  $\beta$ -carotene (c).

## Appendix C - Flavonoid skeleton in spinach



**Figure. C. 1. Flavonoid skeleton in spinach.**

Aglycone	R <sub>1</sub>	R <sub>2</sub>
Patuletin	OH	OH
Spinacetin	OCH <sub>3</sub>	OH
Spinatoside	OH	OCH <sub>3</sub>
Jaceidin	OCH <sub>3</sub>	OCH <sub>3</sub>