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ACCESSING THE BIOAVAILABILITY OF PHYTOCHEMICALS IN CACO-2 CELL MODEL AND DEVELOPING A SENSITIVE METHOD FOR THE DETECTION AND QUANTIFICATION OF THESE COMPOUNDS

A Thesis Presented

by

HANÁ SHATARA SOBERS

Submitted to the Graduate School of the

University of Massachusetts Amherst in partial fulfillment

of the requirements for the degree of

MASTER OF SCIENCE

September 2012

Food Science

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HANÁ SHATARA SOBERS

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It's impossible to express my gratitude in words to my beloved family members – my sister, my three little brothers, my parents (Yuma & Baba), my other parents (Mummy & Da) and my two little baby boys (who were born during the making of this thesis) – for all their love, understanding and encouragement. Last but not least, to my loving husband, THANK YOU!

ABSTRACT

ACCESSING THE BIOAVAILABILITY OF PHYTOCHEMICALS IN CACO-2 CELL MODEL AND DEVELOPING A SENSITIVE METHOD FOR THE DETECTION AND QUANTIFICATION OF THESE COMPOUNDS

SEPTEMBER 2012

HANÁ SHATARA SOBERS, B.S., PURDUE UNIVERSITY

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Directed by: Professor H. Xiao

Numerous studies have found certain unmethylated phytochemicals to possess anti-carcinogenic activity; however, they have been associated with poor oral bioavailability which is a major limiting factor in their usage in chemopreventative treatment. The purpose of this study was to investigate if methylation of a compound would affect bioavailability, in terms of transport and permeability, in a Caco-2 cell model as well as the effect of cell viability and cellular uptake in human colon cancer cell lines. Furthermore, a new analytic method using reversed-phase high performance liquid chromatography coupled with electrochemical detector (HPLC-EC) for the detection and quantification of resveratrol and pterostilbene was developed.

This new method was simple, rapid, and more sensitive compared to other detection methods used to analyze resveratrol and pterostilbene. Linear range, limit of detection (LOD), precision and recovery were used to validate this new analytical

method. There was a significant increase in intracellular uptake and stronger growth inhibitory of pterostilbene in human cancer cells lines in comparison to resveratrol. Resveratrol exhibited a higher and more rapid rate of transport than pterostilbene across the Caco-2 monolayer regardless of the concentration tested and direction. Pterostilbene exhibited little difference in the rate of transport from either direction. The HCT-116 colon cells had intracellular uptake of each of the polymethoxyflavones (PMFs) tested. Transport was observed by all the PMFs and each had different rates of transport. Overall, location and amount of methyl groups had an effect on bioavailablity of a compound and these compounds show promise as chemopreventative agents.

Keywords: Resveratrol; Pterostilbene; Polymethoxyflavones (PMF); Caco-2 cells; Transport; Permeability; Bioavailability; Electrochemical (EC) detection

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS iv

ABSTRACT v

LIST OF TABLES xi

LIST OF FIGURES xii

CHAPTER

1. INTRODUCTION 1

1.1 Cancer 1

1.1.1 Statistics 1

1.1.2 Definition 1

1.1.3 Chemoprevention 3

1.2 Phytochemicals 3

1.2.1 Methylated vs. Unmethylated 3

1.2.2 Polymethoxyflavones 4

1.2.3 Stilbenes 8

1.2.3.1 Resveratrol 9

1.2.3.2 Pterostilbene 11

1.3 Bioavailability 12

1.3.1 Overview 12

1.3.2 Bioavailability of Compounds of Interest 14

1.3.4 Caco-2 cell Monolayer Model 16

2. RAPID METHOD FOR THE DETERMINATION OF RESVERATROL AND PTEROSTILBENE BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION 19

2.1 Abstract 19

2.2 Introduction 20

2.3 Materials and Methods 22

2.3.1 Chemicals and reagents 22

2.3.2 Instrumentation 22

2.3.3 Chromatographic Conditions 22

2.3.4 Preparation of standards 23

2.3.5 Analyses of Juice Samples 23

2.4 Results and Discussion 24

2.4.1 Optimization of Chromatographic Conditions 24

2.4.2 Voltametric Behavior of Resveratrol and Pterostilbene 25

2.4.3 Linearity and Range 28

2.4.4 LOD and LOQ 29

2.4.5 Accuracy/Recovery Studies 29

2.4.6 Precision 30

2.4.7 Analysis of resveratrol and pterostilbene in commercial juice products 31

2.5 Conclusion 32

3. COMPARISON OF THE CELLULAR UPTAKE AND INHIBITORY EFFECTS OF RESVERATROL AND ITS METHYLATED ANALOGUE PTEROSTILBENE ON HUMAN COLON CANCER CELLS 34

3.1 Abstract 34
3.2 Introduction 35
3.3 Materials and Methods 36
3.3.1 Materials 36
3.3.2 Cell culture treatment 37
3.3.3 Cell Viability Assay 37
3.3.4 Cellular Uptake Assay 38
3.3.4.1 Cytosol Faction 38
3.3.4.2 Membrane Fraction 39
3.3.5 HPLC analysis 39
3.3.6 Statistical Analysis 40
3.4 Results & Discussion 41
3.4.1 Growth inhibition on Caco-2 41
3.4.2 Cellular Uptake of 3 Colon Cancer Cell Lines 42
3.5 Conclusion 48

4. COMPARISON OF PERMEABILITY AND TRANSPORT OF RESVERATROL AND ITS METHYLATED ANALOGUE PTEROSTILBENE IN HUMAN INTESTINAL CACO-2 CELLS 50
4.1 Abstract 50
4.2 Introduction 51
4.3 Materials and Methods 52
4.3.1 Materials 52
4.3.2 Cell Culture - Caco-2 53
4.3.3 Transport Experiments 53
4.3.4 HPLC analysis 54

4.3.5 Data Analysis 55
4.4 Results and Discussion 56
4.4.1 Overall 56
4.4.2 Resveratrol 62
4.4.3 Pterostilbene 63
4.5 Conclusion 63

5. COMPARISON OF THE CELLULAR UPTAKE, PERMEABILITY AND TRANSPORT OF POLYMETHOXYFLAVONES (PMFS) 66
5.1 Abstract 66
5.2 Introduction 67
5.3 Materials and Methods 68
5.3.1 Materials and Reagents 68
5.3.2 Cell Culture Treatment 69
5.3.4 Cellular Uptake Assay 69
5.3.5 Transport Experiments 70
5.3.6 Sample Analysis 70
5.3.7 Data Analysis 71
5.4 Results & Discussion 72
5.4.1 Cellular Uptake of PMFs in HCT-116 cancer cells 72
5.4.2 Transport of PMFs by Caco-2 cells 75
5.5 Conclusion 77

6. FUTURE RESEACH 80

BIBLIOGRAPHY 82

LIST OF TABLES

Table Page

Table 1.1 PMFs used in the different experiments 7

Table 2.1 Voltametric behavior of resveratrol and pterostilbene. 28

Table 2.2 Linearity, LOD, and LOQ for resveratrol and pterostilbene. 28

Table 2.3 Accuracy/Recovery data of resveratrol and pterostilbene standards. 30

Table 2.4 Precision data of resveratrol and pterostilbene standards. 31

Table 2.5 Amount of resveratrol and pterostilbene in different juice products. 32

Table 4.1 Apparent permeability coefficients or different concentrations of resveratrol and pterostilbene through Caco-2 monolayers 62

Table 5.1 Apparent permeability coefficients of the PMFs through Caco-2cell monolayers. 77

LIST OF FIGURES

Figure Page

1.1 Cancer arises from a loss of normal growth control 2

1.2 Chemical structure of (a) resveratrol and (b) pterostilbene. 9

1.3 Overview of Bioavailability and ADME. 13

1.4 Determinants of oral bioavailability and a decision-tree to assess their role 14

1.5 Diagram of the Caco-2 cell monolayer cultivated on a permeable filter support 16

1.6 Possible compound transport pathways across the intestinal mucosa, illustrating (1) transcellular and (2) paracellular modes of passive transport, (3) transcytosis, (4) carrier-mediated transport and (5) efflux transport. 17

2.1 Representative chromatograms of resveratrol and pterostilbene at different potentials. 25

2.2 The effect of electrochemical cell potential on the peak area (μC) for (a) resveratrol and (b) pterostilbene. 27

3.1 Growth inhibitory effect of resveratrol and pterostilbene on CaCo-2 human colon adenocarcinoma cells.. 42

3.2 Cellular uptake of resveratrol and pterostilbene in the cytosol of (a)Caco-2, (b)HT-29, and (c)HCT-116 human colon cancer cell lines. Colon cancer cells were incubated with 10μM of (d) resveratrol or (e) pterostilbene in complete medium for various time periods 46

3.3 Cellular uptake ratio of pterostilbene to resveratrol in the cytosol of Caco-2, HT-29, and HCT-116 human colon cell lines. The cells were incubated with 10μM resveratrol or pterostilbene in complete medium for (a) 0.5, (b) 1 and (c) 2 hour 47

4.1 The transport from the apical to the basolateral compartment of (a) resveratrol and (b) pterostilbene across the Caco-2 monolayer 58

4.2 The transport from the basolateral to the apical compartment of (a) resveratrol and (b) pterostilbene across the Caco-2 monolayer.....59

4.3 Cumulative fraction transport from the apical to the basolateral compartment of (a) resveratrol and (b)pterostilbene across the Caco-2 monolayer. 60

4.4 Cumulative fraction transport from the basolateral to the apical compartment of (a) resveratrol and (b) pterostilbene across the Caco-2 monolayer.. 61

5.1 Percentage of cellular uptake in HCT-116 human colon cancer cells of PMF4, 5 and 7 from the PMF mixture in comparison to uptake of PMF3 from the same mixture.....74

5.2 Cumulative fraction transported from the apical to the basolateral compartment for PMF3, PMF4 and PMF7across the Caco-2 cell monolayer.. 76

CHAPTER 1

INTRODUCTION

1.1 Cancer

1.1.1 Statistics

According to the World Health Organization (WHO), cancer accounts for more deaths worldwide than HIV/AIDs, malaria, and tuberculosis combined and will soon surpass heart disease and stroke as the most prevailing cause of death globally (WHO, 2011). In the United States, one in every four deaths is due to cancer exceeded only by heart disease. It is estimated that almost 1.65 million new cases of cancer will be diagnosed this year alone in the United States.

The probability of an American male developing cancer over his lifetime is a one in two while for woman it is a one in three likelihood. In the United States, the most common cancer in men is prostate and for women, it is breast cancer; lung and colorectal cancers are the second and third most common cancers in both men and women (American Cancer Society, 2011). These staggering statistics has sparked a surge in cancer research.

1.1.2 Definition

Cancer arises from the transformation of a normal cell into an abnormal cell that divides without control. In general, this is a multistage process that advances from a pre-cancerous lesion to malignant tumors. Cancer cells can invade and spread to other parts of the body through the blood stream and lymph system. Even though cancer can develop

in nearly any tissue of the body, each type of cancer has its distinctive features yet the fundamental processes that generate cancer are similar in all forms of the disease. A majority of all colon cells are adenocarcinomas, which produce and release mucus and other fluids (Ruoslahti, 1996; Weinberg, 1996). Using natural occurring compounds has materialized as a plausible approach for cancer management and prevention (Francy-Guilford and Pezzuto, 2008; Khan et al., 2008; Surh, 2003; Syed et al., 2007).

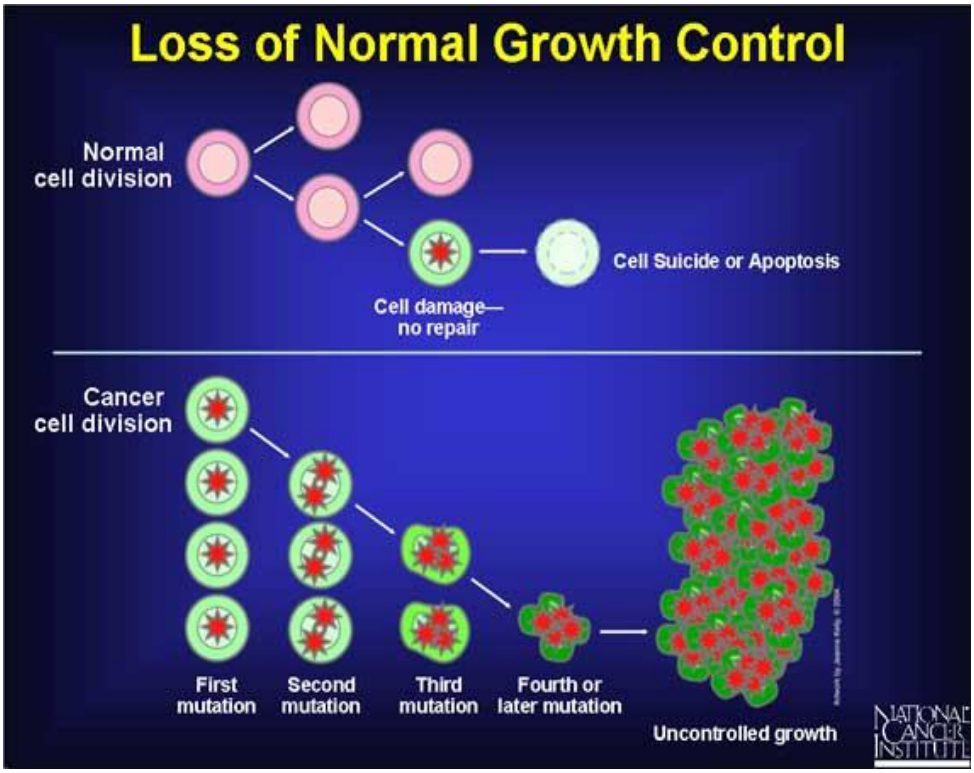


Figure 1.1 Cancer arises from a loss of normal growth control (National Cancer Institute, 2012)

1.1.3 Chemoprevention

Chemoprevention can be defined as managing cancer by which the disease can be prevented, hindered or reversed via administration of one or more compounds of natural and/or synthetic origin (Siddiqui et al., 2008; Sporn and Suh, 2002; Surh, 2003). Numerous epidemiological studies have linked fruit and vegetable consumption with a reduced risk of cancer (Aggarwal and Shishodia, 2006; Center et al., 2009; Gusman et al., 2001; McCullough et al, 2011). Multiple phytochemicals have been identified as potential cancer fighting agents and subsequently, resulted in an explosion in the supplement market selling these compounds to prevent cancer.

There are different ways to approach the use of phytochemicals as chemopreventive agents. One method would be to incorporate foods rich with that compound into ones diet. A whole food approach exist all ready for other disease and aliments such as the use of soy base foods to reduce osteoporosis and cardiovascular disease (Karp et al., 2007; Scheiber et al., 2001). Another approach would be to put the phytochemical in a tablet or a power. Overall chemoprevention can be an easier and cheap strategy to manage cancer in comparison to the more traditional methods (Eg. Chemotherapy, Organ Removal).

1.2 Phytochemicals

1.2.1 Methylated vs. Unmethylated

A phytochemical can be defined as any chemical that is produced naturally by a plant. Many phytochemicals, in particular flavonoids and stilbenes, are plant metabolites

deriving from the phenlypropadnoid metabolism and shikimate pathways with a small number of exceptions (Stafford, 1990; Watts et al., 2006). Unmethylated phytochemicals have been studied most extensively (Pan and Ho, 2008; Walle et al., 2007).

Even though unmethylated phytochemicals have the potential to be great candidates as chemoprevention agents, they have been associated with poor oral bioavailability, which is a major limiting factor in their potency and usage as an additive in food products. These compounds have low bioavailability because of the free hydroxyl groups which gives rise to rapid intestinal/hepatic conjugation and/or sulfation and excretion (Wen and Walle, 2006). The methylation of the free hydroxyl group(s) on compound leads to a reduction in susceptible to glucuronic acid or sulfate conjugation, resulting in improved metabolic stability (Walle, 2007). There is also an improved transport through biological membranes (like intestinal absorption) and an increase in oral bioavailability because of methylation (Walle, 2007; Wilson et al., 2008). Walle and others (2007) have observed an increase in effectiveness by methylated compounds to inhibit cancer cell growth. Despite being present in nature and their great potential in cancer prevention, they lack the anti-oxidant effects that are usually linked with free hydroxyl groups.

1.2.2 Polymethoxyflavones

Polymethoxyflavones (PMFs) refer to a distinctive class of flavonoinds containing two or more methoxy groups on benzo- γ -pyrone skeleton (C_6-C_3-C_6) with a carbonyl group at the C_4 position. In nature, PMFs are present exclusively in the citrus genus, particularly in the peel of mandarin oranges (*Citrus reticulata*) and sweet oranges (*Citrus sinensis*). Cultivation of citrus is thought to have begun around 4,000 years ago

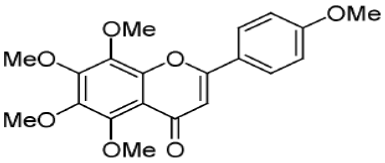
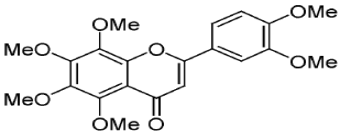
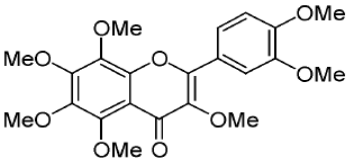
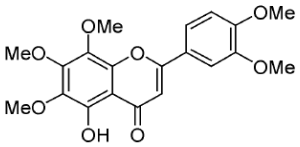
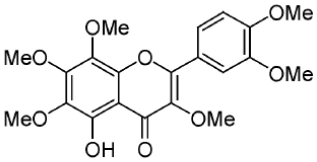
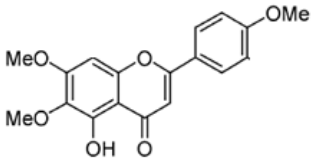
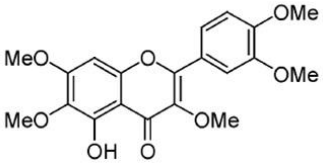
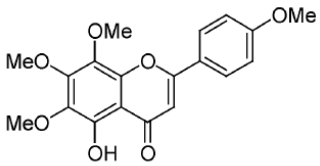
in Southeast Asia and moved gradually west to Northern Africa, the Mediterranean, and then to southern Europe by the Middle Ages (Bruening et al., 2010). In traditional Chinese medicine, they have used citrus peel to treat and alleviate a wide range of aliments like respiratory infections, indigestion and ringworm infections (Ou, 1999). More than 20 PMFs have been isolated and identified from different tissues of citrus plants (Li et al., 2006). (See Table 1.1 for a complete list of all the PMFs that were utilized in this thesis).

The projected forecast for 2012, as of July 2012, on the global production of oranges is 51.1 million metric tons (MMT) and 22.6 MMT for tangerine/mandarin. In the United States, the predicted quantity is 8.1 MMT for productions of oranges and slightly more than 0.5 MMT for tangerine/mandarin. Most of the tangerine/mandarin produced are expected to be entirely consumed leaving only about 6% for processing. Of the oranges grown worldwide, an estimated 28.5 MMT will be consumed leaving the remaindering amount for processing, which is expected to be approximately 22.2 MMT. An anticipated 2.2MMT of orange juice will be produced worldwide which will yield a considerable amount of orange peel by-product that could potentially be used for medical purposes (USDA: Foreign Agricultural Service, 2012).

PMFs have been shown to exhibit an expansive range of biological activities, including anti-atherogenic (Whitman et al., 2005), anti-inflammatory (Chen et al., 2007; Choi et al., 2007; Manthey et al, 2001; Middleton et al., 2000) and anti-oxidant (Li et al., 2007a). PMFs have recently received a lot of attention because they have been shown to have greater anti-carcinogenic activity than other flavones (Li et al., 2007a; Xiao et al., 2009).

Tangeretin, one of the most copious PMFs in citrus peels, has widely been accepted has having excellent anti- carcinogenic activity (Chen et al., 2007; Morley et al., 2007). Sergeev and his colleagues (2006) demonstrated that both PMF3 and PMF4 induce apoptosis by raising the amount of intracellular Ca^{2+} resulting from the depletion of the Ca^{2+} endoplasmic reticulum and infux of Ca^{2+} from the extracellular space in human breast cancer cell. Pan et al. (2007) observed induced growth inhibition of human cancer cells by PMF4 as well as induction of apoptosis in human promyelocytic leukemia cells through modulation of mitochondrial functions by PMF4. There has however been a lag in the investigation on the bioavailability of PMFs.

Table 1.1 PMFs used in the different experiments

Abbreviation	Structure	Name	Molecular Formula	Molecular Mass (g/mol)
Tan (Tangeretin)		5,6,7,8, 4' – penamethoxyflavone	$C_{20}H_{20}O_7$	372
PMF1 (Nobiletin)		5,6,7,8, 3',4' – hexamethoxyflavone	$C_{20}H_{19}O_8$	402
PMF2		3,5,6,7,8, 3',4' – heptamethoxyflavone	$C_{22}H_{24}O_9$	432
PMF3		5 – hydroxyl – 6,7,8,3',4' – pentamethoxyflavone	$C_{20}H_{20}O_8$	388
PMF4		5 – hydroxyl – 3,6,7,8,3',4' – hexamethoxyflavone	$C_{21}H_{22}O_9$	418
PMF5		5 – hydroxyl – 6,7,4' – trimethoxyflavone	$C_{15}H_{13}O_6$	328
PMF6		5-hydroxyl - 3,6,7,3',4' – pentamethoxyflavone	$C_{20}H_{19}O_8$	388
PMF7		5- hydroxyl-6,7,8,4' –tetramethoxyflavone	$C_{19}H_{16}O_7$	358

1.2.3 Stilbenes

The word stilbene was derived from the Greek word *stilbos*, which means shining (Likhtenshtein, 2010). Stilbenes are small molecular weight compounds (between 200 to 300 g/mol) produced in response to excessive ultraviolet exposure; microbial, fungal or viral attack; or injury (Fauconneau et al., 1997). They are created by means of the phenylpropanoid pathway, using phenylalanine ammonia lyase, cinnamate-4-hydroxylase, stilbene synthase and 4-Coumarate-CoA ligase. The core chemical structure of stilbene compounds is 1,2-diphenylethylene (Watts et al., 2006). They are found in a wide range of plants and fruits, including *Vitis* and *Vaccinium* berries (Rimando et al., 2004.) Stilbenes have been shown to possess a wide range of biological activities such as anti-inflammation (Garodia et al., 2007; Juan et al., 2008; Ndiaye et al., 2011) and anti-carcinogenic (Chillemi et al., 2007; Rimano and Suh, 2008).

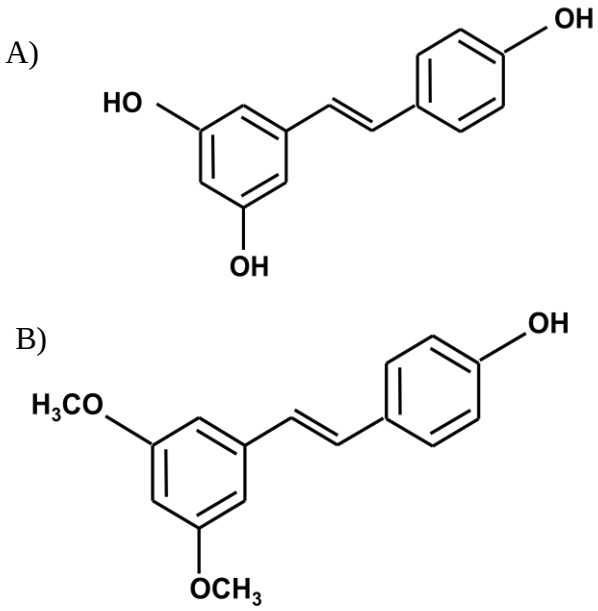


Figure 1.2 Chemical structure of (a) resveratrol and (b) pterostilbene.

1.2.3.1 Resveratrol

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) (Figure 1.2B) is the most extensively investigated stilbene. They can be found in foods like berries, grapes and peanuts (Rimando et al., 2004). There has been a long held notation that resveratrol from red wine is responsible for "French Paradox". It was back in the 1819 when an Irish doctor, Samuel Black, first noticed that the French ate a lot of fatty foods yet stayed healthy. This epidemiological phenomenon is that the French population has a drastically lower incidence of cardiovascular disease than other developed countries despite having a diet high in saturated fats. The French drink a lot of red wine, which resveratrol is a major component (Kopp, 1998; Vidavalur et al., 2006). Numerous studies have shown that resveratrol has the ability to block human platelet aggregation and eicosaoid

snythesies, which may lower the chances of one developing cardiovascular disease (Bertelli et al., 1995; Pace-Asciak et al., 1995; Yoshiyuki et al., 1985).

Each year, hundreds of studies are published on the therapeutic activities of resveratrol ranging from an anti-oxidant (Bhat and Pezzuto, 2002; Chanvitayapongs, et al. 1997) to anti-tumoural (Bishayee, 2009; Jang et al. 1997). It has also been shown to be a powerful anti-carcinogenic agent because of its low toxicity and capability of modulating numerous molecular pathways involved in cancer progression (Athar et al., 2009). These pathways play a key role in anti-apoptosis, angiogenesis, cell cycle progression and tumor invasion. In a mouse study executed by Cui et al. (2010), they reported significant reduction in the incidence and multiplicity of tumors when mice were feed resveratrol. They demonstrated that resveratrol can abate colitis; therefore, decrease the potentially for colon cancer associated with colitis. In ovarian cancer cells, resveratrol has been shown to cause apoptois, cell cycle arrest and detachment (Raj et al., 2008). Jang et al. (1997) demonstrated the capability of resveratrol to block the development of skin cancer at initiation, promotion and progression stages. Resveratrol has also been showed to suppressing cancer progression in other cancers like breast, gastrointestinal tract, lung and prostate (Bishayee, 2009).

Despite all the accolades resveratrol has received as a cancer chemopreventive agent, it has a low systemic bioavailability due to it metabolizing rapidly once it is ingested (Asensi et al., 2002; Delmas et al., 2011; Walle, 2011), which may lessen its efficacy in humans. Therefore, there has been great effort to consider resveratrol derivatives, which may have better bioavailability profiles.

1.2.3.2 Pterostilbene

Pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene) (Figure 1.2A), a naturally dimethylether analogue of resveratrol, has recently gained a upsurge of interest because it has been shown to have similar biological activities as resveratrol (Rimando et al., 2002). It has been proven to be as effective as resveratrol in successfully inhibiting the carcinogensesis in mice epidermis. They were both also able to decreased the expression and activity of COX-2 and inducible nitric oxide synthase (iNOS) (Cichocki, 2008).

In an *in vitro* study done on human gastric carcinoma cells, pterostilbene caused cell cycle arrest and induced apoptosis (Pan et al., 2007). Pterostilbene was capable of suppressing aberrant crypt foci (ACF) formation, which occurs prior to colorectal polyps. ACF is one of the earliest transformation observed in the colon that might lead to cancer (Suh et al., 2007). Other studies have shown pterostilbene has the potential to prevention and treatment of other cancers like breast, liver and lung (Alosi et al., 2010; Pan et al., 2009; Schneider et al., 2010). In a side-by-side comparison of the effects of resveratrol and pterostilbene on human colon cancer, pterostilbene had stronger apoptosis-inducting effect and was more potent in inhibiting colony formation than resveratrol (Nutakul et al., 2011). Methlyation may play a part in why there are these stark differences. From all the research thus far, pterostilbene is an appealing candidate for cancer prevention and treatment.

1.3 Bioavailability

1.3.1 Overview

The bioavailability is an overall result of absorption, distribution, metabolism and excretion (ADME).

- Absorption describes a compound's capability to pass into the systemic circulation following oral administration.
- Distribution explains how well a compound reaches the target tissue.
- Metabolism is the rate that a nutrient/compound is eliminated from the systemic circulation, following its initial absorption.
- Excretion is the rate that a compound is excreted from the systemic circulation and ultimately the body.

Figure 1.3 provides a graphic depiction of the bioavailability/ADME process. Thus, bioavailability is determined by the combination of the rates of all these factors. The bioavailability of a compound can therefore be defined as the amount of nutrient/compound that reaches the blood circulation system and ultimately the target tissue. Therefore, only the unbound fraction of a compound will reach the target tissue to be able to interact with the molecular target (Balani et al., 2005; Van de Waterbeemd et al., 2003).

Since oral administration is a widely employed method for the delivery of drugs and foods, the effectiveness of a compound is dependent on their intestinal absorption to get into systemic circulation to subsequently reach the intented tissue. The intestinal

epithelium is a key determinant for the oral absorption of ingested pharmaceuticals, food ingredients and toxins (Tong and Wen, 2008).

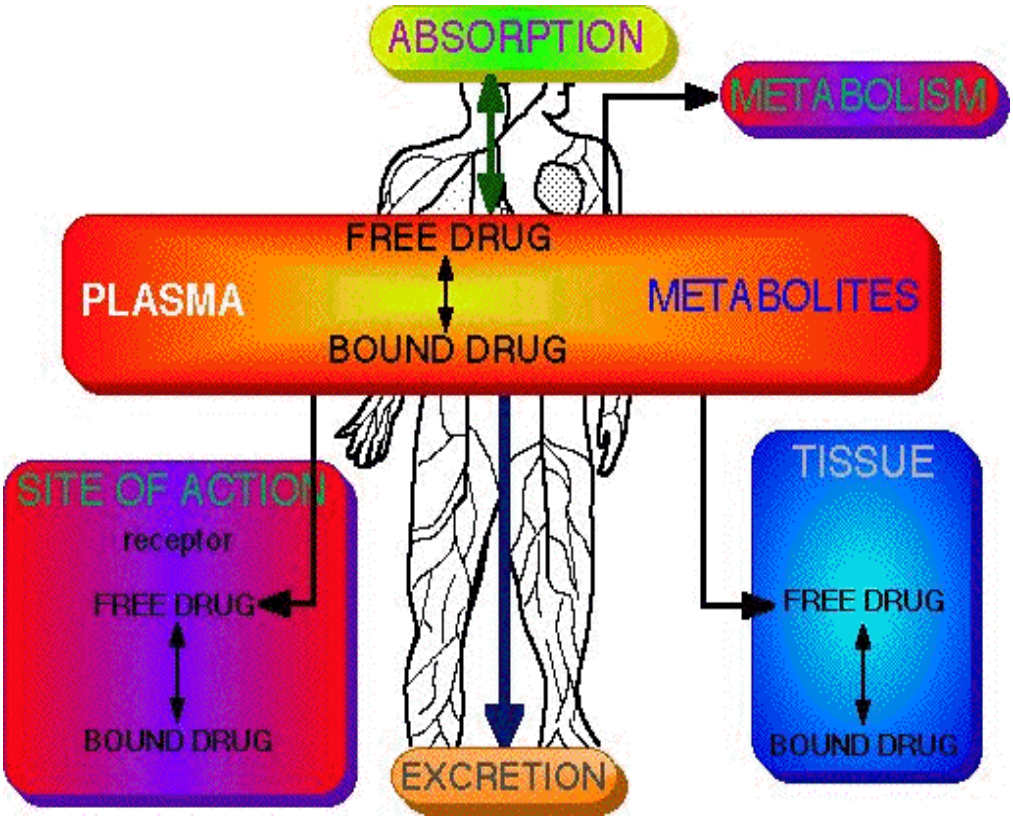


Figure 1.3 Overview of Bioavailability and ADME (Bourne, 2010).

Usually the first sets of experiments executed to understand the bioavailability of a compound are absorption studies (Figure 1.4.). Permeability measures the ability and velocity of a compound to cross through the intestinal membranes into the blood circulation system. Permeability denotes the overall effects of influx and efflux in the body. Intestine like cells such as Caco-2 cells can be used to predict permeability.

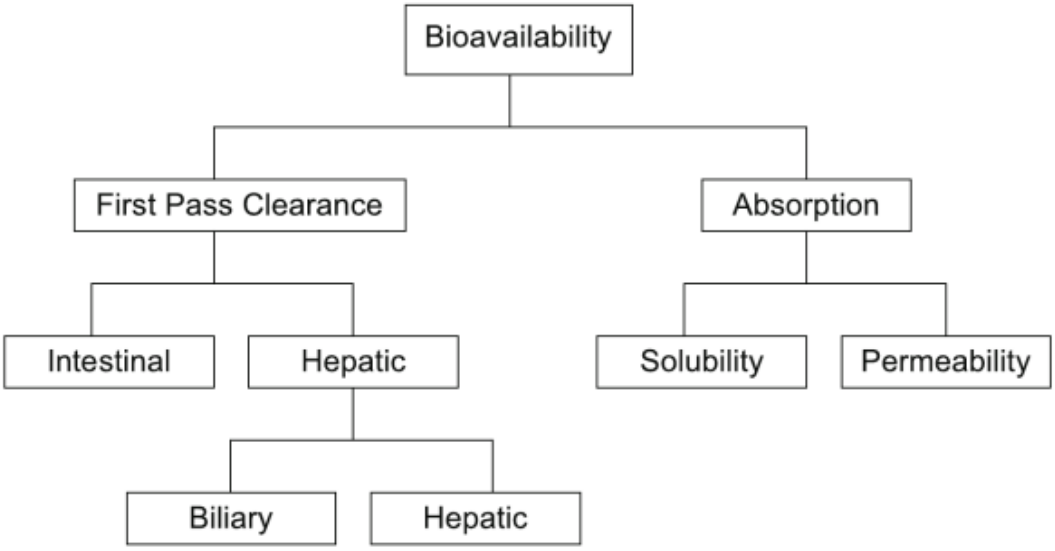


Figure 1.4 Determinants of oral bioavailability and a decision-tree to assess their role (Borchardt, 2006).

1.3.2 Bioavailability of Compounds of Interest

There have been a scarce number of investigations on the bioavailability of PMFs. There have been more studies done on the solubility of PMFs which is a key factor when accessing the absorption and bioavailability of PMFs but it alone doesn't provide a complete picture on overall bioavailability. When Murakami and his colleagues (2001) conducted an *in vitro* study using Caco-2 cell monolayer to evaluate the absorption of PMF1 and luteolin (unmethlyated PMF), more PMF1 was transported to the basolateral compartment than luteolin after 4 hours. Also, PMF 1 accumulated in the Caco-2 cell monolayer while luteolin did not. They concluding PMF 1 had higher permeability.

An *in vivo* study was also carried out on the same two compounds by Murakami et al. (2002). Each compound was given independently by gastirc intubation to male rats SD and after 1, 4 and 24 hours of administration, the concentrations were calculated.

There results revealed that during the 1 to 4 hour periods, PMF1 was detected in stomach, small and large intestines, kidney and liver; in contrast, luteolin was predominantly detected in the gastrointestinal tract of the rats during 1 to 4 hour period following a single dose administration. Also, PMF 1 tended to be localized in the mucous membrane and muscularis at 1 and 4 hours time marks. Overall, PMF 1 had a wider distribution and accumulation in tissues.

Numerous *in vitro* and *in vivo* studies on absorption, transport and bioavailability have been done on resveratrol but not significant amounts have been conducted on pterostilbene. Kapetanvico et al. (2011) demonstrated that when pterostilbene and resveratrol were administered orally to rats, pterostilbene and resveratrol were approximately 80% and 20% bioavailable, respectively. Pterostilbene had greater bioavailability and total plasma levels of both parent compound and metabolites than resveratrol. These differences are a hint that the *in vivo* biological activity of pterostilbene might be greater than that of resveratrol. Also, suggesting that methlyation may affect bioavailability.

Walle and his colleagues at Medical University of South Carolina have conducted a number of studies comparing bioavailability of unmethyalted and methylated flavoniods. In one study, they showed a 5 to 8 fold higher rate of intestinal permeability by methylated flavonoids compared to their corresponding umethlyated counterpart (Walle et al., 2006). In another study, again they demonstrated methylated compound had better absorption and high oral bioavailability as well as tissue accumulation *in vivo* in the rats (Walle et al., 2006).

1.3.4 Caco-2 cell Monolayer Model

Caco-2 cell monolayers have been extensively used for years as a tool to test permeability, assess the oral absorption potential and study the absorption mechanism of compounds (Gan et al., 1993). Caco-2 cells originate from human colonic adenocarcinoma cell lines. Typically after 21 days of growth, the Caco-2 cells differentiate into a monolayer with micro villus structures and many other biochemical and functional characteristics of small intestinal villus epithelium. The cells form tight junctions, express many brush border enzymes, some CYP isosnzymes, and phase II enzymes (such as glucuronidase, glutathione-S-transferases and sulfotranserase) (Hubatsch et al., 2007; Lind et al., 2007).

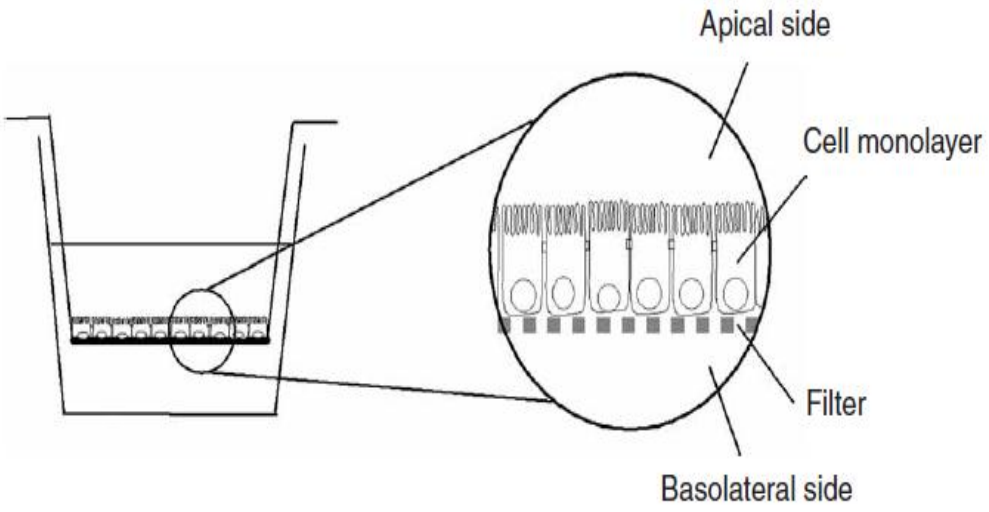


Figure 1.5 Diagram of the Caco-2 cell monolayer cultivated on a permeable filter support. Test compound is place on the apical or basolateral compartments. (Hubatch et al., 2007).

In the transport/permeability experiments, Caco-2 cells are cultivated on permeable filters in which they represent the intestinal environment in that lumen is separated from the bloodstream by the intestinal epithelial monolayer (Figure 1.5). Trans-epithelial passage of molecules from the apical to the basolateral side of the monolayer can be easily measured in different experimental conditions, thus allowing discriminating factors involved in transport mechanisms. To better mimic what is happening in the body, different pH solutions can be used in each compartment. A pH of 6.5 can be used in apical compartment to represent the pH of the upper small intestine under fasted conditions while a pH of 7.4 can be used in the basolateral compartment to replicate the pH of blood in the body (Deferme et al., 2008; Fallingborg, 1989; Lind et al. 2007).

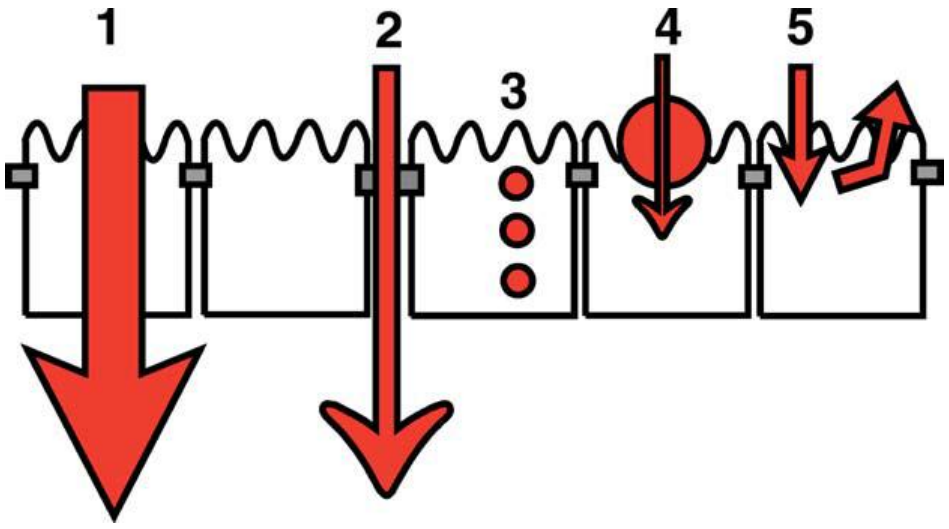


Figure 1.6 Possible compound transport pathways across the intestinal mucosa, illustrating (1) transcellular and (2) paracellular modes of passive transport, (3) transcytosis, (4) carrier-mediated transport, and (5) efflux transport. A combination of these routes often defines the overall transepithelial transport rate of nutrients and drug (Deferme et al., 2008).

Caco-2 cell monolayer expermients provide valuable information regarding (i) intestinal permeability, (ii) transport mechanisms - paracellular, transcellar or active carrier (Figure 1.6), (iii) role of intestinal metabolism and (iv) influence of p-glycoproetin efflux system. There are potentially three datasets that can be obtained from the Caco-2 cell experiments and they are the following:

- The apparent permeability (P_{app}) from apical compartment to the basolateral compartment represents the overall effect of transportation carried out by both absorptive transporters and secrectory transporters.
- The apparent permeability (P_{app}) from basolateral compartment to apical compartment measures the effects of secretory transporters only.
- The ratio of P_{app} from basolateral compartment to apical compartment over the P_{app} from apical compartment to the basolateral compartment evaulates efflux. If the ratio is greater than 3, then there is a greater possible of efflux will occur. This means a compound is being pumped out too quickly from the blood circulation system, which will have an effect on the amount of compound in the sytemtic circulation and thus affect the absorption and bioavailability of the compound.

Therefore, Caco-2 cell model experiments offer vital insight in the prelimianry phase of compound discovery on intestinal permeability, transport, absorption through membrances and overall potential bioavailiablity (Walle et al., 2003) .

CHAPTER 2

RAPID METHOD FOR THE DETERMINATION OF RESVERATROL AND PTEROSTILBENE BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

2.1 Abstract

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) and pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene) both belong to the stilbene family. They have an array of pharmacological properties from anti-atherosclerosis to anti-carcinogenic. The aim of this study was to develop a simple, rapid and highly sensitive method using reversed-phase high performance liquid chromatography (HPLC) equipped with an electrochemical (EC) detector for the quantification of resveratrol and pterostilbene. The developed analytical method was fully validated in terms of accuracy, linearity and precision. The recovery rates were between 96.59% and 109.01%. Both calibration curves showed excellent linearity with correlation coefficient (r^2) greater than 0.999. The limit of detection (LOD) values was lower than those obtained by other detectors published in the literature. The precision for the retention times and peak areas were both below 9%. The method was applied to quantify resveratrol and pterostilbene from commercial juice products. The proposed method could be useful for prospective nutritional, pharmacological and toxicological research on resveratrol and pterostilbene.

Keywords: Resveratrol; Pterostilbene; Stilbene; High performance liquid chromatography (HPLC); Electrochemical detection (EC)

2.2 Introduction

Stilbenes, a small family of polyphenolic compounds, have received increased attention over the past decade due to their array of pharmacological properties, including anti-atherosclerosis (Ko et al., 1999; Luo et al., 2008, Pace-Asciak et al., 1995) and anti-carcinogenic activity in various biological systems (Namasivayam, 2011; Paul et al., 2010; Rimano and Suh, 2008). Of all the stilbenes, resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) has been most extensively investigated for its potential health benefits to humans such being anti-oxidant (Gescher, 2008; Rimando and Suh, 2008). Despite all the accolades resveratrol has received, it has low systemic bioavailability (Asensi et al., 2002; Delmas et al., 2011; Walle, 2011), which may lower its efficacy in humans; therefore, has lead researchers to investigate resveratrol derivatives.

Pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene), a dimethylether analogue of resveratrol, has been shown to have anti-carcinogenic (Paul et al., 2010; Tolomeo et al., 2005), anti-diabetic (Amarnath and Pari, 2006; Pari and Satheesh, 2006; Szkudelski and Szkudelska, 2011) and anti-inflammatory activity (Remsberg et al., 2007). In some cases, it possesses stronger inhibitory activity (Billack et al., 2008; Huang et al., 2007; Paul et al, 2010;) and overall better pharmacokinetic characteristics than resveratrol (Lin et al., 2009).

Numerous analytical techniques have been proposed for the separation, identification, and determination of stilbenes. Researchers overall prefer the employment of high performance liquid chromatographs (HPLC) for qualitative and quantitative measurements of resveratrol and pterostilbene (Buiarelli et al., 2006; Gocan, 2009; Kolouchová-Hanzlíková et al., 2004; Rodríguez-Bernaldo de Quiró et al. 2007). When

compared to other systems, HPLC has higher resolution, columns that can be used again without regeneration or repacking and quicker cycle times. Furthermore, the mobile phase of these systems can be varied during the analysis producing a gradient elution. HPLC coupled with UV detector is the most prevalently used for quantification of stilbenes (Dong, 2005; Lin et al., 2009); however, HPLC coupled with mass spectrometry (MS) and fluorescence (FL) detectors are also commonly used (Buiarelli et al., 2006, Remsberg et al., 2007) for detection of stilbens.

Because of its high selectivity and excellent sensitivity, HPLC with electrochemical (EC) detector has become more popular in the determination of stilbenes in complex matrices (Jandera et al., 2005; Benova et al., 2008). EC detector does not exploit a physical property of an analyte, like UV and fluorescence detectors, but induce a chemical change that results from an electrochemical reaction. EC has the ability to detect resveratrol in the low pg range (Gocan, 2009). However, in the literature, there are few validated analytical methods, if any, for the determination of pterostilbene with HPLC-EC using a multichannel arrary.

In this study, a validated HPLC method with multi-channel EC detection was developed for the quantitative analysis of resveratrol and pterostilbene. The capability of the HPLC system was evaluated with the following factors: peak shape, linearity, detection limits, accuracy, recovery and precision. Potentials of electrodes and mobile phase were also evaluated to see how they can aid in optimizing the analytical conditions.

2.3 Materials and Methods

2.3.1 Chemicals and reagents

All organic solvents utilized, acetonitrile (ACN), methanol (MeOH), tetrahydrofucan (THF), trifluoroacetic acid (TFA) were of HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Ammonium Acetate (99%) was purchased from EMD Chemicals Inc. (Gibbstwon, NJ, USA). Highly purified *trans*-resveratrol (99%) and pterostilbene(98%) were obtained from Quality Phyochemical LLC (NJ, USA).

2.3.2 Instrumentation

The CoulArray® HPLC system was obtained from ESA (Chelmsford, MA, USA) and equipped with a binary solvent delivery system (model 584), an auto-sampler (model 542), a CoulArray® Multi-Channel EC detector (model 6210 - two cells, each cell contains four channels), and a UV detector. Data collection, processing and instrument control were achieved using the CoulArray 3.06 software.

2.3.3 Chromatographic Conditions

Ascentis RP-Amide reversed-phase HPLC column (15 cm x 4.6 mm id, 3 µm) from Sigma-Aldrich (St. Louis, MO, USA) was used to analyze compounds. The mobile phase consisted of 50% water, 40% ACN, 10% THF and 50mM ammonium acetate. The pH of the mobile phase was adjusted with TFA to achieve a pH range between 3.5 to 3.8. Mobile phase was filtered through a 0.45µm membrane filter from Millipore (Bedford, MA, USA). Both EC detector cells (each contains four channels) were used

and detection potentials were set at 200, 300, 400, 500, 600, 700, 800 and 900 mV. The temperature of the auto-sampler was set to 4°C. The flow rate was set to 1.0 ml/min.

2.3.4 Preparation of standards

The stock solution of each compound was prepared in DMSO. These solutions were protected from light and stored in the darkness at 4°C when not in use. The concentrations ranges for both compounds were between 0.001 and 10µL and were prepared by a serial of dilution with 50% MeOH just before each experiment. A 10µL volume was injected in the HPLC.

2.3.5 Analyses of Juice Samples

A total of 6 juices (2 Grape Juices, 2 Blueberry Juices, and 2 Mix Fruit Juices) were analyzed. They were purchased from local supermarkets (Amherst, MA, USA). A volume of 400µL of juice and 100 µL MeOH were votexed together for 1 minute. The juice/MeOH mixture was extracted with equal volume of ethyl acetate for a total of three times. The ethyl acetate collected was pooled together and evaporated to dryness. Samples were reconstituted in 400µL 50% MeOH and analyzed using the HPLC-EC method developed in this study to determine the amount of resveratrol and pterostilbene in each sample.

2.4 Results and Discussion

2.4.1 Optimization of Chromatographic Conditions

The aim of this study was to achieve in a single HPLC run the separation of resveratrol and pterostilbene in a respective short period of time with relatively simple gradient profile. This method was able to successfully attain this goal. A representative chromatogram of resveratrol and pterostilbene at various potentials is illustrated in Figure 2.2.

Mobile phase with water-MeOH-THF and water-ACN-THF using the same gradient profile were evaluated. It was determined that the mobile phase composed of water-ACN-THF attained superior resolution and better retention time than the water-MeOH-THF mobile phase. There were no interfering peaks co-eluted from the mobile phase or with compounds of interest. ACN was also selected as the organic solvent of choice over MeOH due to issues with high pressure associated with MeOH as a solvent. EC detector is a very sensitive detector that can be effected by the ph and ionic strength of the buffer system in the mobile phase (Benova et al., 2008). Therefore, ammonium acetate was added to stabilize the pH value of mobile phase. The retention time of resveratrol and pterostilbene were around 4 and 12.5 minutes, respectively. After evaluation, the optimal conditions were achieved with mobile phase of water-ACN-THF (50:40:10, v/v/v) containing 50mM ammonium acetate and the flow rate of 1.0 ml/min.

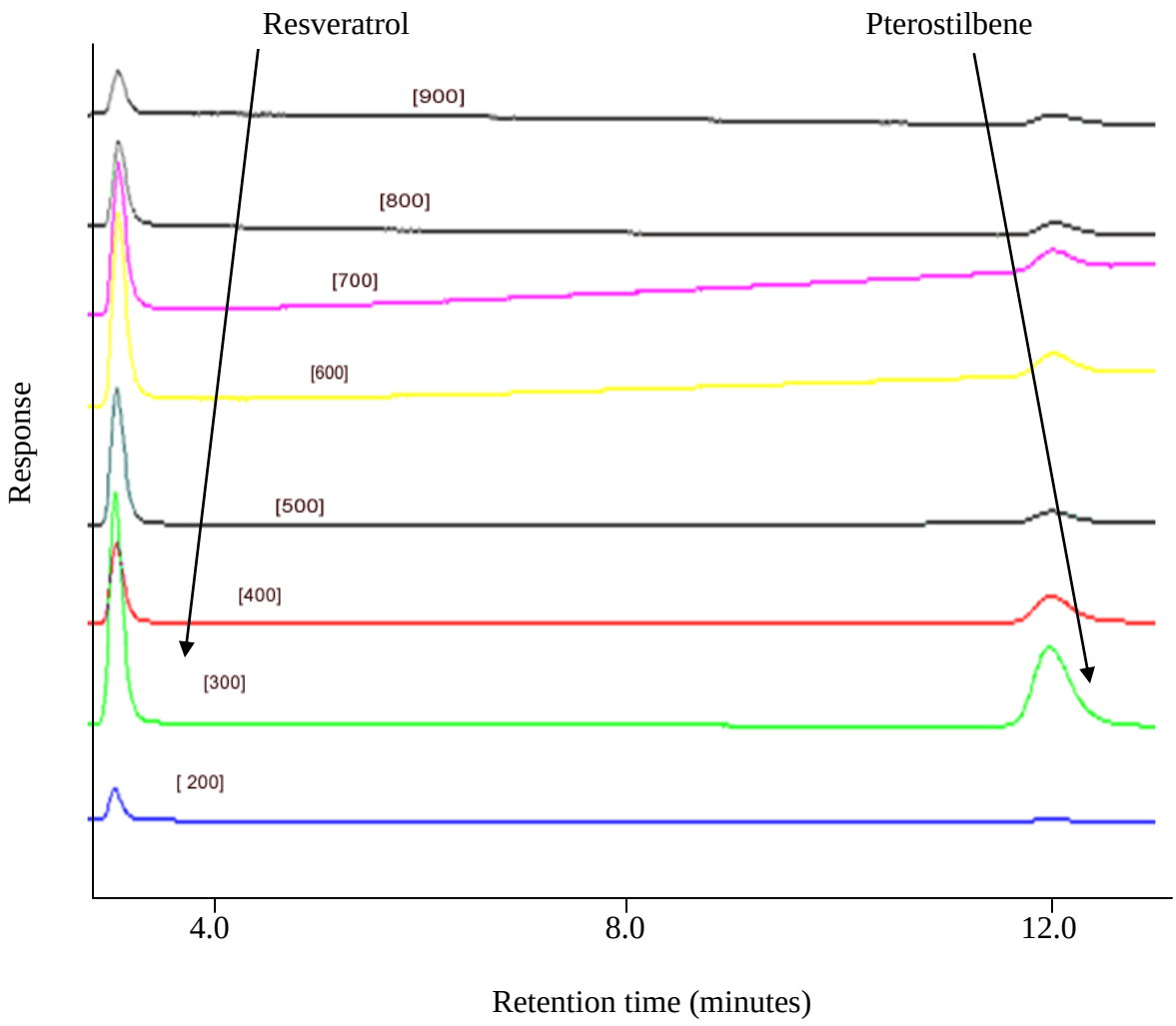


Figure 2.1 Representative chromatograms of resveratrol and pterostilbene at different potentials. Potentials with dominant peaks of compounds are noted on the figure.

2.4.2 Voltametric Behavior of Resveratrol and Pterostilbene

The electrochemical detection offers different and reproducible signal responses to a compound at the oxidation or reduction potential applied across the individual flow-through cells connected in series. The correlation between potential utilized on electrochemical cells and the peak area of the target compound was evaluated. Each

stilbene was subject a series of electric voltages in 100mV increments on EC detector concurrently for HPLC analysis. This allowed for the study to establish optimal potential for resveratrol and pterostilbene based on the observed voltametric behavior of the compounds.

Table 2.1 highlights the ratios of pre/postdominant peak area and electrochemical cell potential of the dominant peak of each compound. The dominant peak area corresponds with the electrochemical cell potential generating the maximum intense signal. The predominant and postdominant peak areas were designated as the signals of the detection cells preceding and succeeding the dominant peak area channel, respectively. As depicted in Figures 2.1 and 2.2, for both resveratrol and pterostilbene, the electric voltage of 300mV generated the largest peak.

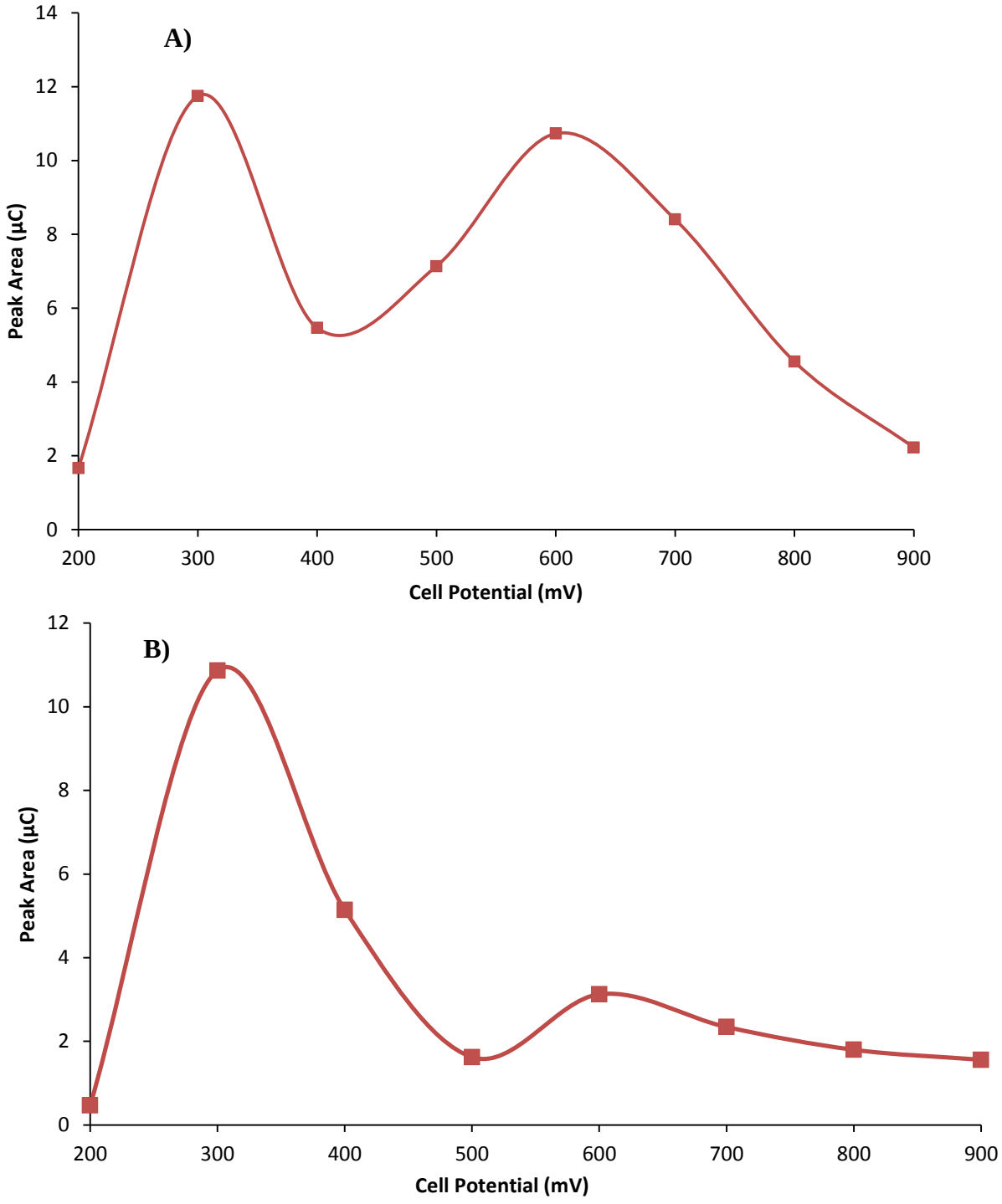


Figure 2.2 The effect of electrochemical cell potential on the peak area (µC) for (a) resveratrol and (b) pterostilbene. The concentration for each compound was 5µM.

Table 2.1 Voltametric behavior of resveratrol and pterostilbene.

Compound	Potential of the Dominant Peak (mV)	Ratio of Peak Area[a]	
		Predominant	Postdominant
Resveratrol	300	0.14	0.04
Pterostilbene	300	0.36	0.33

a. The ratios are associated with the dominant peak area of the compound of interest.

2.4.3 Linearity and Range

The linearity of the detector response was assessed by analyzing the calibration graphs of resveratrol and pterostilbene. The graphs were constructed based on plotting the peak area of the compound of interest against the corresponding concentrations. Linear regression analysis was used to determine the linearity of the analytical method.

Table 2.2 lists the regression equations and correlation coefficients (r^2) for resveratrol and pterostilbene. The concentration range between 0.001µM and 10 µM were utilized. The correlation coefficients (r^2) were greater than 0.999 for both resveratrol and pterostilbene indicating good linearity for the tested concentration ranges for each compound.

Table 2.2 Linearity, LOD, and LOQ for resveratrol and pterostilbene.

Compound	Concentration range (µM)	Linear regression equation	Correlation coefficient,r^2	LOD (ng/ml)	LOQ (ng/ml)
Resveratrol	0.001 - 10	y = 4368.9x + 77.696	0.9997	0.29	1.0
Pterostilbene	0.001 - 10	y = 4530.1x + 71.95	0.9996	2.0	6.7

2.4.4 LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on method outlined by the International Conference of Harmonisation (ICH 2005). The LOD was defined by lowest measured concentration that can be detected above baseline nose with a signal-to-noise (S/N) ratio of 3:1. Similarly, the LOQ was determined S/N of 10:1.

As indicated in Table 2.2, pterostilbene had higher LOD and LOQ values than resveratrol. The LOD and LOQ values presented here were lower than values acquired from other HPLC with EC detector (Benova et al., 2008; Kolouchová-Hanzlíková et al., 2004) as well as to usage of other detectors like FL and UV (Buiarelli et al., 2006; Gocan, 2009; Rodríguez-Bernaldo de Quirós et al., 2007). For resveratrol, this method had LOD value that was at least 100 times lower than with UV detection (Vian et al., 2005). Also, the LOD and LOQ values for pterostilbene in this study were lower than those obtained by other detectors published in the literature (Remsberg et al., 2007; Lin et al., 2009).

2.4.5 Accuracy/Recovery Studies

The accuracy of this HPLC procedure was evaluated on the recovery of known amounts of compound. Accuracy is based on the closeness of the result obtained to the true concentration. The compounds were injected three times at four different concencentration levels (0.005, 0.05, 0.5, and 5µM). The results from accuracy studies for resveratrol and pterostilbene are shown in Table 2.3. The highest concentration (5µM) for both compounds had the lowest RSD values.

For resveratrol, the recovery range was from 96.59% to 109.01% with RSD less than 2.05%. The lower concentrations (0.005 and 0.05µM) had similar recovery

pecentage. The highest concentration (5µM) had highest percentage of recovery of 109.01%. The recovery range for pterostilbene was between 99.53% to 106.29% with the RSD less than 2.35%. The lowest concentration (0.005µM) had highest percentage of recovery 0f 106.29%.

Table 2.3 Accuracy/Recovery data of resveratrol and pterostilbene standards.

Compound	Concentration(µM)	% Recovery	% RSD (n=3)
Reservatrol	0.005	96.59	2.05
	0.05	96.23	0.82
	0.5	104.80	0.40
	5	109.01	0.04
Pterostilbene	0.005	106.29	1.00
	0.05	101.88	2.35
	0.5	99.53	1.31
	5	100.93	0.21

2.4.6 Precision

The precision for this study was determined based on repeatability (intraday variation) and intermediate precision (interday variation) (ICH, 2005). Intraday variation was evaluated by analyzing four different concentration levels (0.005, 0.05, 0.5, and 5µM) with triplicate injection within the same day; whereas, interday variation was analyzing four different concentration levels (0.005, 0.05, 0.5, and 5µM) on three separate days under the same experimental conditions.

For the intraday variation, resveratrol RSD values for retention time were between 0.00 - 0.23% and the RSD values for peak area were between 0.04% to 1.91%. The RSD

values for retention time ranged from 0.04 to 0.49% and the RSD values for peak area were between 0.21% to 2.46% for pterostilbene. (See Table 2.4)

For interday variation of resveratrol, the percent RSD of the retention time and peak were between 1.70% to 2.70% and 0.39% to 7.60%, respectively. For pterostilbene, RSD values for retention time were between 1.86% to 3.36% and the RSD values for peak area were in the range of 4.20 to 8.54%.

Table 2.4 Precision data of resveratrol and pterostilbene standards.

Compound	Concentration (μM)	Intraday variation (%RSD)(n=3)		Interday variation (%RSD)(n=3)	
		Retention time	Peak area	Retention time	Peak area
Reservatrol	0.005	0.23	1.91	1.83	4.29
	0.05	0.23	0.76	2.02	0.39
	0.5	0.13	0.40	2.70	7.37
	5	0.00	0.04	1.70	7.60
Pterostilbene	0.005	0.28	1.34	2.15	8.54
	0.05	0.49	2.45	2.19	4.31
	0.5	0.16	1.35	3.46	4.20
	5	0.04	0.21	1.86	7.83

2.4.7 Analysis of resveratrol and pterostilbene in commercial juice products

The amount of resveratrol and pterostilbene present in the six different brands of juices were determined by the HPLC method developed for this paper. The results listed in Table 2.5. All the samples that contained blueberries juice (Blueberry Juice 1 and 2; Mix Juice Blend 1 and 2) had pterostilbene present. Blueberry Juice 1 had the highest concentration of reveratrol and pterostilbene. Blueberry Juice 1 contained organic

blueberry juice concentrate. Resveratrol can naturally be found in great abundance in grapes and there is a significant amount also found in blueberries. In nature, there is more pterostilbene found in blueberries than in grapes, which would explain why there is little, if any, pterostilbene found in the grape juices samples (Rimando et a.l, 2004).

Table 2.5 Amount of resveratrol and pterostilbene in different juice products.

Juice Sample	Resveratrol (μM)	Pterostilbene(μM)
Grape Juice 1	0.451	Trace Amount
Grape Juice 2	1.988	None
Blueberry Juice 1	6.584	0.021
Blueberry Juice 2	1.963	0.016
Mix Juice Blend 1	2.205	0.005
Mix Juice Blend 2	0.950	0.002

2.5 Conclusion

In conclusion, this method developed, by using HPLC coupled with an EC detector for the determination of resveratrol and pterostilbene, was simple, rapid and highly sensitive. Linear, range, repeatability and intermediate precision, LOD, LOQ and recovery were used to successfully validate this analytical method.

When this method is compared to HPLC methods using other detectors for the detection of resveratrol and pterostilbene, like UV and fluorescence, this method was faster and provides greater sensitivity (Buiarelli an et al., 2006; Gocan, 2009; Lui et al., 2009; Rodríguez-Bernaldo de Quirós et al., 2007; Remsberg, 2007). In our lab, this method has been used to analyze small amounts of resveratrol and pterostilbene for permeability and cellular uptake experiments as well as determine the concentration of resveratrol and pterostilbene in various food products. The application of this method

will allow for analyzing large quantities of samples in a relatively short period of time, in addition to be able to detect trace amounts of resveratrol and pterostilbene for future nutritional, toxicological, and pharmacological research.

CHAPTER 3

COMPARISON OF THE CELLULAR UPTAKE AND INHIBITORY EFFECTS OF RESVERATROL AND ITS METHYLATED ANALOGUE PTEROSTILBENE ON HUMAN COLON CANCER CELLS

3.1 Abstract

Colon cancer is the third most commonly diagnosed cancer. Numerous epidemiological studies have revealed a relation between high consumption of fruits and vegetables with reduced risk of colon cancer. Resveratrol and pterostilbene are stilbene compounds found in fruits such as grapes and blueberries. The aim of this study was to compare the cellular uptake and inhibitory effects of resveratrol and pterostilbene on human colon cancer cells. Pterostilbene had a stronger inhibitory effect than resveratrol on Caco-2 human colon cancer cell line. Pterostilbene IC_{50} value was approximately 2.5 times lower than resveratrol. The cellular uptake of reseveratrol and pterostilbene in three human colon cancer cells (Caco-2, HT-29, and HCT-116) was examined. The cellular uptake of pterostilbene was around 2 to 3 fold higher than of resveratrol in all colon cancer cells. The increased intracellular uptake and stronger growth inhibitory by pterostilbene maybe contributed to the two methyl groups on pterostilbene. Pterostilbene is an appealing candidate for cancer prevention.

Keywords: Resveratrol; Pterostilbene; Uptake; Colon Cancer; MTT

3.2 Introduction

In 2012, the World Health Organization (WHO) projected that cancer will be the most prevailing cause for death worldwide surpassing heart disease and stroke. According to the American Cancer Society (2012), cancer is the second most common cause of death in the United States and accounts for about one in every fourth deaths. The third most commonly diagnosed cancer in the United States is colon cancer and there has been noticeable increase in incidents in adults under the age of 50 (Siegel, 2009).

These staggering statistics has triggered a surge of interest in cancer prevention. Diet and lifestyle has received much attention since they are effective means of prevention. They are both major contributing factors as to whether or not one develops cancer (Center et al, 2009; McCullough et al, 2011). Having a diet abundant in fruit and vegetables is essential in maintaining good health. Numerous epidemiological studies have revealed a relation between high consumption of fruits and vegetables associated with a reduced risk of colon cancer (Rimando and Suh, 2008; Van Duijnhoven et al., 2009).

Reservetrol has been shown to exert anti-carcinogenic properties (Athar et al., 2009). *In vitro* models, Aggarwal et al. (2000) and Juan et al. (2008) have demonstrated reservatrol ability to be anti-angiogenic, anti-porliferative and proapototic. Furthermore, resveratrol has been shown to inhibit tumor progression in multiple sites such as breast, gastrointestinal tract and skin (Bishayee, 2009). In spite of all its potent as anti-cancer agent (Gupta et al., 2011; Li et al., 2010), the bioavailability of resveratrol has been a concern due to its rapidly metabolizing leaving small amounts of unconjugated

resveratrol in the systemic circulation (Asensi et al., 2002 Kapetanovic et al, 2010). This has spark researcher is explore resveratrol derivatives.

Pterostilbene (*trans*-3,5-dimethoxy-4'-hydroxystilbene), a dimethylether analogue of resveratrol, has been shown to exhibit strong inhibitory activity again difference cancer cell lines (Billack et al., 2008; Huang et al., 2007; Paul et al, 2010) and overall better pharmacokinetic characteristics than resveratrol (Lin et al., 2009; Remsberg et al., 2007; Wilson et al., 2008). *In vivo* study, pterostilbene had a longer plasma half-life in comparison to resveratrol (Ferrer et al., 2005), which is why pterostilbene is an interesting compound to investigate as a potential compound for use in chemoprevention.

The objective of this study is to see if the structural differences between resveratrol and pterostilbene would affect their inhibitory effects in Caco-2 - colon cancer cell line as well as compare the cellular uptake of the two compounds in three human colon adenocarcinoma cell lines.

3.3 Materials and Methods

3.3.1 Materials

Highly purified *trans*-resveratrol (99%) and pterostilbene(98%) were obtained from Quality Phyochemical LLC (NJ, USA). All organic solvents utilized were of HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Ammonium Acetate (99%) was purchased from EMD Chemicals Inc. (Gibbstwon, NJ, USA). All three human colon adenocarcinoma cell lines were obtained from American Type Cell Collection (Manassas, VA, USA). Penicillin and streptomycin were obtained from Sigma-Aldrich

(St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), McCoy's 5A media, fetal bovine serum (FBS), *MEM non-essential amino acid*, HEPES and other cell culture supplies were purchased from Mediatech Inc. (Herndon, VA, USA).

3.3.2 Cell culture treatment

Caco-2 cells were maintained in DMEM containing glucose, L-glutamine, and sodium pyruvate, supplemented with 10% fetal bovine serum, 0.1% *MEM non-essential amino acid*, 6mM HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. HCT116 and HT-29 were maintained in McCoy's 5A media supplemented with 5% heat inactivated FBS, 100 U/mL of penicillin, and 0.1mg/mL streptomycin.

All cell lines were kept at 37°C in atmosphere of 5% CO_2 and 95% air. Cells were subcultured at 70-90% confluency with media changed every 2 to 3 days for Caco-2 cells and every other day for HCT116 and HT29. Cell lines used were between 15 and 30 passages. DMSO was utilized as the agent to deliver resveratrol and pterostilbene. The final concentration of DMSO in all experiments did not exceed 0.1%.

3.3.3 Cell Viability Assay

Caco-2 cells were seeded in 96-well plates at a density of 10,000 cells/well. After 24 hours of incubation, media was replaced and cells were treated with serial concentrations of resveratrol and/or pterostilbene in 200µl in complete serum media except for the control samples that contained media with only 0.1% DMSO. At the end of treatment period of 48 hours, cells were subject to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; therefore, the media was replaced with 100µl of fresh complete media containing 0.5mg/ml of MTT (Sigma-Aldrich, St. Louis, MO,

USA). After 2 hours of incubation at 37˚C, the media containing MTT was removed and the reduced formazan dye was solubized by the addition of 100µl of DMSO per well. After lightly tipping the plate to dissolve the DMSO, the absorbance was evaluated at 570nm using a spectrophotemtric microtiter plate reader (Elx800TM absorbance microplate reader, BioTek Instrument, Winooski, VT, USA). Results were expressed as percentage of viable with 100% representing the control cells treated with only DMSO.

3.3.4 Cellular Uptake Assay

The colon cancer cells (400 cells/ml) were suspended in 1ml of culture media containing different concentrations of resveratrol and/or pterostilbene in glass tube and then were incubated at 37°C in atmosphere of 5% CO_2 and 95% air for 0.5, 1, or 2 hours. After incubation, the cell suspension wase centrifuged at 2000 rpm for 2 minutes at 4°C. Supernatant was then removed and cells were rinsed with 1ml of ice-cold PBS (1X; pH 7.25) and centrifuged at 1000 rpm for 1 minute at 4°C. After centrifugation, supernatant was again removed and 1ml of cytosol buffer (pH 7.5, 10mM-Tris-HCL, 1mM EDTA, 1mM MgCl2) was added. The solution was kept on ice for 5 minutes before cells were sonicated with a probe sonicator and transferred to eppendorf tubes. The cells were then centrifuged at 14,000rpm for 25 minutes at 4°C. The supernatant was used to determined protein amount by BCA assay (Foster et al., 2001).

3.3.4.1 Cytosol Faction

After centrifugation, the supernatant was transferred to a new tube. 180µl of supernatant was added to 120µl of MeOH (40% MeOH concentration). The 300µl of ethyl acetated (equal volume to the supernatant/MeOH solution) was added to

suspernatant/MeOH and centrifuged at 10,000 rpm for 5 minutes at 4°C. The ethyl acetate layer (top layer) was collected. Ethyl acetate was added again to the supernantant-MeOH solution and centrifugated at 10,000rpm for 5 minutes at 4°C. Then ethyl acetate was collected again. The collected ethyl acetate extracts were evaporated to dryness and reconstitued in 180µl of 50% MeOH and analyzed using HPLC-EC. The peak areas were compared to standards and the cellular uptake was normalized for cellular protein.

3.3.4.2 Membrane Fraction

The membrane buffer (pH 6.5, 25mM HEPES, 150mM NaCl, 10% Triton, 60mM OCG) was added to the cell pellet. After 30 minutes on ice, 80µl of MeOH was added and solution was votexed. 200µl of ethyl acetate was added to membrane-MeOH solution. The solution was centrifuged at 10,000rpm for 5 minutes at 4°C. The ethyl acetate (top layer) was collected. Ethyle acetate was added again to the membrane-MeOH solution and centrifugated at 10,000rpm for 5 minutes 4°C. Then ethyl acetate was collected. The collected ethyl acetate were evaporated to dryness and reconstitued in 120µl of 50% MeOH and analyzed by HPLC-EC. The peak areas were compared to standards.

3.3.5 HPLC analysis

Samples were analyses on CoulArray® HPLC system, obtained from ESA (Chelmsford, MA, USA), equipped with a binary solvent delivery system (model 584), an auto-sampler (model 542), a CoulArray® Multi-Channel EC detector (model 6210) consisted of two cells, each cell contains 4 channels, and a UV detector. Ascentis RP-

Amide column (15 cm x 4.6 mm id, 3 µm) from Sigma-Aldrich (St. Louis, MO, USA) was used. Data collection, processing and instrument control were achieved using the CoulArray 3.06 software.

The mobile phase used consisted of 50mM ammonium acetate in 50% water, 40% ACN, and 10% THF. The pH of the mobile phase was adjusted with TFA to achieve a pH range between 3.5 to 3.8 and then filtered through a 0.45µm membrane filter from Millipore (Bedford, MA, USA). Both EC detector cells were used and detection potentials were set to 300, 400, 500, 600, 700, 800 and 900 mV. The flow rate was set to 1.0 ml/min. The temperature of the auto-sampler was set to 4°C. This detection method has high selectivity and excellent sensitivity even at low concentrations.

3.3.6 Statistical Analysis

Data were expressed as mean ± standard error mean (SEM). Statistical significance of mean difference between two groups was calculated by using Student's two-tailed t-test. Analysis of variance (ANOVA) model with Dunnett multiple comparison was used when comparing more than two groups' differences. A significance level of $P < 0.05$ was used for all tests.

3.4 Results & Discussion

3.4.1 Growth inhibition on Caco-2

The colorimetric assay MTT was utilized to determine cell viability; therefore, determining the growth inhibition effect of resveratrol and pterostilbene on Caco-2 colon cancer cells. The cell viability is directly proportional to the intensity of the color in the solution in each well (Mosmann, 1983). The results showed that both resveratrol and pterostilbene caused dose-dependent growth inhibition of the cell viability of Caco-2 colon cancer cells (Figure 3.1). Resveratrol IC_{50} value was estimated to be around 207μM while pterostilbene attained a significantly lower IC_{50} value of approximately 79μM. Pterostilbene was around 2.6 times more potent than resveratrol in hindering growth.

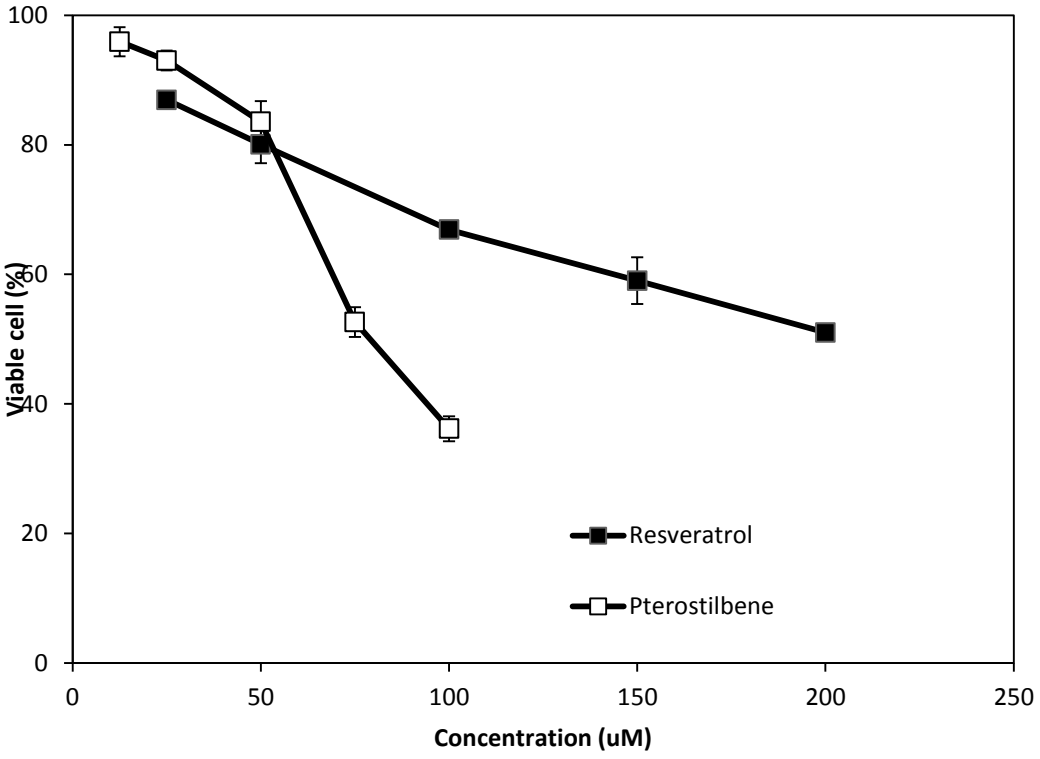


Figure 3.1 Growth inhibitory effect of resveratrol and pterostilbene on CaCo-2 human colon adenocarcinoma cells. Cells were seeded on 96-well plates for 24 hours. After this period of time, cells were treated with serial concentrations of resveratrol or pterostilbene. After 48 hours of treatment, growth inhibition was measured by MTT assay as described in material and method section. Each point represents the mean ± SEM. (n=6).

3.4.2 Cellular Uptake of 3 Colon Cancer Cell Lines

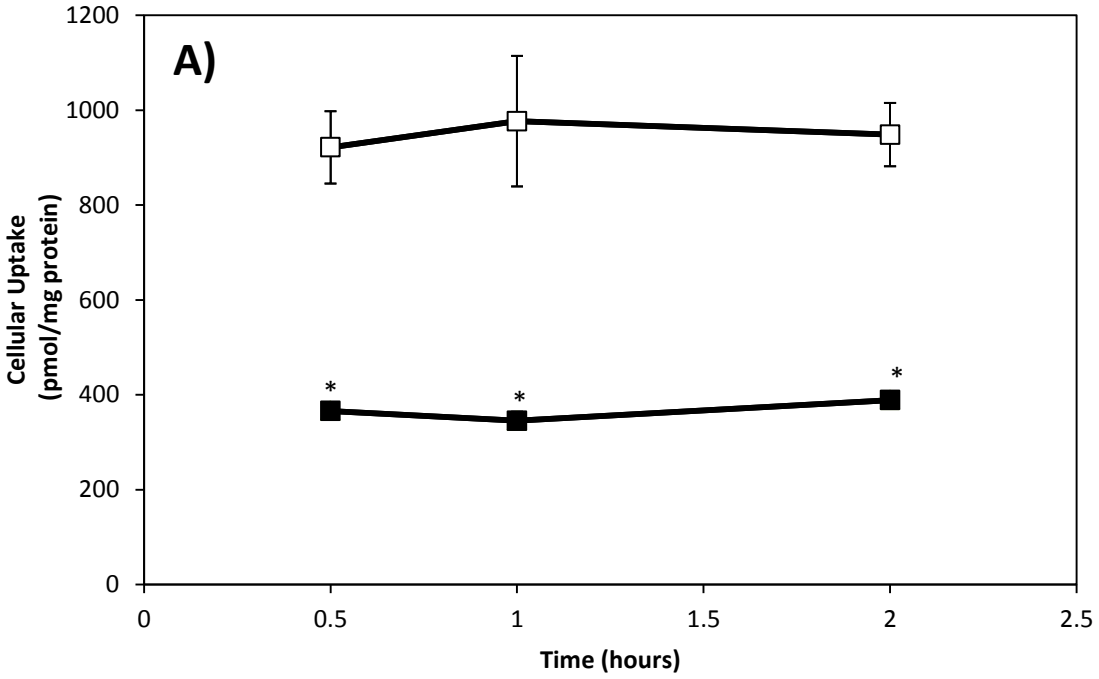
The cellular uptake of resveratrol and pterostilbene in three different human colon cell lines, Caco-2, HT-29 and HCT-116 were evaulated. The results revealed that in all cell lines the cellular uptake of pterostilbene in the cytostol was statistical significantly higher than resveratrol (Figures 3.2A, B and C). Similar results were obtained when the membrane fraction of the cellular uptake of the colon cell lines were examined (Results

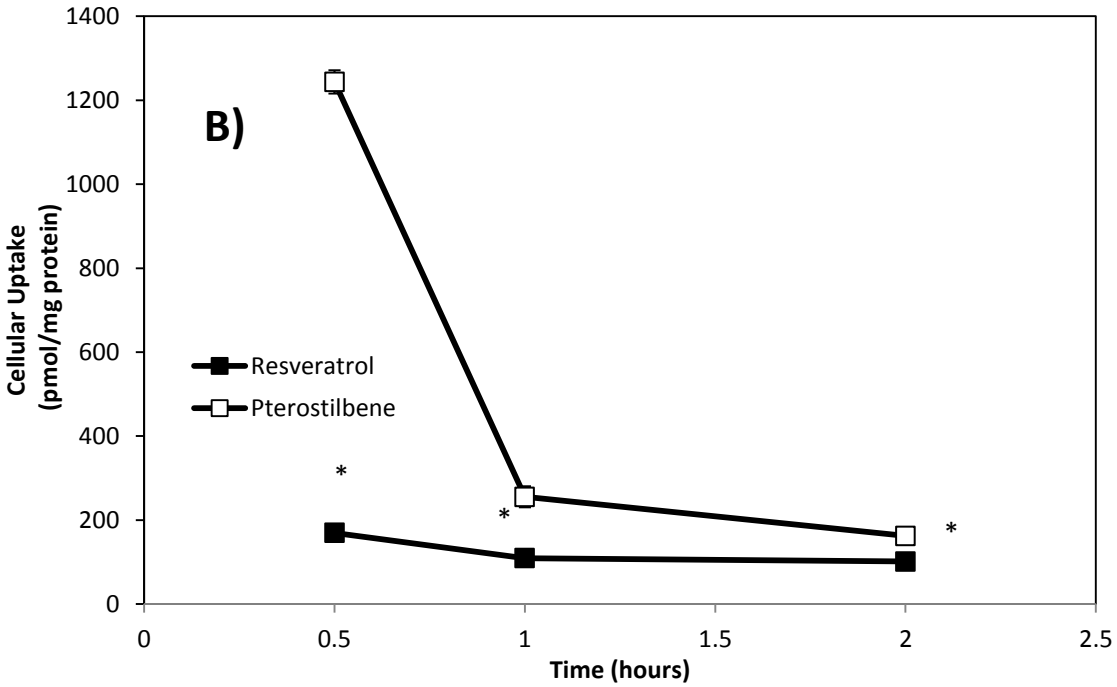
not shown). Also, there were no synergistic or inhibitor effects observed when cells were exposed to both compounds at the same time.

After 0.5 hours of exposure, all cells lines were statistically different from each other in the presences of either compound (Figures 3.2D and E). There was no difference between HT-29 and HCT-116 cellular uptake of resveratrol or pterostilbene at the 1 hour and 2 hour incubation periods. Caco-2 had the most cellular uptake of resveratrol for all treatment times in comparison to HT-29 and HCT-116. However, when cells were exposed to pterostilbene, HT-29 had the most cellular uptake at 0.5 hours but then uptake amounts reduce to that similar to HCT-116 for other treatment times (1 and 2 hour).

In examining the overall cellular uptake ratio of pterostilbene to resveratrol in the different colon cell lines, HCT-116 and Caco-2 had similar ratios (Figures 3.3A, B, and C). HT-29 exhibits a considerably higher ratio of cellular uptake of pterostilbene to resveratrol at 0.5 hours compared to the two other cell lines by approximately four folds. However, HT-29 uptake levels off to similar ratios as the 1 hour and 2 hour time pulls as the other cell lines. The cellular uptake treatments clearly illustrated that pterostilbene was taken up into the cell more readily than resveratrol.

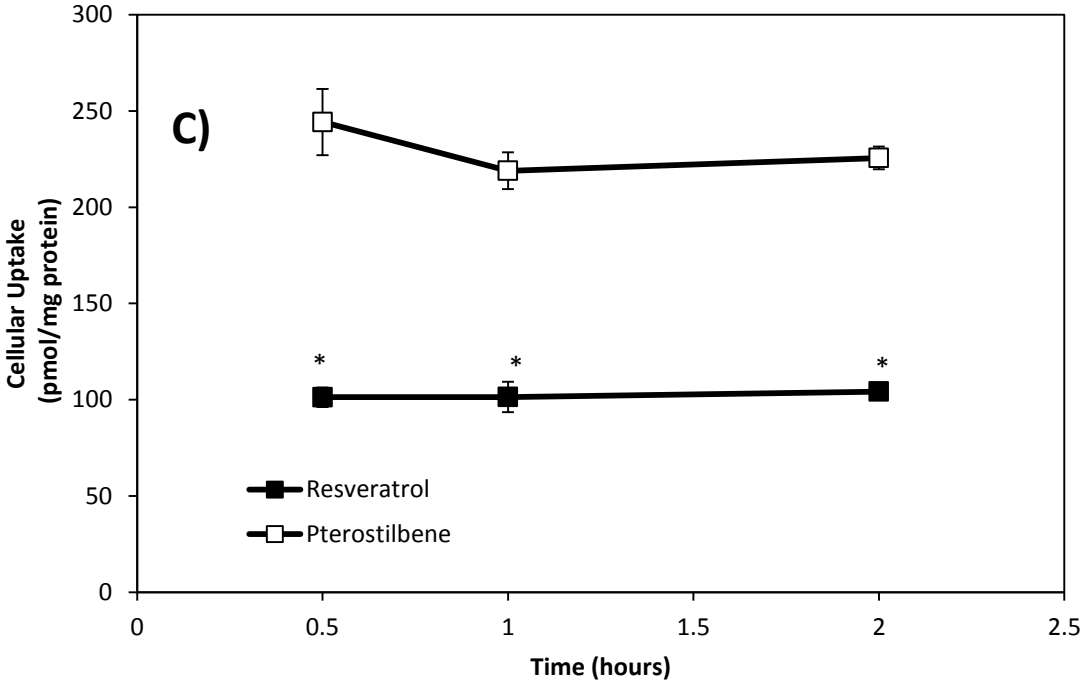
Since a cell suspension was used to determine cellular uptake, resveratrol and pterostilbene had more accessibility to the cells than conventional uptake methods, where cells are grown to confluence in plastic plates that merely allow apical membrane access. Also, this method alleviates any difficulties that may occur from scraping cells from the plastic plates; therefore, decreasing the variation after sample collection. (Henry et al. 2005; Sacclui et al., 2002; Vaidyanathan and Walle, 2003).

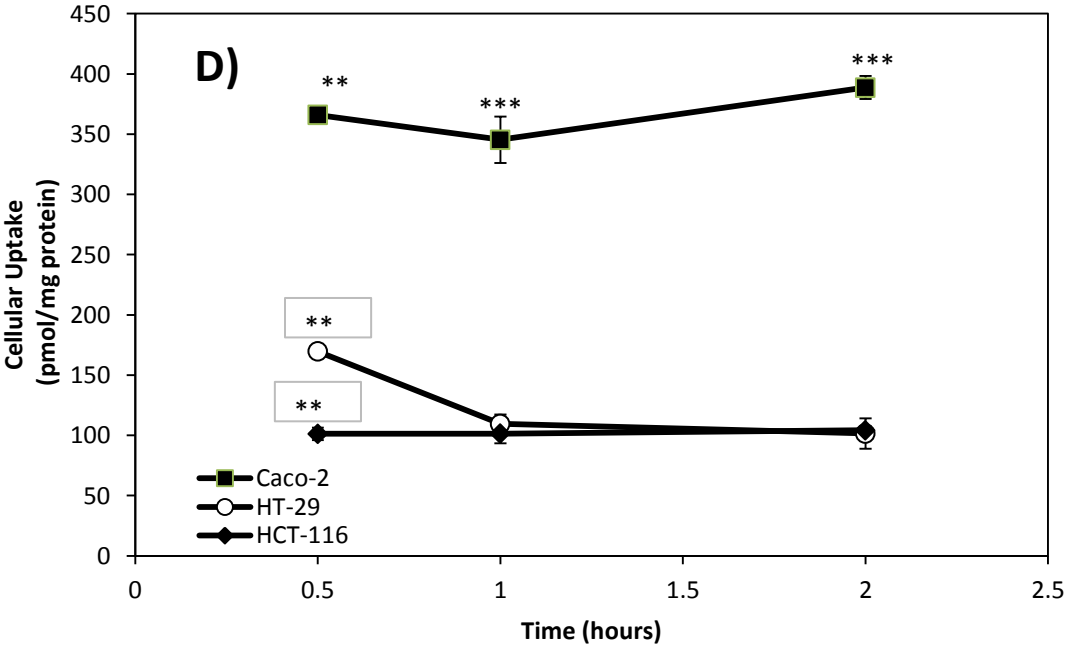




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Figure 3.2, continued





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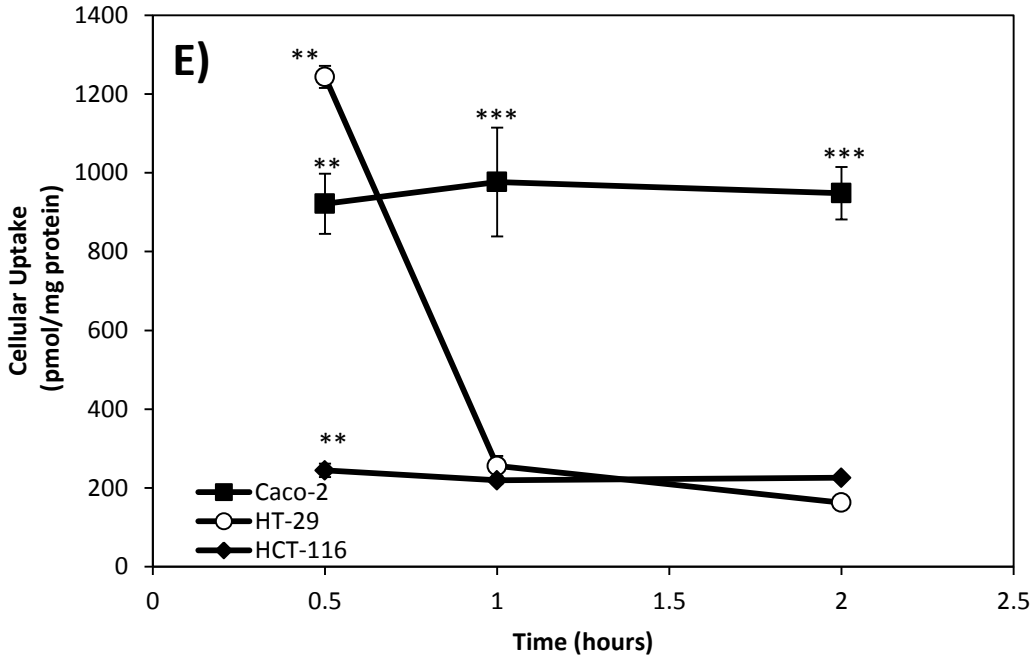


Figure 3.2 Cellular uptake of resveratrol and pterostilbene in the cytosol of (a)Caco-2, (b)HT-29, and (c)HCT-116 human colon cancer cell lines. Colon cancer cells were incubated with 10μM of (d) resveratrol or (e) pterostilbene in complete medium for various time periods. Each point represents the mean (n=3) ± SEM. * indicates a statistical significance between resveratrol and pterostilbene in the same cell line at the same incubation period. ** indicates a statistical significance between all cell lines at the same incubation period. *** indicates only a statistical significance between Caco-2 and other cells lines (HT-29 and HCT-116) but not between each other (HT-29 and HCT-116) at the same incubation period.

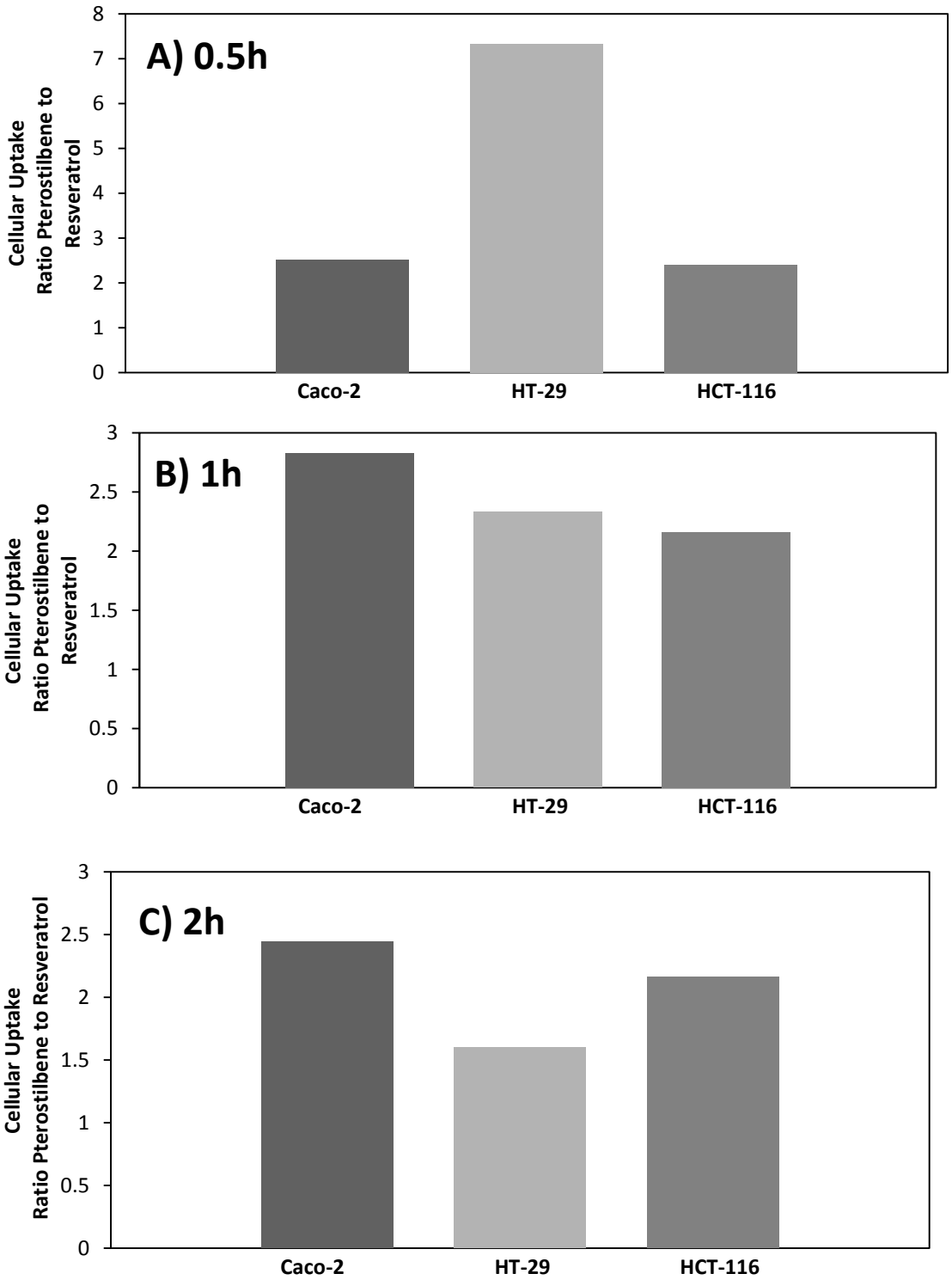


Figure 3.3 Cellular uptake ratio of pterostilbene to resveratrol in the cytosol of Caco-2, HT-29, and HCT-116 human colon cell lines. The cells were incubated with 10μM resveratrol or pterostilbene in complete medium for (a) 0.5, (b) 1, and (c) 2 hours.

3.5 Conclusion

The present study is part of our labs current investigation on accessing the bioavailability and potential health benefits of methalyed flavones. In this study, there was an examination of the effects of resveratrol and pterostilbene on three different human colon cancer cell lines – Caco-2, HT-29, and HCT-116. Both resveratrol and pterostilbene are naturally occurring stilbene that have the same core structure but differ by their functional groups. Pterostilbene has methoxy groups on the 3 and 5 position on the phenyl ring A instead of the hydroxyl groups that resveratrol contains at these positions.

MTT viability assay was used to determine the growth inhibition of the resveratrol and pterostilbene. The assay revealed that pterostilbene had a stronger inhibitory effect than resveratrol on Caco-2. Pterostilbene IC_{50} value was approximately more than 2.5 times lower than that of resveratrol. In our lab has also explored the inhibitor effects of resveratrol and pterostilbene on other human colon cancer lines (HCT116 and HT-29) and similar results were observed (Nutakul et al., 2011).

These findings are in agreement with prior reports that showed the methoxlyated derivative have higher bioactivity and increased totoxic activity (Huang et al., 2007; Wilson et al., 2008). Previous studies have shown that the structural difference of the stilbene compounds contribute to difference in their activities (Jospeh et al. 2008; Ovesna and Horvathova-Kozics, 2005; Stivala et al., 2001). Other methylated flavones have shown to have more potent inhibition of growth versus unmethylated counterparts as well (Walle et al., 2007).

Bioavailability is key factor that can influence the efficacy of bioactive of a compound. Only bioavailable compounds can be accessible to the target cells to be bioactive for these cells. The results obtain suggest greater potency of pterostilbene than resveratrol could potential be because of greater cellular uptake of the pterostilbene. Therefore, we investigated cellular uptake of resveratrol and pterostilbene in three human colon cancer cell lines – Caco-2, HT-29, and HCT-116.

The cellular uptake experiments demonstrated a significant higher uptake of the pterostilbene than resveratrol in all cell lines. The substitution of hydroxy with methoxy group makes pterostilbene more lipophilic than resveratrol (Cichocki, 2008;Paul et al,. 2009), therefore, pterostilbene may be able to permeate the plasma membrane of the cell and in turn enter the cytsol of the cell more readily than that of reservatrol (Tolomeo et al., 2005). As a consequence of better cellular uptake, the higher intracellular levels of pterostilbene can cause more potent inhibitory effects on the colon cancer cells in comparison to resveratrol. There may also be the probability that each colon cancer cell have different preference in taking up pterostilbene and resveratrol.

In conclusion, on the basis of the above findings, pterostilbene has stronger anti-carcinogenic effect and better bioavailabilty in comparison to resveratrol in human colon cancer cells. This study highlights that pterostilbene merits additional investigation as a chemopreventive agent in humans against colon cancer.

CHAPTER 4

COMPARISON OF PERMEABILITY AND TRANSPORT OF RESVERATROL AND ITS METHYLATED ANALOGUE PTEROSTILBENE IN HUMAN INTESTINAL CACO-2 CELLS

4.1 Abstract

Resveratrol and pterostilbene are both structurally related stilbene compounds that have been shown to possess a wide range of therapeutic benefits. Caco-2 cells monolayers were utilized to evaluate the transport of resveratrol and/or pterostilbene from apical to the basolateral compartment and from the basolateral to apical compartment. Samples were analyzed using high-performance liquid chromatography equipped with electrochemical (HPLC-EC). Resveratrol exhibited a higher rapid rate of transport than pterostilbene across the Caco-2 monolayer regardless of the concentration tested and direction. The transport of resveratrol was linear for only an hour; whereas, the transport of pterostilbene stability increased for the first 3 hours and did not reach a plateau until 6 hours into examination. There was little difference in the rate of transport of pterostilbene in either direction. Pterostilbene had an extended period of linear transport than resveratrol, suggesting less extensive pre-systemic metabolism than reservatrol which may lead to greater bioavailability and biological activity than resveratrol. Further research is warranted to investigate how these closely related structures can be utilized effectively for cancer prevention.

Keywords: Resveratrol; Pterostilbene; Stilbene; Caco-2; Permability; Transport

4.2 Introduction

Resveratrol and pterostilbene are both naturally derived polyphenol compounds belonging to the stilbene family. It has long been postulated that resveratrol, which is present in wine, maybe the reason for the "French Paradox". This epidemiological phenomenon is that the French population has a drastically lower incidence of cardiovascular disease despite having a diet higher in fat than other populations (Vidavalur et al., 2006). Resveratrol has been show to block human platelet aggregation, which may lower ones chance of developing cardiovascular disease (Bertelli et al., 1995; Pace-Asciak et al., 1995). Pterostilbene exhibit similar properties as resveratrol as well as its own distinctive therapeutic benefits (Rimando, 2002).

It is not clearly understood how resveratrol and even a greater extent pterostilbene obtain access to an intended cellular site of action. Furthermore, little is known about their absorption and bioavailability in humans. Various studies have demonstrated that resveratrol is well absorbed *in vitro*; yet, *in vivo*, it has very low bioavailability (Goldberg et al., 2003; Walle et al., 2004). Pterostilbene appears to have greater biological activity based on having relatively higher bioavailiability than resveratrol *in vivo* (rats) (Lin et al., 2009; Remerg et al., 2008).

For any compound of interest, their overall absorpiton, distribution, metabolism, excretion and toxicity are crucial in evaulating a compounds prospective as a therapeutic agent (Balani, 2005; Tetko et al., 2006). Since oral administration is a widely employed method for the delivery of drugs and foods, the effectiveness of a compound is dependent on their intestinal absorption to get into systemic circulation to subsequently reach the

intented tissue. The intestinal epithelium is a key determinant for the oral absorption of ingested pharmaceuticals, food ingredients and toxins (Tong and Wen, 2008).

The usage of Caco-2 monolayer to predict the intestinal absorption of compounds is routinely utilized (Lind et al., 2007). Artursson and Karlson (1991) demonstrated a good correlation between the rate of transport across Caco-2 cell monolayer to the amount absorbed in humans by oral administration of a compound. These cells maintain several of the functional and morphological characteristics of the *in vivo* intestinal epithelial cells; therefore, Caco-2 cell monolayer is a vital model for *in vitro* absorption screening (Fossati et al., 2008).

The intent of this study was to compare the permeability and transport of resveratrol and its methylated analogue, pterostilbene, in Caco-2 cell model that were cultured as monolayers on transwells. Samples were analyzed using high-performance liquid chromatography equipped with electrochemical (HPLC-EC).

4.3 Materials and Methods

4.3.1 Materials

Highly purified *trans*-resveratrol (99%) and pterostilbene (98%) were obtained from Quality Phyochemical LLC (NJ, USA). All organic solvents utilized were of HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Ammonium Acetate (99%) was purchased from EMD Chemicals Inc. (Gibbstwon, NJ, USA). The human colon adenocarcinoma cell line, Caco-2, was obtained from American Type Cell Collection (ATCC, Manassas, VA, USA). Polyester (PET) transwell inserts with a pore size of 0.4µm and growth area of 4.67 cm^2 were purchased from Corning Costar

Corporation (New York, USA). Penicillin and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), *MEM non-essential amino acid*, HEPES and other cell culture supplies were purchased from Mediatech Inc. (Herndon, VA, USA).

4.3.2 Cell Culture - Caco-2

Caco-2 were cultured in DMEM containing glucose, L-glutamine and sodium pyruvate, supplemented with 10% FBS, 0.1% *MEM non-essential amino acid*, 10mM HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in an atmosphere of 5% CO_2 and 95% air. The cells were split when 70-90% confluent, using trypsin/EDTA, and media was changed every 2 to 3 days.

Caco-2 cells utilized were between 15 and 40 passages. The culture medium was changed three times a week after seeding and experiments were performed after 21 - 27 days of post-seeding. The final concentration of DMSO in all experiments did not exceed 0.1%.

4.3.3 Transport Experiments

The transwell inserts was seeded with 2.5 X 10^5 cells/ml of Caco-2 cells. The culture medium was changed three times a week after seeding and experiments were performed after 21 to 27 days post seeding. Culture medium was changed 12 to 24 hours prior to performing experiments. The culture medium was removed from both chambers and the cells were washed twice for 30 minutes with pre-incubated (37°C) Hank's balanced salt solution (HBSS). The pH of HBSS in apical compartment was 6.5 and 7.4 for basolateral compartment.

Samples from both compartments were withdrawn at specific times and the volume take from each compartment was replenished to the initial volume with the appropriate HBSS. Each sample was analyzed by HPLC-EC.

4.3.4 HPLC analysis

Samples were analyses on CoulArray® HPLC system, obtained from ESA (Chelmsford, MA, USA), equipped with a binary solvent delivery system (model 584), an auto-sampler (model 542), a CoulArray® Multi-Channel EC detector (model 6210) consisted of two cells, each cell contains 4 channels, and a UV detector. Ascentis RP-Amide column (15 cm x 4.6 mm id, 3 µm) from Sigma-Aldrich (St. Louis, MO, USA) was used. Data collection, processing and instrument control were achieved using the CoulArray 3.06 software.

The mobile phase used consisted 50% water, 40% ACN, 10% THF and 50mM ammonium acetate The pH of the mobile phase was adjusted with TFA to achieve a pH range between 3.5 to 3.8 and then filtered through a 0.45µm membrane filter from Millipore (Bedford, MA, USA). Both EC detector cells were used and detection potentials were set to 300, 400, 500, 600, 700, 800 and 900 mV. The flow rate was set to 1.0 ml/min. The temperature of the auto-sampler was set to 4°C.

This detection method has high selectivity and excellent sensitivity even at low concentration. The range of validation (0.001 - 5µM) was linear with coefficients of correlation more than 0.999 (Dong et al., 2010). Samples were stored at -20º C until analysis.

4.3.5 Data Analysis

Apparent permeability coefficient, P_{app}, (cm/s) of a compound was calculated by using the equation below (Hubatch et al., 2007).

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0}$$

dQ/dt is steady-state appearance rate of compound in receiver compartment (µmol/s)

A is the surface area of the filter (4.67 cm^2)

C_0 is the initial concentration in the donor compartment (µM)

$$FA_{cum} = \frac{1}{A} \sum_{K=1}^{i} \frac{[C_R(t_k) - fC_R(t_{k-1})]V_R}{[C_D(t_{k-1}) + C_D(t_k)]/2}$$

The cumulative fraction transported, FA_{cum}, (cm) for each experiment was also evaluated. See the equation above. This equation takes into account the weighted normalized cumulative amount of a compound transported, where the amount of transported compound in each time pulls is weighted by the inverse average driving force (donor concentration for that time pull) (Hubatch et al., 2007).

Data were expressed as mean ± standard error mean (SEM). Statistical significance of mean difference between two groups was calculated by using Student's two-tailed t-test. Analysis of variance (ANOVA) model with Dunnett multiple comparison was used when comparing more than two groups' differences. A significance level of $P < 0.05$ was used for all tests.

4.4 Results and Discussion

4.4.1 Overall

Caco-2 cell monolayers were utilized to evaluate the transport rate of resveratrol and/or pterostilbene from apical to the basolateral compartment and from the basolateral to apical compartment. The transport of the resveratrol and pterostilbene were monitored over an 8 hour time period at three different concentrations (10, 25 and 50µM). Samples from both compartments were taken at each time pull and replaced with buffer. Samples were subsequently analyzed by HPLC-EC without any further preparation.

The concentration range we employed some claim is slightly lower than the range of a compound in the gastrointestinal lumen following oral dose (Kerns and Di, 2008) while others say slightly higher than expected in the gastrointestinal lumen after drinking a cup of juice or glass of red wine (Fremont, 2000; Gescher, 2008). To better mimic what occurs in the body after oral administration of a compound, a pH gradient was used, where an acidic pH of 6.5 was utilized in the apical compartment and a neutral pH of 7.4 was utilized in the basolateral compartment (Lind et al. 2007). The acidic microclimate models the pH of the upper small intestine under fasted conditions and the neutral pH (7.4) in the basolateral side mimics the pH of the blood (Fallingborg et al., 1989; Deferme et al., 2008).

When either resveratrol or pterostilbene were loaded on the apical or basolateral compartment, they both were detected on the opposite side at the first time pull of 30 minutes and throughout the 8 hours incubation period. Both compounds at all three concentrations evaluated, clearly demonstrated transcellular absorption (Figurres 4.3 and

4.4). When both resveratrol and pterostilbene were added to the apical compartment at the same time together (data not shown), there was no synergistic effected occurred.

Table 4.1 summarizes the P_{app} for the first hour of transport for resveratrol and pterostilbene. There was a distinctive difference between rates that resveratrol and pterostilbene had across the Caco-2 monolayer and how concentration and direction effect transports of these compounds.

The efflux ratio (P_{app} of baterolateral compartment to apical compartment / P_{app} of apical compartment to basolateral compartment) for resveratrol and pterostilbene were both less than 3 indicating that no efflux is occurring.

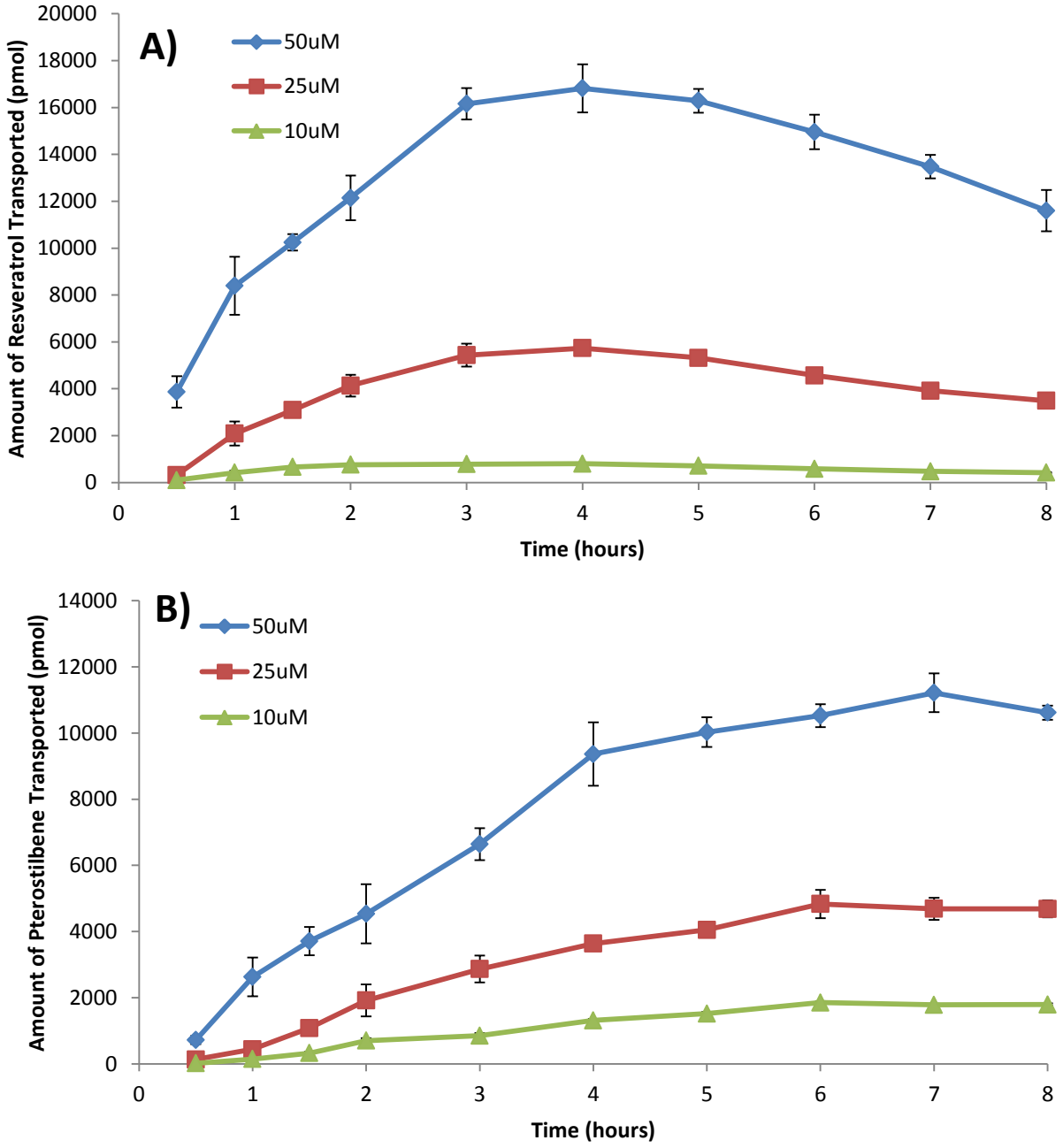


Figure 4.1 The transport from the apical to the basolateral compartment of (a) resveratrol and (b) pterostilbene across the Caco-2 monolayer. Each point represents the mean (n=4) ± SEM.

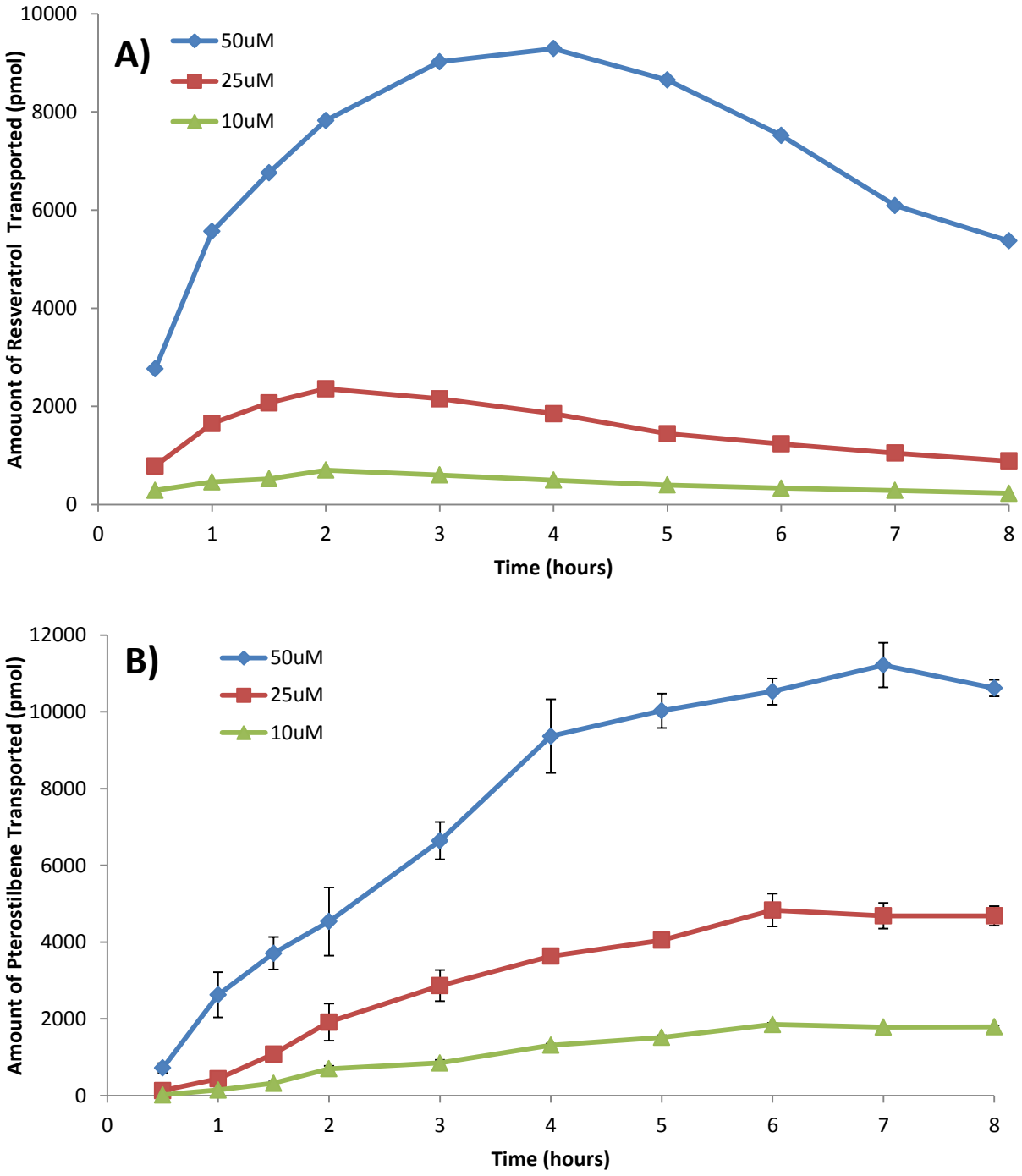


Figure 4.2 The transport from the basolateral to the apical compartment of (a) resveratrol and (b) pterostilbene across the Caco-2 monolayer. Each point represents the mean (n=4) ± SEM.

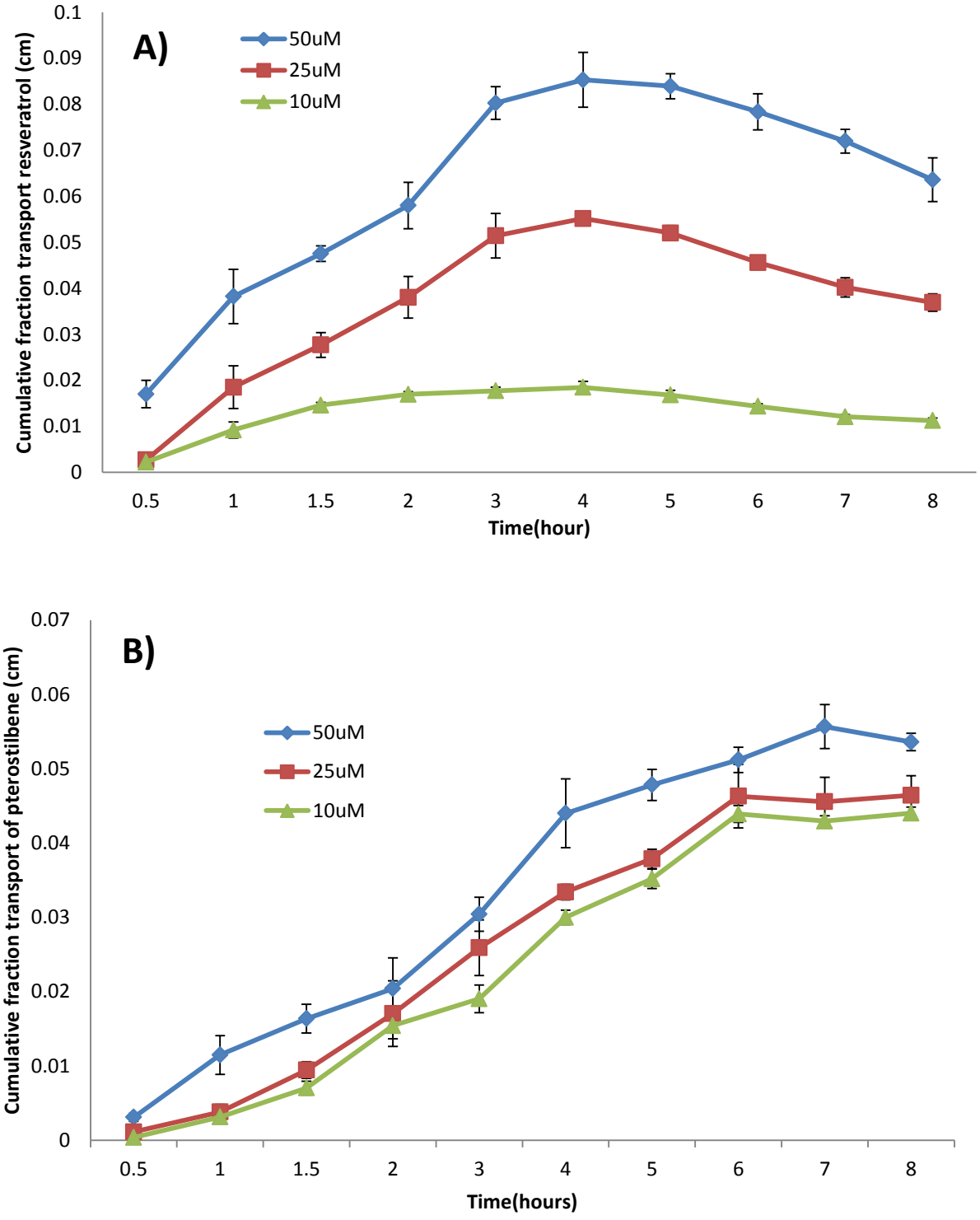


Figure 4.3 Cumulative fraction transport from the apical to the basolateral compartment of (a) resveratrol and (b)pterostilbene across the Caco-2 monolayer. Each point represents the mean (n=4) ± SEM.

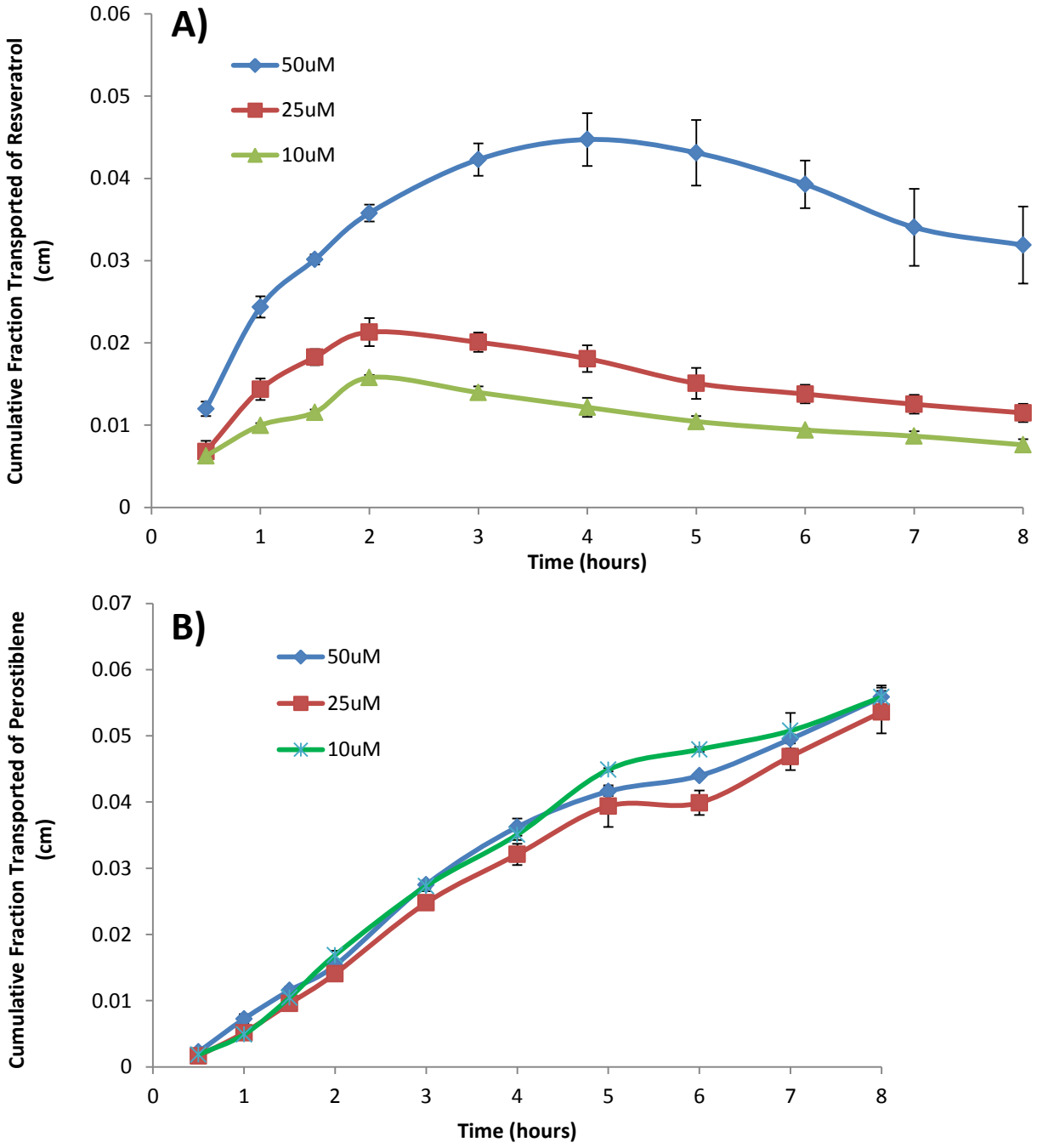


Figure 4.4 Cumulative fraction transport from the basolateral to the apical compartment of (a) resveratrol and (b) pterostilbene across the Caco-2 monolayer. Each point represents the mean (n=4) ± SEM.

Table 4.1 Apparent permeability coefficients or different concentrations of resveratrol and pterostilbene through Caco-2 monolayers

Compound	Concentration (μM)	Transport Direction	
		P_{app}[a]: Apical to Basolateral	P_{app}:[a] Basolateral to Apical
Resveratrol	10	4.4±0.9	2.8±0.8
	25	10.2±0.2	4.5±0.7
	50	12.1±0.6	6.9±0.7
Pterostilbene	10	2.6±0.3	3.0±0.2
	25	3.5±05	2.5±0.3
	50	4.5±1.5	3.0±0.2

a. Papp is expressed in cm/sec (x 10^{-6}). Values are means ± SEM. n=4.

4.4.2 Resveratrol

For the first hour of transport of resveratrol across the Caco-2 monolayers, there was a linear increase in the amount of resveratrol transported when added to either compartment (Figures 4.3A and 4.4A). However after 3 hours of inhibition, there was plateau in transport of resveratrol, regardless of what compartment resveratrol was loaded into. Also, there were slightly lower amount of resveratrol transported when resveratrol was added on the basolateral compartment.

Transcellular absorption from the apical to basolateral compartment was observed at all concentrations and appears to be concentration dependent (Figure 4.5A). As the concentration of resveratrol increase from 10μM to 50 μM, the transport rate from the apical to basolateral compartment more than doubled (Table 5.1).

The basolateral to apical flux of resveratrol was lower than transport from apical to basolateral compartment (Figure 5.6A). Since there was limited linearity in transport

of resveratrol with time, this could imply considerable metabolism of resveratrol by the Caco-2 cells (Walle et al., 2007).

4.4.3 Pterostilbene

Transport of pterostilbene across the Caco-2 monolayer increased steadily for the first three hours (Figure 4.3B and 4.4B). It took twice as long for pterostilbene to reach plateau than resveratrol which was after 6 hours of incubation.

Pterostilbene, unlike resveratrol, did not exhibit concentration dependence when it was added to basolateral compartment (Figure 4.6B). Transport was virtually identical for each concentration and direction which demonstrates the absence of direction-dependent transport (Figures 4.5B and 4.6B).

This suggests that the transport of pterostilbene across Caco-2 monolayers is probably through a passive diffusion mechanism.

4.5 Conclusion

The objective of this study was to examine the transport of resveratrol and pterostilbene across Caco-2 cell monolayer. Even though resveratrol and pterostilbene have the same core structure; they differ in their functional groups. Resveratrol contain 3,5-dihydroxy motif on the phenyl A ring, while pterostilbene has 3,5-dimethoxy motif. Resveratrol is the mostly studied stilbene; however, research on the absorptions of other stilbene, especially ones with methylated groups, are still limited.

The usage of human intestinal cell line Caco-2 cultivated on permeable membranes is a well accepted model of human intestinal absorption. It is critical to understand the bioavailability of stilbenes in order to figure out their potential actions *in*

vivo. Gres *et al.* (1998) determined that *P*app values >2 × 10-6 cm/sec from Caco-2 cell experiments ought to correlate with efficient intestinal absorption. As a result, it is predicted that both resveratrol and pterostilbene can be effectively absorbed in humans. Other stilbene have reported to have lower transport rates than both resveratrol and pterostilbene (Kim et al., 2008)

Resveratrol exhibited a higher and more rapid rate of transport than pterostilbene across the Caco-2 monolayer regardless of the concentration tested (10, 25 and 50μM) and direction. The transport of resveratrol was linear for only one hour; whereas, pterostilbene transport stability increased for the first 3 hours and did not reach a plateau until 6 hours into examination. Regardless of what compartment pterostilbene was put into and concentration examined (10, 25, 50), there was little difference in the rate of transport of pterostilbene.

Previous studies also observed a reduction in transport of resveratrol over time from the apical to basolateral compartment, which was clearly concentration dependent (Kadlas et al., 2003; Maier-Salamon et al., 2006; Li et al., 2003;). Human studies demonstrated peak levels resveratrol were reached at 30 minutes and one hour times after oral dosing and rapidly declined afterwards (Goldberg et al., 2003). Since transport of resveratrol had limited linearity over time, this could possibly be linked with increased amount of resveratrol being metabolized over time. This could indicate low oral bioavailability, due to the fact this has been observed in other polyphenols (Walle et al., 1999).

The transport of pterostilbene in this study clearly demonstrated passive diffusion permeation. Pterostilbene has been reported to not metabolized as quickly and have

longer plasma half-live than resveratrol (Ferrer et al., 2005). Lin et al. (2009) demonstrated pterostilbene has better pharmacokinetic characteristics than resveratrol. Pterostilbene may have higher bioavailability than resveratrol, since the substitution of the hydroxyl with methoxy group increases metabolic stability of most compounds.

In conclusion, this data showed that even though resveratrol absorbed more rapidly than pterostilbene in the Caco-2 cell model, both would be efficiently absorbed in the human intestine. The data revealed that pterostilbene had extended period of linear transport than resveratrol suggesting less extensive pre-systemic metabolism than resveratrol which may lead to greater bioavailability and biological activity than resveratrol. Further research is warranted to investigate how these closely related structures can be utilized for different therapeutic uses.

CHAPTER 5

COMPARISON OF THE CELLULAR UPTAKE, PERMEABILITY AND TRANSPORT OF POLYMETHOXYFLAVONES (PMFS)

5.1 Abstract

Polymethoxyflavones (PMFs) naturally exist in citrus genus and accumulating amount of evidence suggesting they have health-promoting benefits but there has been a lack of research on examining their bioavailability. Samples were analyzed using high-performance liquid chromatography equipped with electrochemical (HPLC-EC). A cell suspension in glass tubes were used to determine cellular uptake in HCT-116 human colon cells. Caco-2 cells monolayers were used to evaluate the transport of PMFs from apical to the basolateral compartment. The HCT-116 human colon cells had intracellular uptake of each of the polymethoxyflavones (PMFs) tested. Transport was observed by all the PMFs and each had different permeability rates. Overall, the results demonstrated that location and amount of methyloxly groups have an effect on intracellular levels of the PMF in HCT-116 and the rate of transport.

Keywords: Polymethoxyflavones, Permeability; Transport Caco-2 cells, Cancer, Bioavailability

5.2 Introduction

Polymethoxyflavones (PMFs) exist almost exclusively in the citrus fruits in nature, particularly in the peel of sweet oranges and mandarin oranges (Li et al., 2006). Currently, more than 20 PMFs have been isolated and identified. For centuries, orange peels have been used in Asian culture to heal varies aliments (Ou, 1999). PMFs have been well documented on their broad array of biological activities, such as anti-atherogenic (Whitman et al., 2005), anti-carcinogenic (Ikeda et al., 2006; Manthey and Najla, 2002) and anti-inflammatory (Li et al., 2006).

It is estimated that 2.2 million metric tons (MMT) of orange juice will be produced worldwide this year (USDA: Foreign Agricultural Service, 2012) which will yield a considerable amount of orange peel by-product. There are some PMFs in commercial juices due to the industrial process being contaminated with peel constituents (Gattuso *et al*., 2007) but most PMFs will come from the peel by-product of the orange juice process which could potential be used for therapeutic purposes.

However, there is a scarce amount of research out on the bioavailability of PMFs. For any compound, the overall effects of absorpiton, distribution, metabolism, and excretion are vital in examining a compounds prospective as a therapeutic agent. The term bioavailability is defined as the fraction of an ingested component that eventually ends up in the blood systemic circulation system and target tissue (Van de Waterbeemd et al., 2003).

Caco-2 cells model is widely used *in vitro* model to examining intestinal transport, biotransformation and bioavailability at the cellular level. They have similar

morphology as human intestinal epithelial cells such as forming polarized monolayers in cultures and differentiate into cells (Fossati et al., 2008; Lind et al., 2007).

This study will focus on the following PMFs:

- 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone (PMF3)
- 5-hydroxy-3,6,7,8,3',4'-hexamethoxyflavone (PMF4)
- 5-hydroxy-6,7,8,4'-tetramethoxyflavone (PMF7)

See Table 1.1 for chemical structure of these PMFs as well as other PMFs used in this study. The object of this study was to investigate if cellular uptake of PMFs occurs in HCT-116 colon cancer cells and to evaluate their transport in Caco-2 cell models.

5.3 Materials and Methods

5.3.1 Materials and Reagents

All organic solvents utilized, acetonitrile (ACN), methanol (MeOH), tetrahydrofucan (THF) and trifluoroacetic acid (TFA), were of HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Ammonium Acetate (99%) was purchased from EMD Chemicals Inc. (Gibbstwon, NJ, USA). Penicillin and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), McCoy's 5A media, fetal bovine serum (FBS), *MEM non-essential amino acid*, HEPES and other cell culture supplies were obtained from Mediatech Inc. (Herndon, VA, USA). Polyester (PET) transwell inserts with a pore size of 0.4µm and growth area of 4.67 cm^2 were purchased from Corning Costar Corporation (New York, USA).

Both human colon adenocarcinoma cell lines, Caco-2 and HCT116, were obtained from American Type Cell Collection (ATCC, Manassas, VA, USA). PMFs were isolated from sweet orange (*Citrus sinensis*) peel extract (cold pressed oil) and dissolved in dimethyl sulfoxide (DMSO). They were identified by MS, UV and NMR (Li et al., 2006). DMSO was used as the vehicle to deliver PMFs and the final concentration of DMSO in all experiments was 0.1% in culture media.

5.3.2 Cell Culture Treatment

HCT-116 were maintained in McCoy's 5A media supplemented with 5% heat inactivated FBS, 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin at 37ºC in an atmosphere of 5% CO_2 and 95% air. Cells were kept sub-confluent and culture media were changed every other day. HCT-116 cells utilized were between 10 and 25 passages.

Caco-2 were cultured in DMEM containing glucose, L-glutamine and sodium pyruvate, supplemented with 10% FBS, 0.1% *MEM non-essential amino acid*, 10mM HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in an atmosphere of 5% CO_2 and 95% air. The cells were split when 70-90% confluent, using trypsin/EDTA, and media was changed every 2 to 3 days. Caco-2 cells used were within 15 to 40 passages.

5.3.4 Cellular Uptake Assay

HCT-116 human colon adenocarcinoma cells (200 cells/ml) were suspended in 1ml of culture media containing different PMFs in glass culture tube and then incubated at 37°C with atmosphere of 5% CO_2 and 95% air for a given period of time (0.25, 0.5, 1 and 2 hours). After incubation, cell suspensions were centrifuged in a benchtop

centrifuges at 12000rpm for 5 minutes. The supernatant was then removed and cells were suspended with 1ml of ice-cold PBS (1x; pH7.25) and then sonicated. The cells suspension was again centrifuged (12000rpm for 5 minute). From the supernatant obtained, some was collected and reserved for the protein analysis by Bicinchoninic Acid (BCA) assay (Wang et al., 2008).

Equal amount of supernatant and MeOH were sonicated and then centrifuge again (12000rpm for 5 minutes). The supernatant obtained was collected for the HPLC analysis to determine the amount of PMF in the cytosol faction.

5.3.5 Transport Experiments

The transwell inserts was seeded with 2.5 X 10^5 cells/ml of Caco-2 cells. The culture medium was changed three times a week after seeding and experiments were performed after 21 to 27 days post seeding. Culture medium was changed 12 to 24 hours prior to performing experiments. The culture medium was removed from both chambers and the cells were washed twice for 30 minutes with pre-incubated (37°C) Hank's balanced salt solution (HBSS). The pH of HBSS in apical compartment was 6.5 and 7.4 for basolateral compartment.

Samples from both compartments were withdrawn at specific times and the volume take from each compartment was replenished to the initial volume with the appropriate HBSS. Each sample was analyzed by HPLC-EC.

5.3.6 Sample Analysis

Samples were analyzed on CoulArray® HPLC system, obtained from ESA (Chelmsford, MA, USA). It was equipped with a binary solvent delivery system (model

584), an auto-sampler (model 542), a CoulArray® Multi-Channel EC detector (model 6210) consisted of two cells (each cell contains 4 channels) and a UV detector. Ascentis RP-Amide column (15 cm x 4.6 mm id, 3 µm) from Sigma-Aldrich (St. Louis, MO, USA) was used. Data collection, processing and instrument control were achieved using the CoulArray 3.06 software.

The mobile phase used consisted of 50% water, 40% ACN, 10% THF and 50mM ammonium acetate. The pH of the mobile phase was adjusted with TFA to achieve a pH range between 3.5 to 3.8 and then filtered through a 0.45µm membrane filter from Millipore (Bedford, MA, USA). Both EC detector cells were used and detection potentials were set to 200, 300, 400, 500, 600, 700 and 800mV. The flow rate was set to 1.0 ml/min. The temperature of the auto-sampler was set to 4°C.

This detection method has high selectivity and excellent sensitivity even at low concentration. The range of validation (0.001 - 5µM) was linear with coefficients of correlation more than 0.999. Samples were stored at -20º C until analysis.

5.3.7 Data Analysis

Apparent permeability coefficient, P_{app}, (cm/s) of a compound was calculated by using the equation below (Hubatch et al., 2007).

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0}$$

dQ/dt is steady-state appearance rate of compound in receiver compartment (µmol/s)

A is the surface area of the filter (4.67 cm^2)

C_0 is the initial concentration in the donor compartment (µM)

$$FA_{cum} = \frac{1}{\mathrm{A}} \sum_{K=1}^{i} \frac{[\mathrm{C_R(t_k)} - \mathrm{fC_R(t_{k-1})}]\mathrm{V_R}}{[\mathrm{C_D(t_{k-1})} + \mathrm{C_D(t_k)}]/2}$$

The cumulative fraction transported, FA_{cum} (cm), for each experiment was also calculated. See the equation above. This equation takes into account the weighted normalized cumulative amount of a compound transported, where the amount of transported compound in each time pulls is weighted by the inverse average driving force (donor concentration for that time pull) (Hubatch et al., 2007).

Data was expressed as mean ± standard error mean (SEM). Statistical significance of mean difference between two groups was calculated by using Student's two-tailed t-test. Analysis of variance (ANOVA) model with Dunnett multiple comparison was used when comparing more than two groups' differences. A probability of less than 0.05 ($p < 0.05$) was deemed statistically significant.

5.4 Results & Discussion

5.4.1 Cellular Uptake of PMFs in HCT-116 cancer cells

The ability of a compound to generate a biological effect depends on if it can first enter the target cell. This study attempts to provide this insight by evaluating the intracellular levels of PMFs in HCT-116 human colon adenocarcinoma cells. The results were normalized with the amount of cytosolic protein (Foster *et al.*, 2001). An array of different cellular uptake experiments were performed. Individual PMFs were examined

and then assessed to see if combined with other PMFs there would be an increase in cellular uptake of the PMF of interest. Some experiments had HCT-116 cells exposed to one PMF for 24 hours prior to treatment and then exposed to another PMF to determine if later PMF would have greater intracellular uptake because cells were exposed to another PMF prior.

Overall, there was no synergistic effect observed when an additional PMF was added to the cellular uptake experiment to see if it would have an effect on the other PMF (the one of interest). There was dose dependent observed; therefore, the intracellular levels of PMF in the cell would increase with a increase in the amount of PMF exposed to the cells (More PMF added the more was uptaken). When PMF4, PMF5 and PMF7 were compared to the uptake of PMF3 from PMF mixture (25uM of each), there was less PMF4, PMF5 and PMF7 uptake in comparison to PMF3 (Figure 5.1). Slightly less PMF7 was uptake in comparison to PMF 4 and PMF5.

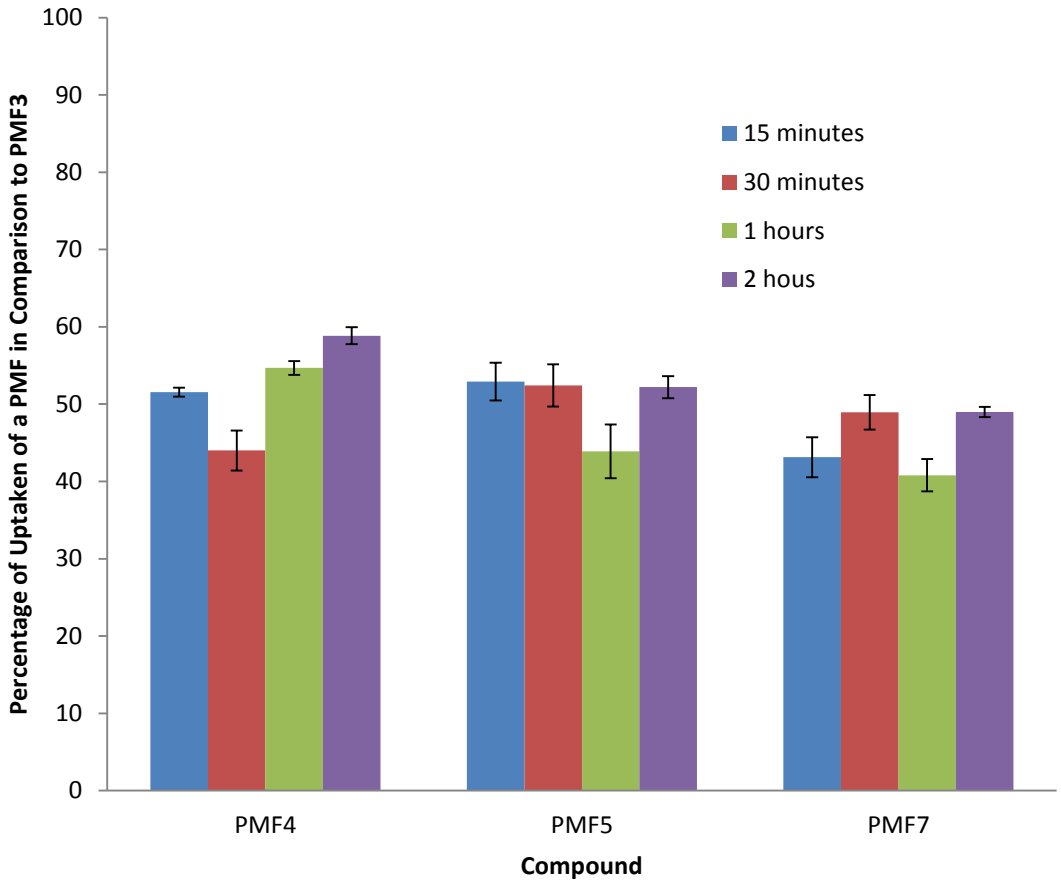


Figure 5.1 Percentage of cellular uptake in HCT-116 human colon cancer cells of PMF4, 5 and 7 from the PMF mixture in comparison to uptake of PMF3 from the same mixture. The values are mean ± SEM (n=3).

5.4.2 Transport of PMFs by Caco-2 cells

A scarce number of studies exist on the transport of PMFs, in particular the PMFs which were examined in this study. The ability of a compound to be effectively absorbed in human intestine is a critical step in determining its bioavailability. Caco-2 cell monolayers were utilized to evaluate the transport of PMFs from apical to the basolateral compartment. The apical-to-basolateral transport of the PMF3, 4 and 7 were monitored over an 8 hour time period using a concentration of 2.5μM. An assortment of other transport experiments was also performed. A pH gradient was used to examine the transport of PMFs because this better mimics what happens in the body after oral administration of a compound (Lind et al. 2007). In the apical compartment, the acidic microclimate (pH 6.5) mimics the pH of the upper small intestine under fasted conditions and the neutral pH (7.4) in the basolateral side imitates the pH of the blood (Fallingborg et al., 1989; Deferme et al., 2008).

When PMFs were individually loaded on to the apical compartment, PMF3 and PMF4 were observed on the opposite compartment at 30 minutes and throughout the 8 hour experiment. However, PMF7 was not detected until 90 minutes into the transport experiment at the same concentration as the other PMFs. Even when the concentration of PMF7 was double to 5 μM, it was not detected on the opposite compartment until 30 minutes into the experiment. There was no significance increase or change overall in the transport of the PMFs when all PMFs were added to the apical compartment in comparison to when only individual PMFs were added to the apical compartment. Other combinations with other compounds (PMF1, PMF2, Resveratrol and Pterostilbene) were

evaluated to see if there would be significance change to transport of the PMF3, PMF4 and PMF7 but no such observation was detected.

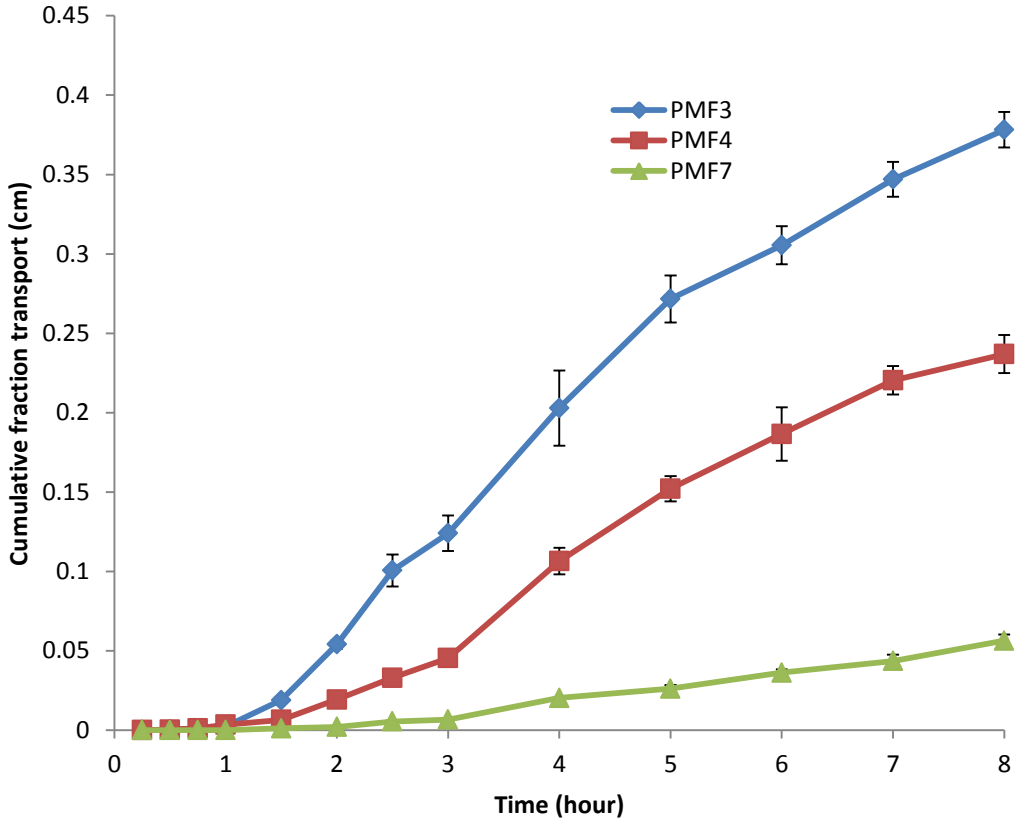


Figure 5.2 Cumulative fraction transported from the apical to the basolateral compartment for PMF3, PMF4 and PMF7across the Caco-2 cell monolayer. Each point represents the mean ± SEM. n=4.

Transcellular absorption clearly occurred for all the PMFs (Figure 5.2). PMF3 had the largest amount of transported to the basolateral compartment and largest apparent permeability coefficient in comparison to PMF4 and PMF7 (Table 5.1). PMF7 had the lowest amount of compound transfer across the Caco-2 monolayer. There was a progressive increase in transport of both PMF3 and PMF4 for the first 5 hours of

transport yet PMF7 had a steady increase in transport throughout the transport experiment.

Table 5.1 Apparent permeability coefficients of the PMFs through Caco-2cell monolayers.

Compound	P_{app}[a]
PMF3	22.3±2.5
PMF4	7.4±0.8
PMF7	1.2±0.2

a. P_{app} is expressed in cm/sec (x 10^{-6}). The values are means ± SEM. n=4.

5.5 Conclusion

This study is part of an ongoing exploration in our lab on the synthesis, mechanisms and potential health effects of PMFs. PMFs have sparked interest due to their wide spectrum of biological effects as an anti-inflammatory (Huang and Ho, 2010; Lai et al., 2011; Li et al., 2007 a & b) and anti-carcinogenic (Morley et al., 2007, Pan et al., 2007; Qui et al., 2011). Despite their health potential, the investigation on the bioavailability of PMFs has lagged behind. This study provides some insight on the cellular uptake of PMFs in HCT-116 colon cancer cells and the transport of these compounds in a Caco-2 cell model.

The cellular uptake of PMF3, PMF4 and PMF7 in HCT-116 colon cancer cells was observed and similar results were obtain in our lab when cellular uptake was examined in another colon cell line, HT-29. Our lab has demonstrated that PMF3, PMF4 and PMF7 have strong inhibitory effects on the growth of the cancer cells in HCT-116, which maybe in part due to their ability to easily be uptaken by the colon cancer cell

lines. Our lab has also shown that these PMFs (3, 4 and 7) have different effects on the cell cycle of colon cancer cells indicating that these three PMFs inhibit colon cancer cell growth by different mechanisms (Qiu et al., 2010 & 2011). The number of methoxy groups and their position may be the reason for why one PMF has higher intracellular levels than another in HCT-116. It is plausible that there is a difference in preference by the colon cancer cells line for uptake of certain PMFs. The exact mechanism by which methoxylation may modulate the bioavailability of PMFs is a topic that warrants more research.

Bioavailability of a compound is the amount of the compound that can reach the blood circulatory system and target tissue (Van de Waterbeemd et al., 2003) . Walle and his colleagues (Walle, 2007; Walle *et al*., 2007; Wen and Walle, 2006) have demonstrated that methoxlyation of PMFs have higher hepatic metabolic stability and intestinal absorption compared to unmethlated polyphenles. The low bioavaiability and poor absorption of these unmethlated flavones is due to extensive conjugative metabolism in the intestine and liver because of the free hydroxyl groups which gives rise to rapid intestinal/hepatic conjugation and/or sulfation and excretion (Wen and Walle, 2006).

There has been investigations done on the *in vitro* absorption of nobiletin, a PMF1, versus luteolin, a polyhydroxyflavonoid (unmethlyated), and results revealed that nobiletin had higher permeability and accumulated in the differentiated Coc-2 cell monolayer while luteolin did not (Murakami *et al., 2001*). Gres *et al.* (1998) reported if a compound has a P_{app} values >2 × 10-6 cm/sec from Caco-2 cell model experiments then the compound should be associated with efficient intestinal absorption. Therefore, PMF 3

and PMF4 can be predicted to be associated with being able to be absorbed in the human intestines, where PMF7 is less likely. More research is necessary to see what will occur if concentrations are increased as well as what would occur if PMFs are placed in the basolateral compartment.

In summary, the results in this study suggest PMFs have the potential to facilitate intestinal absorption *in vivo* in human because of their capability to be transported in the *in vitro* absorption Caco-2 cell model. Along with other results attained from our lab, these observations demonstrate that PMFs as promising agents in cancer therapy and prevention but future research efforts are needed to understand their mechanism of transport.

CHAPTER 6

FUTURE RESEACH

To compliment the research executed in this thesis, future work should continue to focus on exploring the potential of methylated phytochemicals as cancer –fighting compounds. Also, work ought to continue to utilize *in vitro* research models to evaluate bioavailability of compounds. Furthermore, because of its sensitivity and high selectivity, HPLC-EC should be evaluated for usage in the separation, identification, and determination of other phytochemicals.

Based on the work done on the side-by-side comparison of resveratrol and pterostilbene, pterostilbene had higher intracellular uptake on all three cancer cells examined than resveratrol. Also, pterostilbene had stronger inhibition on the cell viability on Caco-2 human colon cancer cells than resveratrol and similar results were observed in other human colon cancer cells lines (Nutakul *et al*., 2011). This data suggests that pterostilbene has superior anti-carcinogeic effect than resveratrol. Thus, it is worth performing other side-by-side comparison of resveratrol and pterostilbene on other cancer cells lines to see if similar results are obtained.

The preliminary experimentation preformed on certain PMFs revealed some new insight on their bioavailability. They have different rates of transport but more research is needed to determine what type of transport is occurring. Since transport experiments on PMFs evaluated only the permeability of PMFs from the apical side to the basolateral side, the next step would be to evaulate transport of PMFs from basolateral compartment to apical compartment. This type of experiment could provide additional information on the effect of secretory trasporters. Also, allows one to calculate the ratio of P_{app} B toA /

P_{app} A to B (P_{app} basolateral compartment to apical compartment over P_{app} apical compartment to basolateral compartment) for each PMF which would aid in determining if efflux possiblity would occurr (Walle *et al*., 2003). Therefore, a better picture could be drawn on the absorption of PMFs.

For all the compounds examined, determining the exact mechanism by which the methoxylation effect intestinal absorption and modulates the bioavailability is an attractive topic for future investigation. The natural progression would be to examine the metabolism of these compounds and assess whether the metabolic products (metabolites) have any biological and pharmacological properties. Knowledge of both a compounds absorption and metabolism can aid in determining a compounds potential biological functions *in vivo* (Masimirembwa et al., 2003; Mohutsky et al., 2006).

Bioavailability is an important factor that can dictate the efficacy of bioactive dietary components. The bioavailability of a these compounds studied in this thesis can be compromised *in vivo* because of their physicochemical properties, such as being highly hydrophobic with poor solubility in both water and oil at body temperature. Therefore, encapsulating and developing a delivery systems for these compounds would aid in enhancing their bioavailability *in vivo* so bioactive compounds can reach the intended target tissue.

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