ANTIHYPERTENSIVE PROPERTIES OF FLAVONOID-RICH APPLE PEEL EXTRACT, SELECTED FLAVONOIDS AND METABOLITES *IN VITRO*

by

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Signature of Author

DEDICATIONS

To my beloved parents, my loving sister and brother,
For holding me from your love,
For hugging me from your thoughts,
For not letting me down at any moment,
Without you this would only be a dream which I could never see infront of my eyes…

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ABSTRACT

Hypertension is a major public health problem rising across the globe. Inhibition of angiotensin converting enzyme (ACE) is identified as a main therapeutic target in controlling high blood pressure. The current research study investigated the ACE inhibitory property of a flavonoid-rich apple peel extract (FAE), its constituents, selected flavonoids and some quercetin metabolites using a biochemical assay of ACE inhibition and a human umbilical vein endothelial cell (HUVEC) model. FAE, all the tested flavonoids except genistein, and two quercetin metabolites (quercetin-3-*O*-glucuronic acid and quercetin-3-*O*-sulfate) significantly ($p < 0.05$) inhibited ACE. Enzyme kinetic analysis proved that flavonoids are competitive inhibitors over ACE. In the HUVEC model FAE, quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronic acid significantly ($p < 0.05$) inhibited ACE. Overall, FAE and most of the flavonoids tested showed ACE inhibition *in vitro* which needs further investigations using animal and human clinical trials.

LIST OF ABBREVIATIONS USED

ACE	Angiotensin converting enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
ARB	Angiotensin receptor blockers
AT_1	Angiotensin II type 1 receptor
AT_2	Angiotensin II type 2 receptor
CRD	Complete randomize design
CVD	Cardiovascular disease
DF	Dilution factor
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulfoxide
DW/l	Dry weight per liter
F	Fluorescence
FAE	Flavonoid-rich apple peel extract
FAPGG	N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine
FBS	Fetal bovine serum
HCl	Hydrochloric acid
HHL	Hippuryl-$_L$-histidyl-$_L$-leucine
His-Leu	Histidine-leucine
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Half maximal inhibitory concentration
JG	Juxtaglomerular apparatus
LC-MS	Liquid chromatography and mass spectrometry
N.D	Not determined
NaOH	Sodium hydroxide
NO	Nitric oxide
PE	Plasma protein extravasation
Pen-strep	Penicillium streptomycin
RAAS	Renin angiotensin aldosterone system
RAEC	Rat aortic endothelial cells
SD	Standard deviation
SHR	Spontaneously hypertensive rats
SH-SY5Y	A cell line derived from human neuroblastoma cells
SIM	Single ion monitoring mode
SNS	Sympathetic nervous system
trypsin-EDTA	Trypsin-ethylenediaminetetra acetic acid
UPLC/MS	Ultra performance liquid chromatography / Mass spectrometry
UV	Ultraviolet

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CHAPTER 1.0 INTRODUCTION

Hypertension is a common progressive disorder leading to chronic diseases like cardiovascular disease (CVD), stroke, renal disease and diabetes. One quarter of the world's adult population is already afflicted with hypertension and the numbers are likely to increase up to 29% within another decade (Mittal and Singh, 2010). As this chronic disorder is asymptomatic in nature, unawareness could lead to fatal conditions such as stroke or heart failure. Frequent measurement of blood pressure is the most convenient way to detect hypertension. In view of the fact that hypertension is a major causative factor for development of chronic diseases, prevention and cure are equally important. Interestingly, hypertension is identified as the most modifiable risk factor in prevention of many chronic diseases (Jiang and Paul, 1997).

Pathogenesis of hypertension could have many causes. Over-activation of the renin angiotensin aldosterone system (RAAS) is a prominent factor among all. Angiotensin converting enzyme (ACE) plays a key role in RAAS by producing angiotensin II (Ang II), which is a potent vasoconstrictor. Therefore, in the past decade inhibition of ACE has become a key therapeutic target in the treatment of hypertension. Common ACE inhibitors currently in use include Captopril, Rampiril, Lisinopril, Enalapril etc. Due to many unacceptable side effects which are associated with the intake of most ACE inhibitory drugs, research continue to seek natural alternatives. The ACE inhibitors are being investigated as dietary supplements which may alleviate hypertension.

Several classes of plant secondary metabolites have shown ACE inhibitory properties *in vitro*. The majority of these compounds belong to the group of plant

compounds known as flavonoids. The basic structure of the flavan nucleus undergoes various chemical reactions (hydroxylation, methoxylation, glycosylation) and gives rise to different classes of flavonoids like flavones, flavonols, anthocyanins, flavan-3-ols, isoflavones and flavanones (Rupasinghe, 2008). The synergistic effect of many compounds present in extracts was found to be effective in ACE inhibition when compared with single compounds (Persson et al., 2009). However, one of the drawbacks in *in vitro* studies is the lack of representation of biological systems. The bioactive molecules are converted into different types of metabolites when ingested into the body. Therefore, studying the metabolites of bioactive compounds *in vitro* could generate more relevant results to biological systems and compensate for drawbacks.

In addition to the circulatory RAAS, several tissues and organs also have shown the ability to synthesize Ang II which is called tissue RAAS. Apart from the chemically driven bio-assays, cell culture model systems are a promising way to investigate both circulatory and tissue RAAS components. Different kinds of endothelial cell lines are widely used in ACE inhibitory studies. Umbilical vein endothelial cells from humans (HUVEC) are the most widely used cell line in many reported studies associated with the RAAS system (Persson et al., 2009 and Ottaviani et al., 2006).

In general, when a therapeutic agent or drug is developed for CVD from plant bioactives, more attention should be paid to the structural properties of the bioactive compounds. Most of the studies conducted on plant-derived ACE inhibitors so far have not focused sufficiently on the relationship between structure and activity or on the type of inhibition. In flavonoids, the presence and position of the hydroxyl groups, the presence and type of the sugar moiety, and the presence of active groups such as

carboxylic groups were found to impact the extent of the enzyme inhibition. Flavonoids were found to interact with the active site of the ACE specifically through the hydroxyl groups which were present (Wagner et al., 1991).

The current research study focused on screening for a potent ACE inhibitor where the sources included flavonoid-rich apple peel extract (FAE), subclasses of flavonoids and selected metabolites of flavonoids. *In vitro* enzyme inhibition assay and a cell culture model were used to study the ACE inhibition. The investigated FAE along with a variety of flavonoids, was designed to assist in identification of promising compounds with the ability to inhibit ACE. Therefore, the research could provide some preliminary understanding for future development of an effective dietary supplement for reducing the risk of hypertension through ACE inhibition.

CHAPTER 2.0 LITERATURE REVIEW

2.1 Hypertension

Hypertension is a rising public health problem across the globe. One-quarter of the world's adult population is afflicted by hypertension and this is likely to increase to 29% by 2025 (Mittal and Singh, 2010). Due to its asymptomatic nature, most people are not aware that they suffer from hypertension. The unawareness could lead to fatal conditions such as stroke or heart attack.

The optimal blood pressure for a healthy individual above 18 years of age is defined as a mean systolic blood pressure < 120 mm Hg and a mean diastolic blood pressure < 80 mm Hg. Blood pressure values greater than these could further be categorized according to the level of severity (Table 2.1). The level of blood pressure varies depending on age, gender, ethnicity, environmental factors and genetics. Average blood pressure is found to be increasing with age and men are reported to have slightly higher levels of blood pressure compared to women (Jiang and Paul, 1997).

Table 2.1: Classification of blood pressure for adults age 18 years or older

Category	Systolic BP (mm Hg)	Diastolic BP (mm Hg)
Optimal	<120	<80
Normal	120-129	80-84
High normal	130-139	85-89
Hypertension		
Stage 1 (mild)	140-159	90-99
Stage 2 (moderate)	160-179	100-109
Stage 3 (severe)	180-209	110-119
Stage 4 (very severe)	>210	>120

Source: He and Whelton, 1997

Hypertension is an independent risk factor for several chronic diseases such as cardiovascular disease (CVD), stroke, renal disease and diabetes. Therefore, it is of great importance to identify and treat hypertension in its early stages. Interestingly, hypertension is identified as the most modifiable risk factor in the prevention of most chronic diseases (Jiang and Paul, 1997). Life style changes, physical exercise, intake of healthy diets and stress management are some common approaches for reducing the risk of hypertension.

2.2 Pathogenesis

The pathology of hypertension could have many causes. For example, increased activity of the renin angiotensin aldosterone system (RAAS) and the sympathetic nervous system (SNS), endothelial dysfunction and genetic influence have been implicated (Oparil et al., 2003). Increased activity of the sympathetic nervous system could trigger high blood pressure by causing rapid contraction of the heart muscle to increase the cardiac output, by increasing the fluid retention in kidneys and by increasing the vascular resistance in blood vessels (Oparil et al., 2003). The impaired production of nitric-oxide (NO) from the endothelium has also been found to increase the risk of hypertension where NO is acting as a vasodilator (Taddei et al., 2000). Among these causes, over activation of RAAS (Figure 2.1) is significant (Hammoud et al., 2007).

2.3 Renin Angiotensin Aldosterone System (RAAS)

The RAAS (Figure 2.1) starts with the production of renin by the juxtaglomerular (JG) cells of the kidney. Production of renin is regulated by several mechanisms. The renal baro-receptor mechanism senses the low blood pressure of the afferent arteriole and generates secretion of renin by JG cells. The changes in chloride ion concentration are

sensed by the macula densa cells and trigger the production of renin by JG cells. Furthermore, sympathetic nervous system activation and negative feedback from angiotensin II (Ang II) initiate renin secretion (Atlas, 2007).

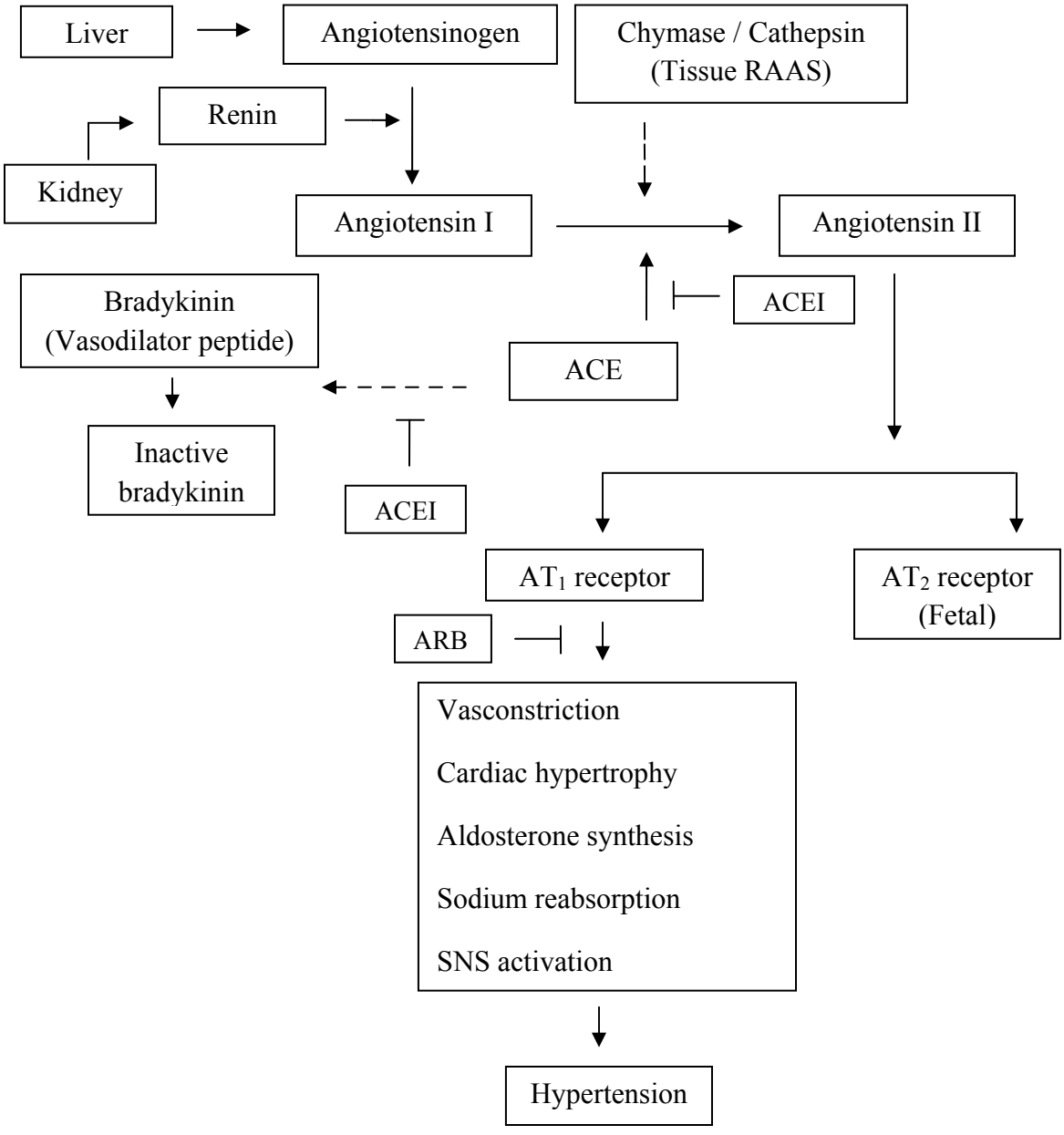


Figure 2.1: A schematic diagram showing the sequential process of the circulatory renin angiotensin aldosterone system (RAAS) (Adopted from Vijayaraghavan and Deedwania, 2011)

Renin cleaves the globular protein, angiotensinogen produced by liver cells, into angiotensin I (Ang I). Angiotensinogen is continuously produced by the liver cells and is present in the circulation at a stable concentration. The conversion of angiotensinogen to Ang I by renin is identified as the rate limiting factor of RAAS where control of renin activity plays a significant role in controlling RAAS (Atlas, 2007).

Ang I does not trigger any blood pressure increasing functions therefore, it is considered as an inactive form. Ang I is hydrolyzed by angiotensin converting enzyme (ACE). The decapeptide (Ang I) is converted into an octapeptide (Ang II) through cleavage by the N terminal amino acids. Ang II is a potent vasoconstrictor (Atlas, 2007). The ACE has the ability to breakdown vasodilator peptides, including bradykinin and kallidin. The overall action of ACE is to promote vasoconstriction. The ACE is a zinc metallopeptidase produced by endothelial cells (Spyroulias et al., 2004) and exists as a membrane bound enzyme but can also be found in the circulatory system. The physiologically important role is provided by the membrane bound form of the enzyme (Atlas, 2007).

The Ang II is responsible for all the blood pressure increasing mechanisms. Ang II receptors are present in various tissues. Several subtypes of Ang II receptors have been identified, of which the type 1 receptor (AT_1) is the most prominent. The type 2 (AT_2) receptors are abundant during fetal development and the expression diminishes in the postnatal period (Atlas, 2007).

The binding of Ang II with AT_1 receptor increases vasoconstriction, cardiac contractility, renal tubular sodium re-absorption, SNS activity and stimulation of aldosterone synthesis (Atlas, 2007), resulting an increased blood pressure. In spite of the

blood pressure increasing mechanisms, Ang II is also responsible for initiating cell growth and proliferation, inflammatory responses and oxidative stress.

The Ang II stimulates the production of aldosterone by stimulating the zona glomerulosa of the adrenal cortex. Aldosterone hormone plays a significant role in maintaining the sodium and potassium balance. Aldosterone facilitates re-absorption of sodium from the distal tubules of the nephrons and collecting ducts. With the absorption of sodium, water is reabsorbed; the resulting blood volume increase ultimately raises blood pressure (Atlas, 2007).

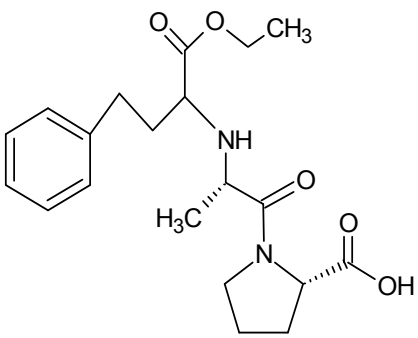
Dysregulation of circulatory RAAS is associated with clinical hypertensive disorders. Therefore, controlling of circulatory RAAS had been identified as a major therapeutic target.

2.4 Tissue RAAS

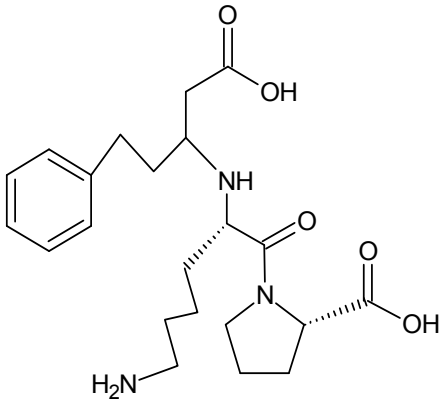
In addition to the circulatory RAAS which plays the prominent role in increasing blood pressure, several tissues and organs (heart, kidney, brain, adrenal gland, adipose tissue) have the ability to synthesize Ang II which is referred as tissue RAAS or local RAAS. The renin enzyme and angiotensinogen are taken up from the circulatory system. The conversion of Ang I to Ang II occurs via non-ACE-mediated pathways. Enzymes like cathepsin G and chymases are involved in this conversion in tissue RAAS (Atlas, 2007). However, there is less evidence concerning the exact role of tissue RAAS in the pathogenesis of hypertension.

2.5 RAAS as a Therapeutic Target

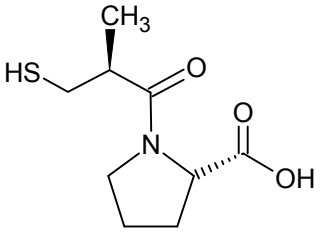
There are several targets identified as blocking the dysregulated RAAS. Inhibition of renin and ACE, blocking the AT_1 and aldosterone receptors are among them. The ACE inhibitors (Figure 2.2) were first developed in the late 1970's as drugs in treatment of hypertension. Renin inhibitors were discovered recently. Aliskiren (TekTurna™) was the first renin inhibitor which was approved by the US Food and Drug Administration (Vijayaraghavan and Deedwania, 2011). Ang II receptor blockers (ARB) target AT_1 receptors. The ARBs currently in use include Candesartan, Irbesartan, Losartan and Telmisartan. Aldosterone antagonists include Spironolactone, Eplerenone and Canrenone (Vijayaraghavan and Deedwania, 2011).



Enalapril



Lisinopril



Captopril

Figure 2.2: Chemical structures of selected ACE inhibitors

2.6 Angiotensin Converting Enzyme (ACE)

Since ACE had been identified as the first and the most popular target for regulating RAAS, researchers were very interested in finding its most effective inhibitor. The structure of the enzyme is the critical factor in designing an inhibitor. The ACE gene gives rise to two types of enzymes, the somatic ACE and the germinal (testis) ACE. Somatic ACE is comprised of a polypeptide chain of 1277 amino acids, where the testis ACE is 701 amino acids long. The polypeptide chain which comprises somatic ACE folds into two structures known as domains N and C which have the ability to function independently. Each domain possesses a zinc binding catalytic site (Natesh et al., 2003). The zinc atom binds to the enzyme via several ligands including histidine and glutamate (Figure 2.3; Corradi et al., 2006). The two domains are similar in amino acid sequence and share common enzymatic properties.

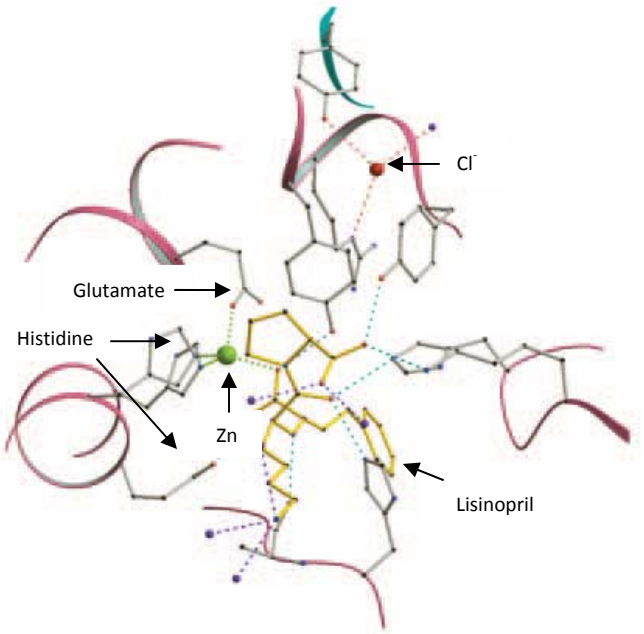


Figure 2.3: Active site of testis ACE with the inhibitor Lisinopril (Adapted from Acharya et al., 2003)

2.7 ACE Inhibition

Drugs were first used as ACE inhibitors and they can be divided into three groups according to their chemical structures, i.e. sulfhydryl containing (Captopril), dicarboxylate containing (Enalpril, Lisinopril, Ramipril) and phosphate containing (Fosinopril; Vijayaraghavan and Deedwania, 2011). The first derived ACE inhibitor was Captopril. The active sulfhydryl group of the drug binds with the zinc atom thereby inhibiting the binding of the substrate. However, intake of Captopril was associated with side effects like proteinuria, altered taste and skin rashes. The carboxyl group of compound inhibitors was produced later as a solution to minimize the side effects. The carboxyl group has shown higher lipophilicity compared to other active groups and this was favoured for the binding with ACE (Atlas, 2007). Though improvements were made, intake of ACE inhibitory drugs causes significant side effects. Other than the above mentioned, dry cough, angioedema, fetal abnormalities, hypotension, deterioration of renal function and hyperkalemia were specified (Atlas, 2007).

Due to the side effects of the currently used drugs, new alternatives have been studied extensively. Research on natural bioactives has gained much attention. Most of the natural bioactives were derived from food components, plant extracts, marine products and microorganisms.

2.7.1 Natural ACE Inhibitors Currently in Use

Different types of naturally derived compounds have been investigated for their ACE inhibitory properties. Protein derivatives are a major group of compounds investigated as potential ACE inhibitors. They can be divided into three categories as animal-derived, plant-derived and microorganism-derived peptides. The animal-derived

category includes peptides from milk, meat, fish and eggs (Hong et al., 2008). Casein, whey protein hydrolysates from milk and ovokinin from eggs are reported to be effective ACE inhibitors in both *in vivo* and *in vitro* studies (Hong et al., 2008 and Yamamoto, 1997). Meat and fish proteins are hydrolyzed using different enzymes such as chymases, and the resulting fractions are used to determine ACE inhibitory properties. Among the fish species used for deriving ACE inhibitory peptides are bonito, sardine, salmon, hake and tuna (Cinq-Mars et al., 2007 and Vercruysse et al., 2005). Plant-derived peptides have also been identified from different sources including soybean, flaxseed, sunflower, rice and corn (Farzamirad and Aluko, 2008, Guan and Phillips, 2009 and Udenigwe et al., 2009). There is less evidence on microorganism-derived peptides.

Secondary metabolites produced in plants are another group of natural compounds which are identified as potential ACE inhibitors. Some terpenoids and polyphenolic compounds including flavonoids, hydrolysable tannins, xanthones, procyanidins, caffeolyquinic acid derivatives are found to be effective as natural ACE inhibitors (Kang et al., 2003 and Loizzo et al., 2007). Most studies have shown that plant extracts rich in phytochemicals were effective in ACE inhibition. However, identification of compounds which specifically inhibit ACE is lacking.

2.7.2 Plant Flavonoids as ACE Inhibitors

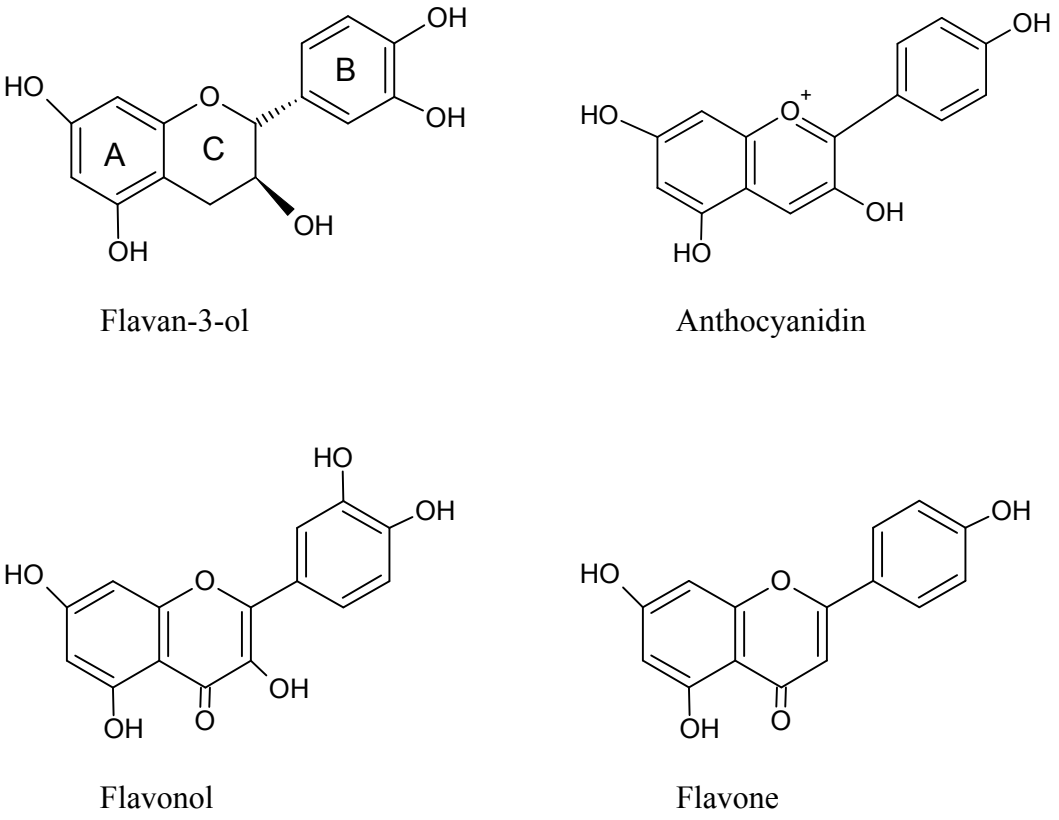
Flavonoids are the largest group of polyphenolic compounds found in higher plants (Croft, 1998). Tea, wine, apples, onions, grapes, and oranges are some foods rich in flavonoids. The biosynthesis of flavonoids occurs in higher plants through the shikimic acid and malonic acid pathways (Rupasinghe, 2008).The common structure of flavonoids is comprised of two phenyl rings (A and C rings) joined with three carbons which make a

closed pyran ring structure (B ring; D'Archivio et al., 2007). Based on the structural differences, flavonoids are further subdivided into six sub-groups namely flavanones, flavones, flavonols, flavan-3-ols, anthocyanins and isoflavones (D'Archivio et al., 2007). The highly diverse structures of flavonoids show numerous functions in biological systems. In plants, flavonoids contribute to insect attraction and repulsion through colour of leaves, fruits and flowers; protection against viral, fungal and bacterial infections and UV light; nodulation in legume roots etc. (Stevenson and Hurst, 2007).

Flavonoids are effective antioxidants in plants as well as in animals (Croft, 1998). Flavonoids in the diet are identified as potential risk reducing components for cardiovascular disease, various cancers, neurodegenerative diseases, etc. (Stevenson and Hurst, 2007). For example, quercetin-3-*O*-glucoside, a flavonoid compound ubiquitous in fruits, has shown a protective effect on human neuroblastoma cells (SH-SY5Y). It limits oxidative stress through a membrane injury recovery mechanism that is involved in the up-regulation of genes involved in lipid and cholesterol synthesis (Soundararajan et al., 2008).

2.7.2.1 Anthocyanins

Anthocyanins are water soluble plant pigments giving rise to red, blue and purple colours of fruits and vegetables. In plants, they occur as anthocyanidins (aglycone form, Figure 2.4) and then conjugate with sugars to form anthocyanins (D'Archivio et al., 2007). Anthocyanins have shown ACE inhibition *in vitro*. Delphinidin-3-*O*-sambubiosides and cyanidin-3-*O*-sambubiosides isolated from Hibiscus (*Hibiscus sabdariffa* L.) extracts have been shown to inhibit ACE in a dose dependent manner (Ojeda et al., 2010).



Flavan-3-ol Anthocyanidin

Flavonol Flavone

Figure 2.4: Basic structures of selected major flavonoids

The IC_{50} (half maximal inhibitory concentration) values of anthocyanins were found to be in 100 to 150 μM range (Table 2.2; Ojeda et al., 2010). Similarly, cyanidin-3-*O*-β-glucoside isolated from rose species (*Rosa damascena*) inhibited ACE *in vitro*. However, other flavonols isolated from rose extract were not effective ACE inhibitors when compared to cyanidin-3-*O*-β-glucoside (Kwon et al., 2010). Bilberry (*Vaccinium myrtillus*) extracts rich in major anthocyanins i.e. cyanidin, delphinidin and malvidin, were investigated for their effect on ACE in a HUVEC model and the ACE activity was significantly reduced after incubation of cells with bilberry extracts (Persson et al., 2009). Dietary administration of anthocyanin-rich (cyanidin-3-glucosides, cyanidin-acyl-glucoside and peonidin-acyl-glucoside) purple corn, purple sweet potato and red radish to spontaneously hypertensive rats (SHR) decreased their systolic and mean blood pressure

(Shindo et al., 2007). The mechanisms behind the reduction of blood pressure by anthocyanins were reported to be due to their antioxidant activity, preservation of endothelial nitric oxide, and prevention of serum lipid oxidation. However, ACE inhibition was not found (Shindo et al., 2007).

The observed ACE inhibitory activity of anthocyanins *in vitro* could be explained by the metal chelating ability of flavonoids with hydroxyl groups at 3, 5, 7 and 3′, 4′ positions (Kwon et al., 2010 and Persson et al., 2009). The planer structure of the anthocyanin molecules were considered to be important in metallopeptidase inhibition (Ojeda et al., 2010). In animals, the absorption rate and the corresponding metabolites of anthocyanins affect enzyme inhibition. However, a strong correlation between ACE inhibition *in vitro* and animal model systems has not been reported.

2.7.2.2 Flavan-3-ols

Flavanols have a saturated C-ring with a hydroxyl group at the C-3 position (Figure 2.4). They do not exist in glycosylated form as in other flavonoids. They can be found in both monomer form as catechins and polymer form as procyanidins (D'Archivio et al., 2007). When ACE was incubated with flavanol-rich food extracts such as chocolates, tea and wine, a significant correlation between the ACE inhibition and the concentration of procyanidin and epicatechin was observed (Actis-Goretta et al., 2006). The ACE inhibition by epicatechins of cocoa could explain the positive relationship between dark chocolate consumption and reduced high blood pressure (Egan et al., 2010). The four major catechins, (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechingallate and (–)-epigallocatechingallate, isolated from tea have also shown a dose dependent ACE inhibition in a HUVEC culture model (Persson et al., 2006).

Pycnogenol, a procyanidin oligomer, isolated from French maritime pine (*Pinus maritime*) was also reported as an effective mediator for blood pressure regulation, possibly by ACE inhibition (Zibadi et al., 2008). These studies suggest that among flavonoids, flavanols and procyanidins could also act as potent inhibitors of ACE *in vitro*.

The relationship between the structure of flavanols and ACE inhibitory properties *in vitro* has been studied (Ottaviani et al., 2006). Increasing numbers of epicatechin units in the procyanidins had increased the enzyme inhibition. When tested using HUVEC cell cultures, tetramers were the most effective enzyme inhibitors compared to dimers and hexamers of procyanidins (Ottaviani et al., 2006). The monomers of flavanols were found to be absorbed in the small intestine (García-Cornesa et al., 2009). However, absorption of procyanidins with higher molecular weight has not clearly been reported. Though the tetramers were shown to be the most effective *in vitro*, the dimers were more effective in biological systems compared to both tetramers and hexamers (Ottaviani et al., 2006).

2.7.2.3 Flavonols

Flavonols (Figure 2.4) are reported to be the most ubiquitous flavonoid sub-group present in foods. Quercetin, kaempferol and myricetin are the three most common flavonols in our diet (D'Archivio et al., 2007). The ACE inhibitory property of many flavonols has been reported. When a bioassay-guided fractionation of extract of stonecrop (*Sedum sarmentosum*) was performed, five purified flavonols were found to possess ACE inhibitory activity (Oh et al., 2004; Table 2.2). Kaempferol-rich stem bark extracts of Cluster Fig (*Ficus racemosa*) had a dose-dependent ACE inhibition property *in vitro* (Ahmed et al., 2010). Based on an *ex vivo* experiment conducted using aortic tissues of male Wistar-Kyoto rats, kaempferol was found to be an effective ACE inhibitor but not

resveratrol (Olszanecki et al., 2008), a polyphenol that is abundant in red wine. The presence of a carbonyl group in the pyran ring of kaempferol is lacking in resveratrol and this could be a reason for the differences in their ACE inhibitory activity.

However, when strawberry extracts rich in flavonoids were tested for ACE inhibition *in vitro*, no ACE inhibition was observed (Pinto et al., 2008). Aqueous extracts of *Gingko biloba,* which had quercetin derivatives as the major flavonoids, had higher ACE inhibitory activity than that of ethanol extracts of *Gingko biloba* (Pinto et al., 2009). The aqueous extracts of red currents (*Ribes rubrum* L.) and black currents (*Ribes nigrum* L.) exhibited ACE inhibition *in vitro* but not the extracts of red and green gooseberries (*Ribes uva-crispa* L.; Pinto et al., 2010). The variation in the ACE inhibitory activity of plant extracts may be due to the type of flavonoids present and their concentrations, the genetic differences of plant materials and the method of preparation of extracts.

Flavonols act as prominent antioxidants in biological systems. Dietary quercetin supplementation at 730 mg/d for 28 d was found to be effective in reducing blood pressure in hypertensive patients in a randomized, double-blind, placebo-controlled, crossover study (Edwards et al., 2007). In another study, Captopril and quercetin treatments were given to male Wistar rats separately, whose hypertensive responses were triggered by Ang I and bradykinin injections. Bradykinin is a physiologically active peptide that causes blood vessels to enlarge. Both treatments significantly triggered the hypotensive responses and quercetin was equally effective to Captopril when given orally or intravenously (Häckl et al., 2002). A significant reduction of plasma ACE due to quercetin pretreatment (88.7 μmol/kg) was reported in this animal study. In contrast, chronic treatment of quercetin aglycone that was given at 10 mg/kg intraperitoneally for

14 d to rats, did not inhibit plasma ACE activity compared to the control group (Neto-Neves et al., 2010).

ACE is found to be involved in plasma protein extravasation (PE), which is an important component in neurogenic inflammation (Willie et al., 2001). It is known that PE can be evoked by substance P which is hydrolyzed by ACE. Similar to the action of Captopril, dietary supplementation of quercetin can potentiate plasma PE induced by substance P in the rat urinary bladder, possibly by the inhibition of the peptidase which hydrolyzes substance P (Nicolau et al., 2003). From the reviewed literature, flavonols showed potential ACE inhibition both *in vitro* and *in vivo*. However, since flavonols are known to produce sulfate, glucuronide and methylated metabolites *in vivo* (Rupasinghe et al., 2010), ACE inhibition by quercetin metabolites (*in vitro)* requires further investigation.

2.7.2.4 Isoflavones

Isoflavones are unique flavonoids as they exhibit structural similarity to mammalian estrogen hormone. They can effectively bind to the estrogen receptor and are often called phytoestrogens (Jackson et al., 2002). Genistein, daidzein and glycetin are the most common isoflavones present in plants (D'Archivio et al., 2007). Among them, genistein is the isoflavone most widely investigated for its health promoting effects. The major isoflavone in soybean is genistein (Wu and Muir, 2008). Genistein has been reported for reducing blood pressure in research animal models. For example, genistein has decreased NaCl-sensitive hypertension in stroke-prone spontaneously hypertensive rats (Cho et al., 2007).Genistein dose-dependently decreased ACE gene expression and enzyme activity in rat aortic endothelial cells (RAEC), serum and aorta tissue (Xu et al.,

2006). *In vitro* studies showed a concentration-dependent ACE inhibition by genistein which was confirmed by others (Montenegro et al., 2009). However, the presence of isoflavones in ACE inhibitory soybean peptide fractions has not shown any enhanced enzyme inhibitory effect when compared with the peptide fractions without isoflavones. Studies have been conducted using research animal models to investigate the *in vivo* activity of isoflavones. Pretreatment with a single intravenous injection of genistein 25 mg/kg demonstrated reduced hypertensive responses in hypertensive Wistar rats. The reduced hypertension was associated with significant reduction of ACE activity in rat plasma (Montenegro et al., 2009). Another *in vivo* study had shown that genistein can down regulate ACE gene expression by interfering with cell signaling pathways (Xu et al., 2006). However, there are no related studies on the ACE inhibitory effect of two other soybean isoflavones, daidzein and glycetin.

2.7.2.5 Flavones

There is less information about ACE inhibitory properties of flavones compared to other types of flavonoids. However, extracts of Roxb (*Ailanthus excels (roxb)*), Japanese cedar (*Cryptomeria japonica*), (*Hibiscus sabdariffa*) and *Senecio* species (Compositae) which are comprised of flavones have shown the ACE inhibitory property (Table 2.3). The two major flavones of Roxb, apigenin and luteolin, have demonstrated a dose dependent enzyme inhibition. Compared to luteolin aglycone, luteolin-7-*O*-glucoside have reduced enzyme activity comprising to a higher IC_{50} value (Table 2.2; Loizzo et al., 2007). The loss of a hydroxyl group at the 7^{th}position could be the reason for the decreased enzyme inhibition by the glycoside. The ethanol extracts of the outer bark of Japanese cedar inhibited ACE *in vitro* and resulted in an IC_{50} value of 16 μg/mL.

The extract was rich in flavan-3-ols and flavones. The enzyme inhibitory effect could be a result of the synergistic effect of all compounds present in the extract (Tsutsumi et al., 1997). Crude hydro alcoholic extract rich in flavones from *Hibiscus sabdariffa* exhibited satisfactory enzyme inhibition on ACE but not elastase, trypsin and alpha-chymotrypsin (Jonadet et al., 1990). As all the studies discussed were investigating the effect of plant extracts containing flavones, the inhibitory effect could also be due to other constituents of the extract. Specific focus on isolated flavone compounds and their ACE inhibitory activity can generate valuable information about the flavones with ACE inhibition properties.

2.7.2.6 Other Flavonoids

Chalcones are precursor molecules of the biosynthetic pathways of flavonoids (Rupasinghe et al., 2008). These consist of two phenyl rings joined by a three carbon open chain. There are numerous evidences concerning beneficial pharmacological properties of chalcones. Chalcones and their pyrazole derivatives inhibited ACE in a concentration-dependent manner *in vitro* (Bonsei et al., 2010). Butein, a chalcone, supplementation through intravenous injection has been found to reduce the arterial blood pressure in anesthetized normotensive rats (Kang et al., 2003). The ACE activity decreased in a dose dependent manner; however, the IC_{50} value of butein seems to be significantly greater than other flavonoids (Table 2.2).

Table 2.2: IC_{50} values of ACE inhibitory flavonoids

Group of Flavonoids	Compound	IC_{50} Value	Reference
Anthocyanins	Delphinidin-3-*O*-sambubioside	142 µM	Ojeda et al., 2010
	Cyanidin-3-*O*-sambubioside	118 µM	Ojeda et al., 2010
	Cyanidin-3-*O*-β-glucoside	139 µM	Kwon et al., 2010
Flavones	Apigenin	280 µM	Loizzo et al., 2007
	Luteolin	290 µM	Loizzo et al., 2007
	Luteolin-7-*O*-glucopyranoside	280 µM	Loizzo et al., 2007
Flavonols	Quercetin glucuronide	200 µM	Kiss et al., 2004
	Quercetin-3-*O*-(6′′-galoyl)-galactoside	160 µM	Kiss et al., 2004
	Quercetin-3-*O*-α—(6′′′-caffeoylglucosyl-β-1,2-rhamnoside)	159 µM	Oh et al., 2004
	Quercetin-3-*O*-α—(6′′′-*p*-coumaroylglucosyl-β-1,2-rhamnoside)	352 µM	Oh et al., 2004
	Isorhamnetin-3-β-glucopyranoside	409 µM	Oh et al., 2004
	Quercetin-3-β-glucopyranoside	709 µM	Oh et al., 2004
	Quercetin-3-α-arabinopyranoside	310 µM	Loizzo et al., 2007
	Kaempferol-3-α-arabinopyranoside	393 µM	Oh et al., 2004
Flavan-3-ols	Epicatechin - tetramer	4 µM	Ottaviani et al., 2006
	Epicatechin - hexamer	8 µM	Ottaviani et al., 2006
Chalcones	Butein	730 µM	Kang et al., 2003

Table 2.3: IC_{50} values of ACE inhibition by plant extracts

Plant Extracts	IC_{50} Value	Reference
Hibiscus sabdariffa (Hibiscus)	91 µg/mL	Ojeda et al., 2010
Camelia synensis (Green tea)	125 µg/mL	Sakaida et al., 2007
Vaccinium asheireade (Blueberry leaf extract)	46 µg/mL	Sakaida et al., 2007
Vaccinium myrtillus (Bilberry)	Log-2.6 mg/mL	Persson et al., 2009
Senecio inaequidens (A perennial herb)	192 µg/mL	Loizzo et al., 2009
Senecio ambiguous subsp. *Ambigus* (Ethyl acetate extract)	219 µg/mL	Loizzo et al., 2009
Senecio ambiguous subsp. *Ambigus* (n-hexane extract)	307 µg/mL	Loizzo et al., 2009
Cryptomeria japonica (Japanese cedar)	16 µg/mL	Tsutsumi et al., 1997

2.8 Enzyme Kinetic Studies

Some studies have focused on identifying the type of enzyme inhibition of flavonoids. All compounds studied were in accordance with the Michaelis-Menten theorem. Anthocyanins have shown a competitive inhibition over ACE. Delphinidin-3-*O*-sambubioside, cyanidin-3-*O*-sambubioside, and anthocyanin rich fractions from *Hibiscus* species were among the samples studied (Ojeda et al., 2010). A kinetic study conducted to find the effect of dimers and tetramers of procyanindins at the presence of chloride ions had found a competitive type enzyme inhibition irrespective of the presence of chloride ions (Ottaviani et al., 2006). The dimers and hexamers of the epicatechins were found to be competitive inhibitors. The inhibition over two types of substrates (HHL: hippuryl-$_L$-histidyl-$_L$-leucine and FAPGG: N-(3-[2-furyl]acryloyl-phenylalaglycy L glycine) was studied and no difference was observed between the substrates (Actis-Goretta et al., 2003). Most flavonoids were reported to be competitive type inhibitors. A group of tannins (procyanidin B-5 3,3'-di-*O*-gallate and procyanidin C-1 3,3',3"-tri-*O*-gallate) isolated from *Rhei rhizoma* showed reversible and non-competitive type of

inhibition over ACE. The inhibitory kinetics in this case were determined using Dixon plots (Uchida et al., 1987). There is little evidence associated with the enzyme kinetics of specific flavonoids compared to other types of natural inhibitors such as peptides (Udenigwe et al., 2009). Flavan-3-ols and anthocyanins were the only groups identified on when kinetic studies had been performed in relation to ACE inhibition (Ojeda et al., 2010).

2.9 Models of ACE Inhibition

By considering the above mentioned studies, it can be noted that both *in vitro* and *in vivo* models have been used to assess the ACE inhibition. *In vitro* assays are the starting point of the experimental process. Based on the results, experiments could be carried out to further confirm the efficacy using *in vivo* models.

In *in vitro* models the enzyme reaction takes place in a controlled environment where optimal conditions for the reaction are provided. A buffer at optimal pH (8.3) is used to facilitate the enzymatic reaction. However, the physiological pH is 7.3. As the enzyme is active at body temperature, the reaction mixture should be incubated at 37 ^{o}C. There are a number of methods used to detect the level of enzyme inhibition. Among them are spectrophotometry, fluorometry, high-performance liquid chromatography (HPLC), radiochemistry and electrophoresis (Alves et al., 2005 and Lahogue et al., 2010). As there is less substrate specificity for ACE, several substrates have been employed for *in vitro* enzyme inhibitory studies. Two commonly used substrates for spectrophotometric and HPLC analysis of ACE inhibitory activity are HHL and FAPGG (Udenigwe et al., 2009 and Wu et al., 2008).The HHL could be used in fluorescence detection methods of ACE inhibition along with fluorescing agents such as *o*-

pthaldialdehyde (Alves et al., 2005). The conversion of internally quenched fluorogenic substrates was reported to be very sensitive in the detection of ACE inhibition. *o*-Aminobenzoylglycyl-p-nitro-phenylalanylproline (Santendreu et al., 2006) and abz-peptidyl-Eddnp (Abz: *ortho* amino benzoic acid. Eddnp: 2,4-dinytrophenyl ethylene diamine) are two examples of fluorogenic substrates (Alves et al., 2005).

Other than *in vitro* studies, cell cultures also have been used widely in ACE inhibition studies. Cell culture system is a cluster of both *in vitro* and *in vivo* model characteristics. The cell is the basic unit of a living organism and the key place where biological reactions occur. Use of cell culture models provides the advantage of studying a biological reaction at its origin. However, as these cells are grown under *in vitro* conditions, it fails to mimic the exact biological condition.

Different kinds of endothelial cell lines are widely used in ACE inhibitory studies. Umbilical vein endothelial cells from humans are the most widely used cell line in most studies (Ottaviani et al., 2006, Persson et al., 2009 and Saijonma et al., 2001). Endothelial cells play a distinct role in responding to physiological stimuli. They maintain vascular tone by releasing vasoconstrictors like Ang II and endothelin, balancing it by releasing vasodilators such as NO (Vijayaraghavan and Deedwania, 2011). The gene regulation of ACE production by the endothelial cells has not yet been fully identified. It has been found that steroids (dexamethasone and aldosterone), platelet activating factor and endothelin-1, induce ACE gene expression in endothelial cells (Saijonma et al., 2001).

The use of umbilical vein endothelial cells has given promising results in ACE inhibitory studies. The HUVEC were found to provide homogenous cell growth and similar physiological characteristics of the endothelium (Johnson and Erdos, 1977). The

abundance of supply as well as the sterility of the veins favor the use of HUVEC. The cell identification could be confirmed by using the positive immune staining of von Willebrand factor and the cobblestone appearance (Ceconi et al., 2007).

CHAPTER 3.0 OBJECTIVES

Hypertension is a progressive disorder leading to many chronic diseases. Over-activation of renin angiotensin aldosterone system (RAAS) plays a significant role in the pathogenesis of hypertension where angiotensin converting enzyme (ACE) plays a key role in producing angiotensin II, which is a potent vasconstrictor. The ACE inhibitors are identified among the most potent antihypertensive drugs. Apart from the treatments on hypertension, this class of drugs is beneficial for several types of cardiovascular diseases (CVD) as well. In addressing this issue, scientists were keen to research new therapeutics with fewer side effects. Plant-based bioactives have gained recognition for their beneficial health effects over a wide range of chronic diseases. Flavonoids are a major group of plant secondary metabolites which have long been recognized for their beneficial health effects. Flavanoids, as well as flavonoid-rich plant extracts, have recently been studied for their ability to inhibit ACE.

The hypothesis of the current study is flavonoids, their metabolites and fruit extracts rich in flavonoids can inhibit ACE *in vitro*. The overall objective is to investigate the ability of flavonoids (representing all subgroups), a set of selected metabolites of flavonoids and an apple peel extract rich in flavonoids (FAE), to act as potential ACE inhibitors, using *in vitro* assays as well as a cell culture model system. The goal of incorporating flavonoid metabolites into the study is to understand the relevance of ACE inhibition by flavonoids *in vitro,* in relation to the *in vivo* systems. The specific objectives include investigation of concentration responsive ACE inhibition, determination of the type of enzyme inhibition and the IC_{50} values of those which show concentration responsive enzyme inhibition. Further, by considering the enzyme activity and the

specific structure of flavonoids, attempts were taken to predict their structure function relationship. A fluorescence-based enzyme inhibition assay was used in determining the enzyme activity over five selected concentrations of test substances. The prescribed drug Captopril, was used for the comparison. The second objective was to select the most promising test material based on the *in vitro* results and detect their ability to inhibit ACE using HUVEC line. The cells were incubated with the test compounds in three selected concentrations and the enzyme activity was determined by analysis of hippuric acid formation using liquid chromatography and mass spectrometry (LC-MS).

CHAPTER 4.0 INHIBITION OF ANGIOTENSIN CONVERTING ENZYME BY FLAVONOID-RICH APPLE PEEL EXTRACT, FLAVONOIDS AND SELECTED METABOLITES

4.1 Abstract

Angiotensin converting enzyme (ACE) is a key component in the renin angiotensin aldosterone system (RAAS) which regulates blood pressure. As the over activation of RAAS is associated with vascular hypertension, ACE inhibition has become a major target for control of hypertension. The research on potential ACE inhibitors is expanding broadly and most is focused on natural bioactives like plant polyphenolics. The current study is investigating the ACE inhibitory property of a flavonoid-rich apple peel extract (FAE) and its constituents, selected flavonoids and some flavonoid metabolites. Enzyme inhibition was determined using a fluorescence based assay in the presence of histidine-$_L$-hippuryl-$_L$-leucine-chloride (HHL) substrate. At the presence of ACE, HHL is cleaved into several products including the dipeptide, histidine-leucine. Histidine-leucine forms a fluorescing adduct at the presence of o-pthaldialdehyde which was quantified fluorimetrically. Among the polyphenolics tested, except for coumarin and genistein, all the flavonoids that were tested showed a significant ($p < 0.05$), concentration responsive enzyme inhibition. Studying on flavonoid structure-ACE inhibition relationships revealed that the presence of hydroxyl groups and ring B in the flavonoid structure is important for the functionality. The lowest IC_{50} values were associated with quercetin-3-*O*-glucoside (71 μM), epicatechin (73 μM) and naringenin (78 μM) respectively. The FAE also exhibited a concentration responsive enzyme inhibition giving an IC_{50} value of 49 μg/ml. Among the three metabolites tested, only quercetin-3-*O*-glucuronic acid and quercetin-3-*O*-sulfate showed concentration responsive enzyme inhibition. Interestingly, the metabolite quercetin-3-*O*-glucuronic acid (27 μM) was the most effective ACE inhibitor among all the flavonoids and the metabolites. Enzyme kinetic analysis proved that flavonoids show a competitive inhibition over ACE. The results demonstrated that flavonoids have the potential to inhibit ACE *in vivo* and the inhibitory property depends on the specific structure of each flavonoid.

Keywords: ACE, RAAS, flavonoids, quercetin-3-*O*-glucoside, hypertension, quercetin-3-*O*-glucuronic acid,

4.2 Introduction

Hypertension is a common progressive disorder leading to several chronic diseases such as cardiovascular disease (CVD), stroke, renal disease and diabetes. Over activation of renin angiotensin aldosterone (RAAS) is found to be a major causative factor in the development of hypertension (Hammoud et al., 2007). The angiotensin converting enzyme (ACE) plays a significant role in RAAS, by converting the precursor angiotensin I (Ang I) into angiotensin II (Ang II) which is the peptide responsible for the mechanisms which increase the blood pressure. Inhibition of ACE is a promising way of controlling the over activation of RAAS. There are prominent ACE inhibitory drugs currently in use for treatment of hypertension, including Captopril, Rampiril and Enalpiril (Quan, 2006). However, research continues to investigate the possibility of inhibiting ACE by natural bioactive molecules. Plant secondary metabolites such as anthocyanins (Ojeda et al., 2010), flavanols (Actis-Goretta et al., 2006) and terpenes (Somova et al., 2003) were found to be exhibiting ACE inhibitory activity. Although there had been research on natural bioactive molecules to determine the ACE inhibitory property of these compounds, less attention has been paid to the type of inhibition, structure-activity relationship and the mode of action of these compounds.

Flavonoids are a diverse group of compounds commonly found in fruits. The highly diverse structures of flavonoids affect numerous functions in biological systems. A recent review reveals that most subclasses of flavonoids, as well as plant extracts rich in flavonoids, are found to be effective ACE inhibitors both *in vitro* and *in vivo* (Balasuriya and Rupasinghe, 2011).

Apples are one of the most popular and frequently consumed fruits in the world. Apples are a rich source of flavonoids with proven health benefits. In North America, 22% of dietary phenolics (mainly flavonoids and phenolic acids) come from consumption of apples (He and Liu, 2008). The polyphenolic content of apple peel was found to be six times higher than the flesh (Boyer and Liu, 2004). Apple peel extracts had higher antioxidant activities compared to apple flesh extracts (He and Liu, 2008). Since the peel is higher in bioactivity compared to pomace, it is interesting to determine the potential of apple peel extract to act as an ACE inhibitor.

Dietary flavonoids are subjected to metabolic conversions when ingested. Their bioactivity determined *in vitro* was found to be different when compared to the bioactivity of metabolically converted forms (Santos et al., 2008). Investigating biological activity of metabolites using *in vitro* systems could generate more reasonable and relevant information rather than studying the bioactive compounds *in vitro*. Flavonoids such as quercetin are subjected to glucuronidation, sulfation and methylation when absorbed by vertebrates (Rupasinghe et al., 2010).

In the current study, a flavonoid-rich apple peel extract (FAE), its constituents, selected flavonoids and metabolites were investigated for their ACE inhibitory property *in vitro*. The enzyme kinetic parameters were determined using the selected best inhibitors.

4.3 Materials and Methods

4.3.1 Chemicals and Reagents

The ACE extracted from rabbit lung, histidine-$_L$-hippuryl-$_L$-leucine-chloride (HHL), histidine-leucine (His-Leu), NaOH, HCl, ethanol anhydrous, quercetin, quercetin-

3-*O*-glucoside, quercetin-3-*O*-galactoside, naringenin, Captopril, chrysin and o-phaldialdehyde were purchased from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada). Cyanidin-3-*O*-galactoside and epicatechin were purchased from Indofine Chemical Company Inc. (Hillsborough, NJ, USA). Cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-rhamnoside and quercetin-4′-*O*-glucoside were obtained from ChromaDex Corporate (Irvine, CA, USA). Catechin and coumarin were from Fluka Chemicals (Buchs, Switzerland) and were purchased through Sigma Aldrich, Canada. Borate saline buffer (100 mM boric acid, 1.5 M sodium chloride, sterile, pH adjusted to 8.3) was ordered from Teknova (Hollister, CA, USA). The *in vivo* quercetin metabolites were kindly provided by Dr. Paul Kroon, Project Leader of Polyphenols and Health, Institute of Food Research, Norwich Research Park, Norwich, UK.

4.3.2 Sample Preparation for Assays

4.3.2.1 Flavonoid-rich Apple Peel Extract (FAE)

The FAE was extracted from ‘Jonagold’ apple peels according to the method described by Rupasinghe et al., (2010). The apple peel extract was in the form of freeze dried powder and the solutions were made by dissolving them in anhydrous ethanol. A stock solution of 1000 mg DW/l (equals 56 mg of phenolics/l) from the extract was prepared first and diluted accordingly. The major constituents of the extract was investigated separately which are discussed in the following sections.

4.3.2.2 Flavonoids

The major flavonoid constituents of the FAE were quercetin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, qurecetin-3-*O*-rutinoside, cyanidin-3-*O*-galactoside, catechin and epicatechin (Table 4.1).

Table 4.1: Composition of the FAE prepared from 'Jonagold' apple peels

Polyphenolic subclasses	Compound	Polyphenolic concentration* (mg/g DW)
Flavanols	Quercetin	1.10 ± 0.1
	Quercetin-3-*O*-galactoside	11.67 ± 0.7
	Quercetin-3-*O*-glucoside	2.33 ± 0.2
	Quercetin-3-*O*-rhamnoside	12.78 ± 0.5
	Qurecetin-3-*O*-rutinoside	1.66 ± 0.1
	Total quantified flavonols	29.50 ± 1.6
Favan-3-ols	Catechin	1.18 ± 0.1
	Epicatechin	7.74 ± 0.5
	Epigalocatechin	0.09 ± 0.0
	Epicatechingalate	0.04 ± 0.0
	Epigalocatechingalate	0.05 ± 0.0
	Total quantified flavan-3-ols	9.11 ± 0.6
Anthocyanins	Cyanidin-3-*O*-galactoside	1.68 ± 0.1
Dihydrochalcones	Phloridzin	7.48 ± 0.4
	Phloretin	0.13 ± 0.0
	Total quantified dihydrochalcones	7.6 ± 0.4
Phenolic acids	Chlorogenic acid	8.52 ± 0.8
Total phenolics analyzed by LC-MS/MS		56.45

*Data are presented as mean ± SD

Flavonoids representing each category of flavonoid group were investigated for ACE inhibition. Quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-4′-*O*-glucoside, quercetin-3-*O*-rutinoside and quercetin-3-*O*-rhamnoside belonged to the flavonol group. Cyanidin, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside and cyanidin-3-*O*-rhamnoside were representing the anthocyanins. Flavan-3-ol group included (±)catechin and epicatechin. Chrysin was representing the flavone

group and naringenin was from the flavanones. Genistein was representing isoflavones. Coumarin which represents A and C rings of flavonoid compounds was also included for comparison purposes.

All compounds were dissolved in anhydrous ethanol. Dilution series of each compound were prepared. The appropriate volumes of compound solutions were transferred into the Eppendorf tubes, N_2 flushed and reconstituted in ethanol and assay buffer.

4.3.2.3 Quercetin Metabolites

The stock solutions (1000 mg/L) of quercetin metabolites were (quercetin-3´-sulphate, quercetin-3-*O*-glucuronic acid and isorhamnetin-3-*O*-glucuronic acid) prepared in anhydrous methanol. The metabolites were reconstituted in ethanol and assay buffer when used in the ACE assay.

4.3.3 Preparation of Enzyme and Substrate

One unit of enzyme was dissolved in 12 ml of sodium tetraborate buffer (assay buffer). The enzyme solution was divided into 1 ml portions and stored at -80 ^{o}C. Thirty microliters of enzyme was used in each experimental unit. The substrate solution (7.8 mM) was prepared by dissolving HHL in the assay buffer and stored at -20 ^{o}C.

4.3.4. ACE Inhibition

4.3.4.1 Enzyme Inhibition Assay

The ACE inhibitory activity of flavonoids, metabolites and FAE were performed according to the methods of Cinq-Mars et al. (2007) and Santos et al. (1985), with some modifications. The substrate (150 µl) was added to reconstituted samples and mixed by tapping the Eppendorf tubes. Next, 2.5 mU of ACE were added to each experimental

unit. The enzyme was mixed by pipetting in and out several times. A blank and a positive control were used for each experiment. There were no ACE inhibitors present in both controls. In blanks, all reagents were added except the enzyme and the inhibitors. Thirty microliters of assay buffer was used to replace the enzyme. In the positive control, all the reagents were added except the ACE inhibitors. During each experimental run, 10 mg/l solution of Captopril was also used for comparison. All the experimental units (controls and samples) were incubated at 37 ^{o}C using a shaker oven (Model: HP 50, Appolo Instrumentation for Molecular Biology, CA, USA) for 1 h. One hundred and fifty microliters of 0.35 M NaOH was added to each Eppendorf tube to stop the enzyme reaction after 1 h.

4.3.4.2 Measurement of Fluorescence

One hundred microliters of o-phaldialdehyde was added to each Eppendorf tube to make the fluorescent adduct. All the experimental units were kept for 10 min at room temperature to facilitate the reaction. Fifty microliters of 3 M HCl was added to stop the reaction. Samples (100 µl) were loaded into a 96-well polystyrene plate. Fluorescence was measured using the FLUOstar OPTIMA plate reader (BMG Labtech Inc., Offenburg, Germany). Excitation and emission wavelengths used were 360 nm and 500 nm, respectively.

4.3.4.3 Calculation of % Enzyme Inhibition

The mean fluorescence values of all controls and samples were obtained. The % inhibition of enzyme was expressed in comparison with the positive control. The equation is as follows.

Percent enzyme inhibition (%) = $\{1-(F_{sample}-F_{blank})/(F_{positive\ control}-F_{blank})\}*100$

(F: fluorescence)

4.3.4.4 Calculation of IC_{50} Values

Using Microsoft Excel (2007) linear regression analysis, the dose response data were used to calculate the IC_{50} values.

4.3.5 Enzyme Kinetic Study

The four test compounds which gave the highest enzyme inhibition (quercetin-3-*O*-glucoside, epicatechin, quercetin-3-*O*-glucuronic acid, and FAE) were selected for the enzyme kinetic study.

4.3.5.1 Obtaining Standard Curve for His–Leu

Concentration gradient (0.125, 0.25, 0.5, 1, 2 mM) of His-Leu was prepared. One hundred microliters of o-phaldialdehyde was added to each and incubated for 10 min at room temperature. Reaction was stopped by adding of 1 M HCl and the fluorescence was measured. The obtained fluorescence values were plotted against the His-Leu concentration to obtain the standard curve (Figure 4.1).

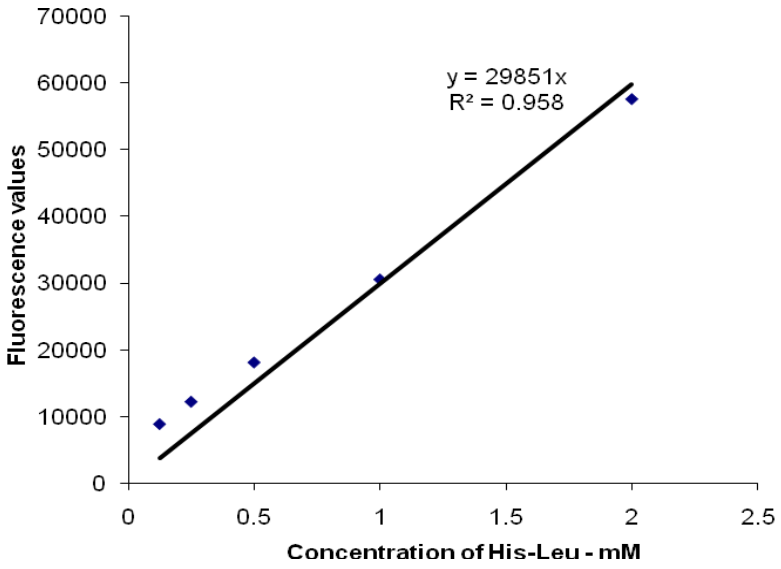


Figure 4.1: Standard curve for His-Leu

4.3.5.2 Enzyme Kinetic Analysis for ACE without Inhibitors

A serial dilution (0.125, 0.25, 0.5, 1, 2, 4, 8 mM) of HHL was prepared. The ACE (2.5 mU) was added to react with 150 µl of each substrate for 1 h at 37 ^{o}C. The reaction was stopped by the addition of 150 µl of NaOH and the formation of fluorescence adduct was carried out as mentioned above. Using obtained fluorescence values, His-Leu concentrations were calculated.

4.3.5.3 Enzyme Kinetic Analysis of Inhibitors

Quercetin-3-*O*-glucoside (21.5 and 215 µM), epicatechin (34.4 and 344 µM), quecetin-3-*O*-glucuronic acid (210 µM) and FAE (10 and 100 mg/l) were selected for the analysis. Each test material was incubated with different concentrations of HHL as described above. Fluorescence values were used to obtain the His-Leu concentrations. Kinetic parameters were determined according to Michaelis Menten kinetic model.

$$V = \frac{V_{max} * [S]}{(K_M + [S])}$$

Where, V: reaction rate, V_{max}: maximum reaction rate, K_M: Michaelis constant, S: substrate concentration

The reaction rate (formation of His-Leu) was plotted against the different substrate concentrations to obtain the saturation curves. Lineweaver Burk plots were derived using the saturation curves to determine the type of inhibition.

$$\frac{1}{V} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}}$$

Where, V: reaction rate, V_{max}: maximum reaction rate, K_M: Michaelis constant, S: substrate concentration,

Kinetic parameters [K_M, V_{max}, K_i (dissociating / inhibition constant)] were calculated using Sigma Plot 12 (Systat software, Inc., Chicago, IL, USA) software. Dissociating constant was determined using the following equation.

$$m_i = m\frac{(1 + [I])}{K_i}$$

Where, m_i: slope from linear plot from inhibited reaction, m: slope from linear plot from non-inhibited reaction, [I]: concentration of inhibitor, K_i: dissociating constant of the inhibitor

4.4 Statistical Analysis

All treatments were carried on triplicate. Each experiment was run independently twice, to check the repeatability. Data were analyzed using SAS V8 (Cary, NC, USA). One way ANOVA was performed in a general linear model at $p < 0.05$. The assumptions i.e. normality and constant variance were analyzed using Anderson Darling test and obtaining the residuals vs fitted values respectively. Independence was achieved through

randomization. Multiple means comparisons were carried out using Tukey's test, and expressed as mean ± standard deviation (SD).

4.5 Results

4.5.1 Inhibition of ACE

The FAE showed a concentration responsive enzyme inhibition (Table 4.2) with an IC_{50} value of 49 μg/ml. When the constituent flavonoids of FAE were investigated separately, all constituents exhibited concentration responsive enzyme inhibition. Most sub-groups of flavonoids also showed a concentration responsive enzyme inhibition. Table 4.3 gives the enzyme inhibition data for quercetin and its glycosides. Among all the glycosides, the highest % inhibition was associated with quercetin-3-*O*-glucoside. Further, several glycosylated forms of cyanidins were investigated for their concentration responsiveness on ACE inhibition (Table 4.4). All four cyanidins which belong to anthocyanins i.e. cyanidin, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside and cyanidin-3-*O*-rhamnoside showed concentration responsive enzyme inhibition. The two flavan-3-ols, catechin and epicatechin (Table 4.5) showed higher % of enzyme inhibition when compared with both groups of flavonols and anthocyanins (Table 4.3 and 4.4). Chrysin (flavone) and naringenin (flavanone) also showed a concentration responsive enzyme inhibition (Table 4.5). However, the % inhibition of chrysin is not as high as that of flavan-3-ols, flavonols and anthocyanins. Coumarin, which is a core skeleton of flavonoids, and genistein (isoflavone) did not show concentration responsive enzyme inhibition (Table 4.5). However, the 100 mg/l sample of coumarin and genistein showed 47% and 60% of inhibition, respectively, where inhibition percentages are similar in value to some other flavonoids. Table 4.7 shows the different structures of flavonoid

compounds. The structural differences which exist within compounds lead to the changes associated with the bioactivity.

Interestingly, among all the flavonoids, FAE constituents and metabolites tested, quercetin-3-*O*-glucuronic acid was found to be the most effective ACE inhibitor (Table 4.6). Among the three metabolites tested, only quercetin-3-*O*-glucuronic acid and quercetin-3′-sulphate showed concentration responsive enzyme inhibition.

Captopril showed a concentration dependent inhibition of ACE (Table 4.2). Interestingly, the concentration range which showed concentration dependent inhibition was the same for both flavonoids and Captopril. However, the drug showed almost 90% enzyme inhibition starting from 1 mg/l concentration and reaching a plateau with the increasing concentrations. In each independent trial, 10 mg/l concentration of drug showed > 90% enzyme inhibition.

Table 4.2: Concentration responsive ACE inhibition by FAE[a] *in vitro*

Concentration (mg/l)	**% Inhibition of ACE**	
	FAE	**Captopril**
0.001	29.9 ± 4.1^{de}	47.6 ± 11.6^{b}
0.01	26.6 ± 6.6^{e}	68.3 ± 9.6^{a}
0.1	37.3 ± 3.9^{cd}	72.9 ± 4.3^{a}
1	42.7 ± 1.1^{bc}	93.6 ± 8.7^{a}
10	45.6 ± 1.7^{b}	91.8 ± 23.7^{a}
100	64.5 ± 0.5^{a}	87.6 ± 21.8^{a}
IC_{50} values	49 μg/ml	0.02 μM

[a] Data are presented as mean ± SD. Mean with different subscripts in each column are significantly different ($p < 0.05$)

Table 4.3: Concentration responsive ACE inhibition by flavonols[a] *in vitro*

Concentration (mg/l)	% Inhibition of ACE					
	Quercetin	**Quercetin-3-*O*-glucoside**	**Quercetin-3-*O*-galactoside**	**Quercetin-3-*O*-rhamnoside**	**Quercetin-3-*O*-rutinoside**	**Quercetin-4´-glucoside**
0.001	19.1 ± 3.6 ^{cd}	42.3 ± 0.8 ^{bc}	21.2 ± 8.6 ^{bc}	8.7 ± 2.5 ^{c}	5.9 ± 4.2 ^{c}	9.8 ± 2.3 ^{bc}
0.01	12.3 ± 3.8 ^{d}	43.4 ± 2.2 ^{bc}	14.3 ± 1.4 ^{c}	9.3 ± 6.0 ^{c}	0.1 ± 2.3 ^{c}	5.7 ± 3.9 ^{c}
0.1	26.8 ± 5.1 ^{c}	39.0 ± 1.1 ^{c}	20.6 ± 2.0 ^{bc}	10.7 ± 1.8 ^{c}	6.7 ± 6.3 ^{c}	9.1 ± 6.5 ^{bc}
1	17.7 ± 1.3 ^{cd}	39.8 ± 4.1 ^{c}	13.2 ± 1.4 ^{c}	12.8 ± 3.6 ^{c}	4.4 ± 4.2 ^{c}	16.7 ± 2.3 ^{bc}
10	30.2 ± 2.5 ^{b}	47.1 ± 1.6 ^{b}	24.5 ± 3.7 ^{b}	19.6 ± 2.1 ^{b}	18.4 ± 3.3 ^{b}	8.9 ± 4.7 ^{bc}
100	80.1 ± 1.8 ^{a}	66.5 ± 3.1 ^{a}	56.0 ± 1.5 ^{a}	49.6 ± 3.2 ^{a}	52.6 ± 1.0 ^{a}	37.2 ± 13.1 ^{a}
IC_{50} values	151 µM	71 µM	180 µM	100 µM	90 µM	211 µM

[a] Data are presented as mean ± SD. Mean with different subscripts in each column are significantly different ($p < 0.05$)

Table 4.4: Concentration responsive ACE inhibition by anthocyanins[a] *in vitro*

Concentration (mg/l)	% Inhibition of ACE			
	Cyanidin	**Cyanidin-3-*O*-glucoside**	**Cyanidin-3-*O*-galactoside**	**Cyanidin-3-*O* rhamnoside**
0.001	21.6 ± 5.8 ^{c}	4.00 ± 5.2 ^{cd}	6.2 ± 3.8 ^{b}	14.4 ± 9.8 ^{b}
0.01	16.5 ± 6.8 ^{bc}	9.0 ± 2.1 ^{bc}	8.0 ± 3.5 ^{b}	12.8 ±7.4 ^{b}
0.1	8.3 ± 3.6 ^{c}	2.4 ± 4.0 ^{d}	8.2 ± 1.3 ^{b}	13.5 ± 1.8 ^{b}
1	18.8 ± 0.8 ^{bc}	5.6 ± 3.5 ^{bc}	8.3 ± 2.1 ^{b}	6.1 ± 7.6 ^{b}
10	27.25 ± 6.8 ^{b}	10.7 ± 1.5 ^{b}	11.1 ± 2.6 ^{b}	13.2 ± 7.4 ^{b}
100	58.1 ± 1.0 ^{a}	32.4 ± 3.8 ^{a}	44.6 ± 4.1 ^{a}	44.5 ± 7.1 ^{a}
IC_{50} values	79 μM	174 μM	206 μM	114 μM

[a] Data are presented as mean ± SD. Mean with different subscripts in each column are significantly different ($p < 0.05$)

Table 4.5: Concentration responsive ACE inhibition by flavan-3-ols, flavonones, flavones and isoflavonoids[a] *in vitro*

Concentration (mg/l)	% Inhibition of ACE					
	Flavan-3-ols		Flavanone	Flavone	Isoflavonoid	
	Epicatechin	Catechin	Naringenin	Chrysin	Genistein	Coumarin
0.001	0 ± 15.4 ^{c}	15.7± 4.2 ^{c}	9.8 ± 3.7 ^{c}	16.8 ± 4.8 ^{b}	17.5 ± 1.1 ^{b}	13.0 ± 11.4 ^{b}
0.01	0 ± 15.1 ^{c}	31.1 ± 2.8 ^{d}	11.8 ± 1.1 ^{c}	9.3 ± 7.1 ^{b}	7.3 ± 2.1 ^{b}	9.7± 0.8 ^{bc}
0.1	9.9 ± 6.2 ^{b}	19.5 ± 3.4 ^{bc}	8.0 ± 5.3 ^{c}	10.3 ± 1.4 ^{b}	2.2 ± 16.0 ^{b}	3.5 ± 1.6 ^{bc}
1	15.1 ± 4.3 ^{b}	19.8± 4.5 ^{bc}	13.2 ± 4.8 ^{bc}	9.4 ± 5.3 ^{b}	5.9 ± 3.2 ^{b}	0 ± 3.04 ^{c}
10	18.3 ± 2.4 ^{b}	23.5± 2.3 ^{b}	24.3 ± 1.8 ^{b}	17.0 ± 3.1^{b}	3.0 ± 6.6 ^{b}	5.7 ± 4.4 ^{bc}
100	65.2 ± 2.9 ^{a}	47.7 ± 4.3 ^{a}	60.1 ± 2.9 ^{a}	37.8 ± 2.9 ^{a}	59.5 ± 1.8 ^{a}	47.3 ± 4.8 ^{a}
IC_{50} values	73 µM	109 µM	78 µM	146 µM	N.D	N.D

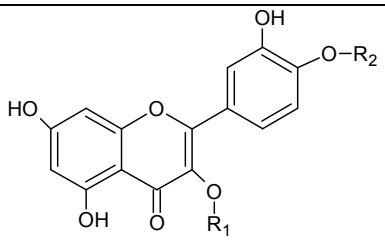
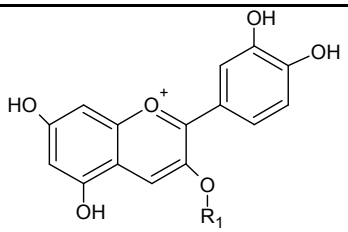
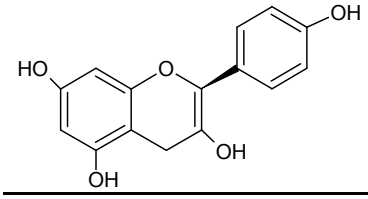
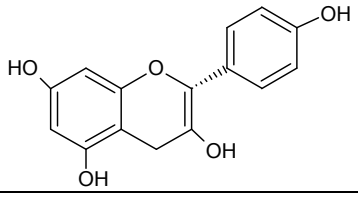
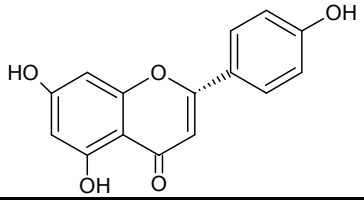
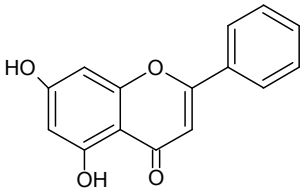
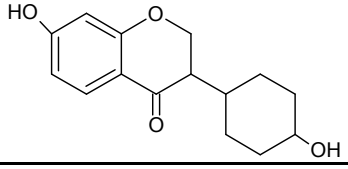
[a] Data are presented as mean ± SD. Mean with different subscripts in each column are significantly different ($p < 0.05$)

Table 4.6: Concentration responsive ACE inhibition by quercetin metabolites[a] *in vitro*

Concentration (mg/l)	% Inhibition of ACE		
	Quercetin-3-*O*-glucuronic acid	**Quercetin-3-*O*-sulphate**	**Isorhamnetin-3-*O*-glucuronic acid**
0.001	43.8 ± 4.1 [c]	10.2 ± 3.1 [b]	10.5 ± 0.9 [ab]
0.01	45.7 ± 2.4 [c]	10.7 ± 11.2 [b]	10.9 ± 2.8 [ab]
0.1	44.4 ± 2.1 [c]	6.5 ± 4.5 [b]	8.6 ± 1.4 [ab]
1	47.5 ± 1.6 [bc]	8.4 ± 2.8 [b]	5.9 ± 6.1 [bc]
10	52.6 ± 4.0 [b]	7.6 ± 5.1 [b]	3.2 ± 3.9 [c]
100	75.1 ± 2.6 [a]	40.4 ± 1.0 [a]	15.9 ± 2.3 [a]
IC_{50} values	27 µM	131 µM	N.D

[a] Data are presented as mean ± SD. Mean with different subscripts in each column are significantly different ($p < 0.05$)

Table 4.7: Molecular structures of flavonoids

Subclass	Structure	R1	R2	Name
Flavonols		H	H	Quercetin
		β-D-glucose	H	Quercetin-3-*O*-glucoside
		H	β-D-glucose	Quercetin-4'-*O*-glucoside
		β-D-galactose	H	Quercetin-3-*O*-galactoside
		β-D-rhamnose	H	Quercetin-3-*O*-rhamnoside
		β-D-rutinose	H	Quercetin-3-*O*-rutinoside
Anthocyanins		H	-	Cyanidins
		β-D-glucose	-	Cyanidin-3-O-glucoside
		β-D-galactose	-	Cyanidin-3-O-galactoside
		β-D-rhamnose	-	Cyanidin-3-O-rhamnoside
Flavan-3-ols		-	-	Catechin
		-	-	Epicatechin
Flavanone		-	-	Naringenin
Flavone		-	-	Chrysin
Isoflavone		-	-	Genistein

4.5.2 IC_{50} Values

The IC_{50} values calculated are shown in Table 4.8. Quercetin-3-*O*-glucuronic acid gave the lowest IC_{50} value.

Table 4.8: IC_{50} values of flavonoids for ACE determined using dose response curves

Compound	IC_{50} value (µM)
Quercetin	151
Quercetin-3-*O*-glucoside	71
Quercetin-3-*O*-galactoside	180
Quercetin-3-*O*-rutinoside	90
Quercetin-3-*O*-rhamnoside	100
Quercetin-4´-*O*- glucoside	211
Cyanidin	79
Cyanidin-3-*O*-glucoside	174
Cyanidin-3-*O*-galactoside	206
Cyanidin-3-*O*-rhamnoside	114
Catechin	109
Epicatechin	73
Naringenin	78
Chrysin	146
Genistein	N.D
Quercetin-3-*O*-glucuronic acid	27
Quercetin-3-*O*-sulphate	131
Isorhamnetin-3-*O*-glucuronic acid	N.D
Coumarin	N.D

N.D: Not determined due to the lack of concentration dependent response

4.5.3 Enzyme Kinetics

The FAE, quercetin-3-*O*-glucoside, epicatechin and queretin-3-*O*-glucuronic acid were selected for enzyme kinetic analysis. The ACE showed a Michalelis-Menten mechanism when enzyme activity was determined using HHL as the substrate (Figure 4.2). Figure 4.3 and 4.4 show the rate of formation of His-Leu, which resulted from the activity of the enzyme over different concentrations of substrate (HHL) in the presence of inhibitors. Since the saturation curves obtained are nonlinear, Lineweaver-Burk plots were created to determine the kinetic parameters (Figure 4.3. C & D and Figure 4.4. C & D). Kinetic parameters i.e. maximum rate of substrate hydrolysis (V_{Max}) and Michaelis

constant (K_M) were calculated (Table 4.9). According to the kinetic parameters and Lineweaver-Burk Plots, quercetin-3-*O*-glucoside, metabolite and FAE showed a competitive inhibition over ACE.

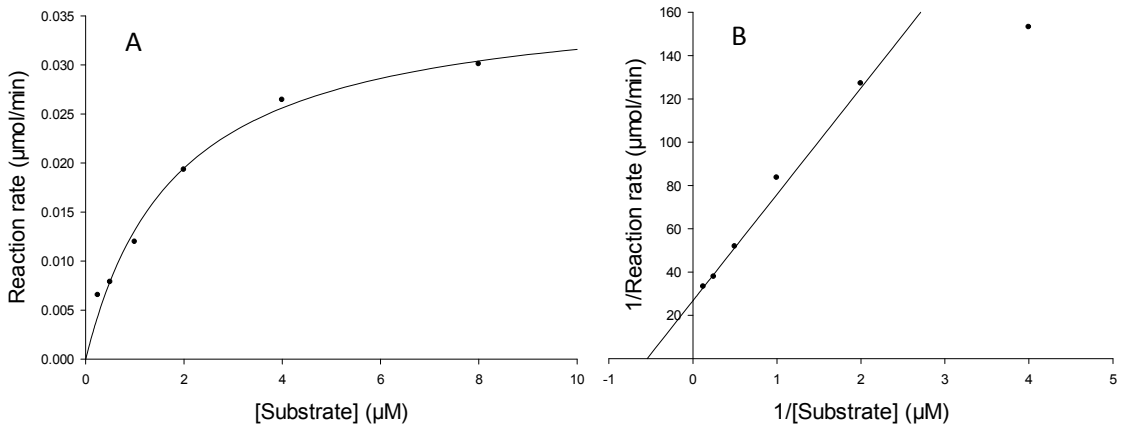


Figure 4.2: Kinetic behaviour of ACE: Michaleis-Menten (A) and Lineweaver-Burk plot (B)

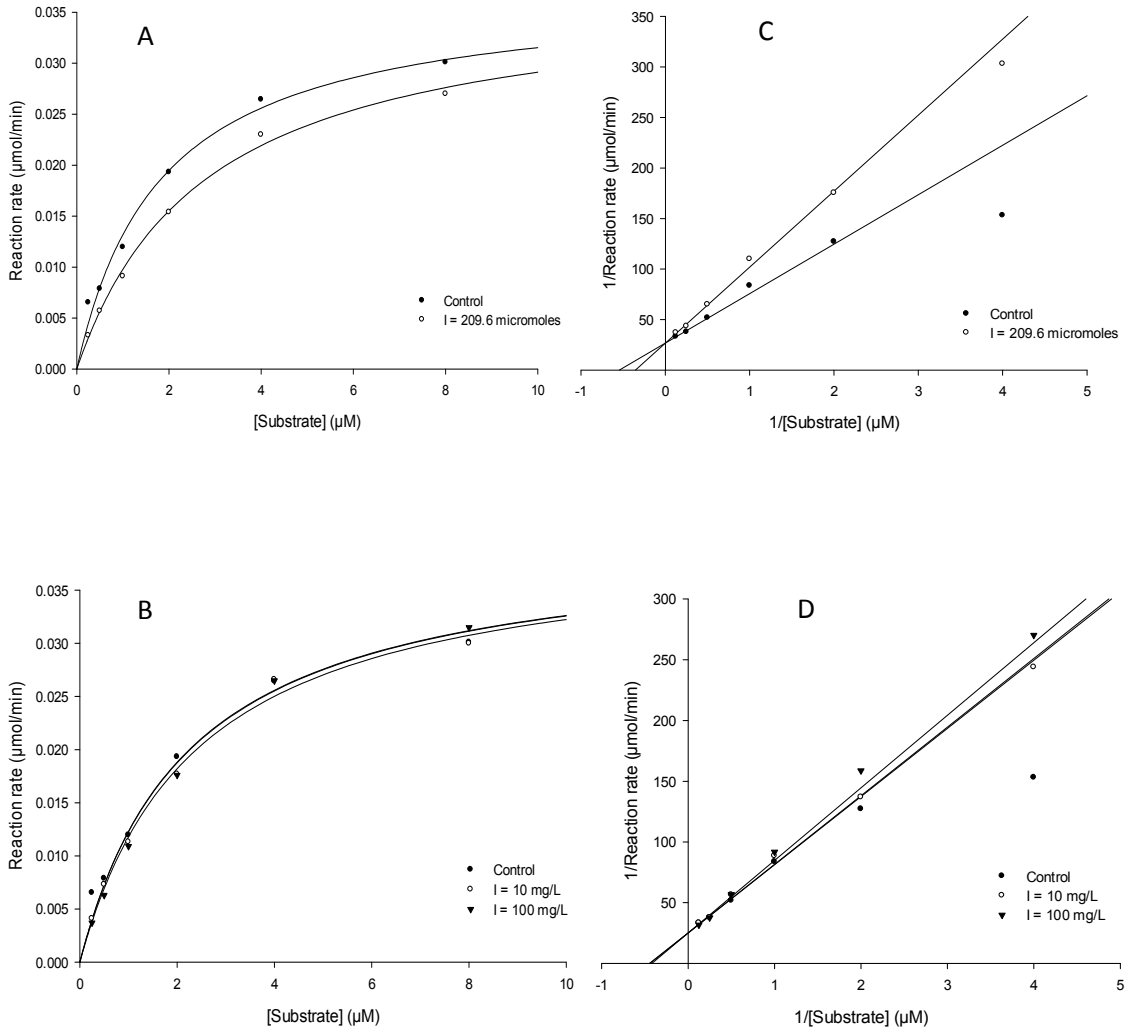


Figure 4.3: Reaction rates for the inhibition of ACE activity by quercetin-3-*O*-glucuronic acid and FAE vs substrate concentration (A and B, respectively) and Lineweaver-Burk plots for quercetin-3-*O*-glucuronic acid and FAE (C and D, respectively)

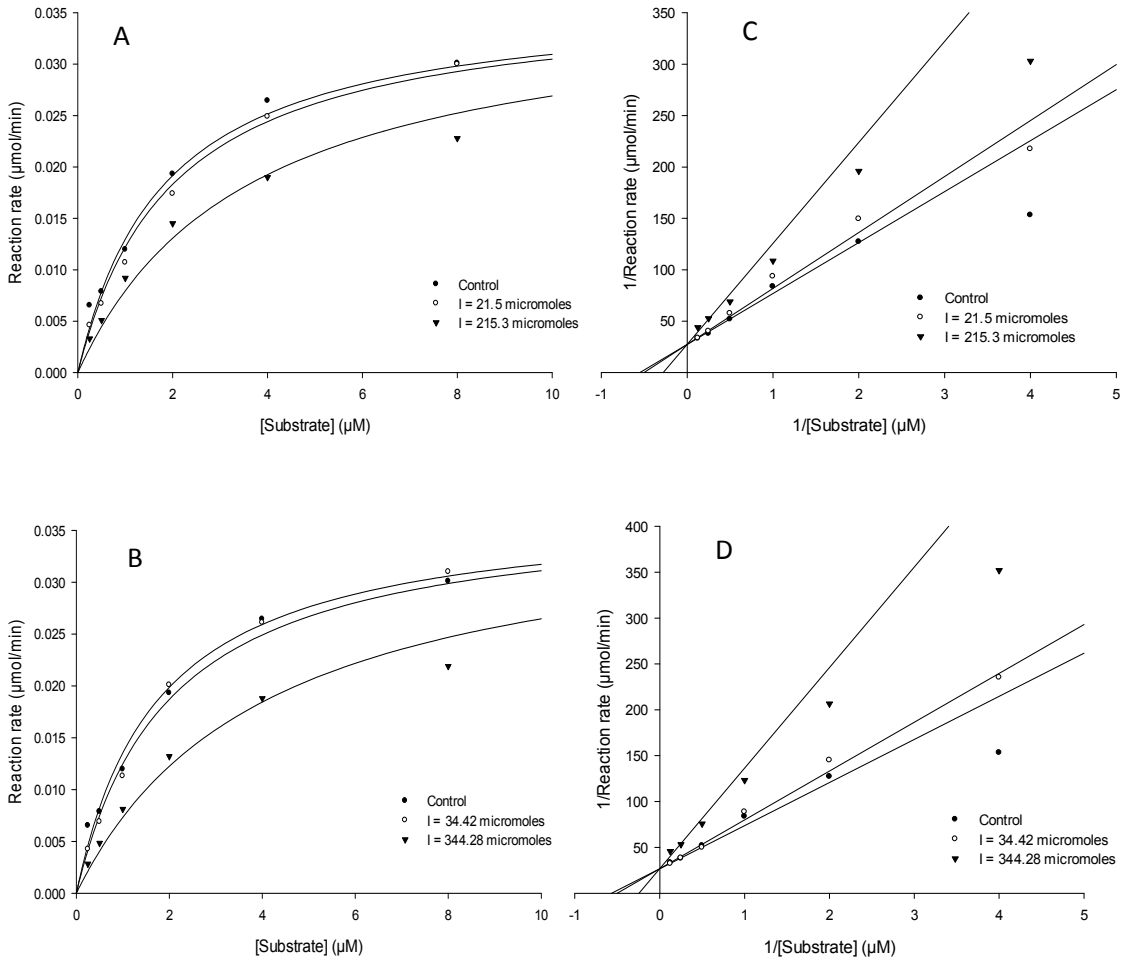


Figure 4.4: Reaction rates for the inhibition of ACE activity by quercetin-3-*O*-glucoside and epicatechin vs substrate concentration (A and B, respectively) and Lineweaver-Burk plots for quercetin-3-*O*-glucoside and epicatechin (C and D, respectively)

Table 4.9: Kinetic parameters of the ACE inhibitory activity of FAE, flavonoids and metabolites

Compound	Concentration	K_M (µM)	V_{MAX} (µM/min)	K_i (µM)
No inhibitor	-	1.8	0.03	-
FAE	100 mg/l	2.6	0.04	-
	10 mg/l	2.6	0.04	-
Quercetin-3-*O*-glucoside	215.3 µM	3.5	0.03	115.4
	21.53 µM	2.1	0.03	19.8
Epicatechin	344.2 µM	4.4	0.04	157.0
	34.42 µM	1.9	0.04	34.8
Quercetin-3-*O*-glucuronic acid	209.6 µM	2.8	0.04	146.6

4.6 Discussion

Hypertension is recognized as a major risk factor for the development of many chronic diseases. Since RAAS plays a significant role in the pathogenesis of hypertension, ACE inhibitors have long been known for their beneficial effects in reducing high blood pressure. Flavonoids were identified as bioactive compounds with cardio-protective effects. In this current study, FAE, different flavonoids and metabolites acted as ACE inhibitors *in vitro*.

Plant extracts had been widely studied for ACE inhibition. Green tea, blueberry, *Hibiscus sabdariffa* and *Senecio inaequidens* extracts were reported to inhibit ACE with IC_{50} values of 125 µg/ml, 46 µg/ml, 91 µg/ml and 192 µg/ml, respectively (Loizzo et al., 2007, Ojeda et al., 2010 and Sakaida et al., 2007). The FAE showed a concentration responsive enzyme inhibition, giving an IC_{50} value of 49 µg/ml. In comparison with most plant extracts reported on ACE inhibition, FAE is an effective ACE inhibitor *in vitro*. It appears that no study has focused on an apple-based extract. Therefore, this is the first report to demonstrate that apple peel extract has a high potential to inhibit ACE *in vitro*.

From the total polyphenolic composition of FAE, more than 50% are flavonoids. Since the constituent compounds also showed concentration responsive enzyme inhibition, it can be postulated that flavonoids are responsible for the bioactivity of FAE. Further, in plant extracts, the synergistic effect of all constituents present i.e. flavonoids, phenolic acids, triterpenes etc. may enhance their bioactivity. In a study conducted to assess the effect of bilberry extracts on the ACE inhibitory property, the extract was found to be a stronger inhibitor compared to the individual compounds alone (Persson et al., 2009).

Since flavonoids being the main target inhibitor for ACE inhibition, flavonoids representing each subclass were incorporated in the study. Quercetin glycosides represented the flavonol group. Quercetin is one of the most studied flavonols and well known for their antioxidant, anti-inflammatory and cardio-protective effects (Erlund, 2004). Quercetin-3-*O*-glucoside was the most effective ACE inhibitor among all the flavonoids with an IC_{50} value of 71 μM. The loss of C-3 hydroxyl group from the aglycone does not appear to have a significant impact on the extent of enzyme inhibition. The aglycone quercetin, as well as other glycosides tested, showed higher IC_{50} values compared with quercetin-3-*O*-glucoside. In previous studies, it was reported that the C-3 hydroxyl group does not play a significant role in ACE inhibition (Tsutsumi et al., 1997). The low ACE inhibitory activity of quercetin-4'-*O*-glucoside could be due to the loss of 4' hydroxyl group. Quercetin-4'-*O*-glucoside is the prominent quercetin glycoside found in onions and whereas quercetin-3-*O*-glucoside is prominent in apples and some other fruits. The common characteristics for all the flavonoid glycosides examined were the presence of heterocyclic oxygen and C-7, C-5 and C-3´ hydroxyl groups. The presence of hydroxyl groups at the C-7 and C-8 positions was found to be effective in ACE inhibition rather than the hydroxyls attached to other positions of the flavonoid structure (Tsutsumi et al., 1997). The type of sugar moiety attached to the aglycone did not show a significant impact on the enzyme inhibition. Since flavonoids are present in glycosylated form in plants, the dietary flavonoids are usually glycosides (Kroon et al., 2004).

Flavan-3-ols are the prominent flavonoids found in all kinds of tea. Consumption of tea has long been recognized for its protective effects on cardiovascular diseases (Rio et al. 2010). Monomeric and polymeric forms of flavan-3-ols were found to be effective

ACE inhibitors *in vitro* (Actis-Goretta et al., 2006). Confirming the previous results obtained for flavan-3-ols, both catechin and epicatechin showed effective inhibition over ACE. Next to quercetion-3-*O*-glucoside, epicatechin showed the lowest IC_{50} value. The tetramers and hexamers of catechins were reported to give IC_{50} values of 4 μM and 6 μM, respectively, which were among the lowest IC_{50} values reported for flavonoids *in vitro* (Ottaviani et al., 2006).

In literature, it has been well reported that anthocyanins and plant extracts rich in anthocyanins showed ACE inhibition in both *in vitro* and cell culture model systems (Ojeda et al., 2010). Among the aglycone and three cyanidin glycosides tested, the lowest IC_{50} value was found with the aglycone cyanidin. The IC_{50} values lie in the same range as flavonols and flavan-3-ols. As in the above mentioned sub groups of flavonoids, in anthocyanidins the positions of hydroxyl groups at C-3, C-5, C-7, C-4′, the ability to make H bonds with the active site, as well as the planer structure of the cyanidin molecules were found to play an important role in ACE inhibition (Kwon et al., 2010 and Ojeda et al., 2010). However, unlike the other two groups, the heterocyclic oxygen molecule is polarized and forms a flavylium cation in anthocyanins (Persson et al, 2009). There is less evidence to understand the role of flavillium cation in ACE inhibition.

Naringenin, chrysin and genistein were representing flavanones, flavones and isoflavones respectively. In all groups, the C-3 hydroxyl group is missing. Both naringenin and chrysin showed concentration dependent enzyme inhibition, giving IC_{50} values of 78 μM and 146 μM, respectively. The obtained IC_{50} values are similar to those reported so far (Loizzo et al., 2007). Genistein showed ACE inhibitory activity in *in vivo*

models (Montenegro et al., 2009). However, in the current study, genistein did not show a concentration responsive inhibition.

Coumarin, where there are no hydroxyl groups and a B ring, did not show any concentration dependent enzyme inhibition. Along with other results, this suggests that in flavonoids, the presence of the B ring and hydroxyl groups is an essential factor in ACE inhibition.

The *in vitro* bioactivity of phytochemicals had been challenged since the *in vivo* metabolism significantly alters the structures of the molecules. However, since *in vivo* experiments such as animal trials and human trials are expensive and time consuming, *in vitro* studies play a vital role in the initial selection of bioactive molecules (Kroon et al. 2004). Bioactive molecules are first subjected to metabolic conversions when absorbed into the enterocytes of the intestinal wall. Glycosylated bioactives are deglycosylated by enzymes like lactase phlorizin hydrolase and β-glucosidases before and after being absorbed into the enterocytes. The absorbed molecules would go through a series of metabolic conversions including methylation, glucuronidation and sulfation (Rupasinghe et al., 2010).

By considering the physiological relevance of metabolites, several quercetin metabolites have been incorporated into this *in vitro* study of ACE inhibition. The quercetin metabolism inside the human body has been well-studied. Therefore, three quercetin metabolites, quercetin-3-*O*-glucuronic acid, quercetin-3-*O*-sulphate and isorhamnetin-3-*O*-glucuronic acid were incorporated for determining ACE inhibition property. Interestingly, quercetin-3-*O*-glucuronic acid gave the lowest IC_{50} value among all the compounds and metabolites tested. It is impressive to observe that one of the

metabolites acts as a potent inhibitor in the *in vitro* system. The structure of quercetin-3-*O*-glucuronic acid is quite similar to quercetin-3-*O*-glucoside except for the carboxylic acid group present in the sugar moiety. Quercetin metabolites were shown to interfere with RAAS and inhibit ACE activity *in vivo*. Several studies reported that quercetin can reduce plasma extravasation by inhibiting ACE and endopeptidases (Cyrino et al., 2002 and Willie et al., 2001). These results could be due to the activity of active metabolites of quercetin such as quercetin-3-*O*-glucuronic acid. However, the other two metabolites did not show a significant impact on ACE inhibition. Though quercetin-3-*O*-sulphate exhibited a concentration responsive enzyme inhibition, the percentage inhibition values were low. Even in previous reports, quercetin-3-*O*-glucuronic acid was identified as the most effective metabolite when compared with different forms of other metabolites (Kroon et al. 2004). Captopril was used as the reference drug throughout the study where the drug was effective even at low concentrations.

In vitro analysis offers the best opportunity to widen the analysis in different directions. Investigating the types of enzyme inhibitions, along with their kinetic parameters could generate valuable information for initiating further developments of these bioactive molecules as therapeutic targets. Therefore, quercetin-3-*O*-glucoside, epicatechin and quercetin-3-*O*-glucuronic acid which were the most effective inhibitors, as well as FAE were subjected to kinetic analysis. Two concentrations were selected for each inhibitor (10 and 100 mg/l) except quercetin-3-*O*-glucuronic acid due to the scarcity of test material.

The V_{max} and K_M for each compound, at different concentrations, were determined to select the type of inhibition. The V_{max} was not significantly altered by each

inhibitor which suggests that it was a competitive type of inhibition. According to the calculated K_i values, the binding affinity of inhibitors to the enzyme did not show much difference.

In our study, most flavonoids, as well as Captopril, demonstrated a concentration responsive enzyme inhibition in a range of 0.001 to 100 mg/l. As for the *in vivo* condition, the physiological concentrations reported for most bioactive molecules were very low, being mostly reported in micromolar or nanomolar range. In a research study, intake of fried onions, which had a quercetin content of 64 mg/g, resulted in 650 nmol/l of metabolites in plasma after 3 h (Hollman et al., 1996). To have these concentrations present in the plasma, the intake of flavonoids would be considerably high since they were metabolized and excreted quickly. After the intake of green tea, catechin metabolites were found to be excreted within 4 h after food intake (Rio et al., 2010).

To support the results in the *in vitro* system further research should include *in vivo* studies. The current results have identified that flavanoids are moderate ACE inhibitors compared to the drug Captopril. The most effective inhibitors identified were quercetin-3-*O*-glucuronic acid, quercetin-3-*O*-glucoside and epicatechin. The FAE was found to be an effective ACE inhibitor *in vitro* when compared with the other plant extracts. The extracts, flavonoids and metabolite showed a competitive type enzyme inhibition where the presence of hydroxyl groups and the B ring were found to be playing an important role on the observed inhibitory activity of flavonoids.

CHAPTER 5.0 EFFECT OF QUERCETIN AND ITS DERIVATIVES ON ACE ACTIVITY IN HUMAN ENDOTHELIAL CELLS

5.1 Abstract

The renin angiotensin aldosterone (RAAS) system is a major pathway for regulating blood pressure in the human body. Angiotensin converting enzyme (ACE) is a key enzyme in RAAS which is responsible for producing angiotensin II which is a potent vasoconstrictor. Inhibition of ACE is a popular target for most antihypertensive drugs currently in use. The ACE is produced by endothelial cells. In the current study, human umbilical vein derived endothelial cells (HUVEC) grown in cultures have been used as a research model system in investigating ACE inhibitory activity of several flavonoids (quercetin, quercetin-3-*O*-glucoside), a metabolite of quercetin (quercetin-3-*O*-glucuronic acid) as well as a flavonoid-rich apple peel extract (FAE) in comparison to the antihypertensive drug Captopril. Captopril showed a dose dependent inhibition of ACE where 0.46 μM and higher concentrations showed more than 90% enzyme inhibition. The FAE, quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronic acid significantly ($p < 0.05$) inhibited ACE when compared to the control. The highest % inhibitions observed for FAE (100 mg/l), quercetin-3-*O*-glucoside (2.1 μM) and quercetin-3-*O*-glucuronic acid (2.1 μM) were 81%, 90%, 89% respectively. However, the low concentrations (0.3 μM and 0.03 μM) of quercetin were not effective in the inhibition of ACE. After the cells were treated with inhibitors, the mean viability of cells was > 85%. Overall, FAE, quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronic acid showed promising results on ACE inhibition but will require further confirmation using an *in vivo* study.

Key words: Renin angiotensin aldosterone system, angiotensin converting enzyme, human umbilical vein endothelial cells**,** quercetin, flavonoid, apple

5.2 Introduction

Hypertension is a leading causative factor for the development of cardiovascular diseases (CVD). Other than being a risk factor for various chronic illnesses, hypertension itself is found to cause extreme damage to internal tissues and organs in humans (Sierra et al., 2011). The underlying mechanisms of pathogenesis of hypertension are closely associated with the endothelium (Shao et al., 2011). The endothelium consists of a cell monolayer which acts as a barrier between the circulating blood and the inner vessel wall cells (Mailloux et al., 2001). Its function is far more than acting as a barrier. The endothelium is also an important endocrine organ in the human body. Endothelial cells produce angiotensin converting enzyme (ACE) which is a key enzyme in the renin angiotensin aldosterone system (RAAS) which is a humoral system involved in the regulation of blood pressure (Atlas, 2007). The ACE produced by endothelial cells is found to be anchored into the plasma membrane of the cell through a hydrophobic region near the C terminus. The enzyme is released to the circulating blood through a regulatory process (Zisman, 1998). Cultured endothelial cells have been used as a research model system to study the activity of ACE (Persson et al., 2009). The presence of the enzyme bound to the membrane, as well as the releasing of enzyme into the medium, provides easy access to the enzyme.

Angiotensin II (Ang II) is the potent vasoconstrictor of RAAS which plays a significant role in increasing blood pressure. Since Ang II is produced by the activity of ACE, it is inevitable that most drugs currently in use are targeting for ACE inhibition (Vijayaraghavan and Deedwania, 2011). Apart from the drugs, dietary intervention of certain peptides and phytochemicals has been identified as a promising way of preventing

hypertension and controlling pre-hypertensive status (Balasuriya and Rupasinghe, 2011 and Farzamirad and Aluko, 2008).

Apples are the most popular tree fruit consumed in North America and have been long known for their health beneficial properties. Apple peel is a rich source of bioactive flavonoids (Boyer and Liu, 2004 and Huber and Rupasinghe, 2009). Consumption of apples was found to reduce the risk of CVD (Boyer and Liu, 2004).

In the present study, a flavonoid-rich apple peel extract (FAE), two constituents of FAE, which are quercetin and quercetin-3-*O*-glucoside and a metabolite of quercetin which is quercetin-3-*O*-glucuronic acid, were also investigated on ACE inhibition *in vitro*.

5.3 Materials and Methods

5.3.1 Human Umbilical Vein Endothelial Cells (HUVEC)

HUVEC were purchased from American Type Culture Collection (ATCC-CRL-2873^{TM}) Manassas, VA, USA. The cells were derived from the umbilical vein endothelium of *Homo sapiens* (human). The cells were categorized under bio-safety level one.

5.3.2 Chemicals and Reagents

Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) which was supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate and 1500 mg/l sodium bicarbonate. To reduce the chances of microbial contamination of cells, penicillin-streptomycin (pen-strep) solution which contained 10,000 IU/ml of penicillin and 10.000 µg/ml of streptomycin was used with the medium. Cell culture tested dimethylsulfoxide (DMSO) was used for cryopreservation of cells and to dissolve the inhibitors. All the

above mentioned chemicals were purchased from ATCC, Manassas, VA, USA. Fetal bovine serum (FBS) was used as a growth supplement with DMEM. A 0.25% trypsin-ethylenediaminetetra acetic acid (trypsin-EDTA) solution was used as the dissociating agent for the detachment of adhering cells to the culture flasks. The FBS, trypsin-EDTA, tryphan blue solution, quercetin-3-*O*-glucoside, hippuryl-$_L$-histidyl-$_L$-leucine (HHL), NaOH, HCl and Captopril were purchased from Sigma Aldrich, Canada Ltd. (Oakville, ON, Canada). Borate saline buffer (100 mM boric acid, 1.5 M NaCl, sterile, pH adjusted to 8.3) was obtained from Teknova (Hollister, CA). Quercetin-3-*O*-glucuronic acid was kindly provided by Dr. Paul Kroon, Project Leader of Polyphenols and Health, Institute of Food Research, Norwich Research Park, Norwich, UK.

5.3.3 Instruments

A class II – type A2 biological safety cabinet (Model LR2-452) was purchased from Esco Technologies Inc. Hartboro, PA, USA. The biological safety cabinet was certified by Con Test Services (Ajax, Ontario) before the starting of experiments. The CO_2 incubator (Model 3074) was purchased from VWR International, West Chester, PA, USA. The incubator was programmed for monitoring the CO_2 levels and temperature inside the chamber. A humidified environment was maintained by using a water tray with mili-Q ultra pure water inside the incubator. An inverted microscope: model ECLIPSE TS 100/ TS 100-F, was purchased from Nikon Instruments Inc. Melville, NY, USA. The microscope was supported with a Lumenara Infinity camera (1-2 USB, 2.9 Megapixel), including infinity capture and infinity analyzing software (Infinity Analyze, Lumenara Corporation, Ottawa, ON, Canada). The cells were preserved in a liquid nitrogen storage

facility using a Biocane system purchased from VWR International, West Chester, PA, USA.

The suppliers of other utensils and equipments were as follows:

Haemocytometer (Bright-Line): Hausser Scientific, Horsham, PA, USA;

Hand held tally counter: Sigma Aldrich, Oakville, ON, Canada;

Stripettor (Stripettor^{TM} Plus): Corning Life Sciences, Lowell, MA, USA;

Gelatin coated flasks (T75): Becton Dickinson Labware, Bedford, USA;

Bottle top filters (500 ml, 0.22 μm): Corning Life Sciences, Lowell, MA, USA;

Sterile disposable pipettes (2 ml, 10 ml, 25 ml): Corning Life Sciences, Lowell, MA, USA;

Cryocanes: Thermo Scientific Rochester, NY, USA;

Cryotubes: Fisher Scientific, Whitby, Ontario, Canada and

Cryogenic vials: Corning Life Sciences, Lowell, MA, USA.

5.3.4 Preparation of Cell Culture Medium

One percent of pen-strep solution and 20% of FBS were added to DMEM. The content was mixed well and sterile filtered using 22 micron bottle top filters. The medium was stored in 250 ml sterile graduated bottles and covered with aluminium foil during storage at 4-8 ^{o}C. The medium was thawed at 37 ^{o}C before it was used in experiments. Serum free medium was prepared by adding pen-strep solution into the DMEM.

5.3.5 Preparation of Cryopreservation Medium

One percent of pen-strep solution, 1% cell culture tested DMSO and 20% of FBS were added to DMEM and sterile filtered. The cryopreservation medium was stored at -80 ^{o}C.

5.3.6 Coating 24-Well Plates with Gelatin

Since gelatin coated 24-well plates were not available from the manufacturer, the plates were manually coated with 0.1% gelatin. The gelatin solution was thawed at 37 ^{o}C and 200 µl of gelatin was poured into each well and kept in the incubator at 37 ^{o}C for 30 min. Excess gelatin was removed and the wells were incubated with the medium.

5.3.7 Initial Cell Preparation

Upon receipt of the cell vial, it was thawed in a water bath heated up to 37 ^{o}C by holding the vial in an upright position and keeping the O-ring out of the water level. The vial was gently shaken during the thawing process for 2 min. The vial was sprayed with 70% of ethanol before placing it into the biological safety cabinet. Seven milliliters of medium was added into a 50 ml centrifuge tube. The cell content was carefully transferred to the centrifuge tube and centrifuged at 125 × g for 10 min. The supernatant was carefully removed without disturbing the cell pellet at the bottom. Ten milliliters of medium was added into a gelatin coated 75 cm^2 flask and thawed at 37 ^{o}C for 30 min. The thawed medium was added into the cell content and mixed by pipetting in and out. The contents were transferred into the gelatin coated flask and incubated at 37 ^{o}C, at 5% CO_2 level. The flask was kept inside the incubator for 24 h without being disturbed. The medium was changed after 4 days from the initial seeding. The cells were observed under 10X magnification with phase contrast optics using the Eclipse TS 100 - Nikon inverted microscope.

5.3.8 First Passage

The first passaging was done after nine days from initial passaging where the cells were grown to a 70% confluence. The cell culture medium was removed and cells were

washed with DMEM without FBS. Since the presence of serum can reduce the activity of trypsin-EDTA, it is necessary to remove the serum before the trypsin-EDTA treatment (Product information sheet – CRL-2873^{TM}, ATCC, Manassas, VA, USA). Two milliliters of trypsin-EDTA solution was added to the flask and rocked several times to spread the trypsin-EDTA solution evenly. The flask was kept inside the incubator at 37 ^{o}C since the trypsin activity is higher at 37 ^{o}C than room temperature. The cells were observed in 10 min intervals. The cells could be completely detached from the bottom of the flask after 25-30 min. After the cells were detached from the bottom, 6-7 ml of DMEM was added into the flask. Upon addition of the medium, the activity of trypsin-EDTA treatment diminished. The cell content was centrifuged at 125 × g for 10 min. The supernatant was removed and 10 ml of fresh DMEM medium was added to the centrifuge tube and mixed by pipetting. Two hundred microliters of the mixed cell suspension were withdrawn for cell count measurements.

The cell contents were further plated in three flasks. The split ratio was 1:3 where the cell contents of one flask was divided into three portions and grown in three separate flasks. Flasks were incubated under the same conditions (37 ^{o}C, 5% CO_2 level). Cells were observed daily and medium replenishment was carried out once in each four days. Once the cells reached a 70% of confluence the cells were passaged for the second time.

5.3.9 Second Passage and Further

The procedure was same as mentioned above. Each flask was passaged into three flasks using a 1:3 split ratio where one flask of cells was preserved. Upon 70% of confluence, the cells were split and plated in 24-well plates where each well contained a cell count of 1.8×10^4 cells/ml. The third passage of cells was grown in 24-well plates.

Later, the frozen cells were re-grown and used with the experiment where those cells were grown up to the fourth passage.

5.3.10 Cryopreservation of Cells

The cells were centrifuged and the supernatant was removed. The cell pellet was resuspended in cryopreservation medium. The cell suspension was separated into 1 ml portions and transferred into 1 ml cryopreservation vials. The vials were labeled and transferred into -80 ^{o}C freezer. After 7 days, the vials were transferred to Biocane liquid nitrogen storage system.

5.3.11 Cell Counts and Viability

Cell counts were taken using the haemocytometer. Twenty microliters of cell suspension were incubated with 20 µl of tryphan blue solution and kept at room temperature. After 10 min, the cell suspension was loaded into the haemocytometer and cells were counted using a hand-held tally counter. Viable cells were clear and bright under 10X – phase contrast optics where the dead cells were blue in colour. The viability counts were obtained using the following equation.

$$\text{Viable cells}\left(\frac{\text{cells}}{\text{ml}}\right) = \left(\frac{\text{cell}_{\text{avg}}}{0.1\ \mu\text{l}}\right) * \frac{1000\ \mu\text{l}}{\text{ml}} * DF$$

Where the cell_{avg} is the mean cell count of four fields of the haemocytometer, 0.1 µl is the volume of one field and DF is the dilution factor. The DF for the experiment was one.

5.3.12 Treatments for the HUVEC

5.3.12.1 ACE Inhibitors

Quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronic acid, FAE and Captopril were investigated for their ability to inhibit ACE in the HUVEC model.

5.3.12.2 Preparation of Samples

Three concentrations were selected for each compound (Quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronic acid, FAE and Captopril), based on the *in vitro* study results (Chapter 4) and the physiologically available concentrations. The selected concentrations were 1, 10 and 100 mg/l. Other than the extract, flavonoids and metabolite, Captopril was used as a standard reference for comparison. The same concentrations were used with Captopril as well.

The compounds were dissolved in a cell culture tested DMSO and the dilutions hundred microliters were made using DMSO as the diluting agent. The drug was dissolved in sodium tetra borate buffer (33.4 mM boric acid, 0.5 M NaCl, pH adjusted to 8.3). A blank (no substrate) was used, and a DMSO control were used in each set of experiments.

5.3.12.3 Cell Treatments

The medium was removed and replaced with FBS free medium 24 h prior to treatments with the inhibitor (Figure 5.1) where each well contained 1 ml of FBS free medium.

Prior to treatments, 350 µl of medium were removed from each well. One of inhibitor at different concentrations, along with the controls, were randomly assigned to each well. The cells with inhibitors were preincubated for 15 min. After 15 min, 250 µl of the substrate (HHL) was added to each well, except the blanks. Cells were incubated for two hours with inhibitors and substrate. After 2 h of incubation, 400 µl of medium were transferred into labeled Eppendorf tubes. The rest of the medium was stored in -80 ^{o}C.

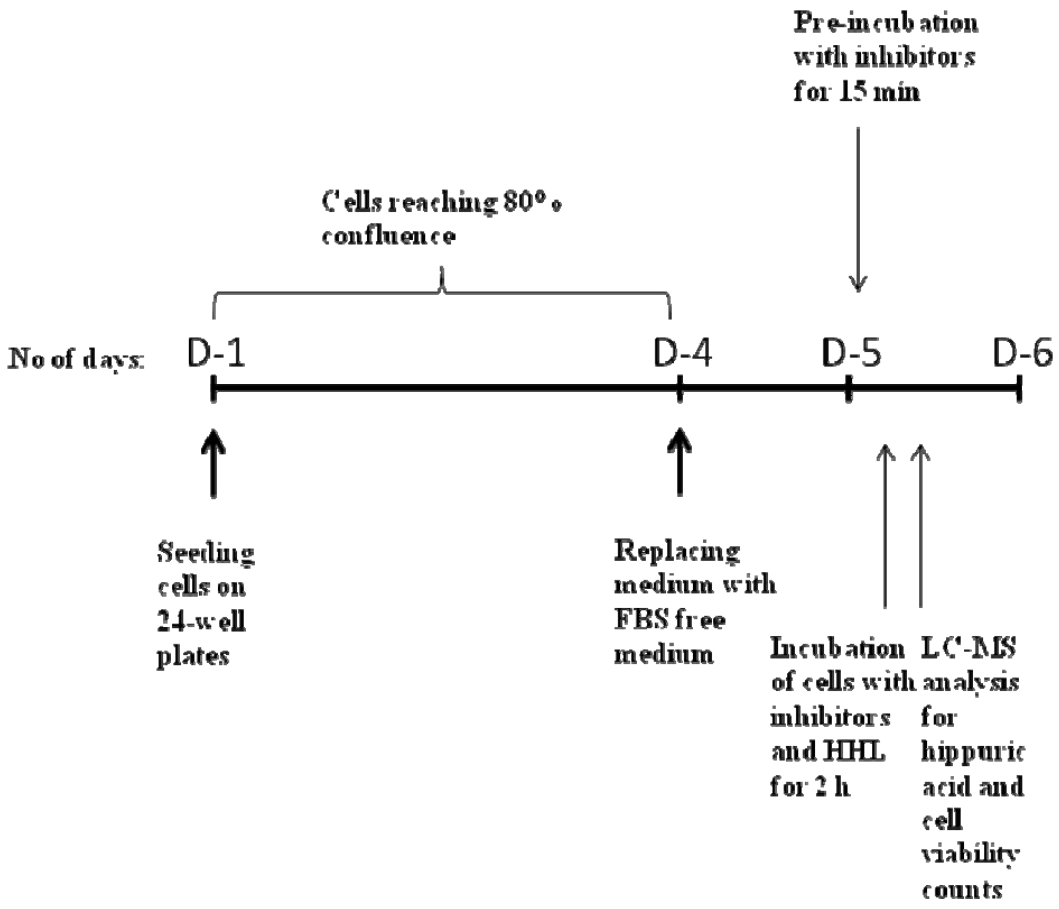


Figure 5.1: The six days experimental setup of treatments for HUVEC

5.3.12.4 Effect of DMSO on ACE Activity

The solubility of flavonoids in aqueous medium is partial. Therefore, the DMSO used to dissolve inhibitors was tested for the effect on the reaction by adding increasing concentrations (0%, 2%, 5% and 10%) of DMSO to culture wells.

5.3.12.5 Cell Morphology

Cell images in each well were captured using the Lumenara Infinity camera attached to the microscope. The cell images were taken at three intervals; prior to treatments with the inhibitors, after the 15 min pre-incubation with the inhibitors and

finally, after 2 h incubation with the substrate respectively. The images were recorded using phase contrast optics under 10X magnification.

5.3.12.6 Determination of Enzyme Inhibition Using UPLC-MS Analysis of the Product

Since ACE is capable of converting HHL into hippuric acid, the formation of hippuric acid was identified and quantified using ultra performance liquid chromatography – mass spectrometry analysis (UPLC/MS).

The analysis was performed using a H-class UPLC system (Model CHA Waters Aquity, Waters Corporation, Milford, MA, USA) equipped with an Aquity BEH C18 (100 mm x 2.1 mm, 1.7 μm) column. The mobile phase consisted of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid (C) in acetonitrile. The flow rate was maintained at 0.3 ml/min where the injection volume was 2 μl. The multi-linear gradient profile used was as follows, (t (min), A%, C%): (0.00, 94.0, 6.0), (2.04, 83.5, 16.5), (2.61, 83.0, 17.0), (3.17, 82.5, 17.5), (3.63, 82.5, 17.5), (4.08, 81.5, 18.5), (4.76, 80.0, 20.0), (6.57, 20.0, 80.0), (8.25, 20.0, 80.0), (12.00, 94.0, 6.0).

Hippuric acid standards were prepared using methanol where the concentrations were 0.1, 1, 10 and 50 mg/l, respectively. The standard curve obtained is shown in Figure 5.2. The culture medium with hippuric acid (samples) was filtered using 0.2 micron nylon filters and 200 μl of the filtrate was loaded into UPLC vials supported with inserts.

The MS analysis was performed using a Micromass Quattro micro API MS/MS system controlled with Masslynx V4.0 data analysis system (Micromass, Cary, NC). The UPLC/MS was operated in electron spray ionization (ESI) negative mode $[M\text{-}H^{+}]^{-}$ where the hippuric acid ion/charge (m/z) = 178.5 was detected using a single ion monitoring mode (SIM). The operating conditions for the MS were as follows: capillary voltage-,

3500 V; cone voltage-, 23 V; extractor voltage-, 3 V; RF lense voltage-, 0.5 V; source temperature-, 110 ^{o}C and desolvation temperature-,175 ^{o}C.

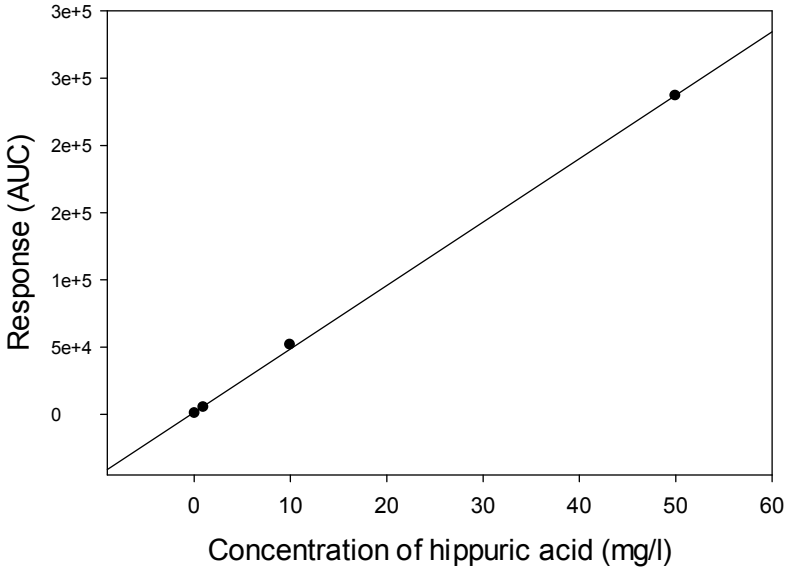


Figure 5.2: Standard curve of hippuric acid

5.3.12.7 Cell Viability Counts

After removing the entire medium, cells in each well were detached using trypsin-EDTA treatment and counted the viable cells and total number of cells was counted using the haemocytometer. The % cell viability was calculated according to the following equation:

$$\% \text{ Cell viability} = \frac{\text{\# of viable cells}}{\text{\# of total cells}} \times 100$$

5.3.13 Statistical Analysis

Treatments were carried out using a complete randomized design (CRD) with four replicates. Data were analyzed using SAS V8 (Cary, NC, USA). One way ANOVA was performed in a general linear model. The assumptions, i.e. normality and constant variance, were analyzed using the Anderson Darling test and obtaining the residuals vs

fitted values respectively. Independence was achieved through randomization. Multiple means comparisons were carried out using Tukey's test with a 5% significance level.

5.4 Results

5.4.1 Growth of HUVEC

The HUVEC were polygonal in shape and were grown as a monolayer attached to the flask bottom. Cells were clearly visible under 10X magnification and phase contrast optics. The time taken for cell doubling increased with the increasing levels of passaging. The initial cells received from ATCC multiplied up to a maximum of four passages.

5.4.2 Effect of DMSO on ACE Activity

DMSO is a commonly used solvent for dissolving most insoluble compounds used in cell-based assays. However, high concentrations of DMSO are toxic to cells. Therefore, different concentrations of DMSO were incubated with the HUVEC to study the toxic effect of DMSO on ACE activity. Three concentrations of DMSO, 2%, 5% and 10% (v/v), were compared with the control without DMSO. The enzyme activity was not influenced by different DMSO concentrations (Figure 5.3). In the current enzyme inhibition assay, 10% (v/v) DMSO was found to be the appropriate solvent in dissolving quercetin and its derivatives. When percentages were less than ten, compounds were partially soluble. Therefore, 10% (v/v) DMSO was selected as the solvent. However, after the cells were treated with either of the three concentrations of DMSO, it could be seen that the morphology of cells was slightly altered since the polygonal shape had become more circular.

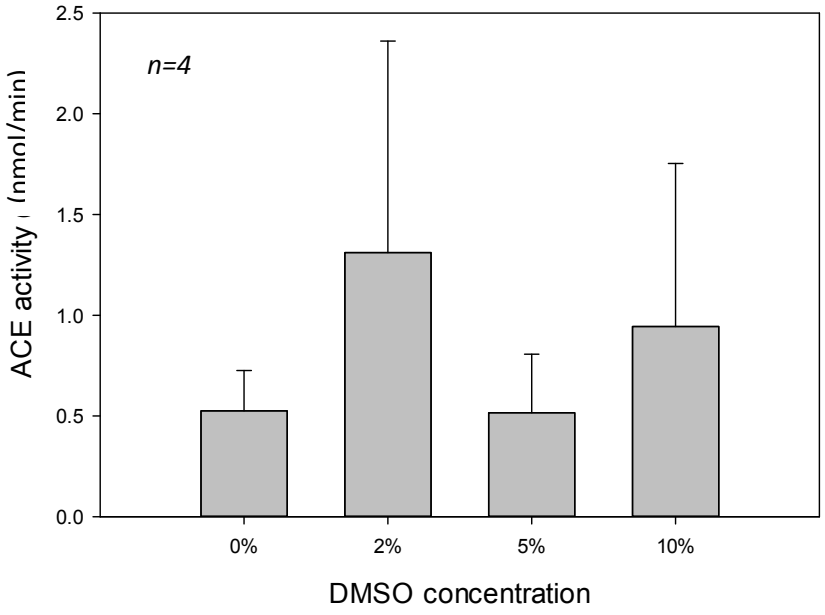


Figure 5.3: ACE activities (mean ± SD) of HUVEC treated at different DMSO concentrations

5.4.3 Detection of Hippuric Acid by UPLC/MS

Hippuric acid was detected at m/z ratio of 178.2. The total ion chromatogram (a) and the mass spectrum (b) of hippuric acid are shown in Figure 5.4.

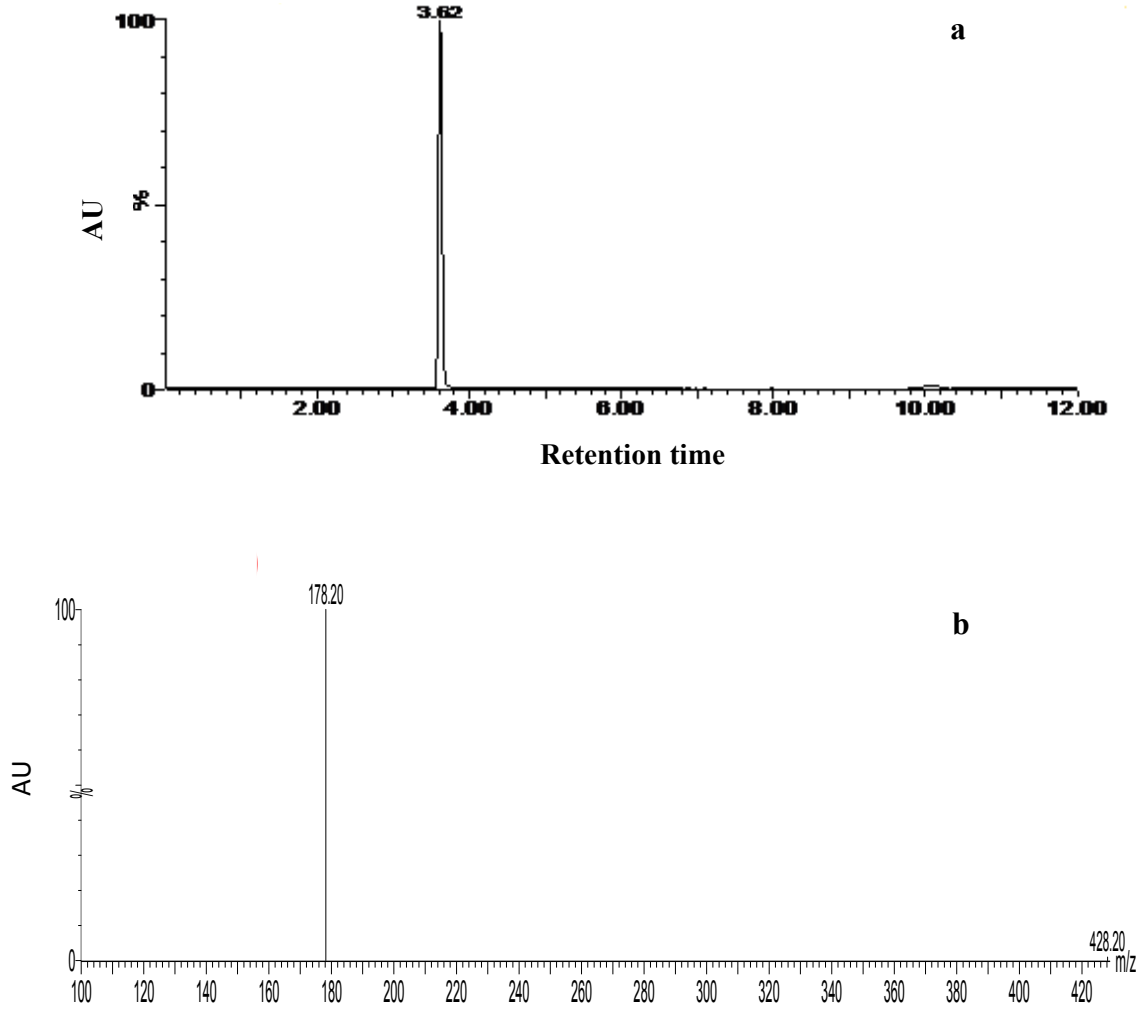


Figure 5.4: (a) The UPLC total ion chromatogram for separation of hippuric acid and (b) The mass spectrum of hippuric acid peak at m/z = 178.2

5.4.4 ACE Inhibition

The three concentrations of FAE (1, 10 and 100 mg/l) showed a significant ($p < 0.05$) inhibition (63%, 89% and 81%, respectively) of ACE in the endothelial cells (Table 5.1). In quercetin, only the highest concentration (3.3 µM) showed a significant ($p < 0.05$) inhibition where the lowest concentrations (0.33 µM and 0.033 µM) did not show significant ($p < 0.05$) inhibition over ACE. Both quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronic acid showed significant ($p < 0.05$) inhibition of ACE. Captopril showed a

dose responsive inhibition of ACE in the HUVEC. FAE and quercetin derivatives did not show a concentration responsive inhibition.

The cell viability counts are given in Table 5.2. An average of 85% of cells was viable after the treatments.

5.4.5 Cell Morphology

The images taken at different times during the treatments are shown in Figures 5.5-5.9. It was observed that the polygonal shape of cells was slightly affected with the inhibitors dissolved in DMSO. However, differences in the morphology were not observed when the buffer was used to dissolve Captopril (Figure 5.4)

Table 5.1: Enzyme activity and the % inhibition relative to controls at the presence of different concentrations of inhibitors[a]

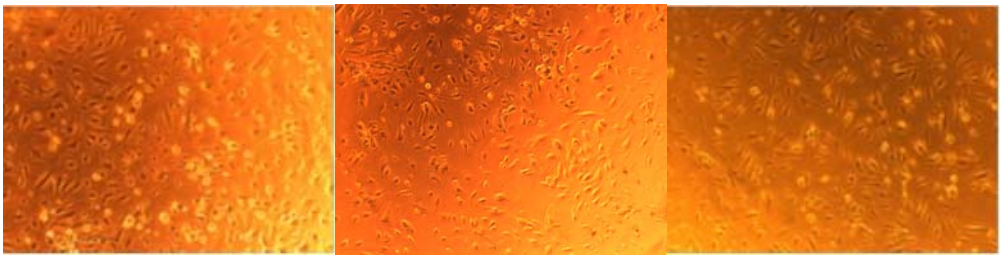
Compound / Extract	Concentration	Enzyme activity $\times 10^{-2}$ (nmol/min)	% inhibition relative to control
Captopril	0	13.4 ± 3.7^{a}	-
	0.046 µM	3.9 ± 0.0^{b}	70.2
	0.46 µM	0.9 ± 0.0^{c}	93.0
	4.6 µM	0.9 ± 0.0^{c}	93.0
FAE[b]	0	6.4 ± 0.4^{a}	-
	1 mg/l	1.3 ± 0.6^{b}	63.5
	10 mg/l	0.1 ± 0.0^{b}	89.1
	100 mg/l	0.6 ± 0.6^{b}	81.2
Quercetin	0	1.8 ± 1.4^{a}	-
	0.033 µM	2.7 ± 2.4^{a}	0
	0.33 µM	1.6 ± 2.2^{a}	11.2
	3.3 µM	0.2 ± 0^{b}	88.3
Quercetin-3-*O*-glucoside	0	1.0 ± 1.1^{a}	-
	0.021 µM	0.2 ± 0.1^{b}	83.6
	0.21 µM	0.3 ± 0.2^{b}	74.9
	2.1 µM	0.1 ± 0.0^{b}	90.6
Quercetin-3-*O*-glucuronic acid	0	32.4 ± 6.5^{a}	-
	0.021 µM	7.9 ± 7.8^{b}	69.3
	0.21 µM	12.6 ± 20.1^{b}	60.7
	2.1 µM	3.3 ± 2.7^{b}	89.8

[a] Data are presented as mean ± SD. Mean with different subscripts in the third column are significantly different ($p < 0.05$)
[b] FAE concentrations are given according to weight basis (mg/l)

A

0 min* 15 min 150 min

B



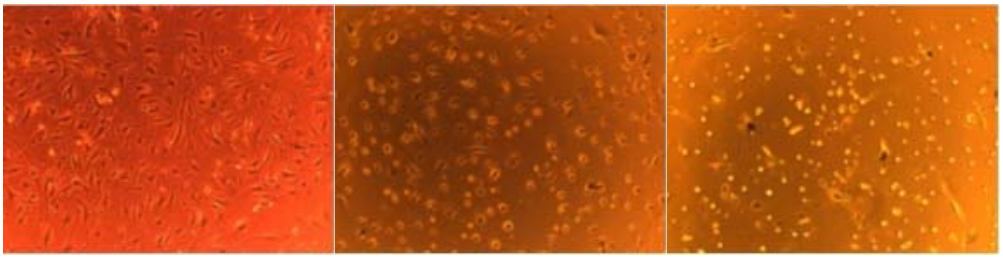
0 min 15 min 150 min

C

0 min 15 min 150 min

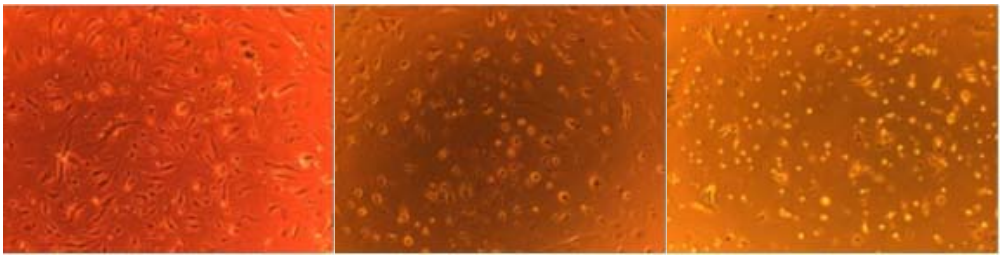
Figure 5.5: Morphology of cells treated with Captopril with 0.046 µM (A), 0.46 µM (B) and 4.6 µM (C) concentrations at different time points (0, 15, 150 min)
*0 min - before inhibitor added; 15 min - before substrate added; 150 min - after incubation with substrate and inhibitor for 120 min

A



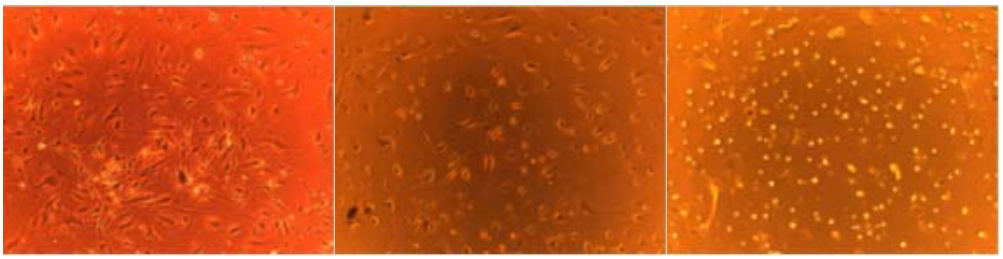
0 min* 15 min 150 min

B



0 min 15 min 150 min

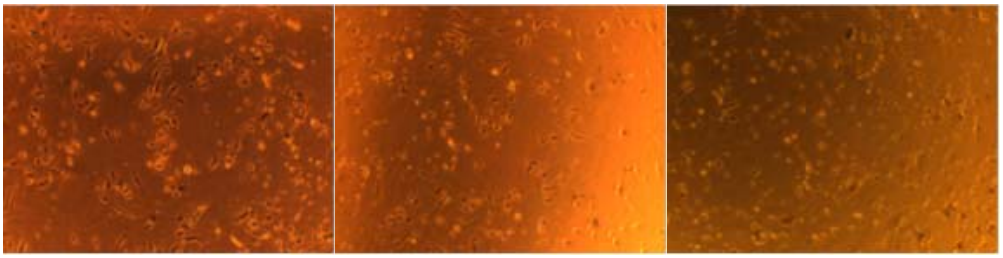
C



0 min 15 min 150 min

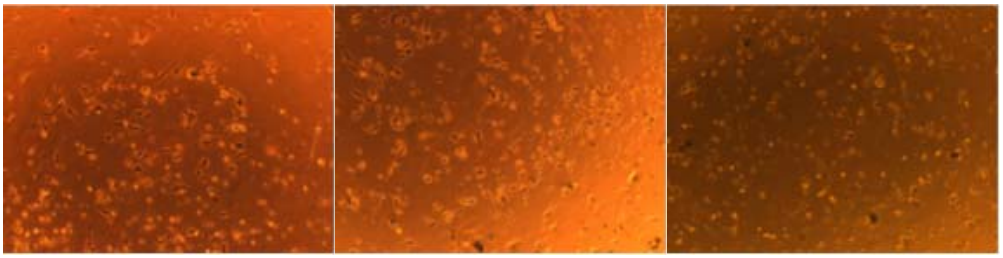
Figure 5.6: Morphology of cells treated with FAE with 1 mg/l (A), 10 mg/l (B) and 100 mg/l (C) concentrations at different time points (0, 15, 150 min)
*0 min - before inhibitor added; 15 min - before substrate added; 150 min - after incubation with substrate and inhibitor for 120 min

A



0 min* 15 min 150 min

B



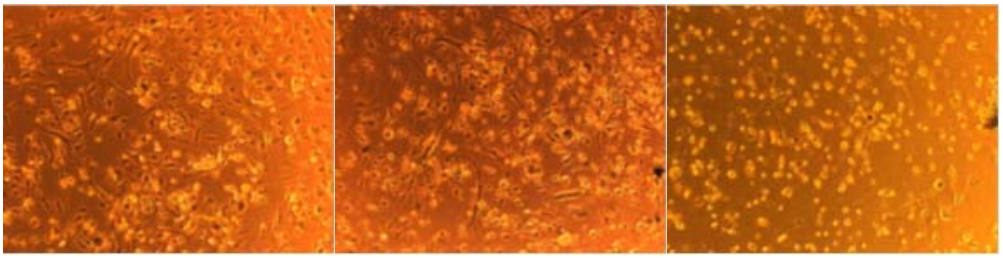
0 min 15 min 150 min

C

0 min 15 min 150 min

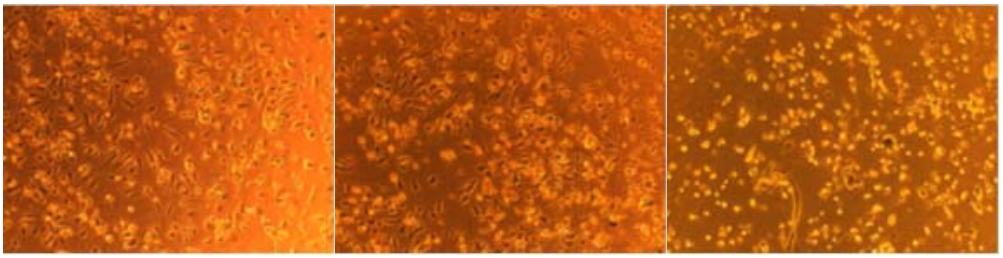
Figure 5.7: Morphology of cells treated with quercetin with 0.033 µM (A), 0.33 µM (B) and 3.3 µM (C) concentrations at different time points (0, 15, 150 min)
*0 min - before inhibitor added; 15 min - before substrate added; 150 min - after incubation with substrate and inhibitor for 120 min

A



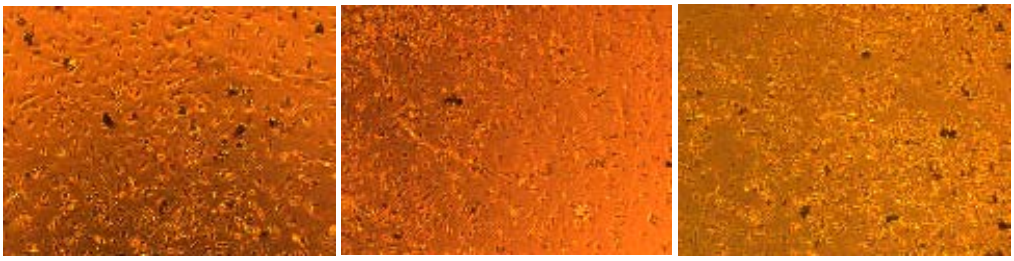
0 min* 15 min 150 min

B



0 min 15 min 150 min

C[a]



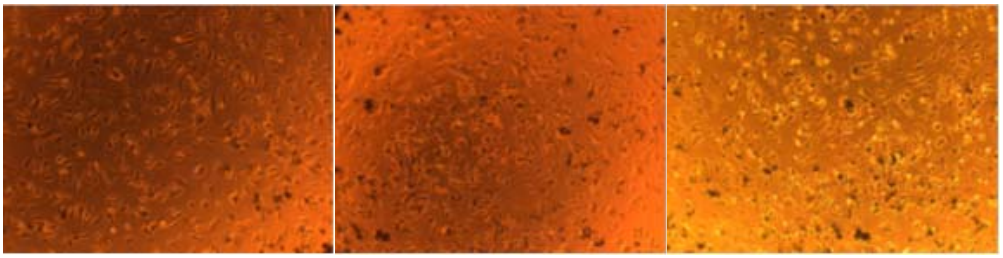
0 min 15 min 150 min

Figure 5.8: Morphology of cells treated with quercetin-3-*O*-glucoside with 0.021 µM (A), 0.21 µM (B) and 2.1 µM (C) concentrations at different time points (0, 15, 150 min)

*0 min - before inhibitor added; 15 min - before substrate added; 150 min - after incubation with substrate and inhibitor for 120 min

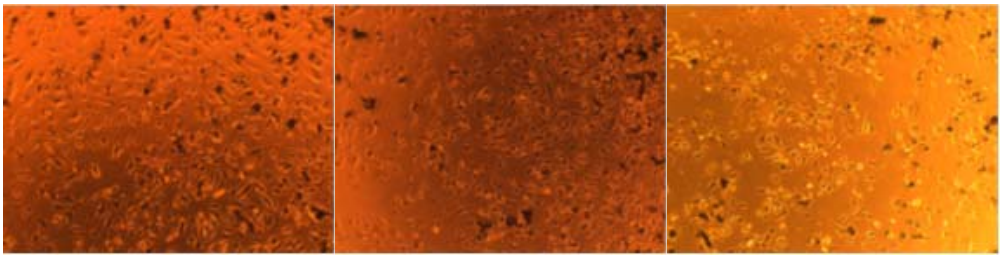
[a] Black dots in the images are dead cells

A[a]



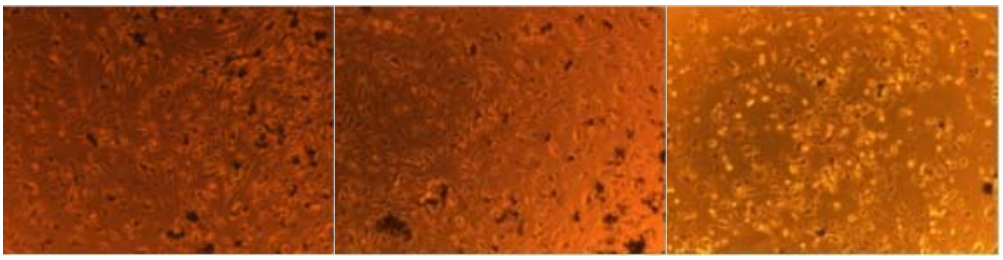
0 min* 15 min 150 min

B[a]



0 min 15 min 150 min

C[a]



0 min 15 min 150 min

Figure 5.9: Morphology of cells treated with quercetin-3-*O*-glucuronic acid with 0.021 µM (A), 0.21 µM (B) and 2.1 µM (C) concentrations at different time points (0, 15, 150 min)

*0 min - before inhibitor added; 15 min - before substrate added; 150 min - after incubation with substrate and inhibitor for 120 min

[a] Black dots in the images are dead cells

Table 5.2: Cell viability (% live cells) of HUVEC after 120 min incubation with inhibitors

Compound / Extract	Concentration	% Live cells
Captopril*	Blank	95
	Positive control[a]	79
	0.046 µM	100
	0.46 µM	83
	4.6 µM	84
FAE*	Blank	92
	Positive control	91
	1 mg/l	82
	10 mg/l	94
	100 mg/l	95
Quercetin*	Blank	80
	Positive control	87
	0.033 µM	75
	0.33 µM	87
	3.3 µM	65
Quercetin-3-*O*-glucoside*	Blank	98
	Positive control	80
	0.021 µM	69
	0.21 µM	100
	2.1 µM	96
Quercetin-3-*O*-glucuronic acid*	Blank	86
	Positive control	93
	0.021 µM	72
	0.21 µM	58
	2.1 µM	75

**n=4,* [a] positive control: no inhibitors

5.5 Discussion

Flavonoids were identified as cardioprotective phytochemicals (Boyer and Liu, 2004) and their effect on ACE inhibition has been reported in several studies (Actis-Goretta et al., 2003 and Ojeda et al., 2010). The *in vitro* ACE activity has been used for initial identification of several compounds with a high inhibitory action on ACE. The effect of the selected inhibitors (FAE, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronic acid and quercetin) of ACE was further assessed using HUVEC in this study. The antihypertensive drug Captopril was used as a reference inhibitor.

Endothelial cell cultures have been widely used for the screening of ACE inhibiting analysis in several research studies (Ottaviani et al., 2006 and Persson et al., 2009). Procyanidins, flavonols as well as several plant extracts i.e. bilberry, tea, ginseng were identified as possible ACE inhibitors in HUVEC models (Ottaviani et al., 2006, Persson, et al., 2009 and Persson et al., 2006,).

The FAE showed a significant ($p < 0.05$) ACE inhibition compared to the positive control. The synergistic inhibitory effect of all types of flavonoids could explain the bioactivity observed. In recent studies, different varieties of apple peel extracts were found to be effective antioxidants (Fattouch et al., 2008), anticarcinogens (Boyer and Liu, 2004) and antimicrobial agents (Fratianni et al., 2011) where the polyphenolics of apple peel played a major role in the observed bioactivity. The lowest concentration of FAE (1 mg/l) showed a 63% inhibition and the highest concentration (100 mg/l) exhibited 81% inhibition, which are noteworthy in an *in vitro* cell model.

The extract FAE was constituted with different types of flavonoids. On a dry weight basis, one gram of FAE contains 30 mg of flavonols, 11 mg of flavan-3-ols and 2 mg of anthocyanins. The presence of flavonols, flavan-3-ols, anthocyanins and dihydrochalcones in FAE possibly facilitate the inhibition of ACE. Flavonols were effective ACE inhibitors in both *in vitro* and *in vivo* ACE inhibition studies (Edwards et al., 2007 and Oh et al., 2004). Tea extracts rich in catechins had inhibited ACE in HUVEC dose dependently (Persson et al., 2006). Chalcones were also found to be effective ACE inhibitors *in vitro* (Bonsei et al., 2010).

However, the bioactivity *in vivo* may vary due to the complexity of constituents of any plant extract. In a study conducted by Boyer and Liu (2004), where the

bioavailability of apple peel extract and onion extract were compared using a caco-2 cell line, higher absorption was found from the onion extract compared to the apple peel extract. High levels of free quercetin and quercetin glucosides, and lesser amounts of glycosides with lower hydrolyzing ability such as quercetin rhamnoside and quercetin xyloside, were proved to be in favor for a high bioavailability. Yet, the available information on bioavailability of apple phytochemicals is scarce and more scientific evidence is needed to understand the metabolism of quercetin glycosides.

Quercetin and quercetin-3-*O*-glucoside were two main constituents of FAE. The effect of these two compounds on ACE inhibition was assessed separately. Quercetin-3-*O*-glucoside significantly ($p < 0.05$) inhibited ACE where in quercetin, only the highest concentrations (0.33 and 3.3 μM) gave 11% and 88% inhibition, respectively. According to the previous studies reported (Chapter 4), quercetin showed a moderate inhibition over ACE when compared with its glucosides. Quercetin was found to be an effective ACE inhibitor *in vivo* (Edwards et al., 2007 and Häckl et al., 2002). Being a more lyphophilic compound, quercetin might not be fully compatible with an aqueous cell system which would reduce its ability to bind with the soluble form of ACE present in the culture medium. Meanwhile, the lyphophilicity favors absorption *in vivo* which could be a reason for the observed bioactivity in *in vivo* studies. Quercetin-3-*O*-glucoside showed 90% enzyme inhibition at its highest concentration (2.1 μM) and 83% at the lowest (0.021 μM). When absorption rates of quercetin and its glucosides were compared, quercetin-4´-glucoside and quercetin-3-*O*-glucoside were proven to be absorbed to a greater extent than quercetin in the small intestine (Boyer and Liu, 2004 and Hollman et al., 1996). Since quercetin-3-*O*-glucoside proved to be an effective ACE inhibitor in *in vitro* models

and proved to be better absorbed in the small intestine, there is a high chance to act as ACE inhibitors *in vivo*. A recent *in vivo* study has demonstrated that the feeding of the male Wistar rats with isoquercitrin (quercetin-3-*O*-glucoside) and isoquercitrin-rich fractions of *Tropaeolum majus* L., a Brazillian medicinal plant, significantly reduced mean arterial pressure and serum ACE activity in Wistar rats (Junior et al., 2011).

The *in vitro* study results of bioactive molecules have always been challenged as opposed to *in vivo* systems due to facts such as absorption, metabolism, bioavailability and bioactivity of the metabolites. Upon ingestion, bioactive molecules are converted into methylated, sulfonated and glucuronidated forms when they are absorbed to the circulation through enterocytes of the small intestine and transferred into the liver cells through the hepatic vein. In order to make *in vitro* studies more biologically relevant, Kroon and others (2004) have suggested incorporating metabolites instead of bioactives in *in vitro* analysis. Many studies have been conducted to investigate the absorption, plasma concentration levels and excretion levels of polyphenolic compounds where a high variability has been observed among various bioactive molecules (Manach et al., 2004).

Quercetin-3-*O*-glucuronic acid is one of the prominent metabolites of quercetin (Kroon et al. 2004). The metabolite significantly ($p < 0.05$) inhibited ACE in HUVEC. The intake dosage and physiologically available concentrations of a metabolite play an important role on bioactivity. Intake of 275 μmol of flavonoid glycosides resulted in 351 nmol/l of the metabolite quercetin-3-*O*-glucuronic acid after 0.6 h in an acute human study (Mullen et al., 2006). In the current study, the lowest concentration of metabolite (0.021 μM) showed a significant inhibition *in vitro*. There were several animal and

human clinical trials where quercetin and quercetin glucoside treatments had demonstrated antihypertensive properties via different mechanisms including ACE inhibition (Edwards et al., 2007, Häckl et al., 2002 and Junior et al., 2011). These study results provide evidence that quercetin and quercetin-3-*O*-glucoside can act as potent hypotensive agents and the metabolite, quercetin-3-*O*-glucuronic acid plays a prominent role in blood pressure reduction in the *in vivo* models. In the current study, Captopril was selected as a reference and it showed a concentration responsive enzyme inhibition where 0.46 µM and 4.6 µM concentrations showed more than 90% enzyme inhibition.

Overall, the HUVEC model provided some preliminary results on the inhibition of ACE by the tested inhibitors. The FAE, quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronic acid showed promising results which need to be confirmed by animal and human clinical trials.

CHAPTER 6.0 CONCLUSIONS

6.1 Objectives of the Study

Hypertension is a key risk factor for the development of cardiovascular diseases (CVD). It is often referred as the 'silent killer' since it has a high tendency to strike without notice. Therapeutics, as well as life style modifications, are equally important in the prevention and cure of this chronic disease condition. The role played by ACE inhibitors in controlling hypertension is enormous. Development of synthetic ACE inhibitory drugs as well as the identification of naturally-derived dietary components, are among the main research focus of hypertension related clinical research.

Plant-based bioactives have long been known for their health beneficial properties. Flavonoid group of bioactives were widely studied as antioxidants and currently, it is believed that apart from their antioxidant activity, they could promote health beneficial properties via different mechanisms. The overall objective of this study was to investigate the ability of flavonoids (representing all subgroups), selected metabolites of quercetin and an apple peel extract rich in flavonoids (FAE), to act as potential ACE inhibitors using an *in vitro* assay as well as a cell culture model system. The overall objective was subdivided into two specific objectives where the first was to investigate the ACE inhibitory activity of the above mentioned compounds and an apple peel extract under *in vitro* conditions. The *in vitro* study included the investigation of concentration responsive ACE inhibition, determining the type of enzyme inhibition and the IC_{50} values of those which showed concentration responsive enzyme inhibition. Based on the results, additional information has been gained in understanding the structure function relationship of flavonoids on ACE inhibition. The second objective

was to select the most promising inhibitors based on the *in vitro* results and to further verify the ability to inhibit ACE using a human umbilical vein endothelial cell (HUVEC) line.

6.2 ACE Inhibition by FAE, Flavonoids and Metabolites

A fluorescence-based assay was used to detect *in vitro* ACE inhibition. Six concentrations were selected to determine the dose responsiveness on the enzyme inhibition. The prescription drug Captopril was used as a reference in each experimental run. The FAE and its constituent flavonoids showed concentration responsive enzyme inhibitions. There has been no previously reported research on the ACE inhibitory effect of an apple-based product or an extract. The current *in vitro* results highlighted the need for further investigations on FAE using *in vivo* model systems. Among the polyphenolics tested, except for coumarin and genistein, all the representative compounds of sub-classes of flavonoids that were tested showed concentration responsive enzyme inhibition. The lowest IC_{50} values were associated with quercetin-3-*O*-glucoside, epicatechin and naringenin. There were three metabolites of quercetin included in this study. Among the three, two metabolites, queretin-3-*O*-glucuronic acid and quercetin-3′-sulphate showed concentration responsive enzyme inhibition. Interestingly, queretin-3-*O*-glucuronic acid was the most effective inhibitor among all the tested compounds which gave an IC_{50} value of 27 μM. It could be concluded that most flavonoids and FAE as well as metabolites are effective ACE inhibitors *in vitro*. The results obtained from the research with metabolites strengthen the evidence that in *in vivo* models, quercetin and its glycosides have high potency to inhibit the circulatory ACE.

The FAE, quercetin-3-*O*-glucoside, epicatechin and queretin-3-*O*-glucuronic acid were selected for the enzyme kinetic analysis. The enzyme activity exhibited the typical Michalelis-Menten enzyme kinetics. According to the kinetic parameters and Lineweaver-Burk plots, qurcetin-3-*O*-glucoside, epicatechin, queretin-3-*O*-glucuronic acid and FAE were competitive inhibitors of ACE. The presence of the B ring of flavonoids and the hydroxyl groups present in the flavonoid structures were found to play an essential role on enzyme inhibition.

6.3 Effect of Selected ACE Inhibitors on Human Endothelial Cell Model

Endothelial cell cultures had been widely used for identification of ACE inhibitors in many research studies. The FAE, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronic acid and quercetin were among the selected inhibitors from the *in vitro* study. The FAE, quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronic acid showed a significant enzyme inhibition when compared with the control, therefore, it can be concluded that these compounds are potent ACE inhibitors in the endothelial cell model. The lowest concentration (0.021μM) of quercetin-3-*O*-glucuronic acid showed 69% enzyme inhibition. This concentration is lower than the reported biologically active concentrations of quercetin metabolite in humans (Mullen et al., 2006). In experiments using quercetin, only the highest concentration (3.3 μM) showed a significant inhibition over ACE. Captopril effectively inhibited ACE where it showed a concentration-dependent enzyme inhibition. The results obtained in the *in vitro* study were reconfirmed using the endothelial cell model. The FAE, quercetin glycoside as well as the metabolite were effective inhibitors over the ACE produced by endothelial cells.

Both *in vitro* models provided evidence that the most flavonoids tested and FAE were able to inhibit ACE. In the *in vitro* study (experiment one), ACE derived from rabbit lung and in the cell model, human endothelial cell derived ACE were used. In literature there had been controversial results associated with flavonoids on ACE inhibition. The current study support that flavonoids show ACE inhibition *in vitro*. Compared to Captopril, the inhibition was at a moderate level. However, the less solubility of most flavonoids in aqueous medium can interfere with the level of enzyme inhibition. In FAE, the synergistic effect of different types of flavonoids could be in favor for the observed bioactivity. The observed enzyme inhibition relating to the metabolite quercetin-3-*O*-glucuronic acid in both models, support the fact that flavonols has a high potential to inhibit ACE *in vivo*. As most of the ACE inhibitory drugs in use, flavonoids competitively inhibit ACE.

6.4 Recommendations for Future Research

Several improvements could be made in relation to the cell culture experimentations. Since the cell membrane integrity was affected by the solvent (10% v/v DMSO), it might be better to substitute another non-toxic solvent system which has the ability to dissolve the flavonoids in an aqueous medium. More attention should be paid to reducing the variability within each experimental unit which may be due to the variation in cell density of experimental units. Using a high initial cell density as well as selecting cells from early passages (eg: passage 3), could be useful in overcoming this weakness.

It is important to know the membrane bound and soluble ACE levels for each experimental unit. A method of quantification of both types of ACE would be beneficial for the cell based experiments. The same experimental set up could be used to investigate

several aspects, including the effect of the inhibitors upon nitric oxide (NO) synthesis and the expression levels of the gene encoding ACE. An increased level of NO promotes vasorelaxation and is responsible for reducing blood pressure. If the flavonoids trigger the production of NO this would become an added advantage apart from the ACE inhibition.

The *in vitro* analysis using two different model systems provided the initial understanding of flavonoids as ACE inhibitors. Development of identified compounds as a neutraceutical to treat hypertension requires many steps, including animal trials and human clinical trials. Rodent models such as spontaneously hypertensive rats are quite popular in hypertensive animal studies. *In vitro* model systems cannot be used in understanding the bioavailability of phytochemicals, the metabolites formed in the body and their biological activity. Therefore, it is essential to confirm the current findings using animal models. Based on the results of animal studies and safety levels of flavonoids, human clinical trials could be performed. These are essential for the development of bioactive-rich apple peel based natural health products or functional foods with beneficial health claims of antihypertensive properties.

Overall, it can be concluded that FAE shows promising ACE inhibitory properties in both *in vitro* biochemical assay and HUVEC cultures where its constituent flavonoids seems to be responsible for the observed bioactivity. The metabolite and the glucoside of quercetin, i.e. quercetin-3-*O*-glucuronic acid and quercetin-3-*O*-glucoside, respectively, were the most effective ACE inhibitors among the tested flavonoids and their derivatives. The flavonoids competitively inhibit ACE where the presence of hydroxyl groups and the

B-ring structure may play a significant role in binding with the active site of ACE which requires further investigations.

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