MAJOR CHLOROGENIC ACID ISOMERS PRESENT IN COFFEE ARE MODULATORS OF REDOX BIOLOGY AND INFLAMMATION IN CACO-2 CELLS

by

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Abstract

Chlorogenic acid (CGA) is a general term used to describe the most abundant group of phenolic acids in coffee. 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA are major CGAs in coffee; but only 5-CQA has been thoroughly studied. The first objective of this thesis was to study interactions between major CGA isomers and chemical changes in coffee brew that affect antioxidant activity noted for coffee. The second overall objective was to study the potential of these six major CGA isomers in modulating oxidative stress and inflammation using a human intestinal Caco-2 cell line. The findings from Chapter 2 suggested that the other five CGA isomers (4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) together account for more than 50% of the total CGA in coffee and contributed to the antioxidant activity of coffee brew. Chapter 3 and 4 addressed the research question of whether these major CGA isomers have a modulating effect on oxidative stress and inflammation in human intestinal Caco-2 cell line. Caco-2 cells were first incubated with, or without, individual CGA isomers, followed by a phorbol 12-myristate 13-acetate plus human interferon gamma challenge. Biomarkers of oxidative stress (intracellular ROS and GSH/GSSG) and inflammation (IL-8) were measured. The results demonstrated that CGA isomers scavenged intracellular ROS in inflamed Caco-2 cells, mitigated the drop in GSH/GSSG ratio and attenuated IL-8 secretion. Dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) had a relatively stronger capacity to evoke protection compared to caffeoylquinic acids (3-CQA, 4-CQA, and 5-CQA). To elucidate the possible mechanism underlying these actions, the effects of CGA isomers on the nuclear factor kappa B signaling pathway, mitogen-activated protein kinase cascades, and nuclear factor (erythroid-derived 2)-like 2 signaling pathway were further investigated. In conclusion, structural differences in six CGA isomers were found to correspond to differences in antioxidant and anti-
inflammation activities. CGA isomers attenuate oxidative stress and inflammation in Caco-2 cells by triggering changes in redox biology parameters, lead to an up-regulation of nuclear factor kappa B signaling at very early stage, mitigation of p38 phosphorylation and up-regulation of antioxidant genes in an intermediate stage, and activation of Nrf2 signaling at a much later stage.
**Lay Summary**

CGA is one of the most abundant groups of polyphenols present in coffee and consists of many different isomers. In this thesis, I studied the interactions between major CGA isomers and chemical changes in coffee brew that affect antioxidant activity noted for coffee. This work was followed by experiments designed to examine the potential of thesis major CGA isomers present in coffee to modify oxidative stress and inflammation using a cultured human intestinal cell line. The results suggested that six major CGA isomers present in significant amounts are major contributors to the antioxidant activity of coffee. Moreover, CGA isomers mitigated oxidative stress and inflammation in an isomer specific manner in cultured human intestinal cells. The findings of this study are important for understanding the potential influence of CGA isomers on human intestinal health as it relates to antioxidant status and inflammation.
Preface

The material presented in the Chapter 1 literature review section 1.2.5 and Table 1.1 have been published in Molecules (2014, 19(11):19180-19208), entitled “Antioxidant property of coffee components: assessment of methods that define mechanisms of action”. Ningjian Liang is the first author who wrote the manuscript and David D. Kitts is the corresponding author who revised the manuscript.

The content presented in the Chapter 1 literature review sections 1.2.1, 1.2.2, 1.2.3, 1.2.4, 1.2.6, 1.2.7, 1.2.8 as well as Table 1.2 & 1.3 and Figure 1.1 have been published in Nutrients (2015, 8(1). Doi: 10.3390/nu8010016), entitled “Role of CGAs present in beverages in the management of oxidative stress”. Ningjian Liang is the first author who wrote the manuscript and David D. Kitts is the corresponding author who revised the manuscript.

A version of chapter 2 of this thesis has been published in Food Chemistry (2016, 213:251-259), entitled “Interactions between major CGA isomers and chemical changes in coffee brew that affect antioxidant activities”. Ningjian Liang is the first author who designed, conducted the experiments, analyzed data, and prepared the manuscript. Wei Xue and Pierre Kennepohl are co-authors who provided technical support in using electron paramagnetic resonance (EPR) spectroscopy. David D. Kitts is the corresponding author who provided guidance on the research and critically revised manuscript.
Some of the findings in Chapter 3 and Chapter 4 has been presented at Experimental Biology conference (San Diego, United States of America, 2016). The title of the presentation is “Capacity of chlorogenic acid isomers to modulate oxidative and inflammatory responses in Caco-2 human enterocyte-like cells”. Paper no. 2115. Ningjian Liang is the first author who designed, conducted the experiments, analyzed data, and prepared the poster. David D. Kitts is the corresponding author who provided guidance on the research.

Some of the findings in Chapter 3 and Chapter 4 has been presented at Fourth International Congress on Cocoa Coffee and Tea (Turin, Italy, 2017). The title of the presentation is “Major chlorogenic acid isomers in coffee ameliorate inflammation and oxidative stress in Caco-2 cells via up-regulation Nrf2/ARE signaling”. Poster no. 0122. Ningjian Liang is the first author who designed, conducted the experiments, analyzed data, and prepared the poster. David D. Kitts is the corresponding author who provided guidance on the research.

Some of the findings in Chapter 3 and Chapter 4 has been presented at 2nd International Conference on Food Chemistry & Nutrition (Vancouver, Canada, 2017). The title of the presentation is “The antioxidant activity of chlorogenic acid isomers is positively correlated with capacity to up-regulate nuclear factor-κB (NFκB) signaling in Caco-2 cells”. Oral presentation. Ningjian Liang is the first author who designed, conducted the experiments, analyzed data, and presented the data. David D. Kitts is the corresponding author who provided guidance on the research.
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change: CGA reduced intracellular ROS, up-regulated Nrf2 signaling, increased GSH/GSSG, and decreased IL-8 secretion.
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<th>Description</th>
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<tr>
<td>3-CQA</td>
<td>3-0-caffeoylquinic acid</td>
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ERK1/2  extracellular signal-regulated kinase 1 and 2
EPR   electron paramagnetic resonance
FBS   fetal bovine serum
GPx   glutathione peroxidase
GR    glutathione reductase
GSH   reduced glutathione
GSSG  oxidized glutathione
GSS   glutathione synthetase
GCLC  glutamate-cysteine ligase catalytic subunit
GCLM  glutamate-cysteine ligase modifier subunit
HMW   high molecular weight
HO·   hydroxyl radical
HPLC  high performance liquid chromatography
IL-1β  interleukin 1β
IL-6   interleukin-6
IL-8   interleukin-8
IL-12  interleukin-12
IBDs   inflammatory bowel diseases
IL-10  interleukin-10
IFNγ  interferon gamma
I/R   ischemia/reperfusion
JNK   c-Jun N-terminal kinases
LDL   low-density lipoprotein
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<td>LPS</td>
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<tr>
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</tr>
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<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MRP</td>
<td>Maillard Reaction Product</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NFkB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>erythroid 2 like 2</td>
</tr>
<tr>
<td>NFE2L1</td>
<td>nuclear factor erythroid 2-related factor 1</td>
</tr>
<tr>
<td>Nrf2</td>
<td>nuclear factor-erythroid-derived 2-like</td>
</tr>
<tr>
<td>ORAC</td>
<td>oxygen radical antioxidant capacity</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>superoxide radical anion</td>
</tr>
<tr>
<td>ONOO-</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>p38</td>
<td>p38 isomers</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PNG</td>
<td>Papua New Guinea</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PC</td>
<td>principal component</td>
</tr>
<tr>
<td>PAH</td>
<td>polyaromatic hydrocarbon</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>real-time PCR</td>
<td>real-time polymerase chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROO·</td>
<td>peroxyl radical</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered-saline-Tween</td>
</tr>
<tr>
<td>t-BOOH</td>
<td>tert-tutyl hydroperoxide</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TEMPO</td>
<td>4-hydroxy-2,2,6,6-tetra-methylpiperidine,N-oxyl</td>
</tr>
<tr>
<td>trolox</td>
<td>2,5,7,8-tetramethylchroman-2-carboxylic acid</td>
</tr>
<tr>
<td>TOF-SIMS</td>
<td>time-of-flight secondary ion mass spectrometry</td>
</tr>
<tr>
<td>XOD</td>
<td>xanthine oxidase</td>
</tr>
</tbody>
</table>
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could not have done this without your unconditional love, enormous sacrifice, and continuing support.
Dedication

To my parents

Mr. Liang and Mrs. Yang
Chapter 1: General Introduction and Literature Review

1.1 Introduction

Chlorogenic acids (CGAs) are phenolic acids that are present in a number of foods. Coffee is the richest source of CGAs (Farah et al., 2008). Major CGAs in coffee include 3-O-cafeoylquinic acid (3-CQA), 4-O-cafeoylquinic acid (4-CQA), 5-O-cafeoylquinic acid (5-CQA), 3,4-O-dicafeoylquinic acid (3,4-diCQA), 3,5-O-dicafeoylquinic acid (3,5-diCQA), 4,5-O-dicafeoylquinic acid (4,5-diCQA), which are the focus of this thesis (Clifford, 2000) (Matei et al., 2012). Additionally, minor CGA isomers including 3-O-feruloylquinic acid (3-FQA), 4-O-feruloylquinic acid (4-FQA), 5-O-feruloylquinic acid (5-FQA), 3-p-coumaroylquinic acid (3-p-CoQA), 4-p-coumaroylquinic acid (4-p-CoQA), and 5-p-coumaroylquinic acid (5-p-CoQA) are also present in traceable amount in coffee (Matei et al., 2012). Various factors that affect CGA isomer profiles in green coffee beans include coffee plant variety, geographic location and the conditions under which the beans are roasted to produce consumable brews (Anthony et al., 1993) (Campa et al., 2005) (Ky et al., 1999). On the other hand, the composition of CGA isomers and other abundant compounds such as melanoidins and caffeine altogether determine the overall antioxidant capacity of coffee brew. The first general objective of this work is to study the impact of geographic location and roasting condition on the CGA isomer profile and to overall antioxidant activity of coffee, and furthermore, to interpret the interactions between major CGA isomers and chemical changes in coffee brew that affect antioxidant activity noted for coffee.
The single-layered gut epithelium is the primary line of defense against luminal stressors. The epithelium in inflamed intestinal segments of patients with inflammatory bowel disease is characterized by an imbalanced redox status, excessive secretion of pro-inflammatory cytokines, and reduction of monolayer integrity (Bhattacharyya et al., 2014) (Katsuta et al., 2000) (Laukoetter et al., 2008). Understanding the potential effect of CGA isomers on intestinal epithelium is of importance because the intestine of coffee drinkers is exposed to dietary CGA isomers. 5-CQA is the most abundant isomer in coffee with notable antioxidant and anti-inflammatory activities demonstrated in both cellular and animal models as listed in Table 1.3 and Table 1.4. However, the bioactive function of the five major CGA isomers (namely, 3-CQA, 4-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) present in coffee have not yet been investigated. The second general objective of this work therefore is to study the effect of CGA isomers to modulate oxidative stress and inflammation on intestinal epithelium model, Caco-2 cell line. Specific questions that were derived to find answers were, whether or not oxidative stress was accompanied with the initiation of inflamed intestinal cells; the relative effectiveness of CGA isomers to alleviate oxidative stress in inflamed intestinal cells. It is also important to determine if CGA isomers are effective to alleviate inflammation in this in vitro model of intestinal inflammation. The underlying mechanisms CGA isomers affect three oxidative and inflammation stress-responsive pathways, nuclear factor-erythroid-derived 2-like 2 (Nrf2) signaling, nuclear factor-κB (NFκB) pathway and mitogen-activated protein kinases (MAPK) signaling pathway were studied in this thesis.
1.2 Literature Review

1.2.1 Chemistry of CGA isomers

CGAs are phenolic acids with vicinal hydroxyl groups present on aromatic residues that are derived from esterification of cinnamic acids (including caffeic acid, ferulic acid, and \( p \)-coumaric acid) and quinic acid (Clifford, 2000) (Farah & Donangelo, 2006). The ester formed between one molecule of caffeic acid and one molecular of quinic acid is called caffeicquinic acid (CQA). There are three isomers within the CQA subgroup, including 3-CQA, 4-CQA, and 5-CQA. The ester formed between two molecules of caffeic acid and one molecular quinic acid is called dicaffeicquinic acid (diCQA). 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA are isomers within the diCQA subgroup that are commonly present in nature. Similarly, feruloylquinic acid (FQA) is the ester formed between ferulic acid and quinic acid. \( p \)-coumaroylquinic acids (\( p \)-CoQA) is the ester formed between \( p \)-coumaric acid and quinic acid (Clifford, 2000). The chemical structures of different CGA isomers are shown in Figure 1.1.
Figure 1.1 Chemical structures of chlorogenic acid isomers (3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, 3-FQA, 4-FQA, 5-FQA, 3-p-CoQA, 4-p-CoQA, and 5-p-CoQA).

1.2.2 Dietary Sources of CGAs

1.2.2.1 Coffee

The most prevalent group of phenolic compounds in coffee are CGAs. CGA isomer composition in green coffee beans is complex and varies in part by coffee variety; geographic location from which it is grown and the processes used in post-harvest, washing/drying procedures, all of which precedes roasting of the coffee beans. Total CGAs may account for 4.0-
14.4% of dry matter basis in green coffee beans and contribute to the final acidity, astringency, bitterness, and overall cup quality of the beverage (Perrone et al., 2010). The most abundant CGA in green coffee beans is 5-CQA, which account for 76-84% of the total CGAs, or approximately 10 g/100 g coffee beans (Farah & Donangelo, 2006) (Perrone et al., 2010). Clifford and Ramirez-Martinez (1976) measured the CGAs composition in two major coffee plant species and reported that green *Coffea Robusta* beans grown at Santos and Sao Paulo contained a higher content of CGAs compared to green *Coffea arabica* beans, grown in Ghana and Uganda. Later research confirmed this finding (Clifford & Ramirez-Martinez, 1991). For example, Perrone et al. (2008) reported that total content of CGAs in green *Coffea Robusta* beans ranged from 63 to 55 milligrams per gram of dry weight. Farah et al. (2005) reported that the total CGAs content in green *Coffea Robusta* beans was 28% higher than the average. In addition to 5-CQA, green coffee beans contain 3- and 4- CGA, dicafeolyquinic acids (3,4-, 3,5-, and 4,5-diCQA), feruloylquinic acids (3-, 4-, and 5-FQA) and *p*-coumaroylquinic acids (3-*p*-, 4-*p*-, and 5-*p*-*CoQA*) (Farah et al., 2008).

Roasting conditions also significantly affect the total CGAs content and profile in processed coffee beans. High temperature roasting will convert some CGAs into flavor and aroma compounds, or alternatively promote CGA to react with other chemical components in the coffee bean through at least five distinct reaction pathways that include epimerization, decarboxylation, acyl migration, lactonisation and dehydration (Jaiswal et al., 2012); notwithstanding a final product being contribution to melanoidins formed in the coffee brew (Bekedam et al., 2008). The theory that CGAs participated the formation of Maillard reaction melanoidin products was initially based on findings that used the Folin-Ciocalteu method for
quantifying phenolics (Bekedam et al., 2006). This has since been confirmed with quantitative analysis of phenolic derivatives recovered from high molecular weight components isolated from coffee brews (Adams et al., 2005). Many, if not all, of these reactions that lead to thermal degradation of CGAs during coffee roasting are dependent on the intensity (e.g. time and temperature) of roasting. The diverse and complex nature of a number of products produced with roasting coffee beans vary from relatively simple decarboxylation of quinic and cinnamic acids to simpler phenolic acids, to more complex formation of chlorogenic lactones, derived from dehydration of the quinic acid moiety. The latter evokes intermolecular ester bond formation when CGA is exposed to high heat treatment. Nucleophilic amine- and thio- groups from peptides are also examples of conjugate additions involving CGAs during heating coffee (Jaiswal et al., 2012). Hence, the greater extent of the transformation occurred as coffee beans are roasted; the lower the content of totals CGAs. Moon et al. (2009) reported that around 45~54% of CGAs were lost in the light roasted coffee beans (230 °C, 12 min) compared to the green beans, whereas more than 99% of CGAs were lost in dark roasted coffee beans (e.g. city roast, 250 °C for 17 min; French roast, 250 °C for 21 min). Although as much as 99% of CGA is lost with dark roasting, 5-CQA still remained as the predominant CGA isomer in dark roasted coffee. It is interesting that isomers, 4-CQA and 3-CQA, increased in some varieties of light roasted coffee beans, which could be attributed to isomerization of CGAs (Moon et al., 2009). The CGA isomer content in roasted coffee beans has been shown to be in decreasing order: CQA > diCQA > FQA > p-CoQA (Perrone et al., 2008). CGAs composition in coffee brew prepared from coffee beans with different roasting degrees has also been comprehensively studied. Total CGAs ranged from 187.7 to 295.6 mg/100 mL brew when prepared from light roasted coffee beans and ranged from 24.2 to 41.3 mg/100 mL brew when prepared from dark roasted coffee beans (Tfouni et al.,
Another study reported that the total CGAs in espresso coffee made from light, medium, and dark roasted coffee beans was 1060 mg/100 mL, 517 mg/100 mL, and 340 mg/100 mL, respectively (Ludwig et al., 2014). Regardless of the roasting degree, the content of the total CGAs in Espresso coffee from different sources ranged from 89 mg/100 mL to 811 mg/100 mL (Crozier et al., 2012). The CGA isomer contents in commercial coffee brew also decreased in the following order, CQA > FQA > diCQA (Fujioka & Shibamoto, 2008) (Mill et al., 2013), which is different from the order reported in roasted coffee beans by Perrone et al. (2008).

The specific procedures used to brew coffee beverages also affect the final content of CGAs, since many factors influence the efficiency of elution and recovery of CGAs from ground roasted coffee beans. Filtered coffee is the most widely consumed coffee brew prepared by pouring boiled water over ground coffee beans that are stationary present on a paper filter. In contrast, Espresso coffeemakers apply high pressure to force a small amount of boiling water through ground coffee beans. The simplest way of making coffee brew is by pouring boiling water over the grounded coffee beans and waiting for the ground to settle. All methods of brewing that vary in the ratio between the hot water and ground coffee beans (v/w), the turbulence, pressure, and the contact surface and contact time will collectively affect the final CGAs profile. Tfouni et al. (2014) reported that brews prepared by boiling water without filtration had a higher content of the CGAs than the corresponding filtered ones. This result might be due to the larger contact surfaces between the added water and the ground coffee when simply boiled, compared to the filtered method. Ludwig et al. (2012) found that espresso coffee brew contained relatively more CGAs compared to filtered coffee. The high pressure applied in Espresso coffee favors the extraction efficiency of CGAs in this brew.
1.2.2.2 Other Plant Sources

In addition to green and processed coffee beans being major sources of dietary CGAs, this group of phenolic compound is also present in fruits and vegetables; again, with 5-CQA being the predominant isomer. Fresh potatoes contain CGAs that range from 0.10 to 0.19 mg of 5-CGA 100 g potato (Dao & Friedman, 1992), which is equivalent to 90% of the total phenolic compounds present in potato tubers (Griffiths & Bain, 1997) (Malmberg & Theander, 1985). 5-CQA, 5-FQA, and 3,4-diCQA, 3,5-diCQA were also detected in different varieties of vegetable Chicorium endivia (Papetti et al., 2008). Genetically modified tomatoes with increased CGA content have also been developed to enhance the antioxidant properties (Niggeweg et al., 2004). Popular citrus fruits such as pear and apple are additional rich sources of CGAs. The content of 5-CQA in pears ranged from 0.02 to 3.72 mg per gram of fresh fruit depending on the ripeness of the fruit (Cui et al., 2005) and type of cultivar (Li et al., 2012). Apples are a rich source of CGAs with the core part having the highest level (2.10 mg per gram of dry fruit), followed by the apple seed (1.10 mg per gram of dry fruit) and then apple flesh (0.48 mg per gram of dry fruit) (Awad et al., 2000). CGAs are also present in some herbs. Wang et al. (2008) reported that 5-CQA and 3,5-diCQA were dominant isomer with 3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, and 4,5-diCQA relatively the minor isomers in beverages prepared from chrysanthemum, purple sweet potato stem, kuding tea, and honeysuckle flower. CGAs are the main phenolic compounds in the tea infusions prepared from the herb Artemisia annua (De Magalhaes et al., 2012).

In summary, CGAs are widely present in the plant kingdom; many plants of which are relevant to our diet. It is interesting to note that the "di-CGA" may contribute different taste
qualities, such as producing a bitter/metallic taste found in certain coffees. The significance of this in respect to the taste profile of coffee could be particularly relevant with Robusta coffees, or blends of coffees that contain a proportion of Robusta beans and hence higher amounts of di-CGAs.

1.2.3 Bioavailability and Metabolism of CGAs

In a cultured gastric epithelial model, several CGA isomers were shown to be transferred intact across the gastric barrier at an acidic apical pH; with di-CQA having a relatively higher permeability coefficient compared with CQA (Farrell et al., 2011). Experiments conducted in a rat model, showed that CGAs are not hydrolyzed in the stomach, but absorbed in the stomach in an intact form (Lafay et al., 2006). This could explain the early detection of CGA in plasma within 30 minutes after coffee consumption. The intestinal absorption of 5-CQA has also been studied in cell culture, using the human colon carcinoma cell line Caco-2, to model the intestinal epithelium in vitro. The absorption rate for 5-CQA was 0.10 ± 0.08% at concentrations relevant to gut lumen concentrations (0.1–1 mM) (Halliwell & Gutteridge, 2007) (Scherbl et al., 2014). The transepithelial flux of 5-CQA was around 0.13-0.59 nmol/min/mg of protein in a Caco-2 intestine epithelia cultured monolayer (Konishi & Kobayashi, 2004). Digestion-balance studies conducted in rats report that around 9.2 ± 6.8% of CGAs can be recovered in the urine 24 hours after consumption of a 50 mg/kg dose of CGAs (Choudhury et al., 1999). CGAs are also hydrolyzed into caffeic and ferulic acids in the small intestine before being absorbed (Lafay et al., 2006). Farah et al. (2008) studied the pharmacokinetic profile and bioavailability of CGAs in health human subjects and found that the apparent bioavailability of CGAs from a green coffee extract was 33 ± 23%. Recovery was principally derived from CQA and di-CQA with poor
absorption from FQA. Similar to the rat study, some of the original CGA dose was recovered as simple phenolics, such as caffeic and ferulic acids. Urine was not the major route for excretion of CGAs in the human trial, but these smaller metabolites recovered suggested that metabolism and excretion kinetics in humans could be quite variable and related to genetic polymorphisms. A similar result (33 ± 17% CGAs) was reported in ileostomy subjects 24 hours after consumption of a high dose (2.8 mmol) of CQAs (Olthof et al., 2001). Further confirmation of the digestion and absorption of CGA has come from a study where plasma CGAs was quantified from subjects and a positive dose-response response describing the absorption efficiency was dependent on the intake level (Renouf et al., 2014). The bioavailability of the CGAs in coffee beverages can also be affected by various factors that are external to the dietary source. For examples, milk fat added to a coffee beverage may increase CGAs bioavailability (Tagliazucchi et al., 2012). Moreover, the concentration of CGAs present in coffee also influences bioavailability of CGAs (Stalmach et al., 2014). At present, the influence of the type of food matrix that can influence CGAs digestion and bioavailability remains unclear.

1.2.4 Assays to Measure Antioxidant Activity

Chemical-based assays, cell-based assays, and animal models have been established to gain different levels of understanding about the antioxidant capacity of dietary compounds.

1.2.4.1 Chemical-based Antioxidant Assays

In chemical-based assays, free radicals are artificially generated to react with tested samples under fixed conditions of time and at defined conditions. At the reaction endpoint, the amount of leftover free radicals is measured to reflect the free radical scavenging capacity of the
tested sample. Various chemical reactions have been used to generate free radicals. Xanthine oxidase (XOD) utilizes hypoxanthine or xanthine as a substrate and O₂ as a cofactor to generate O₂-. Fenton reaction between ferrous iron and H₂O₂ produces ·OH. Sodium nitroprusside breaks down to yield NO· and 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH) constantly generates ROO· at physiological relevant pH.

1.2.4.2 Cell-based Antioxidant Assays

Although chemical-based antioxidant assays provide valuable information regarding the free radical scavenging capacity under optimal conditions for tested samples, the results cannot be totally extrapolated to biological systems. This antioxidant capacity is not limited to only free radical scavenging ability but also includes modulation of oxidative stress related transcription factors and the interplay with antioxidant enzyme activities. Cellular antioxidant activity assays provide biologically relevant methods to measure the activity of antioxidants at the cellular level, because they account for some important factors such as cellular uptake, distribution, and metabolism (Zhang et al., 2013). To show antioxidant activity of coffee components in cell-based model systems, investigators have used a number of different cell lines, stimulators of oxidative stress, and various endpoints that point to specific underlying mechanisms of antioxidant activity or oxidative stress. There are a variety of cell-based in vitro models available for studying antioxidant activity of different food components, where chemical or physical stressors are used to induce oxidative stress prior to or during exposure of cells to a potential antioxidant compound. For example, human colon carcinoma Caco-2 cell (Sessa et al., 2011), human liver Hep G2 cell (Wolfe & Liu, 2007), human vascular endothelia cell line EA. Hy926 (Ziberna et al., 2010), and human macrophage cell line U937 (Roy et al., 2009) have been used.
in cell-based antioxidant mechanism assays. The oxidative stress in these cell-based systems is induced by different stressors, such as chemical (AAPH, hydrogen peroxide, and tert-tutyl hydroperoxide (t-BOOH)), and also physical (radiation and hyperoxia) stimulation. The redox sensor dichlorofluorescein diacetate (DCFH-DA), is commonly used as a probe to indicate the oxidative status in AAPH stimulated cells because DCFH-DA is easily oxidized to fluorescent dichlorofluorescein (DCF) by the peroxyl radicals generated from AAPH. The stronger the antioxidant activity of the tested sample, the less available the peroxyl radical; the consequence is a weaker fluorescent signal. The application of DCFH-DA is not limited to AAPH stimulated peroxyl radical but can also be used with other oxidative stress stimulation (Wolfe & Liu, 2007). Actual measures of cellular metabolism resulting in changes in oxidative stress include determining the concentrations of reduced glutathione (GSH), the presence of a secondary lipid oxidation product, malondialdehyde (MDA), and activities of antioxidant enzymes including GPx, GR, and SOD. GSH is an important intracellular antioxidant that prevents damage to cellular ROS as well as being a substrate for glutathione enzymes. MDA, a product of secondary lipid peroxidation is a useful marker for indicating the extent of lipid oxidation that defines potential subsequent reactions with macromolecules and secondary oxidation products. For example, MDA reacts with DNA to form adducts that represent oxidative stress specific to DNA damage. An important measurement for this event involves detecting oxidized purines, such as 8-hydroxydeoxyguanosine (Cooke et al., 2003). Genomic DNA integrity is another important biomarker of redox status because reactive species continuously attack DNA structure and cause DNA strand breaks, crosslinks, or sister chromatid exchanges, resulting in oxidative damage of DNA (Niu et al., 2015). Furthermore, ROS affect the DNA methylation by oxidizing key enzymes involved in the methylation process (Jena, 2012). Moreover, ROS easily initiate lipid
oxidation *in vitro*, leading to the accumulation of lipid peroxidation products, such as hydroperoxides and MDA, characteristic components of first and second stages of lipid oxidation reactions, respectively. Oxidative stress related transcription factors and antioxidant enzymes are also commonly used measures that reflect the redox status of the cell. The level of Nrf2 that translocate to the nucleus is a useful indicator to show whether the tested sample has an antioxidant effect. Coffee constituents are demonstrated as modulators of Nrf2 nuclear translocation and thus inducing an antioxidant response element-mediated expression of Phase II enzymes can involve a detoxification and antioxidant defense (Boettler et al., 2011).

### 1.2.4.3 Animal-based Antioxidant Assays

Animal studies have also provided a comprehensive understanding on the ability of food constituents to modulate oxidative status *in vivo*. Since oxidative stress is the initiator of many chronic diseases such as inflammation, diabetes, Parkinson’s disease and Alzheimer’s disease, different animal models have been employed to study the specific affinity of coffee to mitigate oxidative stress related to these disorders. Oxidative status in these animal models is determined by measuring the susceptibility of inducing lipid peroxidation, changes in antioxidant enzyme activity, up-regulation of oxidative stress related genes and transcription factors.

### 1.2.5 Coffee Serves as a Source of Dietary Antioxidants

Coffee represents one of the most popular consumer beverages globally. In the last decade, many epidemiological studies have reported coffee consumption to be associated with reduced risk of several chronic diseases that have in common an oxidative or inflammatory underlying mechanism of induction (Andersen et al., 2006) (Van Dam et al., 2006) (Xu et al.,...
The physiological chemistry that underlies these claims has been attributed to a number of bioactive compounds that exhibit antioxidant and anti-inflammatory activities. Green coffee beans are a complex source of multiple bioactive constituents with characteristic free radical or antioxidant activity, that include in varying quantities of caffeine, CGA, trigonelline, cafestol, and kahweol, depending on the source. In addition, the process of roasting coffee beans produces a series of changes to the chemical composition of coffee, leading to the formation of characteristic flavor, aroma and browning pigments. In roasted coffee beans, melanoidins generated from non-enzymatic browning exhibit antioxidant activity. Therefore, the antioxidant capacity of coffee brew is not only contributed to those components originally present in green beans, but also from components that are generated during the conditions of the roasting process. The antioxidant capacities of hydrophilic coffee components such as caffeine, CGA, and melanoidins against different free radicals have been extensively investigated (Liang et al., 2016). Other hydrophobic compounds such as cafestol, kahweol, and trigonelline also showed antioxidant activity in cellular and animal models.

Evaluation of the antioxidant capacity of coffee therefore has been the focus for many studies that have used distinct in vitro chemical and enzymatic assays; some of which employ stable radicals as probes to quantitate free radical scavenging activity. Other assays to be described employ methods that generate non-stable reaction products to assess the radical quenching power of coffee constituents. Former, but still very popular methods are the chemical antioxidant assays that involve chromogen compounds of a radical nature used to simulate ROS. The presence of antioxidant compounds present in coffee leads to the disappearance of radical chromogens and the activity in doing so is calculated from the disappearance of color, or
absorbance readings generated from a specific UV spectrum. Examples of widely used chromogens that have received considerable use in chemical methods of antioxidant detection are the stable free radicals, DPPH· and 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS·) in the DPPH and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS assay, respectively. In the DPPH assay, an odd electron displays a strong absorption band at a wavelength of 519 nm, which loses absorption once the odd electron is paired off by a hydrogen, or electron-donating antioxidant. Other chemical assays use unstable free radicals, such as peroxyl radical (ROO·), superoxide radical anion (O2·-), and hydroxyl radical (HO·-), as examples of ROS, generated at fixed rates over time in chemical reactions that attempt to mimic physiological mechanism in vivo. In these examples, the oxygen radical antioxidant capacity (ORAC), hydroxyl radical scavenging assay, and superoxide radical scavenging capacity assay have all been used, respectively. DPPH, ABTS, Ferric Reducing Ability of Plasma (FRAP) assay, ORAC, Hydroxyl Radical Scavenging Assay, and O2·- Scavenging Capacity Assay have been used to measure antioxidant activity of coffee beans/brew by different investigators. Finally, an important extension from the free radical scavenging power of coffee extracts is to extrapolate these results with potential anti-peroxidation activity, using model lipid systems and end-point measures of primary lipid oxidation (Morales & Jiménez-pérez, 2004). Table 1.1 lists some examples of the application of these assays in evaluating antioxidant activity of coffee beans/brew.
Table 1.1 Chemical Assays That Commonly Used to Evaluate Antioxidant Capacity of Coffee and Examples of Application of These Assays to Measure Antioxidant Activity of Coffee Bean/brew.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Source of free radical</th>
<th>Method of quantification of the targeted free radical</th>
<th>Application to measure antioxidant activity of coffee</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Species of the beans</td>
</tr>
<tr>
<td>DPPH</td>
<td>Dissolve DPPH in ethanol</td>
<td>1. Measure the absorption at 517 nm; 2. EPR</td>
<td>Blend of different varieties</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Green</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Blend of different varieties</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arabica, and Robusta</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Light, medium, and dark</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arabica, and Robusta</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>Green</td>
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<td></td>
<td></td>
<td></td>
<td>Arabic (219 °C for 905 sec); Robusta (228 °C for 859 sec)</td>
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<tr>
<td>ABTS</td>
<td>Oxidize ABTS with potassium persulfate</td>
<td>1. Measure the absorption at 645 nm, 734 nm, or 815 nm; 2. EPR</td>
<td>Blend of 80% Arabica and 20% Robusta</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arabica, Robusta, and a blend of these two</td>
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<td></td>
<td></td>
<td></td>
<td>Arabica</td>
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<td></td>
<td></td>
<td></td>
<td>Green, light, medium, and dark</td>
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<td></td>
<td></td>
<td></td>
<td>Arabica</td>
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<tr>
<td></td>
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<td></td>
<td>Light (225 °C for 3 min); medium (233°C for 3 min); dark (240 °C for 3 min)</td>
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<td></td>
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<td></td>
<td>Arabica, and Robusta</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Arabic (219 °C for 905 sec); Robusta (228 °C for 859 sec)</td>
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<td></td>
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</tbody>
</table>

Reference:
- Ramalakshmi et al. (2008)
- Madzhitov et al. (2008)
- Ludwig et al. (2012)
- Cammerer & Kroh (2006)
- Sacchetti et al. (2009)
- Del Castillo et al. (2002)
- Ludwig et al. (2012)
<table>
<thead>
<tr>
<th>Assay</th>
<th>Source of free radical</th>
<th>Method of quantification of the targeted free radical</th>
<th>Application to measure antioxidant activity of coffee</th>
</tr>
</thead>
<tbody>
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<tr>
<td></td>
<td></td>
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<td>Species of the beans</td>
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<td></td>
<td></td>
<td></td>
<td>Roasting degree of the beans</td>
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<td></td>
<td></td>
<td></td>
<td>Reference</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fe$^{3+}$/tripyrindtriazine complex</td>
<td>Measure the absorption of ferrous at 593 nm</td>
<td>Blend of different species</td>
</tr>
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<tr>
<td>ORAC</td>
<td>Dissolve AAPH in buffer to form peroxy radicals</td>
<td>1. β-phycoerythrin</td>
<td>Arabica</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Arabica and Robusta</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>OH·- Scavenging Assay</td>
<td>1. Fe$^{2+}$ +H$_2$O$_2$</td>
<td>1. Deoxyribose, Benzoate salicylate</td>
<td>Arabica, and Robusta</td>
</tr>
<tr>
<td></td>
<td>2. DMSO+ H$_2$O$_2$</td>
<td>2. EPR with spin trap reagent (DMPO)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Photochemical decomposition of H$_2$O$_2$</td>
<td>3.</td>
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<td>Arabica</td>
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<td>Arabica and Robusta</td>
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<td></td>
<td>Arabica</td>
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<td></td>
<td>Arabica and Robusta</td>
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</table>

Mullen et al. (2011) 190±3 °C for 18-20 min Parras et al. (2007)
<table>
<thead>
<tr>
<th>Assay</th>
<th>Source of free radical</th>
<th>Method of quantification of the targeted free radical</th>
<th>Application to measure antioxidant activity of coffee</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Species of the beans</td>
</tr>
<tr>
<td>O$_2^-$. Scavenging Assay</td>
<td>Hypoxanthine/xanthine+O$_2^+$+uric acid</td>
<td>1. O$_2^-$. reduce the probe NBT to a purple formazan which could be measured at 562 nm</td>
<td>Arabica</td>
</tr>
<tr>
<td></td>
<td>PMS+NADH →O$_2^-$.</td>
<td>2. EPR with spin trap reagent (DMPO or BMPO)</td>
<td></td>
</tr>
</tbody>
</table>
1.2.6 Oxidative Stress and Nrf-2 Cell Signaling

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated endogenously by mitochondrial respiration and are also derived from exogenous exposure to oxidizing agents including ionizing radiation, heavy metals, and hypoxia (Davis et al., 2010) (Jena, 2012). The term ROS/RNS is used to include not only the superoxide anions (O$_2^-$), hydroxyl radicals (·OH), nitric oxide radicals (NO·), and peroxyl radicals (ROO·), but also non-radical oxidants, such as hypochlorous acid (HOCl), singlet oxygen (¹O₂), peroxynitrite (ONOOC·), and hydrogen peroxide (H₂O₂), which are all capable of oxidizing important biomolecules (Halliwell & Gutteridge, 2007). Dietary components such as vitamin E, vitamin C, and phenolic compounds also serve as non-enzymatic antioxidants. Together, the endogenous and dietary derived antioxidants constitute our antioxidant defense system. When there is an imbalance between the generation of ROS/RNS and the capacity of the antioxidant defense system to neutralize reactive molecules, oxidative stress occurs. The Nrf2 is a master regulator of cellular resistance to oxidative stress. Nrf2 plays an essential role in the antioxidant response element-mediated expression of phase II detoxifying enzymes, including glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD) (Motohashi & Yamamoto, 2004) (Zatorski et al., 2015).

1.2.7 Antioxidant Activity of CGAs

1.2.7.1 Evidences Obtained from Chemical-based Assays

All CGA isomers are potent antioxidants, as they possess one to two aromatic rings linked to hydroxyl groups and the one-electron oxidation product of CGAs formed by the reaction with free radicals is rapidly broken down to non-free radical products (Shibata et al.,...
Chemical based assays have shown that CGAs have the capacity to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, superoxide anions (O$_2^-$), hydroxyl radicals (·OH) (Cha et al., 2014) (Zhang et al., 2003), ABTS radicals (Oboh et al., 2013), lipid oxidation (Kono et al., 1997) (Oboh et al., 2013), and peroxynitrite (ONOO-) (Kono et al., 1997). CGAs react with different sources of free radicals at different rate constants. The second-order rate constants for 5-CQA reacting with superoxide, hydroxyl radical, peroxyl radical, and peroxynitrite have been determined to be 0.96±0.01×10$^6$ M$^{-1}$ s$^{-1}$, 3.34±0.19×10$^9$ M$^{-1}$ s$^{-1}$, 1.28±0.11×10$^5$ M$^{-1}$ s$^{-1}$, and 1.6±0.7×10$^5$ M$^{-1}$ s$^{-1}$ respectively (Kono et al., 1997). This result shows that the relative efficiency of 5-CQA to react with free radicals is radical species specific.

Another important activity of CGAs towards ROS-induced oxidative stress involves the radical damage caused to DNA that can be quantified to include DNA strand breakage. Six CGA isomers, namely, 3-CQA, 4-CQA, 5-CQA, 3,5-diCQA, 3,4-diCQA, and 4,5-diCQA, were shown to exhibit a protective effect against H$_2$O$_2$-induced DNA plasmid chromosome breaks (Xu et al., 2012). Secondary by-products of the oxidation reaction are also relevant in initiating a mutagenic response as a result of DNA damage. One example concerns the oxidation of chloride by H$_2$O$_2$, resulting in formation of HOCl, and subsequent reaction with amines to produce NH$_2$Cl, an oxidant and potent mutagen. CGAs, specifically 5-CQA, have been shown to protect against NH$_2$Cl-induced plasmid DNA breakage in cultured neutrophils (Shibata et al., 1999). Another example of the anti-peroxidation activity of 5-CQA comes from the protection against low-density lipoprotein (LDL) oxidation, which is the initial step in the development of atherosclerosis. Studies based on incubating isolated LDL with oxidizing agents in vitro showed that 5-CQA was effective at mitigating both copper-induced LDL oxidation (Gordon & Wishart, 2010) and ferrylmyoglobin-induced LDL oxidation (Laranjinha et al., 1996). These results
correspond to other studies that reported reduced MDA content in brain tissues when pre-treated with 5-CQA (Oboh et al., 2013). Table 1.1 is a summary of the results obtained from chemical-based studies that have examined the antioxidant activity of CGAs.
<table>
<thead>
<tr>
<th>Chemical Assays</th>
<th>End-point Measure</th>
<th>CGA isomer</th>
<th>Concentration/Exposure Time</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH assay</td>
<td>DPPH</td>
<td>5-CQA</td>
<td>5 ~ 80 µM for 3 h</td>
<td>10% ~ 90% inhibition on DPPH</td>
<td>Cha et al. (2014)</td>
</tr>
<tr>
<td>Xanthine/xanthine oxidase system</td>
<td>DMPO/·OOH adducts</td>
<td>5-CQA</td>
<td>20 µM for 2.5 min</td>
<td>↓ 30% ·OOH</td>
<td>Cha et al. (2014)</td>
</tr>
<tr>
<td>FeSO₄⁺ H₂O₂</td>
<td>DMPO/·OH adducts</td>
<td>5-CQA</td>
<td>20 µM for 2.5 min</td>
<td>↓ 51% ·OH</td>
<td>Cha et al. (2014)</td>
</tr>
<tr>
<td>FeSO₄⁺ H₂O₂</td>
<td>DMPO/·OH adducts</td>
<td>5-CQA</td>
<td>100 ~ 400 µM for 1 min</td>
<td>↓ 50% to 80% ·OH</td>
<td>Zhang et al. (2003)</td>
</tr>
<tr>
<td>ABTS assay</td>
<td>ABTS⁻⁺</td>
<td>5-CQA</td>
<td>Serials concentration for 15 min</td>
<td>The ability of 100 g of CGA in scavenging ABTS⁻⁺ is equivalent to 3.7 mmol Trolox</td>
<td>Oboh et al. (2013)</td>
</tr>
<tr>
<td>Rat brain homogenates + sodium</td>
<td>MDA</td>
<td>5-CQA</td>
<td>1.56 ~ 6.25 µg/mL</td>
<td>No significant inhibition of MDA</td>
<td>Oboh et al. (2013)</td>
</tr>
<tr>
<td>nitroprusside system</td>
<td></td>
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</tr>
<tr>
<td>Liposome system containing AAPH</td>
<td>MDA</td>
<td>5-CQA</td>
<td>0.1 ~ 0.5 mM</td>
<td>Second order rate of constant of the reactions of LOO⁻ with CGA is 1.28 ± 0.11 × 10⁵ M⁻¹ s⁻¹</td>
<td>Kono et al. (1997)</td>
</tr>
<tr>
<td>Pulse radiolysis to generate O₂⁻</td>
<td>O₂⁻</td>
<td>5-CQA</td>
<td>0.2 ~ 0.75 mM</td>
<td>Second order rate of constant of the reactions of O₂⁻ with CGA is 0.96 ± 0.01×10⁶ M⁻¹ s⁻¹</td>
<td>Kono et al. (1997)</td>
</tr>
<tr>
<td>Chemical Assays</td>
<td>End-point Measure</td>
<td>CGA isomer</td>
<td>Concentration/Exposure Time</td>
<td>Results</td>
<td>Ref.</td>
</tr>
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<tr>
<td>Fenton type reaction to generate ·OH</td>
<td>·OH</td>
<td>5-CQA</td>
<td>0.1 ~ 0.75 mM</td>
<td>Second order rate constant of the reactions of ·OH with CGA is 3.34 ±0.19×10^9 M⁻¹ s⁻¹</td>
<td>Kono et al. (1997)</td>
</tr>
<tr>
<td>Potassium phosphate to generate ONOO⁻</td>
<td>ONOO⁻</td>
<td>5-CQA</td>
<td>80 µM</td>
<td>Second order rate constant of the reactions of ONOO⁻ with CGA is 1.6 ±0.7×10^5 M⁻¹ s⁻¹</td>
<td>Kono et al. (1997)</td>
</tr>
<tr>
<td>DPPH assay</td>
<td>DPPH</td>
<td>3-CQA, 4-CQA, 5-CQA, 3,5-diCQA, 3,4-diCQA, 4,5-diCQA</td>
<td>5 µg/mL ~ 60 µg/mL</td>
<td>EC₅₀ ³-CQA: 13.4 µg/mL 4-CQA: 13.2 µg/mL 5-CQA: 13.8 µg/mL 3,5-diCQA: 9.3 µg/mL 3,4-diCQA: 9.4 µg/mL 4,5-diCQA: 7.5 µg/mL</td>
<td>Xu et al. (2012)</td>
</tr>
<tr>
<td>ABTS assay</td>
<td>ABTS⁺</td>
<td>3-CQA, 4-CQA, 5-CQA, 3,5-diCQA, 3,4-diCQA, 4,5-diCQA</td>
<td>50 µg/mL ~ 150 µg/mL</td>
<td>EC₅₀ ³-CQA: 91.4 µg/mL 4-CQA: 87.5 µg/mL 5-CQA: 91.5 µg/mL 3,5-diCQA: 77.6 µg/mL 3,4-diCQA: 77.4 µg/mL 4,5-diCQA: 67.3 µg/mL</td>
<td>Xu et al. (2012)</td>
</tr>
<tr>
<td>Chemical Assays</td>
<td>End-point Measure</td>
<td>CGA isomer</td>
<td>Concentration/Exposure Time</td>
<td>Results</td>
<td>Ref.</td>
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<tr>
<td>FRAP assay</td>
<td>Reducing power</td>
<td>3-CQA, 4-CQA, 5-CQA, 3,5-diCQA, 3,4-diCQA, 4,5-diCQA</td>
<td>25 ~ 125 µg/mL</td>
<td>4,5-diCQA &gt; 3,5-diCQA &gt; 3,4-diCQA &gt; 5-CQA = 4-CQA = 3-CQA</td>
<td>Xu et al. (2012)</td>
</tr>
<tr>
<td>DNA damage protective effect assay</td>
<td>DNA damage</td>
<td>3-CQA, 4-CQA, 5-CQA, 3,5-diCQA, 3,4-diCQA, 4,5-diCQA</td>
<td>50 µg/mL</td>
<td>↓ 43.1 to 62.4% DNA damage</td>
<td>Xu et al. (2012)</td>
</tr>
<tr>
<td>Plasmid pUC18 + NH₂Cl</td>
<td>supercoiled DNA, nicked circular DNA and linear duplex</td>
<td>5-CQA</td>
<td>0.01 mM ~ 1.23 mM</td>
<td>Prevented a stepwise conversion of plasmid DNA form supercoiled DNA, nicked circular DNA and linear duplex DNA</td>
<td>Shibata et al. (1999)</td>
</tr>
<tr>
<td>LDL + copper</td>
<td>Conjugated dienes</td>
<td>5-CQA</td>
<td>0.25 ~ 1.0 µM</td>
<td>↑ lag time of LDL oxidation</td>
<td>Gordon &amp; Wishart (2010)</td>
</tr>
<tr>
<td>LDL + metmyoglobin + H₂O₂</td>
<td>ROS</td>
<td>5-CQA</td>
<td>1 molar ratio to metmyoglobin</td>
<td>Effectively blocked LDL oxidation</td>
<td>Laranjinha et al. (1996)</td>
</tr>
</tbody>
</table>

*EC₅₀ represents the concentration of the tested compound that results in half-maximal response.*
1.2.7.2 Evidences Obtained from Cell-based Assays

Research directed at characterizing antioxidant capacity of CGAs using cell-based models has focused mostly on the 5-CQA isomer. 5-CQA protects against H$_2$O$_2$ induced oxidative stress in human HaCaT cells (Cha et al., 2014); can activate Akt phosphorylation and increase the expression of FOXO family genes in mesenchymal stem cells of bone marrow (Li et al., 2012); and reduces apoptosis in primary cortical neurons by up-regulating antioxidant enzymes such as NADPH:quinine oxidoreductase 1 (Kim et al., 2012). The protective effect of 5-CQA against oxidative stress stimulated by various oxidative stressors (e.g. $\ell$-BHP, H$_2$O$_2$, FeSO$_4$) has also been studied in PC12 cells, which used multiple biomarkers such as the reduction of lipid peroxidation product, the extent of ROS formation, and GSH depletion to evaluate the utility of CGAs to prevent oxidation (Pavlica et al., 2005). 5-CQA showed effectiveness in protecting against DNA damage with activity to inhibit methylation of the promoter region of the RAR beta gene through increased formation of S-adenosyl-L-homocysteine in cultured human breast cancer cells, MCF-7 and MAD-MB-231, respectively (Lee & Zhu, 2006). 5-CQA also decreased the DNA damage by 4.49 ~ 48.15% in human blood lymphocytes caused by X-ray irradiation (Cinkilic et al., 2013).

In addition to these examples of chemical induced oxidation, other studies have successfully shown that CGAs are effective at reducing damage caused by exposure to ultraviolet A light (UVB) (Heck et al., 2003). The photo oxidation-protection offered by 5-CQA in mouse epidermal cell line (Feng et al., 2005) and human HaCaT keratinocytes (Cha et al., 2014) were related to the effects of 5-CQA to trigger induction of Nrf2 transactivation and phase II enzyme activities.
There are only a few studies that have investigated the affinity of minor CGA isomers to modulate redox status in biological systems. Three CGA isomers, namely 3-CQA, 4-CQA, and 5-CQA, have affinity to protect against H$_2$O$_2$ induced apoptosis in PC12 cells by suppressing mitochondrial membrane depolarization, caused by oxidative stress (Park, 2013). Recently, another research group reported that 5-CQA and 3,5-diCQA had a protective effect against t-BOOH induced ROS generation in HepG2 cells (Baeza et al., 2014).

1.2.7.3 Evidences Obtained from Animal-based Assays

Given the positive data from cell culture experiments that show antioxidant properties of specific CGAs at both cellular and molecular levels, other studies conducted in rodent models have confirmed these observations by examining the redox status in animals exposed to a variety of forms of oxidative stress when fed CGAs. Furthermore, the efficacy of dietary intake of CGAs to prevent pathogenesis of a wide range of chronic disease states has been examined. One example includes the side effect of hyperglycemia that occurs with diabetes, and which leads to an increase ROS production and increased susceptibility to oxidative stress (Bonnefont-Rousselot, 2002). The antioxidant activity of 5-CQA in diabetic rat models showed that feeding 5-CQA effectively reduced lipid hydroperoxide production and increased the level of non-enzymatic antioxidants, such as reduced glutathione, Vitamins, C and E (Karthikesan et al., 2010) (Pari et al., 2010). Other studies have shown that 5-CQA can alleviate oxidative stress induced by methamphetamine in rats by restoring liver SOD, GPx activities and preventing the accumulation of MDA (Koriem & Soliman, 2014).
The role of CGAs in prevention of environmental toxicity caused by heavy metal pollution, specifically Cd has produced data showing protection against the induction of oxidative stress in the central nervous system (Kasprzak, 2002). Pretreatment of rats with 5-CQA before Cd exposure significantly restored the depleted levels of GSH, vitamin C and vitamin E, and attenuated Cd-induced MDA level in brain tissue (Hao et al., 2015). Other model systems used scopolamine, a muscarinic antagonist which significantly increases MDA levels in the cortex and hippocampus (Jeong et al., 2008). The scopolamine-induced amnesic mouse, an animal model to study Alzheimer’s disease, has produced data to show that 5-CQA decreased MDA level in both frontal cortex and the hippocampus of scopolamine induced anemia in mice (Kwon et al., 2010). The anti-amnesic activity of 5-CQA was attributed to the affinity to reduce lipid peroxidation in addition to reducing free radical scavenging activity (Kwon et al., 2010). Former studies have also examined the role of 5-CQA to enhance detoxification of environmental toxic residues derived from polyaromatic hydrocarbon (PAHs) exposure in mice (Kitts & Wijewickreme, 1994). Dietary CGAs were effective at enhancing gastrointestinal xenobiotic detoxification enzymes that are central to the detoxification of PAHs. The more recent findings that 5-CQA protects against oxidative stress through its activation of Nrf2 nuclear translocation and up-regulation of cellular antioxidant enzymes confirms the observation reported earlier in mice fed PAHs (Feng et al., 2005).

ROS are also implicated in the development of ischemia/reperfusion (I/R) injury in the intestine and pathogenesis of colorectal cancer. An intestinal I/R model and a colorectal cancer model were used to assess the ability of 5-CQA in alleviating oxidative stress in these sections of the gastrointestinal tract. Sato et al (2011) reported in rats with I/R injury induced by sodium
pentobarbital, that dietary intakes of CGA at concentrations ranging from 0.5 ~ 1.0 mM were effective to improve capillary permeability of the small intestine and a capacity to reduce oxidative stress. In the azoxymethane-induced colon cancer mouse model, a 20% reduction in small intestinal GSH levels occurred, pointing to an induced oxidative stress condition. Researchers found that feeding diets containing 0.1% 5-CQA for 20 weeks attenuated azoxymethane-induced oxidative stress by bringing GSH levels back to normal levels (Park et al., 2010).

Ultraviolet radiation and gamma radiation, can also trigger the generation of ROS and consequently cause chromosomal damage in animals (Heck et al., 2003). Intradermal delivery of 5-CQA in guinea pigs during exposure to UVB reduced photooxidation-induced damage of skin attributed to photooxidation stress (Kitagawa et al., 2011). Another study showed that an oral administration of 5-CQA to mice at a concentration of 100 mg/kg body weight before exposure to gamma radiation significantly reduced chromosomal damage (Abraham et al., 1993).

Since oxidative stress is implicated with a wide range of chronic diseases, it is challenging to understand the role of specific antioxidants in different pathological and physiological conditions. Numerous animal models have been used to identify a useful biomarker that will reflect the initiation of oxidative stress so that the quality of the antioxidant can be evaluated. Despite using different methodologies, there is strong evidence that CGAs are effective antioxidants that will protect against oxidation reactions in vivo by up-regulating redox related nuclear transcription factors involved in the expression of antioxidant enzymes. Majority of the studies are focused on the primary isomer of CGA being 5-CQA, with a lesser amount of
information available for other minor CGA isomers. **Table 1.3** is a summary of the cell-based and animal studies describing the antioxidant activity of CGAs reviewed in this section.

### 1.2.8 Phenoxy Radical

Phenolic acid exhibit antioxidant activity through donating electron/electrons to free radicals. For example, phenolic acid traps the chain-carrying peroxy radicals (ROO·), forming a hydroperoxide (ROOH) and a derived resonance-stabilized phenoxy radicals (ArO·), reaction 1 (Foti et al., 1994). The phenoxy radical has the capacity to react with another peroxy radical to form non-radical products, terminating the reaction (Reaction 2) or alternatively react again to produce another peroxy radical (Reaction 3) (Foti et al., 1994).

\[
\begin{align*}
\text{ROO}^\cdot + \text{ArOH} &\rightarrow \text{ROOH} + \text{ArO}^\cdot \quad (1) \\
\text{ROO}^\cdot + \text{ArO}^\cdot &\rightarrow \text{nonradical products} \quad (2) \\
\text{ArO}^\cdot + \text{RH} &\rightarrow \text{ArOH} + \text{R}^\cdot \rightarrow \text{ROO}^\cdot \quad (3)
\end{align*}
\]
Table 1.3 Summary of Studies that Evaluated the Capacity of CGAs to Modulate Oxidative Stress in Cell-based and Animal-based Models.

<table>
<thead>
<tr>
<th>Model</th>
<th>End-point Measure</th>
<th>CGA isomer</th>
<th>Concentration/Exposure Time</th>
<th>Results compared to the control without CGA treatment</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell-based assay</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HaCaT cell + H₂O₂</td>
<td>H₂O₂</td>
<td>5-CQA</td>
<td>20 µM for 20 h</td>
<td>↓ ROS</td>
<td>Cha et al. (2014)</td>
</tr>
<tr>
<td>HaCaT cell + UVB</td>
<td>ROS, DNA damage, cell viability</td>
<td>5-CQA</td>
<td>20 µM for 20 h</td>
<td>↓ ROS, ↓ DNA damage, ↑ 13% cell viability</td>
<td>Cha et al. (2014)</td>
</tr>
<tr>
<td>Mesenchymal stem cell + H₂O₂</td>
<td>Chromosomal condensation, cell apoptosis, ROS</td>
<td>5-CQA</td>
<td>10 mM for 12 h</td>
<td>↓ Chromosomal condensation, ↓ Cell apoptosis, ↓ ROS</td>
<td>Li et al. (2012)</td>
</tr>
<tr>
<td>Primary cortical neurons + H₂O₂</td>
<td>NADPH: quinine oxidoreductase 1, Cell viability</td>
<td>5-CQA</td>
<td>12.5 ~ 100 µM for 1 h</td>
<td>↑ NADPH: quinine oxidoreductase 1, ↑ Cell viability</td>
<td>Kim et al. (2012)</td>
</tr>
<tr>
<td>Differentiated neuronal PC12 cells + H₂O₂</td>
<td>Cell viability, GSH</td>
<td>5-CQA</td>
<td>6.2 ~ 25 µM for 2 h</td>
<td>↑ Cell viability, Attenuated GSH decrease</td>
<td>Pavlica &amp; Gebhardt (2005)</td>
</tr>
<tr>
<td>Differentiated neuronal PC12 cells + FeSO₄</td>
<td>Cell viability, ROS, MDA</td>
<td>5-CQA</td>
<td>6.2 ~ 25 µM for 2 h</td>
<td>Did not change cell viability, ↓ ROS level, ↓ MDA</td>
<td>Pavlica &amp; Gebhardt (2005)</td>
</tr>
<tr>
<td>Differentiated neuronal PC12 cell + t-BHP</td>
<td>Cell viability, GSH</td>
<td>5-CQA</td>
<td>6.2 ~ 25 µM for 2 h</td>
<td>↑ Cell viability, Did not change GSH level</td>
<td>Pavlica &amp; Gebhardt (2005)</td>
</tr>
<tr>
<td>Model</td>
<td>End-point Measure</td>
<td>CGA isomer</td>
<td>Concentration/Exposure Time</td>
<td>Results compared to the control without CGA treatment</td>
<td>Ref.</td>
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<td><strong>Cell-based assay</strong></td>
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<tr>
<td>Human breast cancer cell line MCF-7 +</td>
<td>Global methylation status</td>
<td>5-CQA</td>
<td>1 ~ 20 µM for 8 days</td>
<td>↓ Global methylation</td>
<td>Lee &amp; Zhu (2006)</td>
</tr>
<tr>
<td>Human breast cancer cell line MDA-MB-231 +</td>
<td>Global methylation status</td>
<td>5-CQA</td>
<td>0.2 ~ 20 µM for 3 days</td>
<td>Did not change the global methylation status</td>
<td>Lee &amp; Zhu (2006)</td>
</tr>
<tr>
<td>Human lymphocyte + X-ray radiation</td>
<td>Genetic damage index</td>
<td>5-CQA</td>
<td>0.5 ~ 4 µg/mL</td>
<td>↓ Genetic damage index by 4.49 to 48.15%</td>
<td>Cinkilic et al. (2013)</td>
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<tr>
<td>Mouse epidermal cell line JB6 + UVB</td>
<td>GST, NADPH:quinone oxidoreductase, Nrf2</td>
<td>5-CQA</td>
<td>5 ~ 160 µM for 1 h</td>
<td>↑ GST</td>
<td>Feng et al. (2005)</td>
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<td>↑ NADPH:quinone oxidoreductase</td>
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<td>↑ Nrf2 nuclear translocation</td>
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<tr>
<td>Differentiated neuronal PC12 cell + H2O2</td>
<td>Mitochondrial membrane depolarization</td>
<td>3-CQA, 4-CQA, 5-CQA</td>
<td>10 µM for 20 min</td>
<td>Protected mitochondrial membrane depolarization through ↓ Caspase 9 activation</td>
<td>Park (2013)</td>
</tr>
<tr>
<td>Human hepatoma HepG2 cell + t-BOOH</td>
<td>ROS, GSH, GPx, GR, MDA</td>
<td>5-CQA, 3,5-diCQA</td>
<td>10 ~ 20 µM for 20 h</td>
<td>↓ ROS, ↑ GSH, ↑ GR, ↓ GPx, ↓ MDA</td>
<td>Baeza et al. (2014)</td>
</tr>
<tr>
<td>Model</td>
<td>End-point Measure</td>
<td>CGA isomer</td>
<td>Concentration/Exposure Time</td>
<td>Results compared to the control without CGA treatment</td>
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<td><strong>Animal Models</strong></td>
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<tr>
<td>Type 2 diabetic rat model</td>
<td>Lipid peroxidation, GSH, V&lt;sub&gt;C&lt;/sub&gt;, V&lt;sub&gt;E&lt;/sub&gt;</td>
<td>5-CQA</td>
<td>Oral administration at 5 mg/kg body weight daily for 45 days</td>
<td>↓ Plasma lipid hydroperoxides, ↑ GSH, ↑ V&lt;sub&gt;C&lt;/sub&gt;, ↑ V&lt;sub&gt;E&lt;/sub&gt;</td>
<td>Karthikesan et al. (2010)</td>
</tr>
<tr>
<td>Type 2 diabetic rat model</td>
<td>Lipid peroxidation, GST, SOD, GPx, CAT</td>
<td>5-CQA</td>
<td>Oral administration at 5 mg/kg body weight daily for 45 days</td>
<td>↓ Lipid oxidation, ↑ GST, ↑ SOD, ↑ GPx, ↑ CAT</td>
<td>Pari et al. (2010)</td>
</tr>
<tr>
<td>Methamphetamine induced oxidative stress rat model</td>
<td>NO, MDA, SOD, GPx</td>
<td>5-CQA</td>
<td>Oral administration at 60 mg/kg body weight, single dose</td>
<td>↓ NO, ↓ MDA, ↑ SOD, ↑ GPx</td>
<td>Koriem &amp; Soliman (2014)</td>
</tr>
<tr>
<td>Cd induced brain impairment rat model</td>
<td>SOD, CAT, GPx, GSH, V&lt;sub&gt;C&lt;/sub&gt;, V&lt;sub&gt;E&lt;/sub&gt;, MDA</td>
<td>5-CQA</td>
<td>Intragastric administration, 60 mg/kg body weight daily for 30 days</td>
<td>↑ SOD, ↑ CAT, ↑ GPx, ↑ GSH, ↑ V&lt;sub&gt;C&lt;/sub&gt;, ↑ V&lt;sub&gt;E&lt;/sub&gt;, ↓ MDA</td>
<td>Hao et al. (2015)</td>
</tr>
<tr>
<td>Scopolamine induced brain impairment rat model</td>
<td>MDA</td>
<td>5-CQA</td>
<td>Orally administered at 3 ~ 9 mg/kg body weight, single dose</td>
<td>↓ MDA</td>
<td>Kwon et al. (2010)</td>
</tr>
<tr>
<td>Benzopyrene induced gastrointestinal pathogenesis rat model</td>
<td>GST, Cytochrome P-450,</td>
<td>5-CQA</td>
<td>Eating 0.2% 5-CQA containing diet for 10 weeks</td>
<td>↑ GST, Did not significantly change cytochrome P-450</td>
<td>Kitts &amp; Wijewickreme (1994)</td>
</tr>
<tr>
<td>Model</td>
<td>End-point Measure</td>
<td>CGA isomer</td>
<td>Concentration/ Exposure Time</td>
<td>Results compared to the control without CGA treatment</td>
<td>Ref.</td>
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<tr>
<td>Sodium pentobarbital induced intestinal ischemia-reperfusion rat model</td>
<td>Vascular permeability in the small intestine</td>
<td>5-CQA</td>
<td>Directly administrate 1 mM into jejunum, single dose</td>
<td>Attenuated the increased vascular permeability</td>
<td>Sato et al. (2011)</td>
</tr>
<tr>
<td>Azoxymethane induced colon cancer mice model</td>
<td>GSH/GSSG ratio</td>
<td>5-CQA</td>
<td>Orally administered at 0.1% 5-CQA containing diet for 20 weeks</td>
<td>↑ Hepatic GSH/GSSG ratio</td>
<td>Park et al. (2010)</td>
</tr>
<tr>
<td>UV irradiation induced erythema formation in Guinea pig and Yucatan micropig</td>
<td>Erythema</td>
<td>5-CQA</td>
<td>Intradermal delivery of 5-CQA at 1.49 μmol/g skin</td>
<td>Prevented erythema formation induced by UV irradiation</td>
<td>Kitagawa et al. (2011)</td>
</tr>
<tr>
<td>Gamma irradiation induced chromosomal damage in mice model</td>
<td>Frequencies of micro-nucleated polychromatic erythrocytes</td>
<td>5-CQA</td>
<td>Orally administered at 50 ~ 200 mg/kg body weight, single dose</td>
<td>↓ Frequencies of micro-nucleated polychromatic erythrocytes</td>
<td>Abraham et al. (1993)</td>
</tr>
</tbody>
</table>
1.2.9 Underlying Factors Involved with Inflammatory Responses

Inflammation is a physiological response to tissue injury caused by exogenous or endogenous sources. Exogenous inducers include pathogen-associated molecular patterns, virulence factors, allergens, foreign bodies and toxic compounds (Medzhitov & Janeway, 1997). Endogenous inducers of inflammation arise from cell signaling in response to damaged or malfunctioning tissues (Medzhitov, 2008). Tissue-resident macrophage and mast cells respond to the inflammatory state by producing a variety of mediators, including chemokines, cytokines, prostaglandins and adhesion molecules. These inflammatory mediators serve to promote recruitment of plasma proteins and neutrophils from the circulation to arrive at the site of infection. Subsequently, neutrophils are activated to kill the invading agents by releasing ROS, RNS and enzymes including proteinase 3, cathepsin G and elastase constituents (Nathan, 2006). It is believed that a controlled inflammatory response is required to combat offending agents and result in the return of tissue homeostasis. However, a dysregulated inflammatory response could lead to the failure of effective resolution, thus leading to excessive tissue damage resulting in acute or chronic disease states (Elenkov, 2005).

1.2.10 Forms of Intestinal Inflammatory Disease

The intestinal wall is composed of four layers, the mucosa, the submucosa, the muscular and the serosa. The mucosa barrier is a complex structure that separates the internal milieu from the luminal environment. Intestinal epithelium is the main component of the mucosa barrier and it consists of a single layer of different specialized subtypes of cells: enterocytes, Paneth cells, goblet cells, and enteroendocrine cells and immunity cells (Laukoetter & Nava, 2008). The space between these cells are sealed by three types of junctional complexes, namely, tight junctions,
adherence junctions, and desmosomes, which are necessary for maintenance of intracellular adhesion and to regulate para-cellular transport (Laukoetter & Nava, 2008).

Inflammatory bowel diseases (IBDs), such as Crohn’s disease and ulcerative colitis, represents chronic relapsing of disorders occurring in the gastrointestinal tract that are characterized by an abnormal immune response to antigens of the intestinal content that leads to a persistent inflammatory state (Neurath, 2014) (Xavier & Podolsky, 2007). The cytokine responses are the key pathophysiological elements that govern the initiation, evolution, and ultimately, resolution of IBDs (Strober & Fuss, 2011). Cytokines are small proteins secreted mainly by immune cells that facilitate communication between cells. The mucosal cytokine network is a complex system. IBD patients have a decrease in the levels of anti-inflammatory cytokines such as interleukin-10 (IL-10), and an increase in the levels of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFNγ), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-12 (Neurath, 2014).

1.2.11 IL-8

IL-8 is a pro-inflammatory cytokine that consists of 99 amino acid with a molecular mass of 10 kDa. Evidences from clinical study have shown that mucosal IL-8 protein concentration is not detectable in non-IBD patients and is increased in intestinal mucosa of IBD patients (Nielsen et al., 2009). In 1990s, scientists reported that lamina propria of the colon in IBD patients secret significantly higher IL-8 compared to healthy individual control (Daig et al., 1996). Anti-cytokine approach that target specific pro-inflammatory cytokine offer an effective therapy for different clinical subgroups of IBDs.
1.2.12 Nuclear Factor Kappa B Signaling

The nuclear factor-κB (NFκB) pathway is a key regulator of the release of pro-inflammatory cytokines, chemokines, and adhesion molecules (Lawrence, 2009). Gene knockout studies have shown that NFκB pathway can have both pro- and anti-inflammatory roles. Some studies suggest that NFκB can have anti-inflammatory roles by directly inhibiting the expression of genes that encode pro-inflammatory cytokines. For examples, increased p50 subunit of NFκB expression was shown to suppress TNFα production in LPS tolerance. In contrast, some study reported that NFκB have pro-inflammatory role by enabling prolonged macrophage activation (Moon et al., 2009). These studies suggest that NFκB activation can have both anti-inflammatory and pro-inflammatory roles in regulating inflammation responses depending on the physiological context.

1.2.13 Mitogen-activated Protein Kinases (MAPK) Signaling

Mitogen-activated protein kinases (MAPK) are a group of serine/threonine kinases, which regulate inflammation, cell survival, differentiation and apoptosis. Multiple cell signal pathways are involved in the maintenance of the epithelial barrier through modulating tight junction proteins. MAPK signaling pathway is able to modulate tight junction protein, such as claudins (Lipschutz & Li, 2005). MAPK are Ser/Thr protein kinases that respond to extracellular stimuli and regulate various cellular activities. Several distinct groups of MAPK are well characterized in mammals: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNK) and p38 isomers (p38) (Coskun et al., 2011).
1.2.14 Caco-2 Cell as an In vitro Model of Intestinal Inflammation

The human intestinal Caco-2 cell line has been widely used over the last twenty years to model the intestinal barrier and factors influencing bioavailability of substrates. The Caco-2 cell line was originally obtained from a human colon adenocarcinoma. Under culture conditions, for up to 21 days, cells undergo spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of the mature enterocyte (Sambuy et al., 2005). Our laboratory used a mixture of IFNγ and phorbol myristate acetate (PMA) to stimulate an inflammatory state in the differentiated Caco-2 cell line (Chen & Kitts, 2008). IFNγ is the only member of the type II class of interferons, which are critical for innate and adaptive immunity against antigens. IFNγ primarily signals through the JAK-STAT pathway which involves sequential receptor recruitment and activation of kinases to control transcription of target genes via specific response elements (Schroder et al., 2004). Besides, cellular responses to IFNγ are also activated through its binding to the glycosaminoglycan heparin sulfate at the cell membrane (Schroder et al., 2004). PMA is a potent activator of protein kinase C (PKC) (Goel et al., 2007). Activation of PKC by PMA results in the induction of pro-inflammatory cytokines and release of ROS (Kontny et al., 1999). The treatment of a IFNγ and PMA cocktail triggered an inflammation response in differentiated Caco-2 cells which served as an in vitro model of intestinal inflammation (Chen and Kitts, 2008).

1.2.15 Anti-inflammatory Strategies

Anti-inflammatory drugs have been developed to resolve conditions of dysregulated inflammation by targeting inflammatory mediators or modulating the activity of cell signaling cascades involved in responding to an inflammatory signal. Nonsteroidal anti-inflammatory
drugs (NSAIDs) are the most widely used drugs for the treatment of inflammatory diseases (Laine, 2001). However, side effects of NSAIDs include a predisposition to ulcers and bleeding in the stomach and intestines. Thus, there is increased interest in searching for novel agents that may exhibit anti-inflammatory activity, without experiencing adverse side effects. Different cell lines triggered with pathogen-associated molecular patterns (lipopolysaccharide (LPS)) and pro-inflammatory cytokines (e.g. TNF-α, interleukin 1β (IL-1β), and IFNγ) have been used as cell-based inflammation models to study anti-inflammatory mechanisms. Also, animal models of inflammatory bowel disease, rheumatoid arthritis, and injury-associated inflammation have been successfully used to evaluate the anti-inflammatory effect of different components, such as dietary bioactive compounds.

1.2.16 Anti-inflammatory Activity of CGA

5-CQA has a protective effect against intestinal related inflammation in both cell-based and animal-based models. Table 1.4 is a summary of cell-based and animal studies describing the anti-inflammatory activity of CGAs reviewed in this section.

1.2.16.1 Evidence of Anti-inflammatory Activity from Cell-based Assays

CGA has an anti-inflammatory effect in TNF-α and H2O2-induced human intestine epithelia Caco-2 cells by down regulating IL-8 production (Shin et al., 2015). Studies conducted with tea, Artemisia annua, containing CGAs gave a strong anti-inflammatory effect by decreasing the secretion of pro-inflammatory cytokine, IL-8 and IL-6 in Caco-2 cells stimulated with TNF-α, LPS, IL-1β, and IFNγ (De Magalhaes et al., 2012). CGA also attenuated IL-1β,
TNF-α and IL-6 production in LPS-stimulated murine RAW 264.7 macrophages and in BV2 microglial cells by effectively down-regulating the NF-κB pathway (Hwang et al., 2014).

1.2.16.2 Evidences of Anti-inflammatory Activity from Animal-based Assays

In animal studies, the oral administration of 5-CQA protected against trinitrobenzenesulfonic acid-induced colitis in mice by reducing neutrophil infiltration and inhibition of the NFκB pathway (Zatorski et al., 2015). A similar effect was also observed in the dextran sulphate sodium-induced colitis model in mice (Shin et al., 2015) and in a carrageenan induced paw edema model in rats (Chauhan et al., 2011); in both cases a suppression of pro-inflammatory cytokines was observed.

Rheumatoid arthritis is another chronic inflammatory disorder characterized by the deterioration of cartilage and bone. Chauhan et al. (2012) observed that oral administration of 40 mg/kg of 5-CQA effectively suppressed pro-inflammatory cytokines including TNF-α and IL-1β in a LPS induced-knee joint inflammation rat model. This effect was similar to the standard treatment of administering ibuprofen at 100 mg/kg.

The reduction of inflammation to result in enhanced wound healing has also been reported for CGAs. Oral administration of 5-CQA at a dose of 50 mg/kg/day accelerated wound healing and decreased MDA, and nitric oxide levels, while elevating reduced-glutathione content in rats (Bagdas et al., 2015). Oral administration of 5-CQA also alleviated hepatic ischemia and reperfusion induced liver injury by reducing inflammatory responses and increasing antioxidant defense systems (Yun et al., 2012). There is also evidence that 5-CQA can suppress IL-1β, TNF-
α and IL-6 production in CCl4 induced liver inflammation and fibrosis in rats by inhibition of NFκB activation (Shi et al., 2013).
Table 1.4 Summary of Studies that Evaluated the Capacity of CGAs to Modulate Inflammatory Stress in Cell-based and Animal-based Models.

<table>
<thead>
<tr>
<th>Models</th>
<th>End-point Measure</th>
<th>CGA isomer</th>
<th>Concentration/Exposure Time</th>
<th>Results compared to the control without CGA treatment</th>
<th>Ref.</th>
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<tbody>
<tr>
<td><strong>Cell Models</strong></td>
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<tr>
<td>Caco-2 + TNF-α and H₂O₂</td>
<td>IL-8</td>
<td>5-CQA</td>
<td>0.5 ~ 2 mM</td>
<td>↓ IL-8</td>
<td>Shin et al. (2015)</td>
</tr>
<tr>
<td>Caco-2 + cocktail of inflammatory mediators</td>
<td>IL-6, IL-8</td>
<td>Mixture of all CGAs</td>
<td>Unknown composition of CGAs for 1 h</td>
<td>↓ IL-6, ↓ IL-8</td>
<td>De Magalhaes et al. (2012)</td>
</tr>
<tr>
<td>RAW 264.7 + LPS</td>
<td>NO, IL-1β, TNF-α, cyclooxygenase-2, NF-κB, IL-6</td>
<td>5-CQA</td>
<td>2 ~ 20 µM for 24 h</td>
<td>↓ NO, ↓ IL-1β, ↓ TNF-α, ↓ IL-6, ↓ cyclooxygenase-2, ↓ NFκB</td>
<td>Hwang et al. (2014)</td>
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<td><strong>Animal Models</strong></td>
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<tr>
<td>Trinitrobenzenesulfonic acid induced colitis mice model</td>
<td>Myeloperoxidase, H₂O₂, NF-κB</td>
<td>5-CQA</td>
<td>Orally administration at 20 mg/kg body weight, twice a day for 6 days</td>
<td>↓ Myeloperoxidase, ↓ H₂O₂, ↓ NF-κB</td>
<td>Zatorski et al. (2015)</td>
</tr>
<tr>
<td>Models</td>
<td>End-point Measure</td>
<td>CGA isomer</td>
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<tr>
<td>Animal Models</td>
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<tr>
<td>Dextran sulphate sodium induced colitis mice model</td>
<td>IL-1β, TNF-α, macrophage inflammatory protein 2</td>
<td>5-CQA</td>
<td>Orally administration at 1 mM for 15 days</td>
<td>↓ IL-1β, did not significantly change the levels of TNF-α and macrophage inflammatory protein 2</td>
<td>Shin et al. (2015)</td>
</tr>
<tr>
<td>Trinitrobenzenesulfonic acid induced colitis mice model</td>
<td>Myeloperoxidase, H₂O₂, NF-κB</td>
<td>5-CQA</td>
<td>Orally administration at 20 mg/kg body weight twice a day</td>
<td>↓ Myeloperoxidase, ↓ H₂O₂, ↓ NF-κB</td>
<td>Chauhan et al. (2011)</td>
</tr>
<tr>
<td>Rheumatoid Arthritis rat model</td>
<td>IL-1β, TNF-α, T cells count, Th1 cytokines, Th2 cytokines</td>
<td>5-CQA</td>
<td>Orally administration at 40 mg/kg body weight</td>
<td>↓ IL-1β, ↓ TNF-α, ↓ T cells count, ↑ Th1 cytokines, ↑ Th2 cytokines</td>
<td>Chauhan et al. (2012)</td>
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<tr>
<td>Wound in diabetic rat</td>
<td>Wound healing speed, NO, MDA, GSH</td>
<td>5-CQA</td>
<td>Intraperitoneal injection at 50 mg/kg/day for 15 days</td>
<td>↑ Wound healing speed, ↑ GSH, ↓ NO, ↓ MDA</td>
<td>Bagdas et al. (2015)</td>
</tr>
<tr>
<td>Liver injury rat model</td>
<td>MDA, GSH, TNF-α, NO, cyclooxygenase-2 protein increase</td>
<td>5-CQA</td>
<td>Orally administration at 2.5 ~ 10 mg/kg body weight, twice a day</td>
<td>↓ MDA, ↑ GSH, ↓ TNF-α, ↓ NO, ↓ cyclooxygenase-2 protein increase</td>
<td>Yun et al. (2012)</td>
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</table>
1.3 Hypothesis and Objectives

EXPERIMENT 1. A study on the interactions between major CGA isomers and chemical changes in coffee brew that affect antioxidant activities

Hypotheses:

$H_01$: Roasting degree and origin of geographic region of coffee will affect CGA isomer concentration and other chemical composition in coffee brews.

$H_02$: Changes occurring in coffee brew CGA isomer concentration correspond to changes in antioxidant activities evaluated by different antioxidant assays.

Objective: To characterize the CGA profile in coffee from different geographic location with different roasting degrees, and study their relationships with coffee brew antioxidant properties, evaluated in chemical and cell-based systems.
EXPERIMENT 2. A study on the potential antioxidant and anti-inflammatory activities of CGA isomers in PMA+IFNγ-induced Caco-2 cells

Hypotheses:

$H_{01}$: Six major CGA isomers (3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) have different free radical scavenging capacities.

$H_{02}$: CGA isomers are bioavailable in differentiated Caco-2 cells.

$H_{03}$: Six major CGA isomers present in coffee have isomer specific capacities to ameliorate oxidative stress in PMA+IFNγ challenged Caco-2 cells.

Objectives:

1. To study the free radical scavenging capacity of six major CGA isomers (3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) present in coffee.

2. To study if CGA isomers were bioavailable to Caco-2 cells.

3. To study if PMA+IFNγ cocktail will elevate intracellular ROS levels to a measurable amount in Caco-2 cells. To study if CGA isomers have specific effectiveness to ameliorate oxidative stress in PMA+IFNγ-induced Caco-2 cells.
EXPERIMENT 3. Investigation in determining underlying mechanisms of antioxidant and anti-inflammatory activities of CGA isomers related to NFκB signaling, MAPK cascades, and Nrf2 signaling

Hypotheses:

$H_{01}$: Six major CGA isomers present in coffee vary in their capacities to attenuate IL-8 secretion in PMA+IFNγ challenged Caco-2 cells.

$H_{02}$: Six major CGA isomers present in coffee vary in their effectiveness at alleviating oxidative stress and inflammation by regulating NFκB signaling pathway, affecting phosphorylation of MAPK cascades, and regulating Nrf2 signaling to different extends.

Objectives:

1. To study the effect of six CGA isomers to mitigate the inflammatory response in PMA+IFNγ-challenged Caco-2 cells.

2. To study the relative capacities of specific CGA isomers to modulate NFκB signaling pathway, phosphorylation of MAPK cascade, and Nrf2 signaling pathway.
Chapter 2: Interactions Between Major CGA Isomers and Chemical Changes in Coffee Brew that Affect Antioxidant Activities

2.1 Introduction

Coffee is like ifaconsumed globally, and epidemiological findings have shown that it can reduce the risk of several chronic diseases, including type 2 diabetes (Van Dam et al., 2006), cardiovascular disease (Andersen et al., 2006) and colorectal cancer (Xu et al., 2012). The physiological chemistry that underlies these claims often involves several bioactive compounds that exhibit antioxidant activity. Such activity in brewed coffee is attributed to phenolic compounds that are originally present in green coffee beans as well as those products derived from browning that arise from advanced phenol-nitrogen condensation reactions, or Mallard reaction products (MRP), generated during roasting (Liu & Kitts, 2011). The predominant phenolic compounds in coffee are CGAs. Major CGA isomers present in coffee are 3-CQA, 4-CQA, 5-CQA, 3,5-diCQA, 3,4-diCQA, and 4,5-diCQA (Farah et al., 2008). Various factors that affect CGA isomer profiles in green coffee beans, include coffee plant variety, geographic location and the conditions under which the beans are roasted to produce consumable brews (Anthony et al., 1993) (Campa et al., 2005) (Ky et al., 1999). Some MRP derivatives contribute to flavor and aroma, whereas others are incorporated into high molecular weight coffee melanoidins that provide color and bioactive activities (Bekedam et al., 2008) (Liu & Kitts, 2011).

Comparing the relevance of antioxidant activity in coffee across studies is challenging because various types of chemical assays produce results that are not always consistent. This is
due to the complex composition of coffee component mixtures that are specific to the brews tested in several studies (Liang & Kitts, 2014). For example, 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) decolorization assays monitor the antioxidant activity of water-soluble constituents in coffee brews that react with the ABTS radical cation in a hydrogen atom transfer mechanism (Ramalakshmi et al., 2008). The antioxidant capacity of coffee has also been assessed using ORAC assays that also involves a hydrogen atom transfer mechanism. However, unlike the ABTS assay, peroxyl radicals that are obligatory intermediates of enzymatic peroxidation and lipid autoxidation, are generated via the azo-initiator which oxidizes fluorescein. The Nitric oxide (NO) chemical assay is a reducing assay that can be measured chemically in vitro using Griess reagent. Electron paramagnetic resonance (EPR) spectroscopy is also used to measure the antioxidant capacity of coffee brew by selectively quenching persistent stable radicals such as 4-hydroxy-2,2,6,6-tetramethylpiperidine, N-oxyl (TEMPO) or Frémy’s salt (Brezová et al., 2009). In summary, although such chemical assays are valuable in determining the antioxidant activity of coffee, the radical species involved and methods of redox quantitation must be considered when interpreting the results in a big picture.

Isomers of CGA are easily altered under coffee roasting conditions and exhibit a range of free radical scavenging capacities when transformed to derivatives that arise during high-temperature processing. Thus, the antioxidant capacity of brewed coffee will largely depend on the overall sensitivity of all relevant coffee molecules that can react in the defined mechanism(s) involved in the antioxidant assays used to quantify activity. Hence, the varying composition of CGA isomers among coffee brews and the involvement of coffee browning components are collectively responsible for the wide range of antioxidant activities found in coffee preparations.
The present study was aimed to determine the effects of geographic regions from which Arabica coffee beans are sourced, and different processing factors on profiles of CGA isomers. In addition, principle component analysis (PCA) was applied to interpret the relationship between the composition of CGA isomers as well as the production of browning products and the antioxidant activity of various coffee brews assessed using specific chemical and biological assays. The latter provided a context for the antioxidant activity of brewed coffee that included potential limitations that influence cellular uptake and potential biological responses.

2.2 Materials and Methods

2.2.1 Materials

The following chemicals were purchased from the following: Sigma (St. Louis, MO, USA): ABTS (2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), AAPH (2,2’-azobis(2-amidinopropane) dihydrochl), DCFH-DA, 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein, sulphanilamide, naphthyl ethylenediamine dichloride, sodium nitroprusside, TEMPO, Frémy’s salt; NaNO₂, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), minimum essential medium (MEM), hydrochloric acid, citric acid, phosphoric acid, methanol (HPLC grade), and sodium dodecyl sulfate. Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco® (Grand Island, NY, USA) and CGA isomers, 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA were obtained from Cerilliant Corporation (Round Rock, TX, USA) and Chengdu Must Bio-Technology Co., Ltd (Chengdu, Sichuan, China). Quartz EPR tubes were purchased from Wilmad Lab-Glass (Vineland, NJ, USA).
2.2.2 **Coffee Brew Preparation**

Three batches of *Arabic* green coffee beans were obtained from cultivators in Sumatra, Dominican, Peru, Ethiopia, and Papua New Guinea (PNG). Before roasting, three batches of green coffee beans from each geographic region were pulled together to minimize other variances within that geographic region. Then, the beans were roasted at 210 °C for 12 min (light roast), 223 °C for 14 min (medium roast), and 235 °C for 15 min (dark roast) in a commercial roaster to achieve specific time-temperature thermal processing conditions for each roast. The roasting process was conducted in triplicate for green beans from each geographic region. Coffee brews were prepared by solid-lipid extractions as described with modifications (Anese & Nicoli, 2003). Briefly, coffee beans were ground to a powder, passed through a sieve (diameter < 0.5 mm) and then extracted with hot water (100 °C) at a coffee powder: water ratio of 1:20 (w:v) for 10 min with constant stirring. The brew was cooled on ice and filtered through a membrane (Whalman No. 4) under a vacuum. Final brews were freeze-dried and stored at -80 °C.

2.2.3 **Measurement of Colour and Browning of Coffee Brews**

The color of freeze-dried coffee brew extracts was analyzed using a Colour Quest XE spectrophotometer (Hunter Associates Laboratory Inc., Reston, VA). Color characteristics were expressed in $L^*$ (lightness), $a^*$ (red-green component) and $b^*$ (yellow-blue component) Cielab scale parameters. Freeze-dried extracts of brewed coffee were dissolved in deionized water to a final concentration of 1 mg/ml and absorbance was measured at 420 nm using a Lambda 25 UV-VIS spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD, USA).
2.2.4 Determination of High Molecular Weight (HMW) Maillard Reaction Product (MRP) (> 10 KDa) Content in Coffee Brew Extracts

Freeze-dried extracts of brewed coffee were dissolved in deionized water to a concentration of 2 mg/ml and then fractionated by ultrafiltration (Milipore, Billerica, MA) through a membrane with a 10 KDa nominal molecular mass cut-off membrane as described (Delgado-Andrade et al., 2005). Samples were ultrafiltrated under nitrogen (pressure of 30 psi) and residues were lyophilized to calculate the proportion of HMW MRP content in coffee brew extracts.

2.2.5 Determination of CGA Isomers Profile and caffeine contents in coffee brew extracts by high performance liquid chromatography (HPLC)

CGA isomer content in extracts of coffee brews were quantified by HPLC according the method developed by Fujioka & Shibamoto (2008) using an Agilent 1100 instrument (Agilent Technologies Inc., Palo Alto, CA). CGA isomers were separated by C-18 column (250 mm × 4.6 mm i.d., 5 µm particle size) using a mobile phase that comprising 10 mM citric acid (A) and 100% methanol (B) at a flow rate of 1 ml/min. Injected samples (5 µL) were eluted with a gradient consisting of 85% A from 0 to 5 min, 85%-60% A from 5 to 40 min, maintain at 60% A from 40 to 85 min, 60%-85% A from 85 to 90 min. The detector was set at 325 nm and 276 nm for CGA isomers and caffeine, respectively. The limit of quantification was defined as the minimum concentration at the signal-to-noise ratio (S/N) of 10. All analysis was repeated three times in triplicate.
2.2.6 **ABTS Assay**

The ABTS assay was followed according to the procedure described previously with modifications (Delgado-Andrade et al., 2005) (Re et al., 1999). Radical ABTS cations were generated by mixing ABTS (7 mM) with potassium persulfate (2.45 mM) in distilled water. The ratio between the slopes of the regression equations for the coffee samples and Trolox was defined as coffee antioxidant capacity. The results are expressed as mmol equivalents of Trolox/g of coffee brew extract (dry weight).

2.2.7 **ORAC FL Assay**

The antioxidant activities of coffee brew extracts were assessed using the oxygen radical absorbance capacity fluorescein (ORAC) assay (Kitts & Hu, 2005). The antioxidant activity of coffee brew extracts was determined from the ratio between the regression equations slopes for coffee and Trolox. ORAC values are expressed as mmol Trolox Equivalent/g coffee brew extract.

2.2.8 **Nitric Oxide Radical Scavenging Assay**

The affinity of coffee brew extracts to scavenge nitric oxide radicals (NO⁻) was determined as previously described (Kang et al., 2006). The ratio between the regression equation slopes for coffee samples and for Trolox was defined as nitric oxide radical-scavenging ability and is expressed as mmol equivalents of Trolox/g of coffee brew extract (dry weight).
2.2.9 **EPR Spectroscopy**

The free radical scavenging capacity of coffee brew extracts was assessed using EPR spectroscopy. To quench the TEMPO radicals, coffee extracts (5-20 mg/ml) were mixed with an equal volume of TEMPO (1 mM in distilled water) and the reaction was monitored for 40 min using an Elexsys E500 series continuous wave EPR spectrometer (Bruker Daltonics Inc., Billerica, MA). Samples were analyzed at a frequency of 9.40 GHz (X-band), 100 KHz field modulation, 5 gauss modulation amplitude, 1.28 ms time constant, 5.12 ms conversion time and 0.64 mW microwave power. The ability of coffee extracts to quench TEMPO radicals was calculated as: % Inhibition=\[(I_0-I/I_0)\times100\%\], where \(I_0\) is the intensity of the EPR spectrum of TEMPO at t=0 min mixing; \(I\) is the intensity of the EPR spectrum of TEMPO at 40 min after mixing with coffee extract. The % inhibition vs. coffee extract concentration was plotted to obtain a regression equation, with Trolox being the reference standard to express the antioxidant activity of various extracts of coffee brews. Trolox (0.5 to 5 mM) was reacted with an equal volume of TEMPO (1 mM) and a regression equation was determined for Trolox concentration vs. % of inhibition. The ratio between the regression equation slopes for coffee extracts and Trolox, defined the antioxidant capacity of individual extracts of coffee brew. The results are expressed as mmol equivalents of Trolox/g of coffee brew extract (dry matter). The antioxidant activity of coffee brew extracts towards inorganic radicals was also assessed using Frémy’s salt. Coffee extracts (0.075 to 2.0 mg/mL) were reacted with an equal volume of Frémy’s salt (1 mM in 50 mM phosphate buffer; pH 7.4) and then free-radical intensity was monitored immediately (t = 0) and at 30 (t = 30) min thereafter. The EPR spectrometer operating conditions comprised: field modulation, 100 KHz; modulation amplitude, 3 gausses; time constant, 2.56 ms; conversion time, 10.49 ms and attenuation, 10 dB. The reference was Trolox (0.025 to 0.2 mM) reacted with
an equal volume of Frémy’s salt (1 mM) in phosphate buffer for 30 min. The antioxidant activity of coffee brew extract determined using Frémy’s salt is expressed as mmol equivalents of Trolox/g of coffee brew extract (dry weight).

2.2.10 Cell Culture

The human colon adenocarcinoma cell line, Caco-2 (HTB-37, American Type Culture Collection, Manassas, VA, USA), was cultured in complete MEM containing Earle’s salts supplemented with 10% FBS (Invitrogen, Canada), 100 U/mL of penicillin and 100 µg/mL of streptomycin. Briefly, cells (passage 26-37) were maintained in 75-cm² plates (Corning Inc., Corning, NY, USA) at 37 °C in a 5% CO₂ humidified atmosphere, and the media was changed every 2-3 days. Cultured cells were split (1:5) once they reached confluence by use of 0.05% trypsin-0.5 mM EDTA (Gibco-BRL). For individual experiments, cells were seeded onto 6-well or 96-well plates (Sarstedt AG & Co., Sarstedtstrabe, Numbrecht, Germany) at a density of 1×10⁵ cells/cm². Cells were cultured for 21 days with medium change every 2-3 days to allow for spontaneous differentiation.

2.2.11 Assessment of Cell Viability

MTT assays were performed to assess the cellular metabolic activity, an indirect measure of viability. After being treated with the tested sample at concentration range of 0.125 mg/mL to 1 mg/mL, cells grown in 96-well plates were rinsed with phosphate buffered saline (PBS) followed by incubation with serum-free medium containing 0.5 mg/ml MTT for 4 h in the dark at 37 °C. The SDS (10%) in hydrochloric acid (0.1M) was added to cells for 12 h to dissolve the formazan crystal. The amount of formazan in the plate well was determined by measuring absorbance at
540 nm, using a spectrophotometric plate reader (ThermolabSystem, Chantilly, VA, USA). Cell viability (% control) was calculated from the equation:

\[
Viability\ (% \ of \ control) = \frac{Ab_{sample}}{Ab_{negative\ control}} \times 100\%
\]

where \( Ab_{sample} \) is the absorbance of the treated cells, \( Ab_{negative\ control} \) is the absorbance of the negative control, which are cells incubated with media only.

2.2.12 Intracellular Oxidative Assay in Caco-2 Cells

The effect of coffee brew extracts on peroxyl radical-initiated intracellular oxidation was assessed as described by Hu et al. (2005). Caco-2 cells were seeded in 96-well plates at a concentration of \( 1 \times 10^5 \) cells/mL and grown for 21 days to allow for differentiation. Caco-2 cells were exposed to MEM media (control) or MEM media containing coffee brew extract (0.5 and 1 mg/mL) for 24 h at 37 °C. Caco-2 cells were then rinsed with PBS (pH 7.2) followed by incubating with a DCFH-DA probe (5 µM) in PBS at 37 °C for 30 min. Cells were then rinsed again with fresh PBS and intracellular oxidation was initiated by treating cells with 100 µl of 1 mM AAPH (dissolved in HBSS). Fluorescence emission was measured using a Fluoroskan Ascent™ FL luminometer (Thermo Fisher Scientific, Waltham, MA, USA) at excitation and emission wavelengths of 485 and 527 nm, respectively, one hour after adding AAPH. Data was expressed according to the following equation:

\[
%\ Fluorescence\ Inhibition = \frac{F_{pc} - F_{coffee\ brew}}{F_{pc} - F_{nc}} \times 100\%
\]

Where \( F_{pc} \) is the intensity of the fluorescence emission from the cells that contained the DCFH-DA probe followed by AAPH challenge; \( F_{nc} \) is the intensity of the fluorescence emission from the cells that contained only the DCFH-DA probe; \( F_{coffee\ brew} \) is the intensity of the
fluorescence emission from the cells that were pre-incubated with coffee brew and then incubated with DCFH-DA probe followed by AAPH challenge.

2.2.13 Statistics

All data were analysed using Origin version 9.2 (OriginLab Corporation, Northampton, MA) and are expressed as means ± standard deviation (SD). Multiple comparisons were performed using a one-way analysis of variance (ANOVA) followed by a multiple range of Bonferroni tests to distinguish the source of the differences in means. Significant differences were identified using $p < 0.05$ as being statistically significant unless otherwise indicated. Principle component analysis (PCA) proceeded on a data matrix of sixteen variables comprising nine physiochemical parameters and six antioxidant values of coffee brew extracts to determine correlations between physiochemical characteristics and the antioxidant activities of coffee brew extracts.

2.3 Results and Discussion

2.3.1 Composition of Coffee Brews Along with Assessment of Browning

Figure 2.1 shows the composition of six CGA isomers in brew extracts made from green Arabica coffee beans, processed under light, medium, and dark roasting conditions and derived from Sumatra, Dominican, Peru, Ethiopia, and PNG. The most abundant CGA isomer was 5-CQA, which accounted for 69% to 74% of total CGA in all coffee brew extracts, especially those from unprocessed green beans. The range of differences in 5-CQA content among samples sourced from five different regions was relatively small. The 5-CQA content decreased > 85% in coffee brews prepared from dark roasted beans compared with those prepared from non-roasted
green beans derived from the same region. For example, the total CGA content in coffee brew extracts of light-, medium- and dark-roasted Sumatran beans decreased to 35.60%, 62.91%, and 80.60%, respectively, compared with the original CGA content in green beans. The 3-CQA and 4-CQA contents were higher in brews prepared from light-roasted compared with green beans, other research group has attributed this phenomenon to acyl migration (Deshpande et al., 2014) (Moon et al., 2009).

Table 2.1 presents the total CGAs and caffeine content, along with browning parameters that included color characteristics ($E$), absorbance at 420 nm and the recovery of HMW MRPs in brew extracts of coffee beans. The impact of the geographic region that referred to the source of coffee beans and the degree of roasting that were found to significantly affect the CGA content in *Arabica* coffee brews. This partially explains the relative changes in CGA content among various coffee beans. It was previously reported that CGA losses are the greatest in dark-roasted coffee (Liu & Kitts, 2012), and others have attributed this fact to the conversion of CGA isomers to precursors incorporated in melanoidins (Perrone et al., 2012). Although this observation appears to be true for roasted coffee in general, the magnitude of the CGA disappearance in medium and dark roasted coffee brews did not correspond to a relative difference in various browning parameter measures, such as $E$, 420 nm absorbance and HMW MRP) in the same brews. One explanation for this could be the absence of specific differences in the amount of MRP fractions present in medium and dark coffee brews. Since coffee brews used herein represented only water soluble MRP, it is possible that an underestimation of the complete transformation of CGAs to HMW melanoidins occurred.
Figure 2.1 (A) 3-CQA, (B) 4-CQA, (C) 5-CQA, (D) 3,5-diCQA, (E) 3,4-diCQA, and (F) 4,5-diCQA (mg) in freeze-dried coffee brew extract (g) prepared from coffee beans derived from 5 different regions with different roasting conditions. Data were expressed as Mean ± SD, n=3. abcd denoted significant (p < 0.05) differences in CGA isomer content among coffee brew extracts derived from the beans with different roasting degree within the same geographic region by using Bonferroni post-tests.
Table 2.1 Total CGAs and Caffeine Content with Color Characteristics, Browning and HMW MRPs in Coffee Brew Extracts.

<table>
<thead>
<tr>
<th>Region</th>
<th>Roasting degree</th>
<th>Total CGAs content (mg/g)</th>
<th>Caffeine (mg/g)</th>
<th>E</th>
<th>Absorbance at 420 nm</th>
<th>HMW MRPs (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominican</td>
<td>Green</td>
<td>184.18 ± 7.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.38 ± 0.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4045.24 ± 12.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>118.61 ± 0.76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.89 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1482.56 ± 15.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.55 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.04 ± 0.97&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>68.32 ± 1.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37.04 ± 0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>944.05 ± 12.68&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.59 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37.41 ± 1.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>35.74 ± 1.43&lt;sup&gt;d&lt;/sup&gt;</td>
<td>37.58 ± 1.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>576.88 ± 13.57&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.59 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39.42 ± 0.99&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peru</td>
<td>Green</td>
<td>192.70 ± 1.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.43 ± 0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4060.05 ± 11.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>104.44 ± 0.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.52 ± 0.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1535.59 ± 16.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.55 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.76 ± 1.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>61.37 ± 0.26&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.22 ± 1.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>910.24 ± 18.42&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.61 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35.42 ± 2.24&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Dark</td>
<td>27.40 ± 0.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30.17 ± 1.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>469.46 ± 16.47&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.61 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39.49 ± 1.70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sumatra</td>
<td>Green</td>
<td>183.95 ± 1.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.01 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4013.08 ± 20.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.24 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
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<tr>
<td></td>
<td>Light</td>
<td>85.42 ± 0.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.44 ± 0.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1311.06 ± 19.72&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.53 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.41 ± 1.72&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Medium</td>
<td>56.20 ± 1.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32.42 ± 0.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>814.50 ± 18.37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.58 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35.42 ± 1.56&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Dark</td>
<td>30.36 ± 0.46&lt;sup&gt;d&lt;/sup&gt;</td>
<td>31.51 ± 0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>533.04 ± 16.38&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.58 ± 0.00&lt;sup&gt;d&lt;/sup&gt;</td>
<td>37.16 ± 1.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PNG</td>
<td>Green</td>
<td>166.33 ± 1.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.63 ± 1.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4081.51 ± 15.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.23 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>102.53 ± 0.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.67 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1387.26 ± 14.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.54 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.60 ± 2.25&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Medium</td>
<td>67.55 ± 0.49&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32.50 ± 1.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>898.46 ± 14.73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.58 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.72 ± 2.57&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Dark</td>
<td>33.91 ± 0.37&lt;sup&gt;d&lt;/sup&gt;</td>
<td>33.66 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>464.35 ± 16.93&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.59 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35.52 ± 2.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>Green</td>
<td>186.99 ± 1.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.67 ± 0.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4083.31 ± 15.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>96.2 ± 0.78&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>1155.25 ± 13.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.57 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.26 ± 2.27&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>50.38 ± 0.60&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.58 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37.39 ± 2.09&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Dark</td>
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<td>415.65 ± 11.35&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.59 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.38 ± 1.29&lt;sup&gt;a&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>1</sup>Color characteristic, \( E = (L^2 + (a^*)^2 + (b^*)^2)^{1/2} \); browning = absorbance at 420 nm; HMW MRPs = MW > 10 KDa. \( a,b,c,d \) represent means in columns that are significantly different among different roasting degree within the same geographic region by using Bonferroni post-tests. Level of confidence set at \( p < 0.05 \). ND = not detected in coffee brew extracts.
2.3.2 Antioxidant Activity of Coffee Brew Extract Evaluated Using ABTS, ORAC, and Nitric Oxide (NO) Chemical Based Assays

Table 2.2 shows the antioxidant activities of various coffee brews evaluated by chemical methods. Coffee brew extracts tested for antioxidant activity using chemical based assays exhibited a relatively higher Trolox equivalent in ORAC, compared to ABTS or NO inhibition assays, respectively. Although absolute antioxidant values varied depending on the chemical assay used, trends were similar between green coffee and roasted coffee brews when tested using ORAC and ABTS assays. In fact, free radical scavenging capacity was actually lower ($p < 0.05$) in green bean brews compared with roasted coffee brews. Results from ABTS and ORAC assays also showed that coffee brews derived from medium and dark roasts, respectively, were less effective at scavenging radicals compared with light-roast brews ($p < 0.05$). Other workers have examined the effect of roasting conditions on the antioxidant activity of coffee brews using Folin-Ciocalteu and ABTS assays and have reported maximum antioxidant activity in the light to medium-roasted coffee brews (Smrke et al., 2013). The antioxidant activity in light-roast coffee brews determined using both ABTS assay and ORAC assays and found to be highest in coffee from Dominica and Peru, but not from Sumatra, PNG and Ethiopia; some varietal differences are significant for antioxidant activity. Of interest was the fact that these particular varieties also exhibited modest differences in the generation of MRP parameters. Previous workers have indicated that that a major source of antioxidant activity could be sub-structures of MRP that are not components of the chromophore (Liu & Kitts, 2011). This conclusion explains how changes in MRP parameters, such as in the color and recovery of MW > 10 kDa MRPs do not always follow predicted antioxidant activities. Other workers have also reported that degradation products of CGA isomers in brewed coffee that are not involved in MRP, have antioxidant
activity (Kamiyama et al., 2015). Moreover, we discount the influence of caffeine as a factor in the overall difference observed in antioxidant capacity of different brews, since caffeine concentrations were not affected by our roasting conditions, and they are relatively more stable during the brewing process than CGA isomers (Crozier et al., 2012) (Liu & Kitts, 2012).

Despite notable decreases in total CGA in coffee brews derived from a light roast thermal process, the similar increase in scavenging activities observed with ABTS and peroxyl radicals, respectively in all coffee varieties tested indicated that compounds derived from the browning reactions occurred at a relatively earlier stage of roasting and are more effective than the presence of total CGA in terms of interacting with these specific radical species. Maillard reactions that specifically occur in thermally processed coffee have been attributed to non-covalent interactions between CGA isomers and melanoidins to produce complexes with varying degrees of antioxidant activity (Wolfe & Liu, 2007). Delgado-Andrade et al. (2005) separated melanoidins from coffee brews prepared with different roasts and found that pure melanoidins contributed only 10% to 15% to the total antioxidant activity of coffee brew when evaluated using ABTS assays. Others who have studied the contribution of CGA to the antioxidant activity of coffee brew concluded that the loss of CGA during the roasting process does not yield a decrease of antioxidant activity due to the simultaneous generation of browning products that also exhibit antioxidant activity (Perrone et al., 2012). The present study found similar NO reducing activities between light-roasted and unroasted coffee, but more activity ($p < 0.05$) compared with medium or dark roasted brews, respectively.
Our results using Frémy’s salt and EPR measurements were similar but less striking. Table 2.2 also shows that green coffee did not have the same TEMPO radical scavenge capacity as roasted coffee brew extracts. In particular, TEMPO scavenging was highest among the dark roasted coffees, regardless of the geographic location where the coffee beans were sourced. Taken together, these results indicate that phenolic compounds in green coffee beans that are more reactive in both the NO reducing test and Frémy’s salt were lost during roasting and likely transformed to compounds that were more responsive in the TEMPO radical assay. It is plausible that melanoidins generated during roasting that parallel the loss of phenolics, possess relatively lower hydrogen dissociation enthalpy or reducing capacity, which explains the typically lower antioxidant activity in observed both the NO and Frémy’s salt assays. Hence, browning components generated during extended roasting processes do not possess enough antioxidant capacity to compensate for the loss of phenolic compounds that is attributed to higher antioxidant capacity in both light roasted and non-roasted brews. A shift to lower activity in the Frémy’s salt assay with roasting corresponded to losses of CGA isomers, whereas the increased radical scavenging activity of TEMPO in dark roasts reflected more so the contribution of MRP to total antioxidant capacity.
Table 2.2 Antioxidant activity of coffee brew extract evaluated by ABTS, ORAC, NO, Frémy’s salt, and TEMPO assays.

| Region  | Roasting Degree | Antioxidant Activity (mmol Trolox Equivalent/g) |  |  |  |
|---------|----------------|-----------------------------------------------|  |  |  |
|         |                | ABTS                                         | ORAC | NO | Frémy’s salt | TEMPO |
|         |                | ABTS                                         | ORAC | NO | Frémy’s salt | TEMPO |
| Dominican | Green         | 0.41±0.01<sup>a</sup>                         | 1.51±0.04<sup>a</sup> | 0.62±0.03<sup>a</sup> | 1.16±0.03<sup>a</sup> | 0.00±0.00<sup>a</sup> |
|         | Light          | 0.60±0.03<sup>b</sup>                         | 2.04±0.13<sup>b</sup> | 0.62±0.03<sup>a</sup> | 0.94±0.03<sup>b</sup> | 0.27±0.01<sup>b</sup> |
|         | Medium         | 0.55±0.02<sup>c</sup>                         | 1.87±0.10<sup>c</sup> | 0.33±0.02<sup>b</sup> | 0.66±0.04<sup>c</sup> | 0.37±0.01<sup>c</sup> |
|         | Dark           | 0.54±0.02<sup>c</sup>                         | 1.43±0.07<sup>d</sup> | 0.32±0.03<sup>b</sup> | 0.67±0.04<sup>d</sup> | 0.40±0.01<sup>d</sup> |
| Peru    | Green          | 0.45±0.02<sup>a</sup>                         | 1.69±0.05<sup>a</sup> | 0.62±0.04<sup>a</sup> | 1.28±0.05<sup>a</sup> | 0.00±0.00<sup>a</sup> |
|         | Light          | 0.61±0.03<sup>b</sup>                         | 2.09±0.07<sup>b</sup> | 0.61±0.04<sup>a</sup> | 1.20±0.03<sup>b</sup> | 0.30±0.01<sup>b</sup> |
|         | Medium         | 0.53±0.03<sup>c</sup>                         | 1.73±0.06<sup>a</sup> | 0.35±0.04<sup>b</sup> | 1.03±0.04<sup>c</sup> | 0.34±0.01<sup>c</sup> |
|         | Dark           | 0.53±0.02<sup>c</sup>                         | 1.54±0.06<sup>c</sup> | 0.34±0.04<sup>b</sup> | 0.82±0.04<sup>d</sup> | 0.38±0.02<sup>d</sup> |
| Sumatra | Green          | 0.42±0.03<sup>a</sup>                         | 1.55±0.06<sup>a</sup> | 0.67±0.03<sup>a</sup> | 1.11±0.03<sup>ac</sup> | 0.00±0.00<sup>a</sup> |
|         | Light          | 0.53±0.02<sup>b</sup>                         | 2.14±0.10<sup>b</sup> | 0.69±0.02<sup>a</sup> | 1.15±0.06<sup>ab</sup> | 0.36±0.01<sup>b</sup> |
|         | Medium         | 0.52±0.03<sup>b</sup>                         | 1.93±0.09<sup>c</sup> | 0.45±0.03<sup>b</sup> | 0.91±0.04<sup>c</sup> | 0.39±0.02<sup>c</sup> |
|         | Dark           | 0.51±0.03<sup>b</sup>                         | 1.74±0.05<sup>d</sup> | 0.45±0.03<sup>b</sup> | 0.80±0.04<sup>c</sup> | 0.42±0.02<sup>d</sup> |
| PNG     | Green          | 0.38±0.02<sup>a</sup>                         | 1.65±0.09<sup>a</sup> | 0.58±0.04<sup>a</sup> | 1.17±0.04<sup>a</sup> | 0.00±0.00<sup>a</sup> |
|         | Light          | 0.51±0.03<sup>b</sup>                         | 2.25±0.11<sup>b</sup> | 0.60±0.03<sup>a</sup> | 1.15±0.04<sup>a</sup> | 0.34±0.01<sup>b</sup> |
|         | Medium         | 0.51±0.02<sup>b</sup>                         | 1.97±0.10<sup>c</sup> | 0.38±0.04<sup>b</sup> | 0.93±0.04<sup>b</sup> | 0.35±0.01<sup>b</sup> |
|         | Dark           | 0.49±0.02<sup>b</sup>                         | 1.94±0.07<sup>c</sup> | 0.38±0.03<sup>b</sup> | 0.84±0.03<sup>c</sup> | 0.36±0.01<sup>c</sup> |
| Ethiopia | Green         | 0.41±0.02<sup>a</sup>                         | 1.73±0.08<sup>a</sup> | 0.62±0.05<sup>a</sup> | 1.13±0.03<sup>a</sup> | 0.00±0.00<sup>a</sup> |
|         | Light          | 0.53±0.02<sup>b,c</sup>                       | 2.16±0.11<sup>b</sup> | 0.64±0.05<sup>a</sup> | 1.04±0.04<sup>b</sup> | 0.27±0.02<sup>b</sup> |
|         | Medium         | 0.55±0.03<sup>b</sup>                         | 2.16±0.08<sup>b</sup> | 0.37±0.03<sup>b</sup> | 0.92±0.04<sup>c</sup> | 0.34±0.01<sup>c</sup> |
|         | Dark           | 0.51±0.02<sup>c</sup>                         | 1.66±0.07<sup>a</sup> | 0.35±0.04<sup>b</sup> | 0.81±0.04<sup>d</sup> | 0.38±0.01<sup>d</sup> |

<sup>1</sup> Values represent mean ± SD; <sup>abcd</sup> represent means in columns that are significantly different among different roasting degree within the same geographic region by using Bonferroni post-tests. Level of confidence set at <i>p</i> < 0.05.
2.3.3 Coffee Brew Extracts Protect Caco-2 Cells Against AAPH-induced Intracellular Oxidative Stress

Although chemical-based antioxidant assays are useful for analysing the antioxidant activity of different coffee brews, the results cannot be fully extrapolated to biological systems. Cellular antioxidant activity assays provide biologically relevant data that accounts for important factors, such as cellular uptake, distribution and to some extent, metabolism in the final assessment of activity (Wolfe & Liu, 2007). Cellular antioxidant activity in Caco-2 cells were assessed after differentiation (Engle et al., 1998). Coffee brews ranging in concentration of 0.125 to 1.0 mg/mL did not adversely affect Caco-2 cell viability, the MTT result of coffee brew at 0.5 mg/mL and 1 mg/mL are shown in Figure Appendix A.1. All coffee brews, regardless of the source of the beans had dose-dependent (0.5 to 1.0 mg/mL) reduction in intracellular oxidative stress induced by AAPH-derived peroxyl radicals (Figure 2.2(A) and Figure 2.2(B)). At a concentration of 1 mg/mL, coffee brew extracts generated an intracellular inhibition effect in the order of green coffee > light > medium > dark roasts. Intestinal cell uptake and metabolism of CGA isomers can occur and these relatively small molecules have known peroxyl radical scavenging capacity (Gómez-Ruiz et al., 2007). The decrease in the percent of intracellular oxidation in Caco-2 cells incubated with roasted coffee brew extracts, compared with green coffee brew extracts can be explained by accounting for the proportional loss of CGA isomers during roasting and the relatively reduced capacity of browning products available to quench intracellular peroxyl radicals.
Figure 2.2 Capacity of coffee brew extracts at concentration of (A) 0.5 mg/mL and (B) 1 mg/mL to reduce AAPH-induced oxidative stress determined by DCFH-DA probe in differentiated Caco-2 cells (passage no. 26). Data were expressed as % of inhibition of fluorescence. Different letters indicate significant differences (p < 0.05) among different roasted coffee brew extracts from each geographic region.
2.3.4 The Relationships Between Physiochemical Characteristics and Antioxidant Properties of Coffee Brews

The results of a Pearson correlation analysis confirmed the relevance of physiochemical characteristics of coffee brews that influence antioxidant capacity, estimated using different types of antioxidant assays (Table 2.3). The strong positive correlations ($r=0.748$ to $0.783$) between specific CGA isomers (5-CQA, 3,4-diCQA, 4,5-diCQA, and 3,5-diCQA) and intracellular oxidative capacity contrasted those negative correlations ($r=-0.984$ to $-0.928$) obtained between specific CGA isomers and the finding of assays of radical scavenging capacity using TEMPO.

PCA was also used to determine relationships that exist between the chemical composition of coffee brews and antioxidant activity. Since CGA isomers and browning compounds are major contributors to the antioxidant activity of coffee, concentrations of each of them were included in the PCA, along with antioxidant activities determined from different types of assays. In total, sixteen variables were included in the data set for PCA. The data was standardized by subtracting the means of each variable and dividing the result by the standard deviation of each variable (Hossain et al., 2011). The purpose of standardization was to make sure that each parameter contributed equally to the data set variance, and thus carried equal weight in the principal component calculation. Two principle components, PC1 and PC2, with eigenvalue greater than one, explained 65.01% and 18.00% respectively, of the total variance in the data set.
PCA bi-plot that allows the display of information about both samples (dots) and variables (vectors) as a data matrix is shown in Figure 2.3. The blue scale represents the loading plot in which the projection of variables is reflected in PC1 and PC2 planes. This result indicates that the content of particular CGA isomers (5-CQA, 3,4-diCQA, 4,5-diCQA, and 3,5-diCQA) have strong positive correlation to the intracellular peroxyl radical scavenging capacity and a strong negative correlation to TEMPO radical scavenging capacity and browning. The black scale on the scatter plot provides the location of each sample point where levels of a given component tend to cluster with a particular characteristic. For example, Figure 2.3 shows a cluster of sample points that have the same roasting degree. Green coffee samples had a high content of 5-CQA, dicafeoylquinic acids and more intracellular antioxidant activity. Light-roasted coffee samples also contain high 3-CQA and 4-CQA content which is accompanied by powerful peroxyl radical scavenging activity observed in the ORAC assay. Medium- and dark-roasted coffee samples with melanoidin content clustered with TEMPO radical scavenging capacity. In summary, physicochemical changes in green and roasted coffee beans ultimately are associated with antioxidant values among Arabica coffee brews. The findings, albeit specific to Arabica coffee, but sourced from five geographic locations, imply that the complex mixture of chemical components in coffee brews collectively influences the characteristic antioxidant activity, which in turn is relevant to the underlying mechanisms of action of various types of antioxidant assays.
Table 2.3 Pearson Correlation Coefficients Between Physiochemical Parameters and Antioxidant Activities in Coffee Brews

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ABTS</th>
<th>ORAC</th>
<th>NO</th>
<th>Frémy’s salt</th>
<th>TEMPO</th>
<th>Intracellular Oxidative Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>-0.001</td>
<td>-0.149*</td>
<td>-0.006</td>
<td>-0.242**</td>
<td>-0.095</td>
<td>-0.026</td>
</tr>
<tr>
<td>3-CQA</td>
<td>0.196**</td>
<td>0.487***</td>
<td>0.678***</td>
<td>0.480***</td>
<td>-0.266***</td>
<td>0.402***</td>
</tr>
<tr>
<td>4-CQA</td>
<td>-0.129</td>
<td>0.285***</td>
<td>0.821***</td>
<td>0.655***</td>
<td>-0.595***</td>
<td>0.634***</td>
</tr>
<tr>
<td>5-CQA</td>
<td>-0.705***</td>
<td>-0.281***</td>
<td>0.696***</td>
<td>0.642***</td>
<td>-0.981***</td>
<td>0.783***</td>
</tr>
<tr>
<td>3,4-diCQA</td>
<td>-0.606***</td>
<td>-0.180*</td>
<td>0.748***</td>
<td>0.630***</td>
<td>-0.928***</td>
<td>0.754***</td>
</tr>
<tr>
<td>3,5-diCQA</td>
<td>-0.776***</td>
<td>-0.374***</td>
<td>0.613***</td>
<td>0.593***</td>
<td>-0.984***</td>
<td>0.748***</td>
</tr>
<tr>
<td>4,5-diCQA</td>
<td>-0.704***</td>
<td>-0.247***</td>
<td>0.704***</td>
<td>0.619***</td>
<td>-0.967***</td>
<td>0.771***</td>
</tr>
<tr>
<td>HMW MRPs</td>
<td>0.835***</td>
<td>0.444***</td>
<td>-0.513***</td>
<td>-0.522***</td>
<td>0.951***</td>
<td>-0.690***</td>
</tr>
<tr>
<td>420 nm absorbance</td>
<td>0.799***</td>
<td>0.406***</td>
<td>-0.611***</td>
<td>-0.564***</td>
<td>0.975***</td>
<td>-0.726***</td>
</tr>
<tr>
<td>E</td>
<td>0.499***</td>
<td>-0.040</td>
<td>-0.838***</td>
<td>-0.755***</td>
<td>0.867***</td>
<td>-0.796***</td>
</tr>
</tbody>
</table>

* denoted significance set at $p < 0.05$; ** denoted significance set at $p < 0.01$; *** denoted significance set at $p < 0.001$. 
Figure 2.3 Bi-plot of principal component analysis of antioxidant activity and coffee physiochemical characteristics of green, light, medium and dark roasted coffee brew extracts. The black scale denotes the coffee sample scatter plot and the blue scale denotes the scale for variable projection. Green, light, medium and dark refer the different roasting conditions used to produce the coffee brews.
2.4 Conclusion

The source of coffee beans and the conditions at which they are roasted significantly affected the CGA isomer content and several indices of browning and subsequent antioxidant values. Principal component analysis (PCA) was used to interpret the correlations between physiochemical parameters and functional parameters of coffee. Two principal components (PC1 and PC2) accounted for 83.01% of the total variance in data. The constituents of *Arabica* beans derived from various locations and roasted under different conditions changed in relation to the magnitude of responses determined by measuring antioxidants using specific assays. The content of CGA isomers was positively correlated with assays where nitric oxide assay is reduced in where Frémy’s salt was quenched, and with the extract of intracellular antioxidant capacity in Caco-2 intestinal cells. Correlations obtained from indices of browning were related to both chemical assays using ABTS and ESR measurement of radical scavenging capacity using TEMPO. This study comprehensively analyzed simultaneous changes in the physicochemical and functional qualities of coffee brews and interpretations of antioxidant values obtained using assays that predict the health benefits of coffee.

The complexity of coffee brews, particularly when created by the influence of roasting, precludes using a single assay to adequately describe the antioxidant activity of coffee. Moreover, while chemical assays of free radical scavenging are useful to evaluate the quality attributes of a coffee source, cell-based antioxidant tests are more effective as predictors of bioactive potential that are relevant to human health. The use of PCA in the final interpretation of the data not only allowed for visual displays of the complex relationships among various physiochemical and functional coffee parameters including the CGA isomer content, but also
provided an immediate view of the effects of processing and geographic region on each parameter. I accept the hypothesis 1 and conclude that the source of coffee beans and the conditions at which they are roasted significantly affect the CGA isomer content and several indices of browning and subsequent antioxidant values. I also accept the hypothesis 2 and conclude that the content of CGA isomers were positively correlated with intracellular antioxidant capacity in Caco-2 intestinal cells.
Chapter 3: Characterization Antioxidant Activities of Different CGA Isomers using Chemical and Cell-based Antioxidant Assays

3.1 Introduction

Total CQA are referred to as CGAs and represents a family of polyphenols, that have been recognized to have antioxidant activities. 5-CQA is the most abundant CGA isomer present in plants. As described in Section 1.2.7, 5-CQA has been largely recognized to be a potent antioxidant with an efficacy to modulate crucial signalling pathways. But the biological importance of other major dietary CGA isomers has largely been overlooked. The results in Chapter 2 indicated that the other five major CGA isomers (3-CQA, 4-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) together account for more than 50% of the total CGAs in coffee brew. Therefore, the biological activity of the other five major dietary CGA isomers should not be ignored. This chapter looks to gain a greater understanding on the antioxidant activities of these major dietary CGA isomers in intestinal cells. Differences in the chemical structures of CGA isomers can influence free radical scavenging properties, which in turn could translate to the isomer-specific modulation of oxidative stress and redox biology in intestinal cells. The overall objective of this chapter was to characterize the relative antioxidant properties of CGA isomers in both chemical and also a cultured human intestine cell line.
3.2 Materials and Methods

3.2.1 Materials and Reagents

MTT, MEM, IFNγ, PMA, sodium dodecyl sulfate (SDS), 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH), dichlorofluorescein diacetate (DCFH-DA), Hanks’ balanced salt solution (HBSS), bovine serum albumin (BSA), paraformaldehyde, triton X-100, 4’,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, MO, USA). CGA isomers, 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA were obtained from Cerilliant Corporation (Round Rock, TX, USA) and Chengdu Must Bio-Technology Co. (Chengdu, Sichuan, China). FBS, penicillin and streptomycin were purchased from Gibco® (Grand Island, NY, USA). Human IL-8 Single Analyte ELISA kit was purchased from Qiagen (Hilden, Germany).

3.2.2 ABTS Assay

The protocols of ABTS assay were described in Section 2.2.6. The antioxidant activity of CGA isomers were expressed as mmol Trolox Equivalent/mmol.

3.2.3 ORAC Assay

The protocols of ORAC assay were described in Section 2.2.7. The antioxidant activity of CGA isomers were expressed as mmol Trolox Equivalent/mmol.
3.2.4 Nitric Oxide Assay

The protocols of Nitric Oxide assay were described in Section 2.2.8. The antioxidant activity of CGA isomers were expressed as mmol Trolox Equivalent/mmol.

3.2.5 Cell Culture

The human colon adenocarcinoma cell line, Caco-2 (HTB-37, American Type Culture Collection, Manassas, VA, USA), was cultured in complete MEM containing Earle’s salts supplemented with 10% FBS (Invitrogen, Canada), 100 U/mL of penicillin and 100 µg/mL of streptomycin. Briefly, cells (passage 26-37) were maintained in 75-cm² plates (Corning Inc., Corning, NY, USA) at 37 °C in a 5% CO₂ humidified atmosphere, and the media was changed every 2-3 days. Cultured cells were split (1:5) once they are about 80% confluent by use of 0.25% (w/v) trypsin-0.53 mM EDTA (Gibco-BRL). For individual experiments, cells were seeded onto 6-well or 96-well plates (Sarstedt AG & Co., Sarstedtstrabe, Numbrecht, Germany) at a density of 1×10⁵ cells/cm². Cells were cultured for 21 days with medium change every 2-3 days to allow for spontaneous differentiation.

3.2.6 MTT Assay

The protocols of MTT assay were described in Section 2.2.11.
3.2.7 Method used to Evaluate CGA Scavenging Capacity of AAPH Generated Peroxyl Radical in Caco-2 Cells

The effect of CGA isomers on AAPH-initiated intracellular oxidation was assessed by following the method described in Section 2.2.12, with modifications. Instead of being exposed to media containing coffee extracts, Caco-2 cells were exposed to MEM medial containing individual CGA isomers at concentrations of 0.2 mM, 1 mM, or 2 mM at 37 °C for 24 h.

3.2.8 *In Vitro* Model of Intracellular Oxidation

The *in vitro* model developed to study intestinal inflammation by Chen & Kitts (2008) was used to generate ROS in the current study. Caco-2 cells were seeded on a 6-well plate at a cell density of $1 \times 10^5$ cells/cm$^2$ and cultured in MEM media containing 10% FBS with 100 µg/mL streptomycin and 100 U penicillin for 21 days at 37°C under a humidified 5% CO$_2$ atmosphere. After 21 days of culture, Caco-2 cells were challenged with IFN$\gamma$ (8000U/mL) + PMA (0.1 µg/mL) cocktail for 24 hours to trigger inflammation and to generate ROS.

3.2.9 Cell Treatment

CGA isomers were dissolved in MEM and filtered sterilized using a 2 µm filter before use. Caco-2 cells cultured to differentiation were pre-incubated with individual
CGA isomers in MEM medium for 24 hours. The medium was then replaced with fresh MEM medium, and cells were challenged with PMA+IFNγ in the presence of fresh CGA for different time period corresponding to specific experimental design. Cells not exposed to CGA isomers but treated with a PMA+IFNγ challenge served as the control. The blank was Caco-2 cells without neither CGA isomers treatment nor PMA+IFNγ challenge.

Figure 3.1 illustrates the steps of experiment and explains the source of data points presented in figures at Results & Discussion sections.

Figure 3.1 A diagram that shows the steps of experiment and explains the source of data points presented in figures at Results & Discussion sections.
3.2.10 Confirmation of Cellular Uptake of CGA Isomers by Time-of-flight

Secondary Ion Mass Spectrometry (TOF-SIMS)

Specific culture plates (75-cm² plates, Corning Inc., Corning, NY, USA) and Caco-2 cell density (1×10⁵ cells/cm²) were used to perform cellular uptake of CGA isomers. Caco-2 cells were grown for 21 days to reach differentiation, and then treated with CGA isomer in MEM for 24 hours. After CGA isomer incubation, the cells were washed five times with PBS; CGA isomers were not detectable in the fourth PBS washing. Cells were detached from plates using trypsin (with 0.05% EDTA), then centrifuged for 5 min at 1,500 rpm and 4 °C. The supernatant was removed, and the pellet was freeze-dried to a powder which was re-suspended in 1 mL of 100% methanol and sonicated for 10 minutes using an ice-cold bath sonicator (VWR Aquasonic 75D; West Chester, PA, USA). This procedure burst the Caco-2 cells to recover intracellular CGAs. The suspension was centrifuged at 10,000 rpm at 4 °C for 5 min to recover the supernatant which was then collected and stored at -80 °C. Before performing the TOF-SIMS analyses, the Caco-2 cell methanol extract was dried under a stream of nitrogen onto a silicon plate. The TOF-SIMS analyses were performed in a PHI TRIFT V nano TOF-SIMS spectrometer (Physical Electronics PHI Inc., Japan). A pulsed primary 30 keV Au⁺ ion beam was rastered over a 400 µm × 400 µm area. The total ion dose per spectra was kept constant at approximately 1.4×10¹¹ ions/cm² to ensure static analysis.
condition. The fragmentation patterns of all the samples were determined using a negative ion model.

### 3.2.11 Effect of CGA Isomers on Caco-2 Cell Oxidative Status of Cells Induced by PMA+IFNγ

Caco-2 cells were exposed to MEM media (control) or MEM media containing specific individual CGA isomers at concentrations of 0.2 mM, 1 mM, or 2 mM (treatment) for 24 h at 37 °C. Caco-2 cells were then rinsed with PBS (pH 7.2), followed by incubating with a DCFH-DA probe (5 μM) in PBS at 37 °C for 30 min. Cells were rinsed again with PBS and then challenged with IFNγ+PMA in the presence of fresh CGA for 24 hours. The positive control consisted of cells exposed to IFNγ+PMA, without the presence of CGA. The negative control was cells not treated with either IFNγ+PMA or CGA. Inhibition was expressed according to the following equation:

\[
\% \text{ Fluorescence Inhibition} = \frac{F_{pc} - F_{CGA}}{F_{pc} - F_{nc}} \times 100\%
\]

Where \(F_{pc}\) is the fluorescence intensity of the positive control, \(F_{CGA}\) is the fluorescence intensity of the cells treated with individual CGA isomers in IFNγ+PMA induced cells, and \(F_{nc}\) is the fluorescence intensity of the negative control.
3.2.12 Transepithelial Electrical Resistance (TEER) Measurement

TEER value is a useful functional parameter to monitor the integrity of epithelial cells cultured on filter supports. Caco-2 cells were grown on a semipermeable filter insert Falcon® Permeable Support designed for 24-well plate with 0.4 μm translucent high-density PET Membrane (Corning Life Sciences, Tewksbury MA, USA). Once Caco-2 cells reached differentiation, PMA+IFNγ cocktail was added to both the apical and basolateral compartments. Millicell® ERS voltohmmeter (Millipore Ltd, Etobicoke, Ontario, Canada) was used to measure the electrical resistance between apical and basolateral compartments. One electrode was placed in the upper apical compartment, while the other was placed in the lower basolateral compartment and the electrodes were separated by the Caco-2 monolayer. The procedure included measuring a blank resistance (R Blank) of the semipermeable membrane without cells and measuring the resistance across the cell layer on the semipermeable membrane (R Total). TEER values were reported in units of Ω.cm² and calculated as:

\[
\text{TEER} = (R_{\text{Total}} - R_{\text{Blank}}) \times M_{\text{Area}} \text{ (cm}^2) ;
\]

The effect of CGA isomers on TEER value of Caco-2 cells treated with PMA+IFNγ cocktail was also evaluated. Cells were treated as described in 3.2.9 and then TEER values were measured as described above.
3.3 Results and Discussion

3.3.1 Free Radical Scavenging Capacity of CGA isomers

The free radical scavenging capacities of six CGA isomers on ABTS\(^{\cdot +}\), nitric oxide, and peroxyl radicals are given in Table 3.1. In general, dicaffeoylquinic acids showed significantly higher antioxidant activity compared to caffeoylquinic acids \((p<0.05)\). This result can be attributed to the structural differences of dicaffeoylquinic acids, which contain two more hydroxyl groups in a phenolic moiety compared to caffeoylquinic acids. Significant difference in peroxyl radical scavenging capacity among different dicaffeoylquinic acid isomers was observed in the ORAC assay \((p<0.05)\). The ORAC value for 5-CQA is comparable to the value previously reported (3.5±0.1 mM Trolox Equivalent/mmol) by others (Bakuradze et al., 2010) and our ABTS value for 5-CQA confirm similar values previously reported (1.15 ± 0.05 mM Trolox Equivalent/mmol) (Gómez-Ruiz et al., 2007). Nitric oxide radical scavenging capacity of different CGA isomers have not been reported before, but the data herein shows the reactivity of six CGA isomers toward nitric oxide radical.

Different mechanisms have been proposed to explain the antioxidant properties of phenolic compounds, among which direct hydrogen atom transfer (HAT) mechanism is the most widely recognized one. In the HAT mechanism, a hydrogen atom of hydroxyl group (OH) is transferred from phenol (ArOH) to free radical (X\(^{\cdot}\)) and form an unstable
The phenoxyl radical (ArO·) and the X-H molecular (Leopoldini et al., 2004). The phenoxyl radical (ArO·) eventually becomes a stable phenoxyl radical through conjugative resonance stabilization. According to the theory of HAT mechanism, the free radical scavenging capacity of hydroxycinnamic acids and their derivatives are depended not only on the number of available hydrogen atom but also depended on the bond dissociation energy (BDE) of the hydrogen atom of the phenolic moiety. For example, 5-CQA contains two hydroxyl groups in phenolic moiety and 3,5-diCQA contains four hydroxyl groups in its phenolic moiety, but the ORAC value of 3,5-diCQA is not double that of 5-CQA, suggesting that the BDE of the hydrogen atoms of the phenolic moiety in 3,5-diCQA and in 5-CQA are not a simple two to one relationship. Furthermore, other workers have reported that the secondary reactions of the initially formed phenoxyl radicals (ArO·) also contribute to the differences in the relative reactivity of hydroxycinnamic acids and their derivatives with free radicals (Pino et al., 2006).

Overall, molecular properties, including both the number and the location of hydroxyl groups in the aromatic ring and the conjugated double bond, and the characteristic of the initially formed phenoxyl radicals (ArO·) together affect the kinetics of free radical scavenging reaction of CGA isomers.
Table 3.1 Antioxidant Activity of CGA Isomers Obtained Using ABTS, NO Radical Scavenging, and ORAC Assays

<table>
<thead>
<tr>
<th>CGA Isomers</th>
<th>ABTS Assay (mmol Trolox Equivalent/mmol)</th>
<th>NO Radical Scavenging Assay</th>
<th>ORAC Assay (mmol Trolox Equivalent/mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-CQA</td>
<td>0.97±0.02a</td>
<td>6.62±0.25a</td>
<td>3.44±0.09a</td>
</tr>
<tr>
<td>5-CQA</td>
<td>0.88±0.04b</td>
<td>6.24±0.43a</td>
<td>3.45±0.06a</td>
</tr>
<tr>
<td>4-CQA</td>
<td>1.01±0.01a</td>
<td>6.04±0.97a</td>
<td>3.47±0.09a</td>
</tr>
<tr>
<td>3,5-diCQA</td>
<td>1.88±0.04c</td>
<td>10.10±0.49b</td>
<td>5.07±0.14b</td>
</tr>
<tr>
<td>3,4-diCQA</td>
<td>1.96±0.02cd</td>
<td>9.23±0.36b</td>
<td>5.49±0.15c</td>
</tr>
<tr>
<td>4,5-diCQA</td>
<td>1.86±0.04e</td>
<td>10.18±0.58b</td>
<td>4.53±0.12d</td>
</tr>
</tbody>
</table>

1 Experiments were performed in triplicate and results are expressed as mean ± standard deviation. Differences in antioxidant values of CGA isomers within individual assay were identified (different letters) using one-way ANOVA, followed by Bonferroni post-hoc analysis at \( p < 0.05 \) using GraphPad Prism software.

3.3.2 Cellular Uptake of CGA Isomers by TOF-SIMS

TOF-MS was successful to demonstrate the cellular uptake of different CGA isomers by Caco-2 cells treated with corresponding CGA isomers for 24 hours. The diagnostic peaks used as evidence of successful cellular uptake by Caco-2 cells for these six CGA isomers were summarized in Table 3.2. Chromatogram for 5-CQA is presented in Figure 3.2(a). Chromatograms for 3,5-diCQA are presented in Figure 3.2(b) and (c). Additional chromatograms for uptake of other CGA isomers are presented in Appendix B. Identification of each CGA isomer was confirmed using pure CGA standards. Caco-2 cells pretreated with corresponding CGA isomers showed cellular uptake after 24 hours.
incubation, albeit this was not quantitated due to the content was too low to quantify.

Peaks that are observed in both mass spectra for authentic CGA isomer standards and the extracts of Caco-2 cells treated with corresponding CGA isomer, but not observed in extract of blank Caco-2 cells, is evidence of cellular uptake by Caco-2 cells. For example, cellular uptake of 5-CQA was confirmed by the authentic 5-CQA standard detected at m/z 179 (Figure 3.2(a)). Similarly, 4-CQA, 3,5-diCQA, 4,5-diCQA displayed the same diagnostic fragment ions at m/z 179 (caffeic acid-H) (Figure Appendix B.2, Figure 3.2(b), Figure Appendix B.5). This fragment is derived from the cleavage of C-O bond adjacent to the ester (Ncube et al., 2014) (Shin et al., 2015). Furthermore, the 3,5-diCQA and 4,5-diCQA isomers were distinguished from 5-CQA and 4-CQA due to the presence of another diagnostic peak obtained at m/z 473, while 5-CQA and 4-CQA had only one peak at m/z 179. As for 3,4-diCQA, two diagnostic peaks occurring at m/z 325 and 473, respectively, were detected (Figure Appendix B.3 and Figure Appendix B.4). In Figure Appendix B.1, 3-CQA had a different diagnostic fragment ion (m/z 325) compared to 5-CQA and 4-CQA, respectively, and this peak was used to show cellular uptake of 3-CQA by Caco-2 cells. The same peak m/z 325 was also used as evidence of successful cellular uptake of 3,4-diCQA by Caco-2 cells. The energy distribution differences on quantum level among these molecules explains the different behavior at the same ionization condition (Ncube et al., 2014).
The results demonstrate that Caco-2 cells uptake CGA isomers, but at minimal extend. Previous study reported that phenolic acids are absorbed by the monocarboxylic acid transporter (MCT) in Caco-2 cells (Konishi et al., 2002). A monoaninoic carboxyl group and a nonpolar side chain or aromatic hydrophobic moiety are thought to be necessary components of a substrate for MCT (Rahman et al., 1999). CGA isomers contain a monocarboxylic group in the portion of quinic acid and the aromatic portion inside, this provides a structure foundation of cellular absorbance of CGA isomers by Caco-2 cell. However, the ester group might cause some hindrance effect on interaction with MCT in Caco-2 cells (Konishi & Kobayashi, 2004). Other workers have measured the direct transport of CGA across Caco-2 cell monolayer and found that around 0.06% of 5-CQA were transported from apical side to basolateral side when there is a proton gradient (apical pH, 6.0; basolateral pH, 7.4) (Konishi & Kobayashi, 2004). In the present study, the recovery of CGA isomers in Caco-2 cells indicated that absorption was very small and could not be quantified by HPLC alone. Only traceable amount of CGA isomers were detected using TOF-MS, suggesting the bioavailability of CGA isomers is low, but present in Caco-2 cells.
Table 3.2 A Summary of Diagnostic $m/z$ Ions of Different CGA Isomers$^*$.  

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Diagnostic $m/z$ ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-CQA</td>
<td>325</td>
</tr>
<tr>
<td>5-CQA</td>
<td>179</td>
</tr>
<tr>
<td>4-CQA</td>
<td>179</td>
</tr>
<tr>
<td>3,5-diCQA</td>
<td>179, 473</td>
</tr>
<tr>
<td>3,4-diCQA</td>
<td>325, 473</td>
</tr>
<tr>
<td>4,5-diCQA</td>
<td>179, 473</td>
</tr>
</tbody>
</table>

$^*$ Peaks that are observed in both mass spectra for authentic CGA isomer standards and the extracts of Caco-2 cells treated with corresponding CGA isomer, but not observed in extract of blank Caco-2 cells, are diagnostic peaks and also the evidence of cellular uptake by Caco-2 cells.
Figure 3.2 TOF-SIMS negative ion mass spectra of CGA standard methanol solution and Caco-2 cell methanol extract deposited on a silicon plate. Each figure contains three spectra. The first one is the spectrum of authentic CGA isomer standard, the
second one is the spectrum of the methanol extract of Caco-2 cells treated with corresponding CGA isomer, the third one is the spectrum of the methanol extract of Caco-2 cells only.
3.3.3 Free Radical Scavenging Capacity of CGA Isomers Against AAPH Generated Proxy Radical in Caco-2 Cells

Chemical assays, such as ABTS assay, Nitric Oxide Assay, ORAC assay, only measure chemical conditions that reflect a chemical radical exchange and cannot be completely translated to the cell environment. Therefore, AAPH challenged Caco-2 cells were used as an in vitro model to study antioxidant activity of CGA isomers to scavenge peroxyl radicals. The decomposition of AAPH forms one mole of nitrogen radical and two moles of carbon radical, where reacts with molecular oxygen to generate peroxyl radicals (Noguchi et al., 1998). The intracellular peroxyl radical scavenging activity of CGA after administration of AAPH to Caco-2 cells is shown in Figure 3.3 (a). The results showed that CGA isomers scavenged intracellular peroxyl radicals in a concentration depended manner. Dicaffeylquinic acids had relatively stronger ROS scavenging capacity compared to caffeoylquinic acids. \((p<0.05)\) This finding is in agreement with previous antioxidant studies conducted with pure chemical assays (Xu et al., 2012). At the highest concentration tested (2 mM), 3-CQA, 4-CQA, and 5-CQA were effective at reducing 35% of ROS generated by AAPH, whereas, 3,5-diCQA, 4,5-diCQA, and 3,4-diCQA reduced approximately 60% of ROS. There were no significant differences in free radical scavenging capacity within individual caffeoylquinic acid and dicafeoylquinic acid isomers however.
3.3.4 Protective Effect of CGA Isomers Against PMA+INFγ-induced Oxidative Stress in Caco-2 Cells

ROS are generated intracellularly by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complexes located in cell membranes, mitochondria, endoplasmic reticulum, and peroxisomes (Holmström & Finkel, 2014). To determine whether CGA isomers exert antioxidant effects in inflamed Caco-2 cells, intracellular ROS scavenging activities were measured in IFNγ+PMA-treated Caco-2 cells. Caco-2 cells challenged with IFNγ+PMA cocktail generated ROS at a detectable level and above that observed in non-treated cells ($p<0.05$) (Figure 3.3(b)). This result indicated that inducing an inflammatory reaction was accompanied by an increase in ROS generation, which is in agreement with a previous other study conducted in different cell types. For example, pro-inflammatory cytokines induced ROS in human retinal pigment epithelial cells by promoting the activity of mitochondria and NADPH oxidase (Yang et al., 2007). In this study, incubation of cells with CGA isomers prior to the IFNγ+PMA treatment significantly ($p<0.05$) reduced ROS production to a level similar to, or even lower, than that observed in non-treated cells. The effect of CGA isomers and IFNγ+PMA treatment on the cell viability of Caco-2 cells was evaluated. A more than 85% of cell viability relative to unexposed cells suggests that the treatment had no cytotoxicity effect on the Caco-2 cells (Figure Appendix A.2). Unlike the findings obtained in AAPH treated Caco-2 cells, different CGA isomers did not show significant difference in relative
intracellular ROS scavenging capacity when cells were challenged with IFN\(\gamma\)+PMA. Moreover, a concentration-dependent effects for CGA isomers to produce ROS was not observed within the 0.2 ~ 2 mM range of CGA treated cells, indicating that 0.2 mM was likely the lowest concentration needed to give maximum inhibition of ROS production, when Caco-2 cells were treated with IFN\(\gamma\)+PMA. Other workers showed that lipopolysaccharide (LPS) challenge increased intracellular ROS production in intestinal porcine enterocyte cells compared to cells without LPS treatment; and furthermore, a significant reduced intracellular ROS level were observed when treated with 25 \(\mu\)M CGA (Palócz et al., 2016). Our results and the findings from other research groups are in agreement that ROS are involved in the pathogenesis of inflammation and that CGA isomers are effective at scavenging intracellular ROS in the inflamed cells.
Figure 3.3 Capacity of CGA isomers at different concentrations (0.2, 1, and 2 mM) to prevent (a) AAPH and (b) PMA+INFγ-induced oxidative stress in Caco-2 cells (passage no. 30), determined using DCFH-DA. The significance of the differences between different treatment was analyzed by one-way analysis of variance (ANOVA), using Bonferroni post-test, using Graph Pad Prism software. Value of \(p<0.05\) was considered to be statistically significant. Different letters represent significantly different. Data are representative of three independent experiments.
3.3.5 **TEER Value in Caco-2 Monolayer**

When cultured on Transwell™ inserts, Caco-2 cells form highly differentiated polarized monolayers that feature apical microvilli, functional tight junctions, and expression of both apical and basolateral transporters. In this experiment, the effect of different concentrations of the PMA+IFNγ cocktail on the monolayer integrity of Caco-2 cells was examined. The results showed that a PMA+IFNγ challenge altered the integrity of Caco-2 cell monolayer, as reflected by the decrease in TEER. **Figure 3.4 (a)** showed that the TEER value declined dramatically from 550±22 Ω·cm² to 341±26 Ω·cm² after 8 hours of exposure to PMA (0.1 µg/mL) + IFNγ (8000U/mL) exposure and continued to drop to lower values of 181±18 Ω·cm² after 24 h, in both apical and basolateral compartments. No further significant differences in TEER values were observed when concentrations of PMA+IFNγ were increased. TEER value is a functional parameter to quantify the barrier integrity of epithelial cells cultured on filter support. The association between oxidative stress and alteration of the tight junction proteins has been explored by many researchers. One study reported that H₂O₂ generated by the xanthine/xanthine oxidase system decreases TEER value of Caco-2 and T-84 cell monolayers (Rao et al., 1997). Another study reported that chloic acid (a bile acid) increased intracellular ROS generation in Caco-2 cells and ROS impaired tight junction proteins, and this resulted in a decrease in the TEER value of Caco-2 cell monolayer (Araki et al., 2005).

Inflammation related conditions also have an impact on the physical structures and
properties of filter-grown epithelial cultures. Other workers have reported that incubation of Caco-2 monolayers with TNF-α resulted in marked disruption of barrier integrity with a decline in TEER value by 25% compared to the cells that were not treated with TNF-α (Piegholdt et al., 2014). The findings from these studies suggest that both ROS and inflammatory cytokines impair the functionality of the epithelial barrier, composing of an optimal assembly of tight junction proteins (Capaldo & Nusrat, 2009). This explains the decline in TEER value of Caco-2 cells when treated with the PMA+IFNγ cocktail.

The effects of CGA isomers on PMA+IFNγ treated Caco-2 cell TEER values were evaluated. CGA isomers were effective at reducing the loss of TEER value associated with response to inducer. No significant differences (p < 0.05) were observed between different CGA isomers in preventing loss of TEER value attributed to the PMA+IFNγ challenge, when compared at equal molar concentrations. Therefore, only the data of 5-CQA is presented (Figure 3.4 (b)). At a concentration of 0.2 mM, 5-CQA had a protective effect on monolayer integrity after 16 hours of treatment. 5-CQA improved TEER by 37% compared to the group without 5-CQA treatment. When the concentration of 5-CQA increased from 0.2 mM to 1 mM, the improvement in TEER value was observed after 8 hours treatment. However, no significant (p > 0.05) difference in protection of TEER values were observed between 1 mM and 2 mM treatment. The efficacy of phenolic compounds to protect barrier integrity of intestinal epithelial cells
has also been investigated by other researchers. For example, quercetin has shown to protect against the decline of TEER value of Caco-2 cells caused by indomethacin (Carrasco-Pozo et al., 2013) and CGA protect against the decrease of intestinal permeability of rats caused by LPS (Ruan et al., 2014). Phenolic compounds exert an modulatory effect on TEER, possibly through interacting with receptors on the membrane of epithelia cell and stimulating a series of cell signaling, which play a crucial role in maintain redox biology and inflammation.
Figure 3.4 (a) TEER value of Caco-2 (passage no. 33) challenged by PMA+ IFNγ Cocktail at different concentrations at varying times. (b) Effect of 5-CQA on PMA+IFNγ-induced paracellular permeability. TEER were measured at varying times in Caco-2 cell monolayers treated with PMA+IFNγ, in the presence or the absence of 5-CQA (0.2, 1, and 2 mM). Values are means ± SD. The significance of the differences between different treatment at the same time point was analyzed by one-way analysis of variance (ANOVA), using Bonferroni post-test, using Graph Pad Prism software. Superscript with different alphabets (a, b, c, and d) are significantly different ($p < 0.05$).
3.4 Conclusion

Dicaffeoylquinic acids (3,5-diCQA, 4,5-diCQA, and 3,4-diCQA) had a relatively stronger potency to scavenge free radicals, compared to caffeoylquinic acids (3-CQA, 4-CQA, and 5-CQA) in cell-free chemical assays. In the in vitro cell-based Caco-2 model, CGA isomers were detected and identified in the intracellular compartment, thus suggesting some degree of bioavailability in Caco-2 cells. The absolute concentration of CGA isomers recovered intracellularly was very small and could not be quantified, but their presence existed. In the experiment where oxidative stress was induced using the PMA+IFNγ challenge, six major CGA isomers present in coffee can alleviate oxidative stress in Caco-2 cells triggered by PMA+IFNγ, but do not have isomer-dependent effect.
Chapter 4: Mechanisms That Describe CGA Isomers Modification of Oxidative and Inflammatory Responses in Caco-2 Cells

4.1 Introduction

The single-layered epithelium is the primary line of defense against intestinal lumen stressors. In patients with Inflammatory Bowel Disease (IBD), sections of the intestinal epithelium are inflamed due to an imbalanced redox status and excessive secretion of pro-inflammatory cytokines (Bhattacharyya et al., 2014) (Katsuta et al., 2000) (Laukoetter et al., 2008). At the initiation stage of intestinal inflammation, ROS are over-produced, leading to oxidative stress and are related to a release of excessive amounts of pro-inflammatory mediators that trigger inflammatory responses (Iborra et al., 2011) (Marui et al., 1993). For example, oxidative stress causes oxidation of proteins, such as glutathionylated peroxiredoxin-2, an oxidized protein that can further facilitate progression of inflammation (Salzano et al., 2014). Therefore, inhibiting, or reducing, oxidative stress is a strategy alongside the use of classical anti-inflammatory medications to control IBD. Support for this statement is found with the drug often used in the treatment of IBD, namely 5-aminosalicylic acids (5-ASA), where therapeutic effects are believed to be due to a capacity to scavenge ROS (Punchard et al., 1992).

Studies that have attempted to understand the etiology of IBD have focused on understanding at the molecular level how inflammatory responses are inhibited. The transcription factor, NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) has a crucial role in controlling cellular biological functions by regulating expression of numerous genes that influences stress responses, inflammation and apoptosis (Wang et al., 2002). NFκB is
a complex of structurally related proteins that consist of p50, p52, p65, c-RelA, and RelB in what is known as the Rel family. Stimuli, including free radicals, cytokines (e.g. TNF-α and interleukin 1β), as well as bacterial lipopolysaccharides (LPS) can induce NFκB through a number of cell membrane-bound receptors. Activation of NFκB is mediated through the phosphorylation of its inhibitory subunit IκB kinase complex (Traenckner et al., 1995). Upon the phosphorylation, IκB is degraded, releasing the NFκB subunit p65, which in turn is translocated to the nucleus and binds to specific gene promoters that modulate anti- and pro-inflammatory proteins. Although these mechanisms are known and to explain NFκB activity, there is relatively less information available on how specific phenolic compounds, such as CGA isomers are involved in modulating NFκB.

Regulation of NFκB activity is connected with the activation of upstream protein kinases. MAPKs (mitogen-activated protein kinases) signaling pathway consists of a heterogeneous group of enzymes which phosphorylate sites containing the core consensus motif Ser/Thr-Pro. MAPKs are activated by dual specificity kinases, called MAP kinases kinases (MAPKKK), which in turn are activated by MAP kinase kinase kinase (MAPKKKs) (Coskun et al., 2011). To date, at least three MAPK cascades have been identified, including ERK1/2, JNK, and p38. MAPK signaling can be activated through phosphorylation of particular amino acid sequences in the protein of MAPK components including ERK1/2, JNK, and p38. The phosphorylated MAPKs interact with other downstream components, including NFκB, which is mediated through the phosphorylation of its inhibitory subunit IκB, and subsequent degradation of IκB at the proteasome. Former studies have linked oxidative stress with the activation of JNK, p38, and ERK kinases (Cross and Templeton, 2004), and ROS are thought to be involved in the crosstalk
between NFκB and JNK (Nakano et al., 2006). Antioxidants, such as vitamin E can block NFκB activation, which leads to the association between ROS generation and stimulation of NFκB activity (Elisia and Kitts, 2015) (Wang et al., 2002).

It is also well documented that Nrf2 (nuclear factor (erythroid-derived 2)-like 2) has a key role in regulating oxidant defenses by binding the antioxidant response element promoter and inducing the expression of a number of antioxidant genes (Kabashima et al., 2005). These genes encode for heme oxygenase 1, glutamate-cysteine ligase, NAD(P)H quinone dehydrogenase 1, superoxide dismutase, catalase, glutathione peroxidase family, glutathione S-transferase, and glutathione synthetase, respectively, which collectively regulate cellular oxidative stress, inflammatory responses and apoptosis. Thus, the pharmacological induction of the Nrf2 pathway may constitute a potent strategy to combat against oxidative stresses that are involved in numerous diseases. Nrf2 is essential for the control of inflammation and recent research suggests that dietary compounds that activate the redox sensitive Nrf2 signaling pathway may represent a promising strategy for alleviating of inflammation (Kobayashi et al., 2016).

CGAs are phenolic acids with vicinal hydroxyl groups located on aromatic residues that are derivated from esterification of cinnamic acids, including caffeic, ferulic, and p-coumaric acids with quinic acid. CGAs are the most abundance phenolic compounds in many fruits and vegetables in addition to coffee. The major CGAs in coffee include 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA. Among these isomers, 5-CQA is the most studied isomer, and there is in vitro evidence that show 5-CQA can protect against UVB and H$_2$O$_2$-induced
oxidative stress in human HaCaT cells (Cha et al., 2014), reduces tert-butyl hydroperoxide, H$_2$O$_2$, and FeSO$_4$-induced lipid oxidation in PC12 cells (Pavlica et al., 2005), and evoked up-regulation of cellular antioxidant enzymes and NFkB signaling in PMA-stimulated human lung carcinoma cells (Tsai et al., 2013). In many cell types, agents that trigger ROS generation also lead to activation of NFkB. In fact, sustained oxidative stress will down-regulate NFkB stimulation, while a transient exposure to ROS at lower concentrations will lead to activation of this pathway (Wu et al., 2009). This general understanding on what activates the NFkB pathway is relevant to the role of polyphenols, such as CGAs, which are known to have antioxidant activity and can reduce oxidative stress or contribute to redox balance. In vivo studies performed in rodents have also reported the protective effects of 5-CQA towards inflammation. For examples, 5-CQA successfully attenuated lipopolysaccharide (LPS)-induced mastitis in mice and also protected against acute hepatotoxicity that was induced by LPS in mice (Ruifeng et al., 2014) (Xu et al., 2010). Despite the fact that the bioactivity of 5-CQA has been thoroughly studied, current knowledge on the integration between antioxidant and anti-inflammatory activities of other CGA isomers that are present in common foods or beverages is limited.

In previous chapters of this thesis, CGA isomers were showed to scavenge free radicals in both chemical and cell-based assays. Also, CGA isomers showed isomer-specific capacity to attenuate ROS levels in inflamed Caco-2 cells. In this chapter, experiments were designed to examine the potential anti-inflammatory activity of CGA isomers and underlying mechanisms that link antioxidant and anti-inflammatory activities together for both major and minor CGA isomers. Specifically, experiments were conducted to compare relative affinity of CGA isomers to influence redox biology and related inflammatory responses. To achieve this goal, the
PMA+INF\(\gamma\)-induced Caco-2 cells model was used. The experimental hypothesis tested was that CGA isomers have an isomer specific efficacy to ameliorate oxidative and inflammatory stress under inflamed conditions. In addition, the mechanisms for this response were related to isomer specific affinity to mortify NF\(\kappa\)B signaling, phosphorylation of MAPK kinases, and Nrf2 signaling.

### 4.2 Materials and Methods

#### 4.2.1 Reagents and Materials

MTT, MEM, IFN\(\gamma\), PMA, sodium dodecyl sulfate (SDS), AAPH, DCFH-DA, Hanks’ balanced salt solution (HBSS), bovine serum albumin (BSA), paraformaldehyde, triton X-100, 4’,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, MO, USA). CGA isomers, 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA were obtained from Cerillian Corporation (Round Rock, TX, USA) and Chengdu Must Bio-Technology Co. (Chengdu, Sichuan, China). FBS, penicillin and streptomycin were purchased from Gibco® (Grand Island, NY, USA). Antibodies against Nrf2 (C terminal) and GSH/GSSG Ratio Detection Assay Kit were purchased from Abcam (Cambridge, MA, USA). Nuclear Extraction Kit and Nrf2 Transcription Factor Assay Kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

#### 4.2.2 Cell Culture

Caco-2 cells were cultured on 6-well plate following the protocols described in section 3.2.5 to test the cell signaling associated with NF\(\kappa\)B, MAPKs and Nrf2.
4.2.3 Cell Treatments

Cells were treated with PMA+INFγ and CGA isomers as described in 3.2.9.

4.2.4 Evaluation of Cytotoxicity in Caco-2 cells by MTT Reduction Assay

The protocols of MTT assay were described in Section 2.2.11.

4.2.5 Analysis of p65 Binding Activity by Transactivation Assay

To study the role of different CGA isomers on modulating NFκB signaling in IFNγ+PMA challenged Caco-2 cells, the binding efficacy of the nuclear extract with NFκB consensus binding site, was measured using a NFκB (p65) Transcription Factor Assay Kit (10007889, Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, Caco-2 cells were first recovered with a rubber spatula into ice-cold PBS in the presence of phosphatase inhibitors, after the treatment with CGA isomer as described in Section 3.2.9. The cytosolic fraction and nuclear fraction were extracted and separated using a Nuclear Extraction Kit (10009277, Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer’s instructions. Purity of the nuclear fraction was confirmed with Histone presence only in the nuclear fraction and not in cytoplasm fraction; GAPHD was present only in cytoplasm and not in nuclear fraction. Protein concentrations were determined using the BCA protein assay and of all the samples were adjusted to 2 mg/mL. An aliquot of the cellular protein extract, 10 μL (containing 20 μg of protein), was incubated with immobilized oligonucleotides, containing the NFκB consensus binding site (5’-GGGACTTTCC-3’). The active form of p65 that bound to the oligonucleotides was detected using anti-p65 primary antibody (1:1000 dilution) for 1 hour
followed by incubation with HRP-conjugated secondary antibody (1:1000 dilution) for 1 hour at room temperature. Absorbance values were recorded using a microplate reader set at 450 nm.

4.2.6 Western Blot to Analyze p-Erk 1/2, p-JNK, p-p38, t-Erk 1/2, t-JNK, and t-p38

(p=phosphorylated; t=total)

Western blot is a semi quantitative analytical technique used to detect specific proteins in a sample of tissue homogenate or cell extract. M-PER™ Mammalian Protein Extraction Reagent (Cat. No 78501, Thermo Fisher Scientific, Waltham, MA, USA) (added phenyl methyl sulphonyl fluoride and protease inhibitor) was used to extract Caco-2 cell protein according to the manufacture’s instructors. The total protein extracts (20 µg protein) were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-PROTEAN® II Multiscreen Apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Separated proteins were transferred to nitrocellulose membranes (0.2 µm, 7×8.4 cm; Bio-Rad Laboratories, Hercules, CA, USA) and membranes were incubated with 3% BSA at 20°C for 1 h to block unspecific binding sites, before incubating with different antibodies (for detecting phosphorylated MAPK cascades): anti-phospho-Erk1/2 (phospho Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (1:2000 dilution) (4370S, Cell Signaling Technology, Danvers, MA, USA); anti-phospho-JNK1+JNK2+JNK3 (phospho T183+T183+T221) antibody (1:1000 dilution) (ab124956, Abcam Inc., Ontario, Canada); anti-phospho-p38 MAPK (phospho Thr180/Tyr182) (D3F9) XP® Rabbit mAb (1:1000 dilution) (4511S, Cell Signaling Technology, Danvers, MA, USA) at temperature of 4°C, overnight. Subsequently, the membranes were washed three times using Tris-buffered-saline-Tween (TBST) and then incubated with a secondary antibody, horse radish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1000 dilution) (Invitrogen,
Carlsbad, CA, USA) for one hour. The membranes were then washed three times with TBST, reacted against a Western ECL substrate (Bio-Rad Laboratories Inc., Woodinville, WA, USA) for 3 min. A ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc., Woodinville, WA, USA) were used to detect the proteins. Equal protein loading was demonstrated by stripping the original blot and re-probing with t-ERK1/2, t-JNK, and t-p38 antibodies. Specifically, the membrane that used for the detection of p-Erk1/2 was washed three times with TBST and incubated in a mixture antibody solution that contains both anti-phospho-Erk1/2 (phospho Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (1:2000 dilution) (4370S, Cell Signaling Technology, Danvers, MA, USA) and anti-Erk1/2 (137F5) Rabbit mAb (1:1000 dilution) (4695S, Cell Signaling Technology, Danvers, MA, USA) [this mixture solution is called total Erk1/2 antibody solution]. Similarly, the membrane that used for the detection of p-JNK was washed three times with TBST and incubated in a mixture antibody solution that contains both anti-phospho-JNK1+JNK2+JNK3 (phospho T183+T183+T221) antibody (1:1000 dilution) (ab124956, Abcam Inc., Ontario, Canada) and anti-JNK1+JNK2+JNK3 antibody (1:2000 dilution) (ab208035, Abcam Inc., Ontario, Canada) [this mixture solution is called total JNK antibody solution]. Similarly, the membrane that used for the detection of p-p38 was washed three times with TBST and incubated in a mixture antibody solution that contains both anti-phospho-p38 MAPK (phospho Thr180/Tyr182) (D3F9) XP® Rabbit mAb (1:1000 dilution) (4511S, Cell Signaling Technology, Danvers, MA, USA) and anti-p38 MAPK (D13E1) XP® Rabbit mAb (1:1000 dilution) (8690S, Cell Signaling Technology, Danvers, MA, USA) [this mixture solution is called total p38 antibody solution]. Image Lab 4.1 software (Bio-Rad Laboratories Inc., Woodinville, WA, USA) was used to conduct the densitometry analysis. The expression of p-ERK1/2 is normalized by calculating the relative density of phosphorylated (p-
ERK1/2) to total (t-ERK1/2). Similarly, the relative density of phosphorylated (p-JNK) to total (t-JNK) and the relative density of phosphorylated (p-p38) to total (t-p38) were also calculated. The normalized expression is then compared across samples to detect the impact of CGA isomers on MAPK signaling.

4.2.7 Analysis of Nrf2 Binding Activity by Transactivation Assay

To study the role of different CGA isomers to activate Nrf2 signaling in IFNγ+PMA challenged Caco-2 cells, the binding efficacy of Nrf2 with the ARE consensus binding site was evaluated using a Nrf2 Transcription Factor Assay Kit (600590, Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, Caco-2 cells were first scrapped from plates into ice-cold PBS containing phosphatase inhibitors after receiving previous treatments described in Section 3.2.9. The cytosolic and nuclear fractions were extracted separately using a Nuclear Extraction Kit (10009277, Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer’s instructions. Purity of the nuclear fraction was confirmed as described in Section 4.2.5. The protein concentrations were determined using the bicinchoninic acid (BCA) reagent and all the samples were standardized to 2 mg/mL. An aliquot of the cellular protein extract, 10 µL (containing 20 µg of protein) was incubated with immobilized oligonucleotides containing the ARE consensus binding site (5’-GTCACAGTACTCAGCAGAATCTG-3’). The active form of Nrf2 that bound to the oligonucleotides was detected using anti-Nrf2 primary antibody (1:1000) for 1 hour, followed by incubation with a HRP-conjugated secondary antibody (1:1000) for 1 hour at room temperature. Absorbance values were recorded using a microplate reader set at 450 nm.
4.2.8 Western Blot to Analyze Nrf2

Caco-2 cells were collected with a rubber spatula into ice-cold PBS in the presence of phosphatase inhibitors after the treatment described in Section 3.2.9. Then, the cytosolic and nuclear fractions were extracted and separated with a Nuclear Extraction Kit (10009277, Cayman Chemical Company, Ann Arbor, MI, USA) by following the manufacturer’s instructions. Purity of the nuclear fraction was confirmed as described above. Similarly, protein concentrations were determined using the BCA method described above and protein standardized to 2 mg/mL. A 10 µL aliquot, containing 20 µg of protein, of cell nuclear protein extract were added to each lane and subjected to 8% SDS-PAGE using Mini-PROTEAN® II Multiscreen Apparatus (Bio-Rad Laboratories, Hercules, CA, USA). In this experiment, β-actin was a loading control protein which was used to mathematically compensate for sample-to-sample variation. The separated proteins were then transferred to nitrocellulose membranes (0.2 µm, 7×8.4 cm; Bio-Rad Laboratories, Hercules, CA, USA) and membranes were incubated with 3% BSA at 20°C for 1 h to block non-specific binding, before being incubated with anti-Nrf2 polyclonal primary antibody (1:1000 dilution) (ab31163, Abcam Inc., Ontario, Canada) or anti-β-actin primary antibody (1:2000 dilution) (Sigma, St, Louis, MO, USA) at 4°C overnight. Subsequently, the membranes were washed three times with TBST and then incubated with the secondary antibody, horse radish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1000 dilution) (Invitrogen, Carlsbad, CA, USA), for one hour. The membranes were then washed three times with TBST, reacted against a Western ECL substrate (Bio-Rad Laboratories Inc., Woodinville, WA, USA) for 3 min, and detected using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc., Woodinville, WA, USA). All western blotting images are a representative of three independent experiments. Image Lab 4.1 software (Bio-Rad Laboratories
Inc., Woodinville, WA, USA) was used to conduct the densitometry analysis. The predicted molecular size of Nrf2 was around 66 kDa (Moi et al., 1994) and Nrf2 migrates at ~95-110 kDa area in SDS-PAGE gel under denatured condition (Lau et al., 2013). The expression of Nrf2 is normalized by calculating the relative density of Nrf2 to β-actin. The normalized expression is then compared across samples to detect the impact of CGA isomers on Nrf2 signaling.

4.2.9 Nrf2 Nuclear Translocation Immunocytochemistry

Caco-2 cells were treated according to methods described in in Section 3.2.9. Cell samples were washed with PBS three times and then fixed with ice-cold 2% paraformaldehyde for 20 min. Cells were then treated with 0.1% Triton X-100 for 10 min and washed three more times with PBS. Cells were blocked with 3% BSA in PBS at room temperature for 30 minutes and then incubated with anti-Nrf2 polyclonal primary antibody (1:1000 dilution) (Abcam Inc., Ontario, Canada) at 4°C, overnight. After being washed with PBS four times, the cells were incubated with goat anti-rabbit FITC IgG (1:1000) (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour. After additional washed with PBS, the slide was mounted with fluorescence mounting medium containing 1 µg/ml of DAPI to label the nuclei. Immunofluorescent signals were visualized using a Zeiss fluorescence microscope (Zeiss Group, Oberkochen, Germany).

4.2.10 Real-time PCR Microarrays

Total RNA was isolated from Caco-2 cells using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Isolated RNA was treated using a RT1 First Strand Kit (Qiagen, Valencia, CA, USA) in order to remove the contaminate DNA. Then, RNA was transcribed to cDNA using an RT² Reaction Ready First Strand Synthesis Kit (Qiagen, Valencia, CA) and analyzed using the
custom build RT² Profiler™ PCR Array (Cat. no. CLAH23927, Qiagen, Valencia, CA, USA). The PCR array was designed to focus on gene families relevant to the oxidative stress responses. The details of the array are listed in the Appendix C. Each experiment comprised of three independent replicates of Caco-2 cells incubated with individual CGA isomers and challenged with IFNγ+PMA for 4 or 8 hours. Untreated cells served as the negative control, and the positive control was composed of cells incubated with PMA+IFNγ for the respective incubation periods. Reverse transcription, genomic DNA and positive PCR controls were incubated for each sample. PCR amplification proceeded as described using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Woodinville, WA, USA). cDNA was amplified using RT² SYBR Green qPCR Mastermix (Qiagen, Valencia, CA, USA). PCR thermal cycle conditions were as follows: 95 °C for 10 min, 40 cycles at 60 °C for 60 s and 95 °C for 15 s, with a melting curve from 60 °C to 95 °C to ensure amplification of a single product. Data were normalized using multiple housekeeping genes (β-actin, GAPDH (glyceraldehyde-3-phosphate dehydrogenase)), and analyzed by comparing $2^{(-\Delta\Delta Ct)}$ of the normalized sample. For interpretation of these results, a minimum of a two-fold increase, or decrease, of gene expression compared to the control was regarded as significant (Schena et al., 1996). The $p$-value for each fold change in expression was calculated using a Student’s paired t-test. Results were assumed to be statistically significant when $p < 0.05$.

4.2.11 Assessment of Intracellular GSH and GSSG Levels

Caco-2 cells in six-well plates were treated as described in Section 3.2.9, then washed three times with PBS. Then, 200 µL of buffer (50 mM phosphate buffer, pH 7, containing 1 mM EDTA) was added to each well and then cells were collected using a rubber spatula. Cells were
freeze-thawed three times between -80 °C and room temperature and deproteinized with an equal volume of 10% metaphosphoric acid. Cell lysates were incubated at room temperature for five minutes and then centrifuged at 10,000 × g for 10 minutes at 4 °C; the supernatant was recovered for GSH and GSSG analyses. GSH and total GSH plus GSSG levels were quantified using the GSH/GSSG Ratio Detection Assay Kit (Abcam, MA, USA), following the manufacturer’s instructions. The reduced glutathione was calculated by subtracting values derived from oxidized samples from those of total glutathione. The ratio of reduced to oxidized glutathione (GSH/GSSG) was calculated as an indicator of cellular redox status.

4.2.12 Effect of CGA Isomers on IL-8 Secretion in PMA+INFγ Challenged Cell

Differentiated Caco-2 cells were pre-incubated with medium containing individual CGA isomer (0.2, 1, and 2 mM) for 24 hours. The medium was replaced by followed by challenging with PMA (0.1 µg/mL)+IFNγ (8000U/mL) cocktail in the presence of fresh CGA isomer for another 24 h. Cells without CGA isomer treatment but exposed to a PMA+IFNγ challenge served as the control. The blank was Caco-2 cells with neither CGA isomer treatment nor the PMA+IFNγ challenge. The supernatant was collect at 24 h and the IL-8 level was quantified using the Human IL-8 Single Analyte ELISA kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. The cytokine evaluation of each sample was performed in triplicate.

4.2.13 Statistics

All experiments were performed in triplicate wells for each condition and repeated at least three times. Representative data are presented as mean ± standard deviation (SD). The data were analyzed by a one-way ANOVA using Graphpad Prism software (San Diego, CA, USA).
Significant differences were compared using Bonferroni post hoc tests with $p < 0.05$ representing a statistically significant difference.

4.3 Results and Discussion

4.3.1 CGA Isomers Up-regulated the NFκB Signaling Pathway in PMA+INFγ-challenged Caco-2 Cells

The effect of CGA isomers on NFκB signaling pathway in Caco-2 cells was examined at 1.5 h following the PMA+INFγ challenge. In a previous study, peak activation of NFκB following treatment of Caco-2 cells with the PMA+INFγ challenge occurred after 1.5 hours (Elisa & Kitts, 2015). This result was confirmed in the present study (Appendix E).

It is widely recognized that phenolic compounds have the potential to exert anti-inflammatory activity, by down-regulating NFκB signaling (Chauhan et al., 2011) (Hwang et al., 2014) (Zatorski et al., 2015). However, data herein shows that CGA isomers (at concentrations of 1 mM and 2 mM) were effective to significantly up-regulate NFκB subunit p65 nuclear translocation, by more than 1.5 times compared to the control (Figure 4.1). This occurred relatively early (1.5 h) after PMA+INFγ treatment. Some studies have shown that NFκB activity will increase after exposure to H₂O₂, which was linked to the activation of IκB kinase complex that leads to p65 nucleus translocation (Kamata et al., 2002). To interpret the present observation, it is likely that the effect of CGA isomers to up-regulate NFκB signaling are linked to an affinity to donate electron/electrons to free radicals were induced by the PMA+INFγ challenge. The CGA isomers thus becoming phenoxy radicals (ArO·), act as a modulator on NFκB signaling. Support for this explanation comes from the fact that antioxidant capacity of
plant phenolic is attributed an affinity to trap the chain-carrying peroxy radicals (ROO·), forming a hydroperoxide (ROOH) and a derived resonance-stabilized phenoxy radicals (ArO·) (Reaction 1) (Foti et al., 1994). The derived phenoxy radical has a capacity to react with another peroxy radical to form non-radical products, which terminates the reaction (Reaction 2) or alternatively reacts again to produce another peroxy radical (reaction 3) (Foti et al., 1994).

\[
\begin{align*}
\text{ROO}^\cdot + \text{ArOH} & \rightarrow \text{ROOH} + \text{ArO}^\cdot \quad (1) \\
\text{ROO}^\cdot + \text{ArO}^\cdot & \rightarrow \text{nonradical products} \\
\text{ArO}^\cdot + \text{RH} & \rightarrow \text{ArOH} + \text{R}^\cdot \rightarrow \text{ROO}^\cdot \quad (3)
\end{align*}
\]

Where: ROO· represents peroxy radicals; ROOH represents mono-substituted derivative of hydrogen peroxide; ArOH represents phenolic acid; ArO· represents phenoxy radicals; RH represents an oxidation site adjacent to a double bond in any unsaturated fatty acid; and R· represents free radical formed.

In the present study, following the uptake of CGA isomers by Caco-2 cells, the intracellular ROS induced by PMA+IFNγ challenge was reduced, likely due to the affinity of CGA isomers to donate electron/electrons thus quenching free radicals, such as hydroxyl (OH·), superoxide (O2·), nitric oxide (NO·), peroxy radicals (ROO·) and lipid peroxy (LOO·). These radicals individually or collectively contribute to the measurement of oxidative stress. To describe the antioxidant activity of CGA isomers, it is important to recognize that phenoxy radicals (ArO·) are formed in the reaction (Reaction 1). Previous researchers employing EPR technique demonstrated the existence of phenoxy CGA radicals by showing the EPR spectra of
both a primary and secondary CGA radical in a H$_2$O$_2$ peroxidase reaction (Yamasaki & Grace, 1998). In relative terms, the newly formed CGA phenoxy radical was not as active as other ROS species, such as OH$^·$, O$_2^·$, NO$^·$, ROO$^·$, and LOO$^·$, but could contribute to modifying redox biology. Thus, CGA radicals (ArO$^·$) being a transit, if not reduced will become a low level pro-oxidant and therefore promote NFκB activation, before reacting with peroxyl radicals (ROO$^·$) to form a non-radical product. This explanation supports why there was an increase in NFκB initially when CGAs were incubated with Caco-2 cells that had undergone an induced oxidative stress.

Figure 4.1 Effect of CGA isomers on p65 nucleus translocation (% of control) in PMA + INF$_γ$ induced Caco-2 cells (passage no. 34). Experiments were performed in triplicate and results were expressed as mean ± standard deviation. The significance of the differences between different treatment was analyzed by one-way analysis of variance (ANOVA), using Bonferroni post-test, using Graph Pad Prism software. Value of $p<0.05$ was considered to be statistically significant. Superscript with different alphabets (a, b, c, d, and e) are significantly different ($p < 0.05$).
4.3.2 The Effects of PMA+IFNγ Challenge in MAPK Signaling in Caco-2 cell.

MAPKs pathways are involved in regulation of gene expression of several proteins involved in inflammation. To further investigate the mechanisms underlying the anti-inflammatory effects of CGA isomers, western blot analysis was performed as a measurement of the activation of MAPKs. Timing is critical for studying cell signaling. It was unknown whether MAPKs were activated upon PMA+IFNγ challenge in Caco-2 cells and if so, to what extent and when were the optimal times to study the effect of CGA isomers on MAPKs signaling. Therefore, a time-course experiment was performed in differentiated Caco-2 cells in the presence of PMA+IFNγ that consisted of 0, 1, 2, 6, 8, and 24 h time points, used to monitor MAPK signaling.

The expression of different phosphorylated forms of MAPKs (p-ERK1/2, p-p38, and p-JNK) and total ERK1/2 (t-ERK), total p38 (t-p38), and total JNK (t-JNK) were analyzed by western blot. Data obtained were expressed as relative densities of p-ERK/t-ERK (Figure 4.2); p-p38/t-p38 (Figure 4.3); and p-JNK/t-JNK (Figure 4.4). The full gels are presented in Appendix F. The phosphorylation of ERK 1/2 was first observed at 1 hour following a PMA+IFNγ challenge and reached its maximum expression after 2 hours, before disappearing at 6 hours following the challenge. The observed effect on p38 phosphorylation began within the first hour, reached its maximum expression at 2 hours of PMA+IFNγ challenge, and gradually decreased thereafter 6 hours. The expression of p-JNK declined slightly during the first hour of the PMA+IFNγ challenge and reached its highest level at 2 h, before being maintained until 6 h. At 8 hours after challenge, the expression of p-JNK declined to the level observed in normal healthy cells.
MAPK cascades are activated by a family of dual-specificity kinases that phosphorylate MAPK cascade at specific Thr and Tyr amino acid residues. A broad range of extracellular stimuli, including cytokines, mitogens, growth factors, and environmental stressors, are known to activate the phosphorylation of MAPK cascades in differentiated Caco-2 cells. Activation of MAPKs is controlled via membrane-associated signaling complexes and involves a network that includes Ras proteins(s), the Raf family of serine kinases and MAPK kinases. Previous work has shown that phorbol 12-myristate 13-acetate (PMA) can activate the ERK1/2 and p38 phosphorylation in differentiated Caco-2 cells (Jiang & Fleet, 2012). Another study reported that INFγ also activate MAPK signaling in macrophage (Valledor et al., 2008). In the present study, it was demonstrated that the cocktail of PMA+IFNγ was effective at triggering the phosphorylation of ERK1/2, p38 and JNK cascades in differentiated Caco-2 cells. The next step of the study was to characterize how CGA isomers would influence oxidative stress and intertwined inflammation.
Figure 4.2 (a) Western Blotting analysis showing the time-dependent effects of PMA+IFNγ (inducer) on the expression of ERK1/2 (p=phosphorylated, t=total, ERK1 is 42 kDa, ERK2 is 44 kDa) in Caco-2 (passage no. 33). (b) Densitometry analysis determined from western blots. Phosphorylated (p-ERK1/2) relative to total (t-ERK1/2). Values represent mean ± SD (n=3). * represents significant difference compared to 0 h by student’s t test, p<0.05.
Figure 4.3 (a) Western Blotting analysis showing the time-dependent effects of PMA+IFNγ (inducer) on the expression of p38 (p=phosphorylated, t=total) in Caco-2 (passage no. 33). (b) Densitometry analysis determined from western blots. Phosphorylated (p-p38) relative to total (t-p38). Values represent mean ± SD (n=3). * represents significant difference compared to 0 h by student’s t test, p<0.05.
Figure 4.4 (a) Western Blotting analysis showing the time-dependent effects of PMA+IFNγ (inducer) on the expression of JNK (p=phosphorylated, t=total) in Caco-2 (passage no. 33). (b) Densitometry analysis determined from western blots. Phosphorylated (p-JNK) relative to total (t-JNK). Values represent mean ± SD (n=3). * represents significant difference compared to 0 h by student’s t test, p<0.05.

4.3.3 Modulation of MAPK Signaling by CGA isomers

The effect of individual CGA isomer on MAPK signaling was studied in PMA+IFNγ challenged Caco-2 cells. Based on the results obtained from the time course study, 2 h, 2h, and 6 h were chosen to evaluate the efficacy of CGA isomers on the phosphorylation of Erk1/2, p38, and JNK cascade, respectively. There were no significant differences from controls, observed for REK phosphorylation and JNK phosphorylation, when CGA isomers were administrated to Caco-2 cells. However, CGA isomer treatment did show a concentration dependent, and isomer
independent, decrease in p38 phosphorylation when expressed as p-p38/t-p38, compared with the control group (Figure 4.5). The full gels are presented in Appendix F. No significant impact on p38 phosphorylation was observed for all CGA isomers at 0.2 mM. Increasing the concentration to 1 mM, all resulted in the six CGA isomers having significantly ($p<0.05$) attenuated p38 phosphorylation by ~40% compared to the control group. No further decrease was observed by increasing CGA concentration to 2 mM. Moreover, no significant differences were observed for p-p38/t-p38 among the six individual CGA isomers when treated at the same concentration.

Solid evidence exists that the modulation of p38 plays a critical role in inflammatory responses (Hommes et al., 2002) (Li et al., 2006). A major function of p38 is to control the production of pro-inflammatory cytokines. In IBD patients, for example, the expression of p-p38 increases (Hommes et al., 2002). After the phosphorylation of p38, pro-inflammatory cytokines, such as interleukin 1β and TNF-α are raised which leads to a stimulation in pro-inflammatory cytokine production (Hommes et al., 2002). This will result in further p38 phosphorylation and as a result activate inflammatory responses (Li et al., 2006). Therefore, controlling p38 phosphorylation has been one effective strategy to control inflammation. The efficacy of the p38 inhibitory compounds on controlling the secretion of pro-inflammatory cytokines in IBD patients has been attempted. Docena et al (2010) showed that p38 inhibitory drugs could reduce pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) from lamina propria mononuclear cells and biopsies. The results presented in Figure 4.5 suggest that CGA isomers are potent inhibitors of p-p38, which could lead to suppression of cytokines that are involved in inflammation. To test this idea, further experiments were planned to determine the effect of different CGA isomers to attenuated IL-8 secretion in PMA+IFNy challenged Caco-2 cells.
Figure 4.5 (a) Representative Western Blotting gel showing the effects of different CGA isomers on the expression of p38 (p=phosphorylated, t=total) in Caco-2 (passage no. 34). The complete gels are presented in Appendix E.
Figure 4.5 (b) Densitometry analysis determined from western blots. Phosphorylated (p-p38) relative to total (t-p38). Values represent mean ± SD (n=3). The significance of the differences between different treatment was analyzed by one-way analysis of variance (ANOVA), using Bonferroni post-test, using Graph Pad Prism software. Superscript with different alphabets (a and b) are significantly different ($p < 0.05$).
4.3.4 CGA Isomers Up-regulated the Keap 1-Nrf2/ARE Signaling Pathway in IFNγ+PMA challenged Caco-2 cells

Since the Keap 1-Nrf2/ARE signaling pathway is essential for regulating antioxidant responses and modulating the expression for numerous antioxidant enzymes, including GCLC, GCLM, SOD1, GSS, and GSR, the effect of different CGA isomer treatments on Nrf2/ARE signaling pathway in PMA+IFNγ-challenged Caco-2 cells was examined. Under normal conditions, Nrf2 is present in the cytoplasm as an inactive complex with its repressor Keap1. Upon stimulation by inducers, such as ROS, Nrf2 dissociates from Keap 1 and translocates to the nucleus where it binds to antioxidant response elements present in the regulatory regions of phase II antioxidant defense genes. In order to study the effects of CGA isomers on the function of Nrf2 pathway, the amount of Nrf2 protein translocated to the nucleus was assessed using Western blot. The results shown in Figure 4.6 revealed that CGA isomers increase ARE binding activity in a concentration-dependent manner. At 2 mM, 3-CQA, 4-CQA and 5-CQA all increased the Nrf2 binding activity approximately 1.5 times compared to the control. The diCQAs, 3,4-diCQA, 4,5-diCQA, and 3,5-diCQA, respectively, increased the Nrf2 binding activity by around 2.4 times compared to the control. Western blot results presented in Figure 4.7 showed that 3-CQA, 4-CQA, and 5-CQA at 1 mM concentration significantly (p<0.05) increased the Nrf2 protein level in Caco-2 cells. In addition, 3,4-diCQA, 4,5-diCQA, and 3,5-diCQA at 2 mM concentration significantly (p<0.05) increased the Nrf2 protein level in Caco-2 cells. The nuclear translocation of Nrf2 induced by CGA isomers was confirmed using confocal microscopy (Figure 4.8). This finding is in agreement with the results of a recent study, where CGA was shown to protect against hydrogen peroxide-induced oxidative stress in MC3T3-E1 cells through the Nrf2 signaling pathway (Han et al., 2017). The emerging role of Nrf2 signaling
in oxidative stress-related pathologies makes it a novel therapeutic target for treating many chronic diseases. For example, mice induced by dextran sodium sulphate were used as an in vivo model of colitis and small Nrf2 activator called 3-(3-Pyridylmethylidene)-2-indolinone prevented the DSS-induced colitis via inducing Nrf2 activation (Wang et al., 2015). The result of the study herein shows that CGA isomers significantly up-regulated Nrf2 signaling, therefore being active to mitigate oxidative stress and inflammation at a molecular level.

Previous studies have reported that coffee rich in CGA effectively induced phase II-enzymes by activating Nrf2 signaling (Boettler et al., 2003) (Boettler et al., 2011). The coffee-dependent Nrf2-activation reported in these studies might not solely be contributed to CGA, but also could overlap with other bioactive constituents, such as browning products, trigonelline and catechol. There are no studies that have attempted to show anti-inflammatory activities attributed from CGA isomers in Caco-2 cells. Moreover, by exposing Caco-2 cells to a cytokine cocktail prior to CGA treatment, the results obtained are relevant to the affinity of CGA isomers to activate Nrf2 signaling in inflamed cells. The possible involvement of Nrf2 responding to inflammatory stimulus and how Nrf2 contributes to the anti-inflammatory process has been investigated by many laboratories. For instance, one group reported that Nrf2 in mice during carrageenan-induced pleurisy displayed persistent inflammatory responses with a characteristic that the recruitment of macrophages was delayed (Itoh et al., 2004). Another study found that inflammatory stimuli-activated endothelial expression of pro-inflammatory mediators (monocyte chemoattractant protein-1 and vascular cell adhesion protein) could be inhibited by the presence of antioxidant such as pyrrolidine dithiocarbamate (Marui et al., 1993). These findings together
show that Nrf2 signaling has an important role to mitigate inflammatory response while also increasing intracellular antioxidant capacity.

Figure 4.6 Effect of CGA isomers on Nrf2 nuclear translocation (% of Control) in PMA+IFNγ induced Caco-2 cells (passage no. 35). Experiments were performed in triplicate and results are expressed as mean ± standard deviation. The significance of the differences between different treatment was analyzed by one-way analysis of variance (ANOVA), using Bonferroni post-test, using Graph Pad Prism software. Superscript with different alphabets (a, b, c, and d) are significantly different (p < 0.05).
Figure 4.7 (a) Representative Western Blotting gel showing the effects of different CGA isomers on the expression of Nrf2 in Caco-2 (passage no. 35). β-actin served as loading control. The complete gels are presented in Appendix E.
Figure 4.7 (b) Densitometry analysis determined from western blots. Nrf2 relative to β-actin (control is set as 1 in order to compare the western blot results with the ELISA result of Nrf2 analysis). Experiments were performed in triplicate (n=3) and results were expressed as mean ± standard deviation. The significance of the differences between different treatment was analyzed by one-way analysis of variance (ANOVA), using Bonferroni post-test, using Graph Pad Prism software. Superscript with different alphabet (a, b, c, and d) are significantly different (p < 0.05).
Figure 4.8 Immunocytochemistry of cells showing different individual CGA isomers (a) 3-CQA, 5-CQA, and 4-CQA, and (b) 3,5-diCQA, 3,4-diCQA, and 4,5-diCQA at 0.2 mM enhance Nrf2 localization into nucleus in Caco-2 cells (passage no. 31). Localization of Nrf2 was performed by double immunofluorescence staining in cells with only PMA+IFNγ treatment, cells with individual CGA isomer treatment before PMA+IFNγ challenge. Background represents cells treated with PMA+IFNγ but without a primary antibody during the immunofluorescence staining. The control represents cells treated with PMA+IFNγ, and with both primary and secondary
antibodies during the immunofluorescence staining. Nrf2 protein were stained in green, nuclei were stained with DAPI (blue). The merged image showed the nuclear location of Nrf2 in nuclei.

4.3.5 Impact of CGA Isomers on Gene Expression Profiling

The toxicity of all the cytokine inducing treatments on Caco-2 cells was evaluated by MTT assay. The treatment conditions used in this study to induce biochemical changes in differentiated cells had no effect on the viability of Caco-2 cells.

In this experiment, the effect of a PMA+IFNγ challenge on the expression of 21 genes that are known to respond to oxidative stress in Caco-2 cells, was performed. In addition, the relative effectiveness of both 5-CQA and 3,5-diCQA to modulate the expression of these genes in Caco-2 cells challenged with PMA+IFNγ were studied, at 4 and 8 hours, respectively, Table 4.1 and Table 4.2. Treatment of Caco-2 cells with PMA+IFNγ for 4 hours produced a significant (p<0.05) up-regulation in three genes, namely Nrf2 nuclear transcription factor, kelch like ECH associated protein 1 (KEAP1) and nuclear factor erythroid 2 like 2 (NFE2L2). Corresponding to these changes, were the down-regulation of genes associated with phase II detoxification enzymes, such as glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), and superoxide dismutase 1 (SOD1) at 4 hours. This result confirms
that the challenge of Caco-2 cells with PMA+IFNγ was successful to trigger an increase in oxidative stress, which in turn initiated responses in cellular antioxidant defenses.

PMA+IFNγ treatment of Caco-2 cells also resulted in a 9.8-fold down-regulation of GCLC expression. The presence of 5-CQA in this treatment reduced the down-regulation to a level that was similar to healthy cells. In comparison, treatment of cells with 3,5-diCQA lead to a 5.9-fold up-regulation of GCLC. Various signaling pathways have been found to be involved in the regulation of both subunits of glutamate-cysteine ligase, the catalytic subunit (GCLC) and the modifier subunit (GCLM), of which Nrf2 is one of them (Zhang & Forman, 2012). Therefore, the data herein, shows that 5-CQA and 3,5-diCQA can effectively modify the expression of GCLM and GCLC genes in contrasting ways through the Nrf2 signaling pathway. Glutathione peroxidase (GPx), is a critical antioxidant enzyme family known to function to reduce organic hydroperoxides and hydrogen peroxide when expression of genes (GPX1, GPX2, and GPX4) are up-regulated. This experiment showed that only the expression of GPX2 was significantly (p<0.05) up-regulated by both 5-CQA and 3,5-diCQA. A similar result was published previously in which CGA increased the liver GPx activity in rats pre-treated with paraquat (Tsuchiya et al., 1996). Extending the CGA exposure time to eight hours following the PMA+IFNγ challenge, produced less profound changes in the up-regulation of KEAP1 and NFE2L2, compared to that observed at 4 h. The transcription of SOD1,
glutathione synthetase (GSS), and glutathione reductase (GSR) genes were also downregulated after 8 h of PMA+IFN\(\gamma\) challenge. This result coincided with the fact that no effect on the expression of GCLM gene was observed.

Glutathione reductase, an enzyme that is important to catalyze the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH) was also sensitive to CGA modification of cytokine induced changes. A reduced expression of GSR will cause a decrease in GSH and an increase in GSSG activities (Zhao et al., 2009).

Incubating Caco-2 cells with CGA isomers following the 8 hours of PMA+IFN\(\gamma\) challenge showed that 5-CQA and 3,5-diCQA both attenuated the down-regulation of GSR. 5-CQA and 3,5-diCQA also up-regulated nuclear factor erythroid 2-related factor 1 (NFE2L1) mRNA transcription by 4.46 and 9.09-fold, respectively. Many of these genes, specifically superoxide dismutase, glutathione synthetase, and glutathione reductase were altered in the presence of 5-CQA and 3,5-diCQA under the control of the transcription factor, Nrf2.
Table 4.1 Effects of 5-CQA and 3,5-diCQA at Concentration of 0.2 mM on Modulation of Transcription of Genes in Redox Signaling in PMA+INFγ treated Caco-2 Cells (passage no. 37) after 4 h. Fold regulation is expressed as relative to untreated control cells*.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Treatments</th>
<th>PMA+INFγ only</th>
<th>5-CQA and PMA+INFγ</th>
<th>3,5-diCQA and PMA+INFγ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fold regulation</td>
<td>p-Value</td>
<td>Fold regulation</td>
</tr>
<tr>
<td>GCLC</td>
<td></td>
<td>-9.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.002</td>
<td>-1.16&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>GCLM</td>
<td></td>
<td>-13.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001</td>
<td>1.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KEAP1</td>
<td></td>
<td>2.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.241×10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>5.84&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NFE2L2</td>
<td></td>
<td>2.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.004</td>
<td>3.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SOD1</td>
<td></td>
<td>-6.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.140×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>-2.48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>0.169</td>
<td>7.22&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td></td>
<td>1.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.104</td>
<td>5.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSS</td>
<td></td>
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<td>0.083</td>
<td>1.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GSR</td>
<td></td>
<td>-2.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.155</td>
<td>1.59&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*The p values are calculated based on Student’s t-test of the triplicate 2^ΔCt values for each gene in the control group (cells without anything) and treatment groups. Different letters indicate significant (p < 0.05) differences observed between treatment groups analyzed by one-way ANOVA followed by Bonferroni post-hoc analysis at p < 0.05 using GraphPad Prism. The full name and function of the genes in this table are listed in Appendix B.
Table 4.2 Effect of 5-CQA and 3,5-diCQA at Concentration of 0.2 mM on Modulation of Transcription of Genes in Redox Signaling in PMA+INFγ treated Caco-2 Cells (passage no. 37) after 8 h. Fold regulation is expressed as relative to untreated cells.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Treatment</th>
<th>PMA+INFγ only</th>
<th>5-CQA and PMA+INFγ</th>
<th>3,5-diCQA and PMA+INFγ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold regulation</td>
<td>p-Value</td>
<td>Fold regulation</td>
<td>p-Value</td>
</tr>
<tr>
<td>GCLC</td>
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<td>0.041</td>
<td>1.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.364</td>
</tr>
<tr>
<td>GCLM</td>
<td>1.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.141</td>
<td>2.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.101</td>
</tr>
<tr>
<td>KEAP1</td>
<td>2.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.003</td>
<td>7.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>3.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.031</td>
<td>4.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.003</td>
</tr>
<tr>
<td>SOD1</td>
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<td>0.120×10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>-2.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.445×10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPX2</td>
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<td>0.142</td>
<td>8.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.039</td>
</tr>
<tr>
<td>NFE2L1</td>
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<td>0.129</td>
<td>4.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.024</td>
</tr>
<tr>
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<td>1.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.665</td>
</tr>
<tr>
<td>GSR</td>
<td>-2.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.034</td>
<td>1.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.370</td>
</tr>
</tbody>
</table>

*The p values are calculated based on Student’s t-test of the triplicate 2^(-ΔCt) values for each gene in the control group (cells without anything) and treatment groups. Different letters indicate significant (p < 0.05) differences observed between treatment groups analyzed by one-way ANOVA followed by Bonferroni post-hoc analysis at p < 0.05 using GraphPad Prism. The full name and function of the genes in this table are listed in Appendix B.*
4.3.6 CGA Isomers Alleviated GSH to GSSG Ratio in PMA+INFγ challenged Caco-2 Cells

GSH is regarded as an important cellular antioxidant for its role in glutathione enzymes and its overall reducing activity. GSH is oxidized to glutathione disulfide (GSSG), which can be regenerated back to GSH by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent GSH reductase. Preserving the GSH to GSSG ratio is important for maintaining oxidative status and inflammation. The later has been shown to be associated with glutathione depletion in the colon (Sido et al., 1998). The gene expression data in Table 4.2 shows that the expression of GSR is down-regulated after eight hours of PMA+IFNγ challenge. To examine the hypothesis that the level of oxidative stress can be elevated in Caco-2 cells when treated with the PMA+IFNγ cocktail, both the content of reduced glutathione (GSH) and total glutathione (GSH+GSSG) were determined and expressed as a GSH/GSSG ratio (Figure 4.9). In untreated cells, the GSH/GSSG ratio was 101.0±9.0, compared to 60.5±4 in cells that were challenged with PMA+IFNγ. This effect represented an approximately 40% decrease in GSH/GSSG ratio ($p<0.05$). Since the GSH/GSSG ratio is an indicator of cellular redox status, this result indicates that the PMA+IFNγ treatment induced oxidative stress in Caco-2 cells. In a second experiment, the relative efficacy of different CGA isomers to return the GSH/GSSG ratio back to normal levels in cells treated with PMA+IFNγ cocktail was performed. Results indicate that 3-CQA, 4-CQA, and 5-CQA, at low concentration (0.2 mM) produced no significant effect on the GSH/GSSG ratio, when compared to the control (Figure 4.9). However, 3,5-diCQA, 4,5-diCQA, and 3,4-diCQA present at the same concentration were equally effective at returning the GSH/GSSG ratio back to normal levels. Increasing the concentration of CGA further to 1 mM for 3,5-diCQA, 4,5-diCQA, and 3,4-diCQA was also sufficient to return the GSH/GSSG ratio
back to normal healthy levels. This results also suggest that dicaffeoylquinic acids have greater potency than caffeoylquinic acids to restore glutathione related antioxidant statues.

Figure 4.9 The effect of CGA isomers at different concentrations (0.2, 1, and 2 mM) on GSH/GSSG ratio in IFNγ+PMA challenged Caco-2 cells (passage no. 31). Experiments were performed in triplicate (n=3) and results were expressed as mean ± standard deviation. The significance of the differences between different treatment was analyzed by one-way analysis of variance (ANOVA), using Bonferroni post-test, using Graph Pad Prism software. Superscript with different alphabets (a, b, and c) are significantly different (p < 0.05).
4.3.7 Impact of CGA Isomers on IL-8 Secretion in PMA+INFγ-challenged Caco-2 Cells

To gain insight on the effect of these CGA isomers to mitigate the inflammatory response in Caco-2 cells, additional experiments were designed to expose cells with different concentrations of individual CGA isomers for 24 h in the presence of PMA+IFNγ challenge. The 24 h time point was chosen as the study point because the level of IL-8 in the medium reached the highest level after 24 h of PMA+IFNγ challenge. This result was originally reported by Chen & Kitts (2008) and confirmed in this thesis (data are shown in Appendix D).

All six CGA isomers significantly ($p<0.05$) reduced the secretion of IL-8 level in PMA+IFNγ challenged Caco-2 cells (Figure 4.10). Increasing the concentration of 3-CQA from 0.2 mM to 2 mM, resulted in a IL-8 secretion that was 82% and 50% of PMA+IFNγ challenge control cells, respectively. Repeating the experiments with 3,5-diCQA, 3,4-diCQA, and 4,5-diCQA at 2 mM showed that more than 90% of the IL-8 secretion was inhibited, compared to controls. This result demonstrates that the anti-inflammatory activity of CGA isomer occurs in a concentration-dependent manner for all CGA isomers. Moreover, dicaffeoylquinic acids had a significantly higher ($p<0.05$) anti-inflammatory activity compared to caffeoylquinic acids at concentrations of 1 mM and 2 mM, respectively. This finding supports other studies, where the anti-inflammatory effect of 5-CQA on TNF-α and H2O2 induced Caco-2 cells was reported over a concentration range of 0.5 to 2 mM (Shin et al., 2015). Our results confirm and extend the knowledge that an anti-inflammatory activity for different CGA isomers exists with differentiated Caco-2 cells. The finding also suggests that oxidative stress and inflammation are closely related physiological processes, one of which can be easily induced by the others. If oxidative stress appears as the primary abnormality in an organ, inflammation will eventually
develop and will further accentuate oxidative stress. Conversely, if inflammation is the primary event, oxidative stress will develop as a consequence which will further exaggerates inflammation.

Figure 4.10 The effect of CGA isomers at different concentrations (0.2, 1, and 2 mM) on IL-8 secretion in PMA+ IFNγ challenged Caco-2 cells (passage no. 27) at 24 h time point. Experiments were performed in triplicate and results were expressed as mean ± standard deviation. The significance of the differences between different treatment was analyzed by one-way analysis of variance (ANOVA), using Bonferroni post-test, using Graph Pad Prism software. Superscript with different alphabets (a, b, c, d, e, and f) are significantly different ($p < 0.05$).
4.4 Conclusion

This chapter investigated three possible underlying mechanisms that describe protective effects of CGA isomers on oxidative stress and inflammation in a relevant mammalian intestinal cell model. CGA isomers played different roles in regulating the redox biology and also inflammatory responses at different stages of multiple cell signalling pathways in differentiated Caco-2 cells. CGA isomers attenuated oxidative stress and inflammation by up-regulating NFκB signaling at very early stage of response, which was followed by down-regulating p38 phosphorylation (at 2 h). Subsequently, up-regulation of the expression of antioxidant genes at 4 and 8 h preceded the activation of nuclear translocation and ARE binding activity of Nrf2 at 24 h. The alleviated oxidative stress and associated changes in inflammation corresponded to an increased GSH/GSSG ratio and decreased IL-8 level.
Chapter 5: General Discussion and Conclusions

5.1 Major Findings

5.1.1 Dietary CGA Composition in Coffee

Coffee is the richest source of CGA in human diet. Although 5-CQA is the most abundant form of CGA, other isomers such as 4-CQA, 3-CQA, 3,5-diCQA, 4,5-diCQA, and 3,4-diCQA are also present in significant amounts. Roasting parameters typical of coffee processing influenced the final CGA composition of coffee beans. With increased roasting of the coffee beans, a notable decrease in the total CGA content occurred; this in part due to the fact that CGA is partially transformed to coffee melanoidins and other flavor compounds. For example, the total CGA content in coffee brew extracts of light-, medium- and dark-roasted Sumatran beans decreased to 35.60%, 62.91%, and 80.60%, respectively, compared with the original CGA content in green beans. It is worthwhile to point out that 3-CQA and 4-CQA contents were higher in brews prepared from light-roasted, compared to those prepared from green beans. This result was also observed by other workers who attributed it to acyl migration and formation of an ortho-ester intermediate (Deshpande et al., 2014) (Moon et al., 2009). The content of CGA isomers in coffee brew prepared from roasted beans decreased in the order of 5-CQA > 4-CQA > 3-CQA > 3,4-diCQA > 4,5-diCQA > 3,5-diCQA. This result was comparable mostly to previous studies where the relative isomer content in commercially blended and brewed coffee were studied (Fujioka & Shibamoto, 2008).

5.1.2 CGA as a Contributor of Coffee Antioxidant Activity

Underlying mechanisms of action between coffee components with antioxidant activity toward free radicals that exist due to common cellular metabolic activity will depend on
differences in the molecular structures specific to antioxidant components and the chemical characteristics of the free radical under investigation, not to mention also the assay system used to quantify antioxidant activity. In general, free radicals vary in terms of relative affinity to interact with atoms that have the highest electron density. The peroxyl radical used for ORAC testing interacts with CGA at hydroxyl groups attached to the carbon atom with the largest electronic density present in the benzene ring. This is different to the ABTS radical, for which the interaction with CGA depends on the degree of ionization with carboxyl and phenolic hydroxyl groups. The results herein showed that CGA isomers present in green coffee beans react to Frémy’s salt, an inorganic stable radical, to a degree that is specific to the isomer. On the contrary, MRP generated partly from CGA transformation during roasting had stronger reactivity with the TEMPO radical, compared with Frémy’s salt. Thus, the complex chemical composition of coffee brew being the basis for different atoms each possessing distinct electron densities, will ultimately govern the overall response depending on different chemical antioxidant assay measurements used to evaluate activity. Factors that include the variety, or source of the coffee bean, exact roasting conditions, and brewing times and procedures collectively influenced the final composition of the coffee brew. These factors in turn were found to react differently with specific radicals used in specific chemical assays. This finding supports the research of other investigation using different assays (Sacchetti et al., 2009) (Vignoli et al., 2011).

5.1.3 Cellular Uptake of CGA Isomers by Caco-2 Cells

Results reported in this thesis also indicated that it is not only the molecule structure of bioactive compounds, but also their isomeric configuration that can affect their absorption. For example, the cis-isomer of lycopene has greater absorption are than the all-trans isomer form
because of the greater solubility of cis-isomers in mixed micelles in crossing the intestinal tract (Boileau et al., 2002). There is a paucity of knowledge on the bioavailability, absorption and metabolism of CGA isomers in humans, and it is likely that different CGA isomers have different pharmacokinetic properties. In human tissues, there are no esterases available to release caffeic acid from chlorogenic acid (Plumb et al., 1999). In the cell culture experiments performed in this thesis, it was important to verify if CGAs were indeed bioavailable for intracellular metabolism, thus transported from culture medium to intracellular space. The presence of CGA recovered from Caco-2 cells following 24 h of incubation was confirmed using TOF-SIMS. This analysis showed that CGA isomers are taken up by Caco-2 cells, however, the extent of uptake for each CGA isomer was very limited and could not be quantified by standard HPLC method used in this work. Never the less, the availability of all CGA isomers for Caco-2 cells metabolism was confirmed.

5.1.4 Structure-function Relationship of CGA isomers

The free radical scavenging capacity of CGA isomers depends on the availability of phenolic hydrogens and the possibility of stabilization of the resulting phenoxy radicals that are formed after hydrogen donation (Mathiesen et al., 1997) (Rice-Evans et al., 1996). Caffeoylquinic acid contains one cinnamic acid, whereas dicaffeoylquinic acid contains two, indicating that dicaffeoylquinic acid contains more phenolic hydrogens. The presence of the double bond (–CH=CH-COOR) groups linked to the phenyl ring has an important role in stabilizing the radical by resonance. This structural characteristic explains the relatively stronger ROS scavenging capacity of dicaffeoylquinic acid compared to caffeoylquinic acid.
In some cases, the evidence of antioxidant properties due to a specific chemical structure could easily open the avenue for a biased, or over-simplified approach, used to the understand of the role and functions of molecules like CGA in human health and disease (Virgili and Marino, 2008). But the fact is that, in the biological system, the structure-function relationship become more complicated. Many plant-derived phytochemicals with diverse structures have been shown to be efficacious in reducing oxidative stress and inflammation. Many of these phytochemicals have a striking propensity to modulate a series of cell signaling events that controls cellular homeostats by affecting the structure of receptors or modulating transcription factors. For example, two critical cysteine residues present in Keap1, C273 and C288, are required for Keap1-dependent ubiquitination of Nrf2 (Dinkova-Kostova et al., 2002). The well-characterized chemical inducer, sulforaphane, present in broccoli is capable of modifying Keap1 cysteines and lead to dissociation of Keap1-Nrf2 interaction (Hong et al., 2005). This suggests that the mechanism by which CGA isomers up-regulate the Nrf2 nuclear localization is unlikely to be only related to free radical scavenging capacity. Instead, a more complex transduction of signaling cascade events triggered by CGA isomers from membrane to nucleus might be the underlying mechanism of action.

5.1.5 Antioxidant and Anti-inflammatory Activities of CGA Isomers

This is the very first *in vitro* study that comprehensively investigated the potential of different CGA isomers in protecting against oxidative stress and inflammation. Results showed that CGA isomers reduced ROS generation, attenuated GSH/GSSG decline, and mitigated IL-8 secretion in PMA+IFNγ challenged Caco-2 cell line. Transcriptional responses concerning oxidative and inflammatory defense, NFκB, MAPK, Nrf2, along with 21 genes that are related to
these signal mediators, were investigated at different time points to elucidate the possible underlying molecular mechanisms of action.

Timing for identifying activity of specific cell signaling mediators induced by the cytokine inducer was an important initial component of this thesis. Before studying the effect of CGA isomers on modulating oxidative and inflammatory responses, much effort was given to characterize the relative time courses of NFκB nucleus translocation, MAPK cascade phosphorylation, and Nrf2 nucleus translocation upon a PMA+IFNγ challenge. The data showed that optimal time points for studying the effect of CGA isomers on NFκB, MAPK, Nrf2 signaling were 1.5 h, 2h ~ 6h, and 24 h, respectively. Hence, the subsequent strategy to study the effect if CGA isomers to modulate specific cell signaling pathways was examined in four different time phases described as: (1) initiation phase (Figure 5.1), (2) early phase (Figure 5.2), (3) intermediate phase (Figure 5.3), and (4) late phase (Figure 5.4).

During the initiation phase of inflammation, the 21-day old, differentiated, Caco-2 cells is in basal state, and p65 is localized in the cytoplasm by its inhibitor IκB protein. Nrf2 during this stage is also localized in the cytoplasm by its inhibitor Keap1 protein. ERK1/2, p38, and JNK cascades are also not phosphorylated by Raf and MAPKK (Figure 5.1).

During the early phase (0 ~ 1.5 h), the control group receiving a PMA+IFNγ challenge produced the required extracellular signal needed to transmit to Raf and MAPKK, which in turn resulted in triggering the phosphorylation of ERK1/2, p38, and JNK. Meanwhile, p65 was dissociated from IκB and translocated to the nucleus; this resulting in the secretion of IL-8
cytokine. Accompanied with the inflammation, ROS were also generated during this phase. When Caco-2 cells were treated with a CGA isomer, a reduction in ROS was observed; which likely corresponded to the formation of a CGA radical (or phenoxy radical). Phenoxy radicals derived from CGA are less reactive and interact with other peroxyl radicals, as illustrated in reaction (2) in section 4.3.3. During the time gap between the formation and disappearance of CGA phenoxy radical, a signal for up-regulation of NFκB to promote changes in redox biology and inflammation occurred (see proposed mechanism in Figure 5.2).

Responses from control Caco-2 cells treated with the PMA+IFNγ challenge during the intermediate phase (1.5 ~ 8 h) showed no change in NFκB activity. During this intermediate phase (1.5 ~ 8 h), the phosphorylation of ERK1/2, p38, and JNK, however, became more dominant and hence produced a detectable response. Oxidative stress and inflammation in Caco-2 cells treated with the PMA+IFNγ challenge increased as evidenced by the elevation in ROS generation and increase in IL-8. The exposure Caco-2 cells to CGA isomers enabled CGA isomer uptake, which was related to significant down-regulation of the phosphorylation of p38. This signal may have partially contributed to attenuating IL-8 secretion at a later stage. It was noted that CGA isomers significantly up-regulate genes encoding for antioxidant enzymes (SOD, CAT, GPX2, and GSS) during the intermediate phase; thus, contributing in part to the decrease in the intracellular ROS level observed at a later stage (see proposed mechanism shown in Figure 5.3).

Cellular responses by Caco-2 cells occurring during the late phase (8 ~ 24 h), following the PMA+IFNγ challenge, underwent a reduction in the phosphorylation of ERK1/2, p38, and
JNK, thesis suggesting that these events were no longer in play. However, Caco-2 cells exhibited a reduction in the GSH/GSSG ratio attributed to PMA+IFNγ challenge during this time. Corresponding to this event was the activation of Nrf2 to reduce oxidative stress. In cells treated with CGA isomers, upregulation of Nrf2 signaling occurred and this corresponded to events that regulated the oxidized glutathione (GSSG) back to reduced form of glutathione (GSH), resulting in a recovery of the GSH/GSSG ratio occurred. CGA isomers were also shown to gradually alleviate oxidative stress and inflammation as characterized by reduced intracellular ROS level and decreased IL-8 secretion; both of which occurred during the late phase (shown in Figure 5.4).
Figure 5.1 Starting point (0 h) of event in (A) the control group: PMA+IFNγ challenged Caco-2 cells and (B) in the treatment group: pre-incubated with individual CGA isomer for 24 h, and then challenged with PMA+IFNγ in the presence of fresh CGA isomer.
Figure 5.2 Early stage (0 ~ 1.5) of event in (A) the control group: PMA+IFNγ challenged Caco-2 cells and (B) in the treatment group: pre-incubated with individual CGA isomer for 24 h, and then challenged with PMA+IFNγ in the presence of fresh CGA isomer. Noted change: CGA isomers scavenged intracellular ROS and the newly formed CGA radicals possibly up-regulated NFκB signaling.
Figure 5.3 Intermediate stage (1.5 ~ 8h) of event in (A) the control group: PMA+IFN$_\gamma$ challenged Caco-2 cells and (B) in the treatment group: pre-incubated with individual CGA isomer for 24 h, and then challenged with PMA+IFN$_\gamma$ in the presence of fresh CGA isomer. Noted change: CGA isomers decreased p38 phosphorylation, increased expression of multi genes (SOD, CAT, GPX2, and GSS).
Figure 5.4 Late stage (8 ~ 24h) of event in (A) the control group: PMA+IFNγ challenged Caco-2 cells and (B) in the treatment group: pre-incubated with individual CGA isomer for 24 h, and then challenged with PMA+IFNγ in the presence of fresh CGA isomer. Noted change: CGA reduced intracellular ROS, up-regulated Nrf2 signaling, increased GSH/GSSG, and decreased IL-8 secretion.
5.2 Final Conclusion

5-CQA is the dominant CGA isomer in coffee, and along with five other CGA isomers (3-CQA, 4-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA), represent a major component of total CGAs. All six CGA isomers exhibited antioxidant activity and contributed to this finding observed in coffee. Structural differences in six CGA isomers were found to correspond to differences in antioxidant activity, with dicaffeoylquinic acids (3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) have relatively stronger capacities to quench free radicals compared to caffeoylquinic acids (3-CQA, 4-CQA, and 5-CQA). Studies conducted in cell-based model using Caco-2 cells showed CGA specific affects in modulating redox biology and inflammatory responses. The results collectively demonstrated that CGA isomers attenuate oxidative stress and inflammation in Caco-2 cells by triggering changes in redox biology parameters, lead to an up-regulation of NFκB signaling at very early stages (at 1.5 h) of response, followed by a down-regulation of p38 phosphorylation, up-regulation of antioxidant genes (at 4 and 8 h), and activation of nuclear translocation and ARE binding activity of Nrf2 (at 24 h). The alleviated oxidative stress (increased GSH/GSSG) observed accompanied reduced inflammation (decreased IL-8 level) at 24 h. NFκB, MAPK, and Nrf2 signaling pathways are integrated to resolve the oxidative stress and inflammation. It is likely that other signaling pathways are also involved in these responses.

5.3 Significance and limitation

The findings from this thesis provide some fundamental knowledge on the bioactivity of different CGA isomers present in many natural health products and foods. The findings from this thesis are also useful to develop future animal and clinical studies that will confirm a role for CGA isomers in controlling oxidative stress and intestinal inflammation. The limitation of this
study is that it only tested the effect of CGA isomers on an in vitro cell model of human intestinal inflammation. Hence, the findings in this in vitro model raise the possibility of using mouse models to study the effects of different CGA isomers on intestinal inflammation.
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Appendices

Appendix A  MTT Results

Figure A.1 The effect of green, light, medium, and dark roasted coffee prepared from beans from Dominican, Peru, Sumatra, PNG, and Ethiopia at concentration of 0.5 mg/mL (A) and 1 mg/mL (B) on cell viability (expressed as % of control) of differentiated Caco-2 cells.
Figure A.2 The effect of incubating individual CGA isomer at different concentrations (0.2, 1, and 2 mM) for 24 h on cell viability (expressed as % of control) of PMA+INFγ challenged Caco-2 cells.
Appendix B  TOF-SIMS Negative Ion Mass Spectra

3-CQA Standard

Caco-2 cells treated with 3-CQA

Caco-2 cells only
Figure Appendix B.1 TOF-SIMS negative ion mass spectra of 3-CQA standard methanol solution and Caco-2 cell methanol extract deposited on a silicon plate. Each figure contains three spectra. The first one is the spectrum of authentic 3-CQA standard, the second one is the spectrum of the methanol extract of Caco-2 cells treated with 3-CQA, the third one is the spectrum of the methanol extract of Caco-2 cells only.
Figure Appendix B.2 TOF-SIMS negative ion mass spectra of 4-CQA standard methanol solution and Caco-2 cell methanol extract deposited on a silicon plate. Each figure contains three spectra. The first one is the spectrum of authentic 4-CQA standard, the second one is the spectrum of the methanol extract of Caco-2 cells treated with 4-CQA, the third one is the spectrum of the methanol extract of Caco-2 cells only.
Figure Appendix B.3 TOF-SIMS negative ion mass spectra (300 ~ 350 m/z range) of 3,4-diCQA standard methanol solution and Caco-2 cell methanol extract deposited on a silicon plate. Each figure contains three spectra. The first one is the spectrum of authentic 3,4-diCQA standard, the second one is the spectrum of the methanol extract of Caco-2 cells treated with 3,4-diCQA, the third one is the spectrum of the methanol extract of Caco-2 cells only.
Figure Appendix B.4 TOF-SIMS negative ion mass spectra (450 ~ 500 m/z range) of 3,4-diCQA standard methanol solution and Caco-2 cell methanol extract deposited on a silicon plate. Each figure contains three spectra. The first one is the spectrum of authentic 3,4-diCQA standard, the second one is the spectrum of the methanol extract of Caco-2 cells treated with 3,4-diCQA, the third one is the spectrum of the methanol extract of Caco-2 cells only.
Figure Appendix B.5 TOF-SIMS negative ion mass spectra (150 ~ 200 m/z range) of 4,5-diCQA standard methanol solution and Caco-2 cell methanol extract deposited on a silicon plate. Each figure contains three spectra. The first one is the spectrum of authentic 4,5-diCQA standard, the second one is the spectrum of the methanol extract of Caco-2 cells treated with 4,5-diCQA, the third one is the spectrum of the methanol extract of Caco-2 cells only.
Figure Appendix B.6 TOF-SIMS negative ion mass spectra (450 ~ 500 m/z range) of 4,5-diCQA standard methanol solution and Caco-2 cell methanol extract deposited on a silicon plate. Each figure contains three spectra. The first one is the spectrum of authentic 4,5-diCQA standard, the second one is the spectrum of the methanol extract of Caco-2 cells treated with 4,5-CQA, the third one is the spectrum of the methanol extract of Caco-2 cells only.
### Appendix C  Information of the Genes Included in the Custom Real Time PCR Array

**Appendix Table C: List of Genes Included in Custom Real Time PCR Array**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
<th>Functions of encode protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMOX1</td>
<td>Heme oxygenase 1</td>
<td>Catalyzes the degradation of heme</td>
</tr>
<tr>
<td>GCLC</td>
<td>glutamate-cysteine ligase catalytic subunit</td>
<td>first rate-limiting enzyme of glutathione synthesis</td>
</tr>
<tr>
<td>GCLM</td>
<td>glutamate-cysteine ligase modifier subunit</td>
<td>the first rate limiting enzyme of glutathione synthesis</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H quinone dehydrogenase 1</td>
<td>prevents the one electron reduction of quinones that results in the production of radical species</td>
</tr>
<tr>
<td>KEAP1</td>
<td>kelch like ECH associated protein 1</td>
<td>Interact with Nrf2 and is important for the amelioration of oxidative stress</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>nuclear factor, erythroid 2 like 2</td>
<td>A basic leucine zipper protein that regulates the expression of antioxidant proteins that protect against oxidative damage</td>
</tr>
<tr>
<td>SOD1</td>
<td>superoxide dismutase 1</td>
<td>An enzyme that catalyzes the dismutation of the superoxide radical into oxygen or hydrogen peroxide.</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
<td>An enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen.</td>
</tr>
<tr>
<td>GPX1</td>
<td>glutathione peroxidase 1</td>
<td>A member of the glutathione peroxidases family that reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water.</td>
</tr>
<tr>
<td>GPX2</td>
<td>glutathione peroxidase 2</td>
<td>A member of the glutathione peroxidases family that reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water.</td>
</tr>
<tr>
<td>GPX4</td>
<td>glutathione peroxidase 4</td>
<td>A member of the glutathione peroxidases family that reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water.</td>
</tr>
<tr>
<td>NFE2</td>
<td>Transcription factor NF-E2 45 kDa subunit</td>
<td>Regulates the expression of antioxidant proteins that protect against oxidative damage</td>
</tr>
<tr>
<td>NFE2L1</td>
<td>Nuclear factor erythroid 2-related factor 1</td>
<td>Regulates the expression of antioxidant proteins that protect against oxidative damage</td>
</tr>
<tr>
<td>OXSR1</td>
<td>Oxidative stress responsive 1</td>
<td>Oxidative stress responsive</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Function</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>cytochrome P450 family 1 subfamily A member 1</td>
<td>Involved in phase I xenobiotic and drug metabolism.</td>
</tr>
<tr>
<td>GSTA1</td>
<td>glutathione S-transferase alpha 1</td>
<td>Add glutathione to target electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins, and products of oxidative stress.</td>
</tr>
<tr>
<td>GSTK1</td>
<td>glutathione S-transferase kappa 1</td>
<td>Catalyzes the conjugation of glutathione to a wide range of hydrophobic substrates facilitating the removal of these compounds from cells</td>
</tr>
<tr>
<td>GSTO1</td>
<td>glutathione S-transferase omega 1</td>
<td>Glutathione-dependent thiol transferase and dehydroascorbate reductase activities.</td>
</tr>
<tr>
<td>GSS</td>
<td>Glutathione synthetase</td>
<td>Glutathione biosynthesis</td>
</tr>
<tr>
<td>GSR</td>
<td>Glutathione reductase</td>
<td>Reduction of GSSG to GSH</td>
</tr>
<tr>
<td>ACTB</td>
<td>Beta-actin</td>
<td>Housekeeping gene</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>Housekeeping gene</td>
</tr>
</tbody>
</table>
Appendix D  Time-dependent IL-8 Secretion upon PMA+INFγ-challenge in Caco-2 Cells

Appendix Figure D.1. IL-8 level secreted by Caco-2 cells after being treated by PMA+INFγ for 0, 1, 2, 4, 8, and 24 hours. Data were expressed as mean ± Standard Deviation.
Appendix E  Time-dependent Activation of NFκB Signaling upon PMA+INFγ-challenge in Caco-2 Cells

Appendix Figure E.1. Time Course Activation of NFκB Signaling by PMA+INF in Caco-2 cells. Caco-2 cells (4.5 × 10^5 cells/mL) were seeded in six well plates and differentiated for 21 days. Cells were stimulated with PMA+INFγ for various incubation times and then nuclear protein was extracted as described in Chapter 5. P65 binding capacity was determined using Trans-Am ELISA kits as described in Chapter 5. * denotes significant difference at p<0.05 vs. time zero. Results are expressed as mean ± standard deviation (n=3).
Appendix F  Full Image of Representative Western Blot Gels

(1) Lane 1  2  3  4  5  6

Appendix Figure F.1 Western blot to exam the time-dependent effects of PMA+IFNγ on the expression of p-ERK1/2 in Caco-2 cell. Caco-2 cells were collected after PMA+IFNγ challenge at 0 (lane 1), 1(lane 2), 2 (lane 3), 6 (lane 4), 8 (lane 5) and 24 (lane 6) hours.
Appendix Figure F.2 Western blot to examine the time-dependent effects of PMA+IFNγ on expression of t-ERK1/2 in Caco-2 cell. Caco-2 cells were collected after PMA+IFNγ challenge at 0 (lane 1), 1 (lane 2), 2 (lane 3), 6 (lane 4), 8 (lane 5) and 24 (lane 6) hours.
Appendix Figure F.3 Western blot to exam the time-dependent effects of PMA+IFNγ on the expression of p38 in Caco-2 cell. Caco-2 cells were collected after PMA+IFNγ challenge at 0 (lane 2), 1(lane 3), 2 (lane 4), 6 (lane 5), 8 (lane 6) and 24 (lane 7) hours. Lane 1 is the protein ladder marker.
Appendix Figure F.4 Western blot to exam the time-dependent effects of PMA+IFNγ on expression of t-p38 in Caco-2 cell. Caco-2 cells were collected after PMA+IFNγ challenge at 0 (lane 1), 1 (lane 2), 2 (lane 3), 6 (lane 4), 8 (lane 5) and 24 (lane 6) hours.
Appendix Figure F.5 Western blot to examine the time-dependent effects of PMA+IFNγ on the expression of p-JNK in Caco-2 cells. Caco-2 cells were collected after PMA+IFNγ challenge at 0 (lane 2), 1 (lane 3), 2 (lane 4), 6 (lane 5), 8 (lane 6) and 24 (lane 7) hours. Lane 1 is the protein ladder marker.
Appendix Figure F.6 Western blot to exam the time-dependent effects of PMA+IFNγ on expression of t-JNK in Caco-2 cell. Caco-2 cells were collected after PMA+IFNγ challenge at 0 (lane 1), 1(lane 2), 2 (lane 3), 6 (lane 4), 8 (lane 5) and 24 (lane 6) hours.
Appendix Figure F.7 Western blot to exam the effect of CGA isomers on the expression of p-p38 in PMA+IFNγ challenged Caco-2 cells.
Lane 1: cells treated without CGA isomer and without PMA+IFNγ challenge
Lane 2: cells treated without CGA isomer and with PMA+IFNγ challenge
Lane 3: cells treated with 3-CQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 4: cells treated with 3-CQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 5: cells treated with 3-CQA at concentration of 2 mM and with PMA+IFNγ challenge
Lane 6: cells treated with 5-CQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 7: cells treated with 5-CQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 8: cells treated with 5-CQA at concentration of 2 mM and with PMA+IFNγ challenge
Appendix Figure F.8 Western blot to exam the effect of CGA isomers on the expression of t-p38 in PMA+IFNγ challenged Caco-2 cells.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cells treated without CGA isomer and without PMA+IFNγ challenge</td>
</tr>
<tr>
<td>2</td>
<td>cells treated without CGA isomer and with PMA+IFNγ challenge</td>
</tr>
<tr>
<td>3</td>
<td>cells treated with 3-CQA at concentration of 0.2 mM and with PMA+IFNγ challenge</td>
</tr>
<tr>
<td>4</td>
<td>cells treated with 3-CQA at concentration of 1 mM and with PMA+IFNγ challenge</td>
</tr>
<tr>
<td>5</td>
<td>cells treated with 3-CQA at concentration of 2 mM and with PMA+IFNγ challenge</td>
</tr>
<tr>
<td>6</td>
<td>cells treated with 5-CQA at concentration of 0.2 mM and with PMA+IFNγ challenge</td>
</tr>
<tr>
<td>7</td>
<td>cells treated with 5-CQA at concentration of 1 mM and with PMA+IFNγ challenge</td>
</tr>
<tr>
<td>8</td>
<td>cells treated with 5-CQA at concentration of 2 mM and with PMA+IFNγ challenge</td>
</tr>
</tbody>
</table>
Appendix Figure F.9 Western blot to examine the effect of CGA isomers on the expression of p-p38 in PMA+IFNγ challenged Caco-2 cells.
Lane 1: cells treated without CGA isomer and without PMA+IFNγ challenge
Lane 2: cells treated without CGA isomer and with PMA+IFNγ challenge
Lane 3: cells treated with 4-CQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 4: cells treated with 4-CQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 5: cells treated with 4-CQA at concentration of 2 mM and with PMA+IFNγ challenge
Lane 6: cells treated with 3,5-diCQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 7: cells treated with 3,5-diCQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 8: cells treated with 3,5-diCQA at concentration of 2 mM and with PMA+IFNγ challenge
Appendix Figure F.10 Western blot to exam the effect of CGA isomers on the expression of t-p38 in PMA+IFNγ challenged Caco-2 cells.
Lane 1: cells treated without CGA isomer and without PMA+IFNγ challenge
Lane 2: cells treated without CGA isomer and with PMA+IFNγ challenge
Lane 3: cells treated with 4-CQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 4: cells treated with 4-CQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 5: cells treated with 4-CQA at concentration of 2 mM and with PMA+IFNγ challenge
Lane 6: cells treated with 3,5-diCQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 7: cells treated with 3,5-diCQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 8: cells treated with 3,5-diCQA at concentration of 2 mM and with PMA+IFNγ challenge
Appendix Figure F.11 Western blot to exam the effect of CGA isomers on the expression of p-p38 in PMA+IFN\(\gamma\) challenged Caco-2 cells.
Lane 1: cells treated without CGA isomer and without PMA+IFN\(\gamma\) challenge
Lane 2: cells treated without CGA isomer and with PMA+IFN\(\gamma\) challenge
Lane 3: cells treated with 3,4-diCQA at concentration of 0.2 mM and with PMA+IFN\(\gamma\) challenge
Lane 4: cells treated with 3,4-diCQA at concentration of 1 mM and with PMA+IFN\(\gamma\) challenge
Lane 5: cells treated with 3,4-diCQA at concentration of 2 mM and with PMA+IFN\(\gamma\) challenge
Lane 6: cells treated with 4,5-diCQA at concentration of 0.2 mM and with PMA+IFN\(\gamma\) challenge
Lane 7: cells treated with 4,5-diCQA at concentration of 1 mM and with PMA+IFN\(\gamma\) challenge
Lane 8: cells treated with 4,5-diCQA at concentration of 2 mM and with PMA+IFN\(\gamma\) challenge
Appendix Figure F.12 Western blot to examine the effect of CGA isomers on the expression of t-p38 in PMA+IFNγ challenged Caco-2 cells.
Lane 1: cells treated without CGA isomer and without PMA+IFNγ challenge
Lane 2: cells treated without CGA isomer and with PMA+IFNγ challenge
Lane 3: cells treated with 3,4-diCQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 4: cells treated with 3,4-diCQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 5: cells treated with 3,4-diCQA at concentration of 2 mM and with PMA+IFNγ challenge
Lane 6: cells treated with 4,5-diCQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 7: cells treated with 4,5-diCQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 8: cells treated with 4,5-diCQA at concentration of 2 mM and with PMA+IFNγ challenge
Appendix Figure F.13 Western blot to examine the effect of CGA isomers on the expression of Nrf2 in PMA+IFNγ challenged Caco-2 cells.
Lane 1: protein ladder
Lane 2: cells treated without CGA isomer and without PMA+IFNγ challenge
Lane 3: cells treated without CGA isomer and with PMA+IFNγ challenge
Lane 4: cells treated with 3-CQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 5: cells treated with 3-CQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 6: cells treated with 3-CQA at concentration of 2 mM and with PMA+IFNγ challenge
Lane 7: cells treated with 5-CQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 8: cells treated with 5-CQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 9: cells treated with 5-CQA at concentration of 2 mM and with PMA+IFNγ challenge
Appendix Figure F.14 Western blot to examine the effect of CGA isomers on the expression of actin in PMA+IFN\(\gamma\) challenged Caco-2 cells.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>Protein ladder</td>
</tr>
<tr>
<td>2</td>
<td>Cells treated without CGA isomer and without PMA+IFN(\gamma) challenge</td>
</tr>
<tr>
<td>3</td>
<td>Cells treated without CGA isomer and with PMA+IFN(\gamma) challenge</td>
</tr>
<tr>
<td>4</td>
<td>Cells treated with 3-CQA at concentration of 0.2 mM and with PMA+IFN(\gamma) challenge</td>
</tr>
<tr>
<td>5</td>
<td>Cells treated with 3-CQA at concentration of 1 mM and with PMA+IFN(\gamma) challenge</td>
</tr>
<tr>
<td>6</td>
<td>Cells treated with 3-CQA at concentration of 2 mM and with PMA+IFN(\gamma) challenge</td>
</tr>
<tr>
<td>7</td>
<td>Cells treated with 5-CQA at concentration of 0.2 mM and with PMA+IFN(\gamma) challenge</td>
</tr>
<tr>
<td>8</td>
<td>Cells treated with 5-CQA at concentration of 1 mM and with PMA+IFN(\gamma) challenge</td>
</tr>
<tr>
<td>9</td>
<td>Cells treated with 5-CQA at concentration of 2 mM and with PMA+IFN(\gamma) challenge</td>
</tr>
</tbody>
</table>
Appendix Figure F: Western blot to examine the effect of CGA isomers on the expression of Nrf2 in PMA+IFNγ challenged Caco-2 cells.

- **Lane 1:** Protein ladder
- **Lane 2:** Cells treated without CGA isomer and without PMA+IFNγ challenge
- **Lane 3:** Cells treated without CGA isomer and with PMA+IFNγ challenge
- **Lane 4:** Cells treated with 4-CQA at concentration of 0.2 mM and with PMA+IFNγ challenge
- **Lane 5:** Cells treated with 4-CQA at concentration of 1 mM and with PMA+IFNγ challenge
- **Lane 6:** Cells treated with 4-CQA at concentration of 2 mM and with PMA+IFNγ challenge
- **Lane 7:** Cells treated with 3,5-diCQA at concentration of 0.2 mM and with PMA+IFNγ challenge
- **Lane 8:** Cells treated with 3,5-diCQA at concentration of 1 mM and with PMA+IFNγ challenge
- **Lane 9:** Cells treated with 3,5-diCQA at concentration of 2 mM and with PMA+IFNγ challenge
Appendix Figure F.16 Western blot to exam the effect of CGA isomers on the expression of actin in PMA+IFNγ challenged Caco-2 cells.
Lane 1: protein ladder
Lane 2: cells treated without CGA isomer and without PMA+IFNγ challenge
Lane 3: cells treated without CGA isomer and with PMA+IFNγ challenge
Lane 4: cells treated with 4-CQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 5: cells treated with 4-CQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 6: cells treated with 4-CQA at concentration of 2 mM and with PMA+IFNγ challenge
Lane 7: cells treated with 3,5-diCQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 8: cells treated with 3,5-diCQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 9: cells treated with 3,5-diCQA at concentration of 2 mM and with PMA+IFNγ challenge
Appendix Figure F.17 Western blot to exam the effect of CGA isomers on the expression of Nrf2 in PMA+IFNγ challenged Caco-2 cells.
Lane 1: protein ladder
Lane 2: cells treated without CGA isomer and without PMA+IFNγ challenge
Lane 3: cells treated without CGA isomer and with PMA+IFNγ challenge
Lane 4: cells treated with 4,5-diCQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 5: cells treated with 4,5-diCQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 6: cells treated with 4,5-diCQA at concentration of 2 mM and with PMA+IFNγ challenge
Lane 7: cells treated with 3,4-diCQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 8: cells treated with 3,4-diCQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 9: cells treated with 3,4-diCQA at concentration of 2 mM and with PMA+IFNγ challenge
Appendix Figure F.18 Western blot to exam the effect of CGA isomers on the expression of actin in PMA+IFNγ challenged Caco-2 cells.
Lane 1: protein ladder
Lane 2: cells treated without CGA isomer and without PMA+IFNγ challenge
Lane 3: cells treated without CGA isomer and with PMA+IFNγ challenge
Lane 4: cells treated with 4,5-diCQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 5: cells treated with 4,5-diCQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 6: cells treated with 4,5-diCQA at concentration of 2 mM and with PMA+IFNγ challenge
Lane 7: cells treated with 3,4-diCQA at concentration of 0.2 mM and with PMA+IFNγ challenge
Lane 8: cells treated with 3,4-diCQA at concentration of 1 mM and with PMA+IFNγ challenge
Lane 9: cells treated with 3,4-diCQA at concentration of 2 mM and with PMA+IFNγ challenge