

© 2014 Shorma Bianca Bailey

A COMPARATIVE STUDY OF WHITE, BLACK AND GREEN TEA SOLUTIONS
AS POTENTIAL ANTIVIRAL AGENTS IN RHESUS MONKEY KIDNEY CELLS
INDUCED WITH PORCINE ROTAVIRUS

BY

SHORMA BIANCA BAILEY

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Environmental Engineering in Civil Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

Adviser:

Professor Thanh (Helen) Nguyen

ABSTRACT

Rotavirus, in humans or animals, is an important global health issue; porcine rotavirus not only affects developing countries, but developed countries as well; this may be a result of the transport of porcine rotavirus through the environment, in addition to the lack of sanitary resources. This study seeks to compare and determine, the inhibition of Group A porcine rotavirus survival in African green monkey kidney cells (MA-104), in an aqueous environment, when exposed to three different concentrations of aqueous teas: White, Black, and Green.

Characterization of White, Black and Green Teas were conducted before virus inhibition experiments. Total polyphenol content, of each tea, was determined using Folin-Ciocalteu Method, with gallic acid (GA) as a standard in milligram equivalents to GA per milliliter. Total polyphenols were highest in Green Tea, 4.50 ± 0.58 mg GA equivalent/mL. Black and White teas were lower in total polyphenol content with values 3.80 ± 0.22 mg GA equivalent/mL, and 2.60 ± 0.23 mg GA equivalent/mL, respectively.

Antioxidant capacities were determined using the oxygen radical absorbance capacity (ORAC) assay. The values of antioxidant capacity for all teas ranged from (31.6 ± 0.71 to 46.8 ± 0.14 mM Trolox equivalents/ml); Green Tea had the highest antioxidant capacity when compared to other teas.

Analysis of aqueous tea extract, using LC-MS, suggested the presence of polyphenols, and their range concentrations in tea, including: gallic acid (GA) (1.9 - 7.6 $\mu\text{g}/\text{mg}$ SE), (+)-catechin (CT) (1.0 - 4.8 $\mu\text{g}/\text{mg}$ SE), (-) epicatechin (EC) (4.3 - 16.5 $\mu\text{g}/\text{mg}$ SE), (-)-epigallocatechin (EGC) (9.6 - 76.9 $\mu\text{g}/\text{mg}$ SE), gallocatechin gallate (GCG) (15.1 - 43.6 $\mu\text{g}/\text{mg}$ SE), and (-)-epigallocatechin gallate (EGCG) (48.6 - 151.0 $\mu\text{g}/\text{mg}$ SE). EGCG was found to have the highest concentration in White, Black, and Green Tea solutions.

Prior to inactivation experiments, toxicity of tea samples on MA-104 monkey kidney cells were tested. None of the tea concentrations tested, were found to be toxic to the kidney cells.

White, Black, and Green Tea solutions with concentrations of 50, 500, 1000 $\mu\text{g SE/mL}$ in water, were allowed to react with porcine rotavirus, and the inhibition of tea extract was quantified using the Focus Forming Unit Assay (FFU), which measured virus particles in FFU/mL. The removal of virus (log) for all concentrations of teas ranged from 1.02 ± 0.49 to 3.16 ± 0.66 . All previously mentioned teas were able to inactivate porcine rotavirus. Furthermore, results suggested a statistical difference ($P < 0.05$) amongst the teas. Green Tea, at 1000 $\mu\text{g SE/mL}$, recorded the highest log removal of virus.

In conclusion, White, Black and Green Teas inhibited porcine rotavirus in MA-104 monkey kidney cells. Green Tea showed the highest values for total polyphenol content, antioxidant capacity and removal of porcine rotavirus. The various types of polyphenols and their presence in White, Black, and Green Teas may play a role in their antiviral activity, against rotavirus survival in aqueous environments. Further studies are needed to understand the mechanisms between tea extract, polyphenols, and their anti-viral capacity to inhibit porcine rotavirus.

ACKNOWLEDGEMENTS

This thesis is based upon work supported by the National Science Foundation (NSF) and the United States Department of Agriculture (USDA). I acknowledge and appreciate the Graduate College of University of Illinois Urbana-Champaign, the National GEM Consortium and Saint-Gobain GYPSUM; for their financial support, graduate fellowship and internship experiences. It is with great respect and dignity, that I express profound gratitude to my adviser, Professor Helen Nguyen, for turning my potential knowledge about environmental engineering into, working knowledge. Her mentoring and academic encouragement has been a constant reassurance. Within the Nguyen Research Group, I would like to personally thank Ofelia Romero, Nora Sadik, and Hanting Wang, their wise ways words and actions. In addition, I would also like to thank Professor De Mejia, co-advisor in Food Sciences, for her wealth of knowledge in different types of teas, in addition to her provided lab space. It is with gratitude that I extend a special thanks to Dr. Kimberly Jones, Chair of the Civil and Environmental Engineering Department at Howard University. As an alumna of the UIUC Environmental Engineering department, Dr. Jones was influential in my decision to attend UIUC. I also, thank Girls Incorporated, for their communal support throughout my personal and academic journey. I would also like to thank the First Lady Michelle Obama for her mentorship and shining example. A special acknowledgement is given to Mr. Stanley Smith for his great assistance, in the editing and preparation of this manuscript. I would like to dedicate this special thanks to my father, sister and brothers, Trazell, Ski, Donta, Torma and Trevion, for their love and encouragement every day. Lastly, I would like to send love and a very special thanks to my mother in her absence on this earth; for being the silent gentle wind pushing me along the way. I miss you Maat and hope to grow up to be the loving, kind and smart woman you once were.

TABLE OF CONTENTS

CHAPTER 1: LITERATURE REVIEW.....	1
1.1 SIGNIFICANCE OF RESEARCH.....	6
1.2 HYPOTHESIS AND OBJECTIVES.....	7
CHAPTER 2: INTRODUCTION.....	9
CHAPTER 3: MATERIALS & METHODS.....	11
3.1 CHEMICALS.....	11
3.2 BIOLOGICAL MATERIALS.....	11
3.3 PREPARATION OF TEAS FOR SOLID EXTRACTION.....	12
3.4 PREPARATION OF AQUEOUS TEA SAMPLES.....	12
3.5 ANTIOXIDANT CAPACITY.....	13
3.6 TOTAL POLYPHENOL CONTENT.....	14
3.7 HPLC AND LC-MS ANALYSIS OF AQUEOUS TEA SOLUTIONS.....	14
3.8 PROPAGATION OF MA-104 MONKEY KIDNEY CELLS.....	16
3.9 PROPAGATION AND CONCENTRATION OF ROTAVIRUS.....	16
3.10 CELL CYTOTOXICITY USING TEA EXTRACT.....	17
3.11 VIRUS INACTIVATION EXPERIMENTS.....	18
3.12 FOCUS FORMING UNIT ASSAY.....	19
3.13 STATISTICAL ANALYSIS.....	20
CHAPTER 4: RESULTS & DISCUSSION.....	21
4.1 ANTIOXIDANT CAPACITY.....	21
4.2 TOTAL POLYPHENOL CONTENT.....	23
4.3 HPLC AND LC-MS ANALYSIS.....	24

4.4 CELL CYTOTOXICITY USING TEA EXTRACT.....	30
4.5 VIRUS INACTIVATION EXPERIMENTS.....	31
CHAPTER 5: CONCLUSION.....	39
CHAPTER 6: REFERENCES.....	41
APPENDICES.....	48
APPENDIX A-ANTIOXIDANT CAPACITY STANDARD CURVE.....	48
APPENDIX B-TOTAL POLYPHENOL CONTENT STANDARD CURVE.....	49
APPENDIX C-HPLC CHROMATOGRAM OF PURE STANDARDS.....	50
APPENDIX D-CHROMATOGRAM OF MIXED PURE STANDARDS.....	52
APPENDIX E-CHROMATOGRAMS OF WHITE TEA.....	53
APPENDIX F-CHROMATOGRAMS OF BLACK TEA.....	54
APPENDIX G-CHROMATOGRAMS OF GREEN TEA	55
APPENDIX H-LC-MS CHROMATOGRAMS OF PURE STANDARDS.....	56
APPENDIX I-STANDARD CURVES FOR PURE STANDARDS.....	59
APPENDIX J-QUERCETINE CHROMATOGRAM FOR WHITE TEA.....	63
APPENDIX K-QUERCETINE CHROMATOGRAM FOR BLACK TEA.....	64
APPENDIX L-QUERCETINE CHROMATOGRAM FOR GREEN TEA.....	65
APPENDIX M-ACTUAL CONCENTRATIONS OF POLYPHENOLS IN TEAS.....	66
APPENDIX N-CYTOTOXICITY STATISTICS.....	67

CHAPTER 1

LITERATURE REVIEW

PORCINE AND HUMAN ROTAVIRUS IN THE ENVIRONMENT

Porcine rotavirus can be transmitted to humans and animals directly by contact or indirectly by contamination of water or food (Bicudo et. al., 2003). Group A porcine or human rotavirus infection takes control of the intestinal track and induces diarrhea, causing gastroenteritis, and malnutrition (Alfajaro et al., 2012). Additionally, the excessive application of animal manure for agricultural nutrients, provides a means of environmental transport (Bicudo et. al., 2003), during rain events that lead to water runoff. Following the application of these wastes to land, the potential exists for environmental contamination in soil and ultimately water courses, may subsequently be used as catchments areas, where water supplies are located (Bicudo et al., 2003; Mawdsley et al., 1995). On the other hand, the role of livestock in most waterborne bacterial outbreaks has often been difficult to clarify, since both humans and various wildlife species, can shed the same microorganisms and thereby serve as sources of infection (Bicudo et al., 2003).

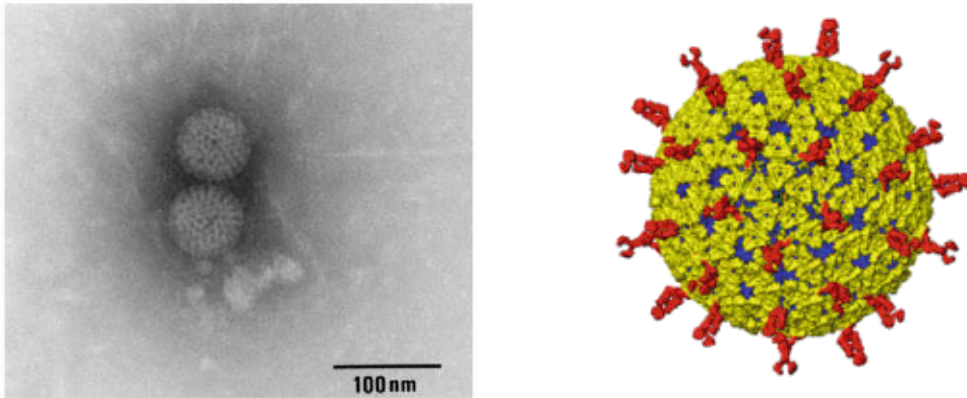


Figure 1-DIAGRAM OF ROTAVIRUS STRUCTURE AND SURFACE PROTEINS

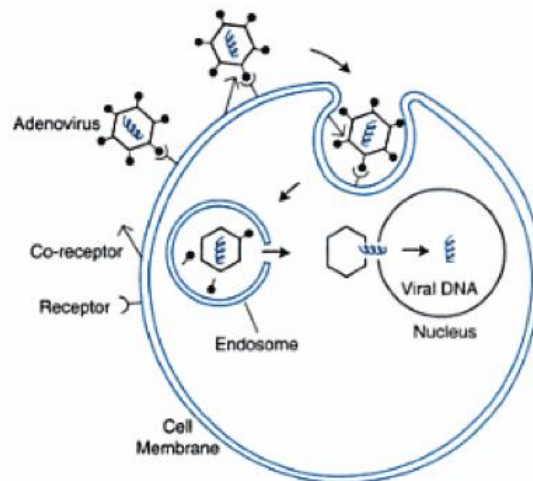


Figure 2-DIAGRAM DISPLAYING A VIRUS INFECTION IN A CELL

VACCINES FOR PORCINE AND HUMAN ROTAVIRUS

Consumption of food-borne disease are associated with the ingestion of infected animal products, much attention has been given from the media, in North America and Europe (Bicudo et al., 2003). Porcine rotavirus vaccines, are commercially available for suckling pigs, via oral route (Theil, 1990). Similarly, human rotavirus vaccines are also prevalent. Before rotavirus vaccines, each year rotavirus killed more than 500,000 children and hospitalized millions, according to the World Health organization (WHO, 2009). Human rotavirus vaccines are said to prevent (85%-98%) of illnesses associated with rotavirus, as published by the Center for Disease Control (Waddington et al., 2009). However, vaccine prices in Brazil and Cambodia are US \$7 and US \$5 respectively (WHO, 2007; Lopman et al., 2012). In developing countries 1.2 billion people live on less than US \$1.25 a day (World Bank, 2014). Thus, rotavirus vaccines may be costly and less effective for the developing than the developed worlds at present (Knipping et. al, 2012). Additionally, poverty continues to reinforce the need to research alternative approaches to control rotavirus disease (Knipping et. al, 2012).

TEA FERMENTATION PROCESS AND PHENOL YIELD

Tea is grown in over 30 countries across the world; which includes developing countries such as: Ethiopia, Sudan, Brazil and China. After water, tea is the most consumed beverage around the world (Graham, 1992). The differences in phenolic content are related to fermentation of tea, with green (non-fermented), white (lightly-fermented) and black (fully fermented) (Lin et al., 2008). A therapeutic and potential study of natural polyphenols or catechins, in antiviral activity discusses inhibition of cytopathic effect (CPE), of five human rotavirus species (Mukoyama et al, 1991). The CPE of the five human rotavirus species was inhibited 100%, at a concentration of 125 µg/ml of (-)-epigallocatechin gallate (EGCG), (Mukoyama et. al, 1991). Polyphenolic

compounds found in Green Tea include: (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), these were responsible for inhibiting influenza virus in chicken red blood cells (Song et al., 2005). A previous study demonstrates that edible plant extracts have antiviral activity against rotavirus (Knipping et. al, 2012). A specific herbal tea called Ardisia species, has been shown to relieve oxidative stress, associated with the onset and progression of cancer (Newell et al., 2010). The protective effect of tea consumption against colorectal cancer was also studied in Yu et al. (2012). Tea has also been used as a chemo-preventative agent in liver cancer cells, and it has now been suggested that tea polyphenol potency, induces apoptotic cell death (Newell et al., 2010; Chen et al., 2008). Another study presented that at 500 µg/mL of green tea extract and 100 µM of EGCG, completely inhibited influenza virus in MDCK cells (Song et al., 2005).

FERMENTATION AND PROCESS OF TEA MANUFACTURING

It is important to note, that White, Black and Green Teas, are harvested from the leaves of *Camellia sinensis* (Song et al., 2005); through different stages of oxidative processing or fermentation. Teas, are classified into un-fermented (White and Green), semi-fermented (Oolong) and fully-fermented (Black), (Almajano et al., 2008; Tanaka et al., 2003). The essential difference in processing tea, is the fermentation before heating, which results in the oxidation of leaf polyphenols, through a multi-stage enzymatic process (Dufresne et al., 2000). Green tea is produced by using young tea leaves (bud and two leaves underneath—a flush). Furthermore, In order to make Black Tea, the leaves of *Camellia sinensis*, are allowed to ferment for several hours, before being either smoke fired, flame fired or steamed. After withering, steaming or pan firing, drying and grading, the tea is packaged and sold for consumption (De Mejia et al., 2009). Among various tea products, Black Tea is the most popular around the world, accounting for almost 80% of the world tea production

(Tanaka et al., 2003). Black tea is a fully-fermented type of tea. On the other hand, post-fermented tea is the type of microbial fermented tea (Chen et al., 2008).

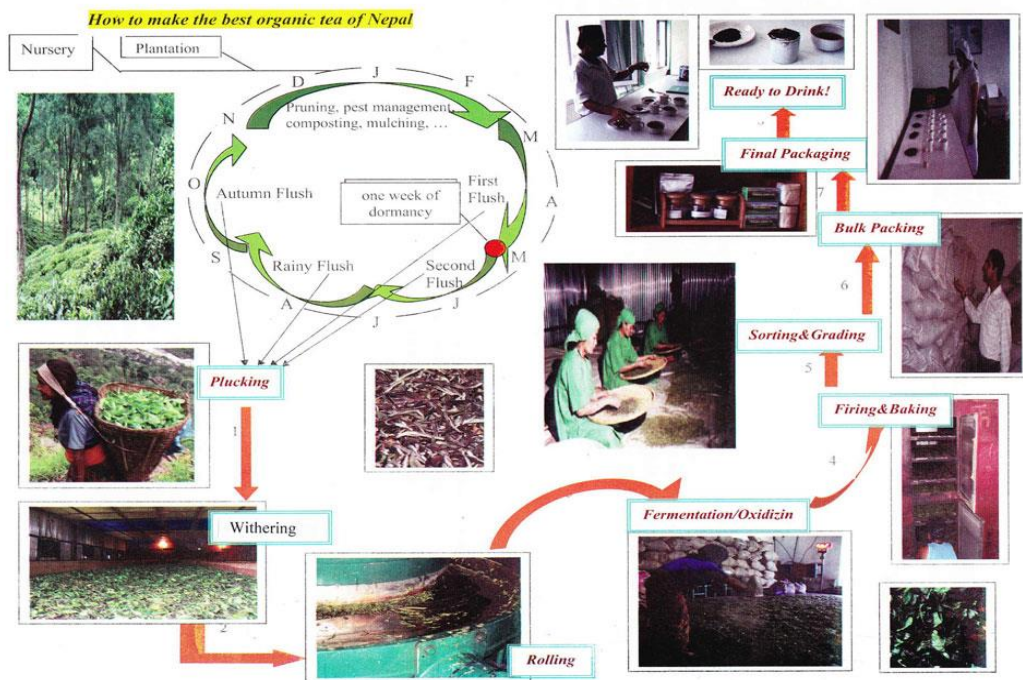


Figure 3-FERMENTATION AND OXIDATION PROCESS OF TEA

1.1 SIGNIFICANCE OF RESEARCH

- a. Porcine rotavirus vaccination, have continued to be recalled, a standard cure for the enteric virus still unmet (Alejandro et al., 2011).
- b. There generates a need for an alternate antiviral option with regards to porcine rotavirus vaccination.
- c. Potential polyphenols from different kinds of tea extract; have been used as an organic medicine since 2700 B.C. (Baker, 1949).
- d. Research amid tea, indicate antioxidants and polyphenols are prevalent in tea; evident factors which, contribute to antiviral abilities, as well as a cytotoxic protective agent.
- e. Polyphenols found in tea, advocate inhibition of rotavirus infection, in the kidney and intestines of humans and animals.
- f. Investigating the scavenging mechanisms of polyphenols in tea, suggest inactivation of porcine rotavirus in monkey kidney cells.
- g. Learning about the chemical characteristics of White, Black and Green Tea phenolic compounds, is important in order to further understand tea as a natural sanitizing agent.
- h. Further studies of reactive oxygen species (ROS), pertains importance, as it relates to oxidation-reduction reactions, which occur with phenols, in various environments.

1.2 HYPOTHESIS AND OBJECTIVES

A. Hypothesis

Due to different compositions of polyphenols, Green Tea has a higher rotavirus inactivation mechanism, then Black Tea and White Tea.

B. Objectives

1. General Objective

To investigate the inactivation capacity of Green Tea, by comparing its phenolic composition and biological activities, to those of Black, and White Teas.

2. Specific Objectives

- a. Determine the total polyphenol content of White Tea, Black Tea and Green Tea. The total polyphenol content measures, phenols, which have been regarded as molecules, with the highest potential to neutralize free radicals (Sánchez-Rangel et al., 2013).
- b. Determine oxygen radical absorbance or antioxidant capacity of White, Black and Green Teas. Antioxidants continue to emerge as therapeutic agents, which scavenge free radicals; otherwise known as reactive oxygen species; and prevents the damage caused by them.
- c. Identify and quantify actual concentrations of phenolic compounds, found in White, Black and Green Teas.
- d. Evaluate cytotoxic concentrations of White, Black and Green Teas when exposed to MA-104 monkey kidney cells; this provides a range of non-toxic tea concentrations, that can be used in virus infectivity experiments. In another study, plant and tea extract exposure MA-104 monkey kidney cells

proved to be non-toxic, in addition cell proliferation was observed (Gu et al., 2000).

- e. Compounds found in natural plants, including tea, may be ideal candidates for use, as preventive and therapeutic drugs against rotavirus (Gu et al., 2000). Moreover, this study will obtain and compare inactivation and inhibition of porcine rotavirus when exposed to White, Black and Green Teas.
- f. Discuss scavenging mechanisms of similar studies in regards to White, Black and Green Teas. In addition, Black Tea solutions, containing antioxidants, were found to scavenge free radicals, resulting in an inhibition of reactive oxygen species. (Almajano et al., 2008; Pal et al., 2013).

CHAPTER 2

INTRODUCTION

Rotavirus infection kills over half a million people globally, according to the World Health Organization (WHO, 2009). The enteric virus causes dehydration, diarrhea, and electrolyte imbalance. Furthermore, pathologic investigations of patients who died of rotavirus infection, have proposed that an electrolyte imbalance, is believed to be the major cause of death (Lynch et al., 2003). The World Health Organization, reported diarrhea was the leading cause in morbidity in 1997, and the sixth leading cause of mortality in 1998 (WHO, 1998). Further reports, have declared 88 percent of diarrheal disease is attributed to unsafe water supply, inadequate sanitation, and hygiene (WHO, 2004).

Humans in addition to animals may obtain rotavirus, through various transmission routes. The first detection of rotavirus, was in 1973; also in swine and other animals (Zimmerman et al., 2006). In addition, it has been reported that over 150 microbial pathogens were identified from various animal species (USDA, 1992) that may induce rotavirus infection. These pathogens may become contaminated upon flooding of ground and surface water, which have obtained rotavirus through fecal properties (USDA, 1992; USEPA, 1998).

Manure farms of swine, have been tested all over the world, from India, North/South Korea, China, Japan, United States of America, and Vietnam, which tested the stool quality of the swine, they have produced to sell (WHO, 2014; Matthijnsens et al., 2012; Hong Anh et al., 2014). Along with rotavirus, porcine rotavirus has been detected in the stool of swine. Porcine rotavirus are classified as one of seven distinct groups, labeled (A-G), in the genus of rotavirus, which can be found in the family of Reoviridae (Zimmerman et al., 2006). Moreover, humans or animals may come into contact with porcine rotavirus; via ground water that has been contaminated through infected manure, liquids, stool, and solids discharged from animal

production facilities; or poor sanitation at day care centers (Gabbay et al., 1999); vaccination and water treatment of porcine rotavirus, become an urgent investigation (WHO, 2009).

The earliest recorded knowledge of water treatment, were found in Egyptian inscriptions and Sanskrit medical lore (Baker, 1949). Yet, due to inadequate research, the cure and cause of waterborne disease was still unknown in developed, and developing countries (Ford, 1999). In addition, Chinese folklore insisted their water be boiled to infuse tea; knowingly or not, had provided themselves with a “large measure of protection from water-borne diseases.” “(Baker, 1949).” Further research suggests, compounds found in natural plants, including tea, may be ideal candidates for use, as a preventive and therapeutic drug against rotavirus (Gu et al., 2000).

The objectives of this study was to compare and determine, the inhibition of Group A porcine rotavirus survival in African green monkey kidney cells (MA-104), in an aqueous environment, when exposed to three different concentrations of White, Black, and Green Tea solutions.

CHAPTER 3

MATERIALS & METHODS

3.1 CHEMICALS

The following chemicals were of the purest grade available from commercial sources and were used, as received, from Sigma Aldrich (St. Louis, MO): (+)-catechin hydrate (CT) ($\geq 98\%$, HPLC), (–)-epigallocatechin gallate (EGCG) ($\geq 80\%$, HPLC), (–)-epigallocatechin (EGC) ($\geq 95\%$, HPLC), (–)-gallocatechin gallate (GCG) ($\geq 98\%$, HPLC), caffeine (CAFF) (99%), (–)-caffeic acid (CA) ($\geq 98\%$, HPLC), quercetin hydrate (QT) ($\geq 95\%$), formic acid ($> 98\%$, Fluka). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) and gallic acid monohydrate (98%) were used as standards. A cell proliferation kit I (MTT) was purchased from Roche Diagnostics (Mannheim, Germany); HPLC reagents: methanol (99.9%, HPLC grade: Sigma), were also used as received. Solutions were prepared with deionized water and purified using Barnstead nanopure water system.

3.2 BIOLOGICAL MATERIALS

MODEL CELL LINE AND VIRUS CULTURE:

Porcine rotavirus OSU (ATCC #VR-892), were obtained from the American Type Culture Collection (ATCC). African green monkey kidney cell lines (MA104; ATCC #CRL-2378.1) were obtained from Sigma Aldrich.

DESCRIPTIONS OF TEAS:

In this study three different types of teas, including White Tea, Black Tea and Green Tea are characterized and tested.

CHINESE WHITE TEA (BAI MUDAN TEA)

The Bai Mudan was a kind gift from the Lv Family of (Fujian Province, China). Bai Mudan Tea is a type of white tea that means white flower. This tea is typically comprised of one to two leaves attached to a leaf bud. The suggested polyphenol content from the manufacturer is 157 mg/g from the white tea company.

NEPALI KANCHANJANGH BLACK AND GREEN TEA:

Green Tea and Black Tea, were kind donations from Kanchanjangha Tea Estate (KTE), Nepal's first organic certified tea garden. Batch numbers for both Green and Black Tea were (License No. 8024), as reported from the manufacturer.

3.3 PREPARATION OF TEAS FOR SOLID EXTRACTION

The extraction of crude tea components from tea leaves will follow the method from (Chandra et al., 2004). The storage and preservation of dry tea leaves were kept in a plastic bag, and placed in a refrigerator at 4 °C, without the presence of light. The crude tea extract was prepared from 2.5g of dry tea leaves and 250 mL of boiling water (100°C), for 10 min. The solid extract (SE) was allowed to sit and cool, until it reached about room temperature (25°C). The SE was vacuum filtered into a 1.0 L glass bottle with a 0.45-µm filter. The filtered SE, was then placed in specialty tubes, sealed with parafilm, and protected from light while covered in foil. Then SE was stored in (-70°C), then (-80°C) for a day each, then freeze dried. The same standardized procedure was carried out for White, Black and Green Tea.

3.4 PREPARATION OF AQUEOUS TEA SAMPLES

Prior to assays, aqueous tea extract solutions were prepared by dissolving 10 mg SE in 1 ml of nanopure water (10 mg SE/mL). Desired concentrations for example, 50, 500, 1000 µg SE/mL, were diluted from the prepared stock solution of 10 mg SE/mL. Dilutions were calculated using

dilution **Equation 1**, ($C_1V_1=C_2V_2$). From this stock solution further dilutions were done, in order to obtain smaller concentrations as necessary. Lastly, samples were vortexed for complete mixing of solid tea extract, in nanopure water. Preparation of previously mentioned pure chemical standards including: EGCG, EGC, GCG, CT, GA, CAFF, CA, QT, were dissolved in 80% methanol for HPLC and LC-MS analysis. Following dissolving of desired pure standards, desired concentrations were accomplished by dilutions calculated using **Equation 1**.

3.5 ANTIOXIDANT CAPACITY

Total antioxidant capacity was obtained by measuring the oxygen radical absorbance capacity (ORAC assay) of White, Black and Green tea samples. The ORAC assay measures the ability of each tea sample to absorb radicals, while protecting fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one), in the presence of free radicals produced by AAPH (Carloni et al., 2013; Chandra et al., 2004). The assay was conducted in black-walled 96-well plates, to prevent degradation of light sensitive compounds (Fisher Scientific, Hanover Park, IL). Each well had a final volume of 200 μ L. The following reactants were added in the order: 50 mg of Trolox in 50 mL of a μ L of 75 mM phosphate buffer pH 7.4; Trolox standards (4 μ M final concentration stock) 100 μ L of fluorescein (70 nM final concentration); and 60 μ L of AAPH (12 mM final concentration). As a blank, 25 μ L of 75 mM phosphate buffer pH 7 was added instead of Trolox or samples. Immediately after addition of AAPH, plates were placed in an FL_800 fluorescence plate reader (Bio-Tek Instruments, Winooski, VT), set with excitation filter 530/25 nm and emission filter 590/35 nm, and then read every 2 min, for 2 h to reach a 95% loss of fluorescence. Final fluorescence measurements were expressed relative to the initial reading. Results were calculated using Trolox (4-240 μ M) as a standard, $y = 0.09x - 1.15$, $R^2 = 0.98$. The standard curve for antioxidant capacity can be found in

Appendix A. Antioxidant capacity of teas are expressed as mM of Trolox equivalents (TE)/g dry leaves (DL).

3.6 TOTAL POLYPHENOL CONTENT

Total polyphenol contents, in teas, were measured using Folin-Ciocalteu method, as described in (Newell et al., 2010). The method determines the reduction of Folin-Ciocalteu reagent by electrons, from phenolic compounds, found in tea. A 1 ml solution of 1 N Folin-Ciocalteu reagent was added to 1 ml sample of tea extract and this mixture was allowed to stand for 2–5 min, before the addition of 2 ml of 20% Na_2CO_3 . The solution rested for 10 min before reading at 730 nm in a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). The total polyphenol content was expressed as milligram equivalents, to the standard used per milliliter, or gram of dry leaves (DL). Three replicates of each sample of White, Black, Green Teas were analyzed. The concentration of total polyphenol content was determined based on the following equation standard curves for gallic acid: $y = 0.02x - 0.10$, $R^2 = 0.99$. The standard curve for total polyphenol content can be found in **Appendix B**.

3.7 HPLC AND LC-MS ANALYSIS OF AQUEOUS TEA SOLUTIONS

Individual chromatograms for 3 different concentrations (1, 3, 5 mg/mL SE) of White, Black and Green Tea samples, were produced using high-pressure liquid chromatography (HPLC). This method was conducted as described in (Chandra et al., 2004). Aqueous tea samples were analyzed by a reverse phase HPLC 1100 Series and Agilent Eclipse XDB-C18 (4.6×150) mm column with $5\mu\text{m}$ particle diameter (Agilent Technologies, Palo Alto, CA, United States). The HPLC includes a gradient pump, a photodiode array detector for UV/VIS spectra. The desired wavelength for polyphenol detection was optimal at 280 nm. The initial conditions were ambient temperature 20°C and constant flow rate (1 ml/min), with injection volume of 10 μl , using

solvent A and B. Solvent A consisted of water (79.9%), methanol (20%), formic acid (0.1%) and Solvent B consisted of methanol (99.9%) and formic acid (0.1%). In addition, solvents were degassed for 1-2 min each and column is flushed for 5 min before the first sample is taken. Solvent A initially began with 100% gradient, with a decreasing linear trend to 48% of A in 52 min, to 20% A in 5 min, and held at 20% A for 3 min, then a linear increase to 100% A in 5 min. Between each run the needle was cleaned 9 times in methanol to reduce cross contamination of samples. Individual chromatograms for White, Black and Green Tea can be found in **Appendix E, F, G**, respectively.

LC-MS ANALYSIS:

Quantification of the selected pure chemical standards was performed via LC-MS in negative ionization mode (mass range m/z 70–700) on an Agilent MSD Trap XCT Plus mass spectrometer (Agilent Technologies, Palo Alto, CA, United States); with an electrospray ionization (ESI) interface (the capillary voltage was set to 3500V, nebulizing gas flow rate of 30 L/h, drying gas temperature of 350°C), and Multiple Wavelength Detectors (MWD). The system consisted of an Agilent 1100 series liquid chromatograph, capillary pump, an auto-sampler, a degasser and a column thermostat. Reversed phase separation was performed on an Agilent Eclipse XDB-C18 4.6×150 mm column with 5 μ m particle diameter (Agilent Technologies, Palo Alto, CA, United States). Data acquisition and analysis was carried out in an Agilent ChemStation (version B.01.02). Solvent A was 79.9% water, 20% methanol and 0.1% formic acid and solvent B consisted of 99.9% methanol and 0.1% formic acid. The flow rate was set to 0.3 mL min⁻¹, column temperature was set at room temperature and the injection volume was 5 μ L. A binary gradient was 0.0–15 min 0% B, from 1.5 to 30 min 10% B, from 30 to 40 min 30% B, from 40 to 50 min 60% B, from 50 to 60 min 80% B, from 60 to 65 min 80% B, and then back to the initial conditions of 0% B. The MWD

was set at 270 and 280 nm. Quantification was done by comparison with authentic and pure standards (calibration ranged was 0.5-50 µg SE/mL). Actual concentrations of standards present in tea were calculated from equations listed in **Table 1**.

Table 1-Standard Curves used in LC-MS

Standards	Standard Curve Equations	R ²
Gallic Acid Monohydrate (GA)	$y = 803149x - 146305$	0.99
(-)-Epigallocatechin (EGC)	$y = 2E+06x + 6E+06$	0.96
(+)-Catechin hydrate (CT)	$y = 4E+06x - 2E+06$	0.99
(-)-Epigallocatechin gallate (EGCG)	$y = 3E+06x + 4E+06$	0.97
Caffeic Acid (CA)	$y = 2E+06x + 367349$	0.99
Gallocatechin gallate (GCG)	$y = 2E+06x - 483407$	0.99
Epicatechin (EC)	$y = 4E+06x - 761165$	0.99
Quercetin hydrate (QT)	$y = 9E+06x + 9E+06$	0.99

3.8 PROPAGATION OF MA-104 MONKEY KIDNEY CELLS

Cell culture of MA-104 rhesus monkey kidney lines were maintained as described in Gillespie (1984). Briefly, MA-104 cell lines were maintained in a series of Corning flasks (75 cm²) in Eagle's minimal essential medium (MEM) with a 10% bovine serum. Monolayers of MA-104 monkey kidney cells were confluent over the course of 2-5 days. Before confluent monolayers of MA-104 cell cultures were used for porcine rotavirus propagation, the cell cultures were washed three times with phosphate buffer solution (PBS).

3.9 PROPAGATION AND CONCENTRATION OF ROTAVIRUS

Porcine rotavirus OSU (catalog #VR-892) were obtained from the American Type Culture Collection (ATCC). Porcine rotavirus was propagated in African monkey kidney cell lines (MA-104; ATCC #CRL-2378.1). After infection of virus in monkey kidney cells, rotavirus solution was removed from cell culture and collected as explained in Rolsma et al. (1994). Prior to purification step, rotavirus solution went through a sequential freezing (-80°C) and thawing (4°C) process three times. Virus solution was then centrifuged at 1000 rpm for 10 min (20°C) to

remove cell debris. Thereafter, rotavirus was filtered through a filtration membrane (Whatman Nucleopore, USA) of pore size 0.45 μm to remove cell debris; permeate was collected. For further purification of virus, permeate from the 0.45 μm membrane, was concentrated in 1 mM NaCl and 0.5 mM CaCl_2 during 100 kDa ultrafiltration. This step is important in order to protect the outer capsid proteins of the virus so that it is still intact and can attach to cell surface for infection (Ruiz et al., 1999). The ultrafiltration membrane consisted of (polymer polyvinylidene fluoride; Koch Membrane). Again, the purified rotavirus stock was filtered through a 0.45 μm membrane. The final porcine rotavirus was around 10^6 - 10^7 (FFU) per mL and stored in 4°C fridge in the dark.

3.10 CELL CYTOTOXICITY USING TEA EXTRACT

Various concentrations of White, Black and Green Tea samples were subjected to cytotoxicity assays, using a Cell Proliferation Kit I (Roche Diagnostics, Basel, Switzerland) according to the manufactures instructions. This cytotoxicity assay was similar to the method used in Ueda et al. (2013) with adjustments for this study. The significance of this assay is to determine which concentrations of aqueous tea samples maybe toxic to the MA-104 monkey kidney cells. In this assay a 96 well plate of confluent MA-104 monkey kidney cells were exposed to aqueous tea samples with varied concentrations. Before MA-104 cells were exposed to aqueous tea samples cell concentration was determined. The concentration of MA-104 cells in the 96 well plate must be evenly distributed to each well. This quantification is to ensure that each well in the 96 well plate has approximately the same number of cells to yield a consistent absorption readings for each individual well during the assay. MA-104 monkey kidney cells were seeded in a 96 well plate with at least a concentration of 5×10^5 cells/well in a volume of 100 μL of MEM. The concentration of the cells in the flask was determined by using In-cyto-C-Chip. Two 10 μL

samples, were taken from the T-150 flask and injected into slot A and slot B slowly to allow for even distribution of the cells in the cyto-chip. The cells were then counted using a microscope with a 10X objective. Concentration of cells were calculated using the following **Equation 2**: Concentration of Cells in cyto-chip/ml (average # of cells counted from each square) x (10^4) . The average concentration was 6×10^5 cells/mL. This cell concentration meets the basic protocol recommended in the Cell Proliferation kit instructions. After cell concentration per well was determined preparation of tea samples were completed as described previously. Cells were then exposed to a volume of 50 μ L including 25 μ L of aqueous solid extract (with concentration range: 100 μ g SE/mL-300 μ g SE/mL) and 25 μ L of MEM. In addition tea-free controls were prepared with only 50 μ L of MEM complete pipetted in 4 wells of confluent monolayer of cells. The well plates were incubated for 24 h at 37°C with 5% CO₂. After this incubation 10 μ L of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) labeling reagent were added to each well and then re-incubated for 4 h. At the end of the 4 h period the 100 μ L of solubilization solution was added into each well and allowed to stand overnight in the incubator. This plate was taken out the next day and analyzed by a plate reader at 650 nm. The viability of MA-104 cells at different concentrations of aqueous tea samples (μ g SE/mL) were estimated by comparing values of White, Black and Green Tea samples in MA-104 cells, with that of tea-free controls as indicated before.

3.11 VIRUS INACTIVATION EXPERIMENTS

Porcine Rotavirus solutions were exposed to three different prepared stock concentrations of aqueous White, Black and Green Teas samples at 50, 500, 1000 μ g SE/mL of water. In order to activate rotavirus, a trypsin concentration of 100 μ g/mL was added to a volume of 200 μ L of virus and maintained in an incubator with 5% CO₂ at 37°C for 30 min. For rotavirus inactivation

experiments, labeled centrifuge tubes contained 50 μL for each individual aqueous tea solution and 8 μL volume of virus at 1×10^5 Focus Forming Units (FFU) per ml. Each reactor contained a 50 μL volume of individual tea solutions plus virus volume of 8 μL for a total volume in reactor 58 μL of tea extract and virus. After tea samples were prepared, they were added to the rotavirus solution resulting in a 14% dilution for each prepared stock of 50, 500, 1000 $\mu\text{g SE/mL}$; resulting in 43, 431, 862 $\mu\text{g SE/mL}$, respectively. The reactors containing the tea and virus solution were incubated in the dark at 5% CO_2 at 37°C , with reaction time of 20 min.

3.12 FOCUS FORMING UNIT ASSAY

The Focus Forming Unit Assay (FFU) is an immunochemical infectivity assay described by Romero-Maraccini et al. (2013). This assay was used to determine rotavirus particle concentration after treated with tea samples. Prior to the FFU assay, MA-104 monkey kidney cells were infected with a volume of 150 μL solutions. These solutions included concentrations of tea samples, porcine rotavirus particles, and MEM non-complete. The given solutions were plated on a confluent monolayer of MA-104 cells, in a 24 well plate, and incubated with 5% CO_2 at 37°C for 30 min. After this incubation step, the 150 μL solutions of tea, rotavirus and MEM non-complete, were vacuum collect form each well, and the confluent monolayer of cells were washed twice with 1mM sterile phosphate buffer (PBS). The PBS wash buffer was vacuum collected after two rinses. Following vacuuming, each of the 24 wells were filled with 150 μL of MEM non-complete. The 24 well plate was maintained in an incubator with 5% CO_2 at 37°C for 18-24 h. After porcine rotavirus exposure to MA-104 monkey kidney cells, the FFU assay procedures were followed as described before (Romero-Maraccini et al., 2013). Rotavirus infections occurring in the MA-104 cells, after tea treatment, were quantified using light microscopy (Arnold and McDonald, 2009). This FFU assay identifies fluorescence foci through

the application of antibodies that react with rotavirus infected cells, creating a circular dark-brown shape under the light microscope. Images of stained cells, suggesting rotavirus infection, were quantified under 10X and 20X objective lens. Images of stained cells after FFU assay are displayed in **Figure 13**.

3.13 STATISTICAL ANALYSIS:

The results are expressed as means \pm standard deviation from at least 3 independent measurements. Statistical analyses were conducted through GraphPad Prism® software and performed by One-way ANOVA utilizing Tukey's test. A probability of $P < 0.05$ indicated a statistical difference. The concentration response analysis were performed by non-linear regression (curve fit) and generated by OriginPro.

CHAPTER 4

RESULTS & DISCUSSION

4.1 ANTIOXIDANT CAPACITY

The antioxidant capacities of White, Black and Green Tea are summarized in **Table 2**.

Antioxidant capacity of each tea, were expressed as mM equivalents of Trolox/mL. All teas when compared to each other, showed antioxidant capacity values that were statistically different ($P < 0.05$). The measured antioxidant capacity values were highest for Green Tea (46.8 ± 0.07 mM equivalents of Trolox/mL). Black Tea was the second highest in antioxidant capacity (44.0 ± 0.29 mM equivalents of Trolox/mL), while White Tea reported the lowest antioxidant value. Green Tea antioxidant capacity and total polyphenol content values reported the highest for all teas tested. A similar study (Carloni et al., 2013) reported phenolic and antioxidant content in Green Tea was the highest, when compared to Green and Black Tea. Phenols are known to be responsible for antioxidant properties (Pal et al., 2013). The studies from (Ndhlala et al., 2010) displayed that high catechin levels correlate with antioxidant capacity. These findings support the data described in **Tables 2**. This technique, determining antioxidant capacity, involves a hydrogen atom transfer reaction (Huang et al., 2005; Chandra et al., 2004). In addition, the reducing power of phenolic compounds in regards to ROS, in teas, relates to its electron transferability, dependent upon antioxidant activity (Pal et al., 2013). Further studies must be conducted in order to understand the functional properties of teas in cellular and viral systems in relation to reactive oxygen species.

Table 2-Antioxidant Capacity of White, Black Tea and Green Tea¹

Tea Types ¹	Antioxidant Capacity for Teas mM eq.TE/mL ²
White Tea	31.6 ± 0.71 ^a
Black Tea	44.0 ± 0.29 ^b
Green Tea	46.8 ± 0.14 ^c

¹Antioxidant Capacity relative to 1 µM Trolox. Values are the average of 3 fresh tea preparations in triplicates. Concentration of antioxidant capacity was expressed as mM equivalents of TE milligram equivalents per milliliter or gram of Dry Leaves.

²Values with different letters in the same column are statistically different, (P<0.001). Abbreviations: DL, dried leaves, TE, Trolox equiv.

4.2 TOTAL POLYPHENOL CONTENT

Table 3 demonstrates the total polyphenol content from freshly prepared solid extracts of White Tea, Black Tea, and Green Tea solutions. Total polyphenol content values are expressed as mg equivalent of gallic acid/mL or as mg equivalent per gram of dry leaves (DL). The total polyphenol content values of Green Tea and Black Tea, suggest the values are statistically different when compared to each other ($P < 0.05$). Green Tea showed the highest polyphenol content value of 4.50 ± 0.58 mg eq. GA/mL. Total polyphenol content for White Tea displayed the lowest value of 2.60 ± 0.23 mg eq. GA/mL. The value for White Tea suggests a statistical difference occurred in total polyphenol content when compared to Green and Black Tea ($P < 0.05$).

Table 3-Total Polyphenol Content of White, Black and Green Tea¹

Tea Type ¹	Fresh Tea, mg eq. GA/mL ²	Fresh Tea, mg eq. GA/g Dry Leaves
White Tea	2.60 ± 0.23^a	186 ± 17.0^a
Black Tea	3.80 ± 0.22^b	283 ± 16.0^b
Green Tea	4.50 ± 0.58^c	317 ± 42.1^c

¹Values are the average of 3 independent fresh tea preparations in triplicates.

²Values with different letters in the same column are statistically different, ($P < 0.001$). Concentration of phenolic compounds are expressed as mg equivalents of milligram equivalents of GA per milliliter or gram of Dry Leaves
Abbreviations: DL, dried leaves: GA, gallic acid equiv.

4.3 HPLC AND LC-MS ANALYSIS

For complete understanding of the anti-viral mechanism conferred by polyphenols found in tea, knowledge regarding the composition and chemical properties is essential (Pal et al., 2013). Therefore, HPLC and LC-MS analysis were used to identify polyphenols and actual concentrations in White Tea, Black Tea and Green Tea samples. The chemical structures of polyphenols tested in this study are shown in **Figure 4**. A preliminary characterization of pure standard phenolic compounds found in tea, was conducted, in order to quantify the phenolic composition in relation to that of White Tea, Black Tea and Green Tea samples. A chromatogram displays the pure phenolic standards displayed in **Figure 5**, with mass spectra (MS) diagrams found in **Appendix H**. Retention times and quantification of recommended standard phenols and other compounds of interest are presented in **Table 4**. Chromatograms showing identified phenolics in White, Black and Green Tea are reported in **Figures 6-8**. Concentration of phenolics i.e. (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG), (-)-epigallocatechin (EGC), epicatechin (EC), were quantified in each tea shown in **Table 5**. Molecular weight (MW), retention time and wavelength of absorbance for pure standard polyphenols were taken into consideration when calculating actual phenolics in tea chromatograms. Identification of caffeine in teas, using HPLC and LC-MS was determined; however caffeine was not detected in tea solutions prepared in water. Caffeine has a low water solubility and with a peak retention time of about 15min (Morikawa et al., 2013). Caffeine was not present at around 15 min in previously mentioned tea chromatograms **Figure 6, 7, 8**. Therefore, caffeine concentrations in tea profiles are below the detection limit. In addition, caffeic acid was found in **Figure 6** for pure standards, at 35.8 min, yet this peak was not present in White, Black and Green Tea chromatograms.

ABSORBANCE AND CONCENTRATION:

Chromatograms were obtained at 280 nm, for each tea shown in **Figures 6-8**. This analysis demonstrates the possible presence of similar polyphenol composition found in White, Black and Green Tea chromatogram profiles. Although, tea chromatograms seem to have a similar profile, suggesting similar polyphenols exists; concentrations of these specific phenols are different. Comparing the concentrations of the different tea chromatograms to the mAU units, suggested a relative association between absorbance of tea sample and sample concentration **Appendix E-G**.

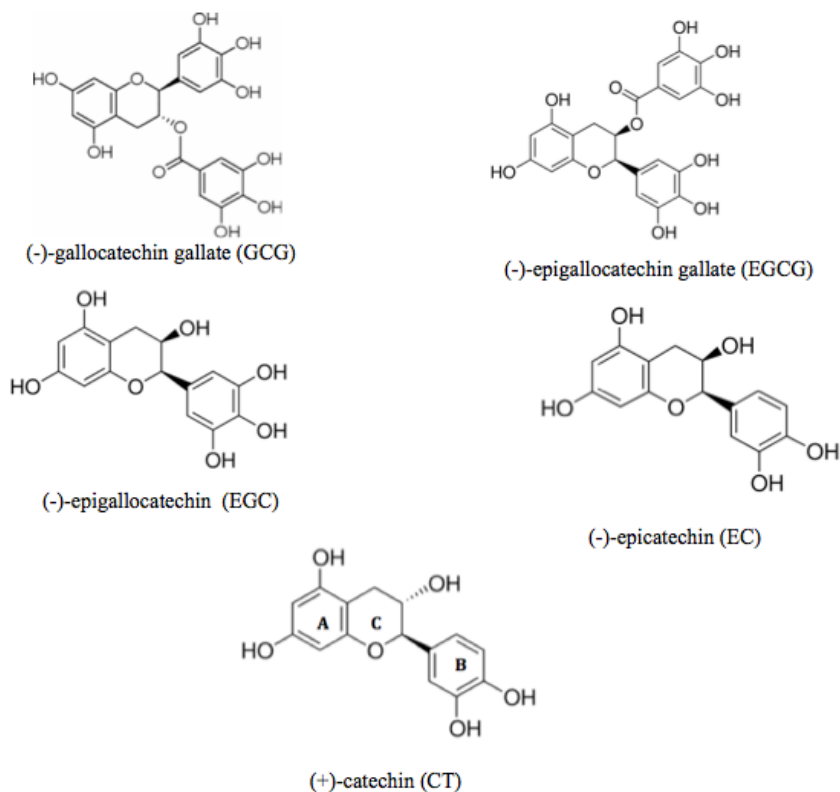


Figure 4-Chemical Structure of polyphenols: (-)-epigallocatechin gallate (EGCG), (-)-gallocatechin gallate (GCG), (-)-epicatechin gallate (ECG), Epicatechin (EC), (+)-catechin (CT)

Figure 5-CHROMATOGRAM OF STANDARD PHENOLICS

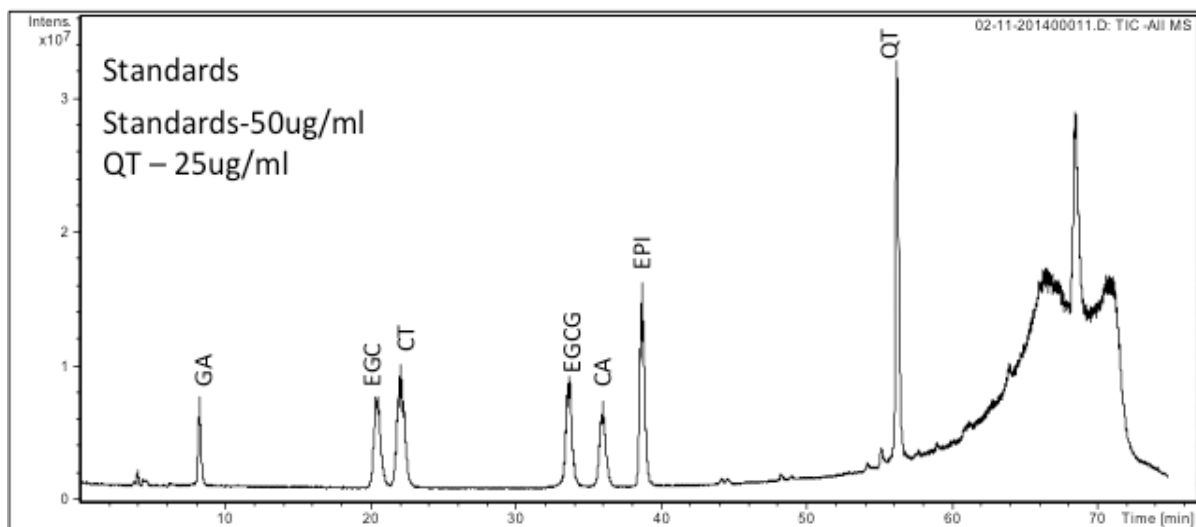


Table 4-The identities of phenolics and other compounds in Teas.

Standard Name	m/z (-) Value	Retention Time [min]	UV-VIS Spectra
Gallic Acid Monohydrate (GA)	169	8.2	280
(-)-Epigallocatechin (EGC)	305	20.3	280
(+)-Catechin hydrate (CT)	289	21.9	280
(-)-Epigallocatechin gallate (EGCG)	457	33.4	280
Caffeic Acid (CA)	179	35.8	280
Galocatechin gallate (GCG)	457	37.9	280
Epicatechin (EC)	289	38.5	280
Quercetin hydrate (QT)	301	56.2	280

Table 5-Actual concentrations of phenols and other compounds in teas

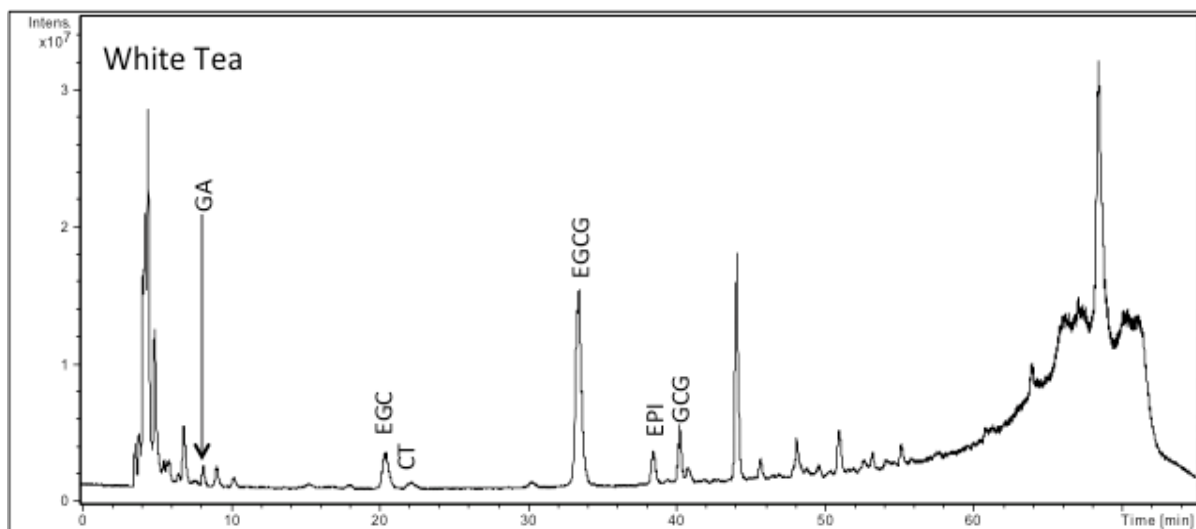
	GA ¹	EGC ¹	CT ¹	EGCG ¹	GCG ¹	EC ¹	QT ¹	Total ² Polyphenol Weight
White Tea	7.6	9.6	1	48.6	15.1	4.3	0.7	71.8
Black Tea	7.1	25.9	3.8	112.1	32.4	11.1	0.9	160.9
Green Tea	1.9	76.9	4.8	151	43.6	16.5	0.7	251.8

¹ The units for these compounds are expressed as µg of (polyphenol name)/mg SE i.e.µg of (GA or EGC, etc.)/mg SE. ² The units for the sum of all the total polyphenol weight, in each tea, is expressed as µg of Total Polyphenol/mg SE.

WHITE TEA COMPOUNDS

In this study, White Tea proved to be the lowest in total polyphenol content, compared to that of Green Tea and Black Tea. The White tea chromatogram is presented in **Figure 6**. This is in agreement with a study reported before, (Carloni et al., 2013); concluding that low oxidation and harvesting process of White Tea, may be the reason phenolic content is lowest when compared to that of Green Tea and Black tea. Although White Tea has the lowest overall polyphenol concentration **Table 3**, it does present a higher concentration of gallic acid than found in Green and Black Tea. The EGCG compound presented the highest concentration of polyphenols identified in the White Tea profile. White Tea also has the lowest in absorbance (mAU) in HPLC chromatogram compared to other teas **Appendix 6, 7, 8**.

Figure 6—CHROMATOGRAM OF WHITE TEA

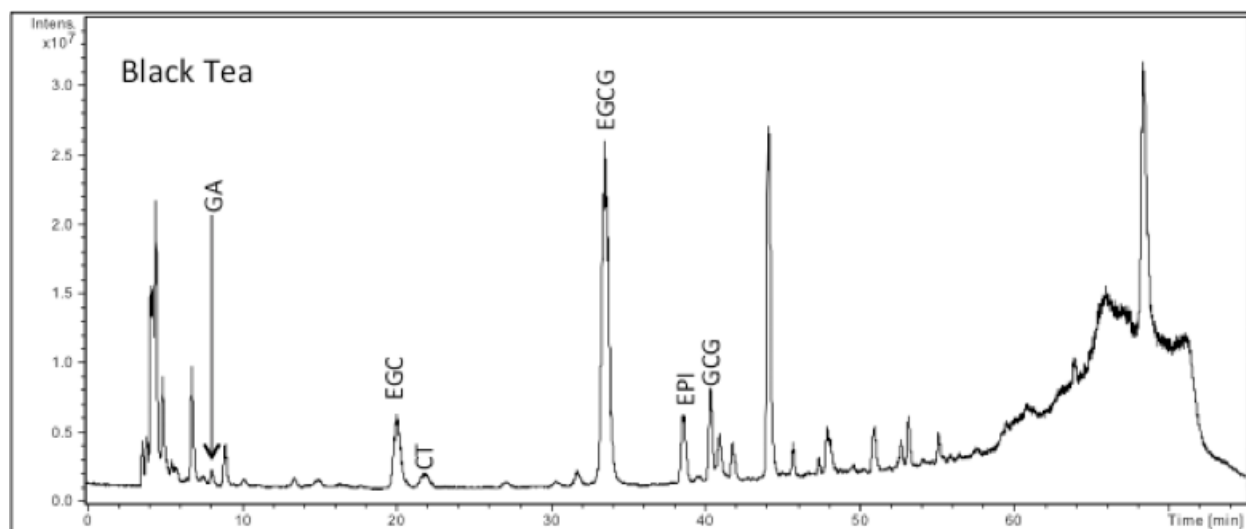


BLACK TEA COMPOUNDS

Chromatogram for Black Tea is presented in **Figure 7**. Black Tea aqueous samples analyzed by LC-MS and HPLC, suggests the same phenolic trend as Green Tea; in regards to EGCG, GCG, EGC, EC, GA, CT concentrations in decreasing order. EGCG represented approximately 45% of the 161 μg of total phenol concentration, including other compounds of interest. Although Black Tea is second in phenol concentration, Black Tea absorbance values (mAU) in chromatograms at 1, 3, 5 mg of SE/mL, **Appendix F**, are relatively higher than the absorbance values (mAU) of White Tea and Green Tea at the same concentrations. This may suggest other compounds, not tested in this study, exist in Black Tea. Quercetin and kaempferol are rich in Black Tea and also contain chemical structures similar to polyphenols such as EGCG, EGC, EC, (Pal et al., 2013). Quercetin, a flavonol tested in this study, presented the highest percentage 0.35% in Black Tea; compared to Green and White Tea quercetin values. Therefore, quercetin can be identified as a polyphenol found in Black Tea. Quercetin's presence may attribute to the high absorption values (mAU) reported for this compound. In a similar study, quercetin and gallic acid were shown to

have a higher value in Black Tea than in Green Tea (Del Rio et al., 2004). The composition of Black Tea, suggested a balance between catechins, flavonols and theaflavins.

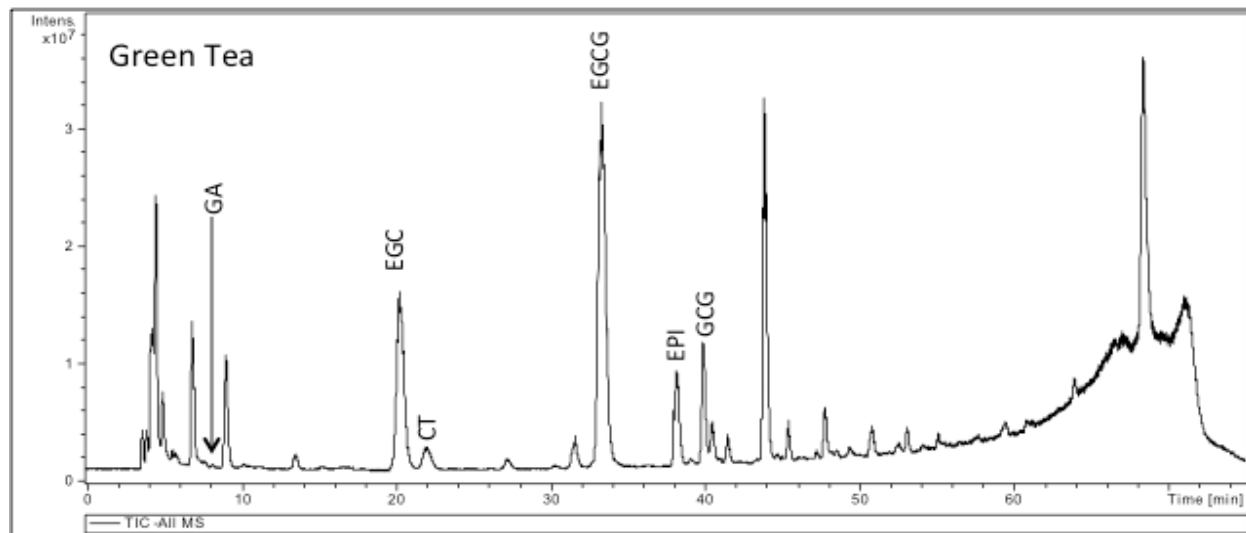
Figure 7-CHROMATOGRAM OF BLACK TEA



GREEN TEA COMPOUNDS

Green Tea chromatogram is presented in **Figure 8**. Green Tea was shown to contain a higher percentage of overall polyphenols and compounds of interest than Black Tea and White Tea, in accordance with Carloni et al. (2013). Out of the total solid extract concentration of Green Tea analyzed, 25% of the weight was attributed to polyphenols. The phenol with the highest concentration in all teas included, EGCG. In addition, EGCG represented about 60% of the total phenol weight for Green Tea. This data suggests that EGCG is the main catechin or polyphenol by weight in each Green Tea, Black Tea and White Tea found in **Table 5**. Results from LC-MS analysis suggest the most abundant polyphenols in Green Tea are EGCG, EGC, GCG, EC, CT in decreasing order, refer to **Table 5**.

Figure 8-CHROMATOGRAM OF GREEN TEA



4.4 CELL CYTOTOXICITY USING TEA EXTRACT

Cytotoxic effects of White Tea, Black Tea and Green Tea were observed on the MA-104 monkey kidney cells **Appendix N**. When compared against the control of a confluent monolayer of cells with MEM only, no tea, cell inhibition was not observed. In fact, complete growth or increase in cell growth was observed in tea treated cells with concentrations 100, 150, 200, 300 $\mu\text{g SE/mL}$. A statistical comparison of White, Black and Green Tea, at 100, 150, 200 $\mu\text{g SE/mL}$, suggest no statistical difference ($P>0.05$) in the cell proliferation values of the teas. However, at 300 $\mu\text{g SE/mL}$, White and Black Tea demonstrated a statistical difference ($P<0.05$) when compared to Green Tea cell proliferation values. Amongst the three teas, Green Tea showed the highest cell growth increase of 27% at 300 $\mu\text{g SE/mL}$ compared to that of the control. At 300 $\mu\text{g SE/mL}$ for Black Tea, a 13% increase in cell growth occurred. White Tea, presented the least cell growth of with 8% increase compared to that of the control. This suggests that neither tea is toxic to MA-104 monkey kidney cells tested at these concentrations. Similar studies showed an increase in cell growth after addition of tea or plant extract (Luce, 2011). This correlates with the catechins and

antioxidants that encouraged the cell to grow and take in nutrients (Luce, 2011). These concentrations suggest that tea at various concentrations reported is not harmful to the monkey kidney cells and any concentration can be used for virus infectivity experiments.

4.5 VIRUS INACTIVATION EXPERIMENTS

Table 6 shows the antiviral activity of White, Black and Green Tea, at three different concentrations, on Group A porcine rotavirus. White, Black and Green Tea concentrations in **Table 6** and **Figure 9** are reported as 50, 500, 1000 µg SE/mL for prepared stock solutions, however actual concentrations after dilutions yielded a 33% decrease in concentration of prepared stock solutions. All teas, including White Tea, Black Tea and Green Tea all showed antiviral activity against porcine rotavirus at each concentration tested in this study **Figures 10, 11, 12**, respectively. The removal of virus (log) for 50, 500, 1000 µg SE /mL of tea samples ranged from (1.02 ± 0.49 to 1.26 ± 0.13), (1.08 ± 0.34 to 2.83 ± 0.56), (1.30 ± 0.11 to 3.16 ± 0.66), respectively. Comparison of all tea log removal values at 50 µg SE/mL showed no statistical difference ($P>0.05$). However at 500 µg of SE/mL, Green Tea was the only value that showed a statistical difference ($P<0.05$), when compared to that of Black and White Tea log removal values. At tea concentration of 1000 µg SE/mL, Green and Black Tea log removal values suggest a similar trend with reported ($P>0.05$). White Tea log removal values at 1000 µg SE/mL, when compared to Green and Black Teas show a statistical difference ($P<0.05$). Porcine rotavirus removal in aqueous tea solutions, under each condition, was determined in log value by the equation listed in **Equation 3**. Green Tea showed the highest antiviral activity at concentrations of 500 and 1000 µg SE/mL, with reported virus log removals of 2.83 ± 0.56 , 3.16 ± 0.66 , respectively. Black Tea, on the other hand, reported at concentration of 50 µg SE/mL, a log removal value of 1.30 ± 0.36 log removal of porcine rotavirus. This value is higher than both

Green and White Tea solutions at concentration of 50 µg SE/mL. Black Tea reported the second highest log inactivation values at 500 µg SE/mL and 1000 µg SE/mL, when compared to all teas in this study shown in **Table 6** and **Figure 9**. White Tea, at 1000 µg SE/mL, when compared to Green and Black Tea, was about two time less effective at porcine rotavirus log removal. The low removal of porcine rotavirus using White Tea extract solutions could be dependent upon its low polyphenol and antioxidant capacity, in addition to its low concentrations of catechin structures tested in this present study **Table 5**. Presented in **Figure 13** are of actual images of rotavirus inhibition by tea treatments in MA-104 kidney cells. Green Tea also demonstrated the highest value in total polyphenol content and antioxidant capacity, **Table 2, 3**. High inhibition of Green and Black tea activity suggests its dependence on the composition and concentration of polyphenols: EGCG, EGC, GCG, EC, GA and CT, **Table 5**. In a previous study, scavenging effects of the catechin structures on ROS, were higher in more gallolated catechins like EGC, GCG and EGCG (Nanjo et al., 1996). This may suggest an explanation for Green Tea's high inactivation values for porcine rotavirus, when compared to that of Black and White Tea inactivation.

Equation 3-Porcine Rotavirus Log Removal after Treated with Tea

$$Porcine\ Rotavirus\ Removal\ (logs) = -\log \frac{Porcine\ Rotavirus_{post\ tea\ treatment}}{Porcine\ Rotavirus_{initial\ (before\ tea\ treatment)}}$$

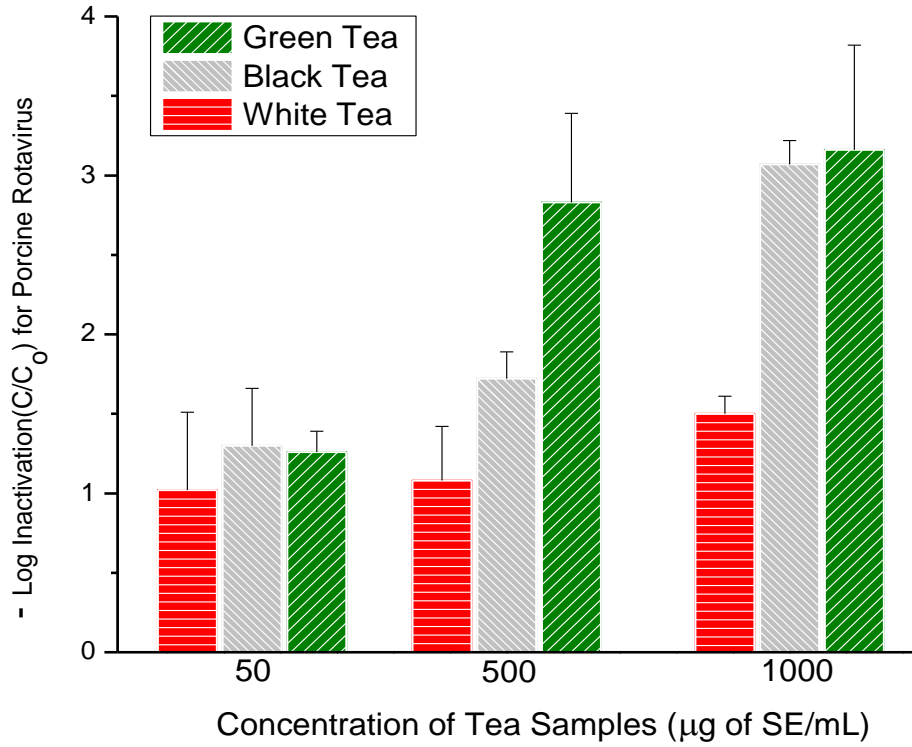
Table 6-Log Removal of Porcine Rotavirus using White, Black and Green Tea¹

Log Inactivation of Porcine Rotavirus			
Tea Concentrations	50 µg SE/mL ²	500 µg SE/mL ²	1000 µg SE/mL ²
White Tea	1.02 ± 0.49	1.08 ± 0.34	1.30 ± 0.11 ^a
Black Tea	1.30 ± 0.36	1.72 ± 0.17	3.07 ± 0.15
Green Tea	1.26 ± 0.13	2.83 ± 0.56 ^a	3.16 ± 0.66

¹log inactivation values are the average of fresh tea preparations in triplicates (N=3). Tea concentrations are expressed as µg Solid Extract (SE)/mL of water.

²Values with different letters in the same column are statistically different, (P<0.001).

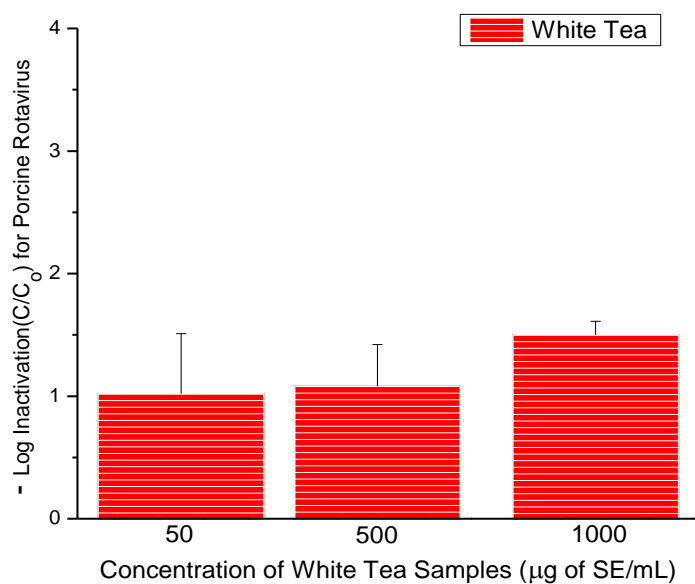
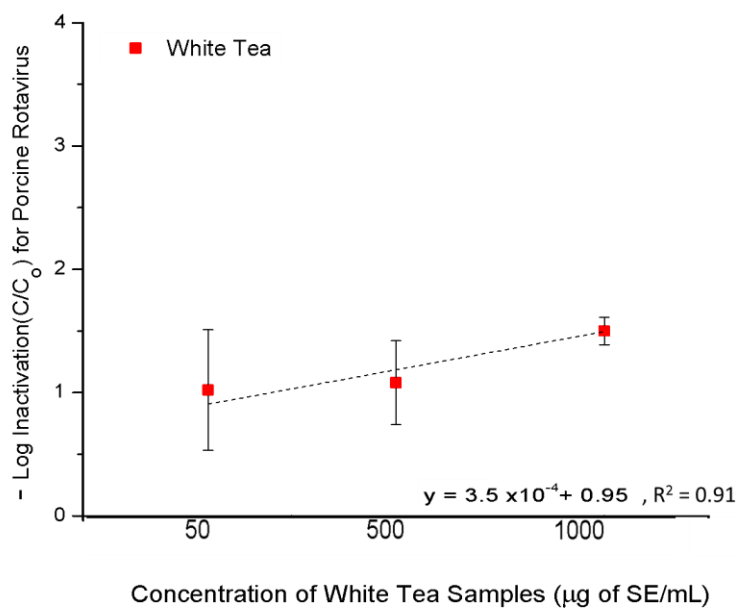
Figure 9-Log Removal of Porcine Rotavirus using White, Black and Green Tea



Tukey's Analysis	50 µg SE/mL	500 µg SE/mL	1000 µg SE/mL
Green vs Black	ns	P<0.05	ns
Green vs White	ns	P<0.05	P<0.05
Black vs White	ns	ns	P<0.05

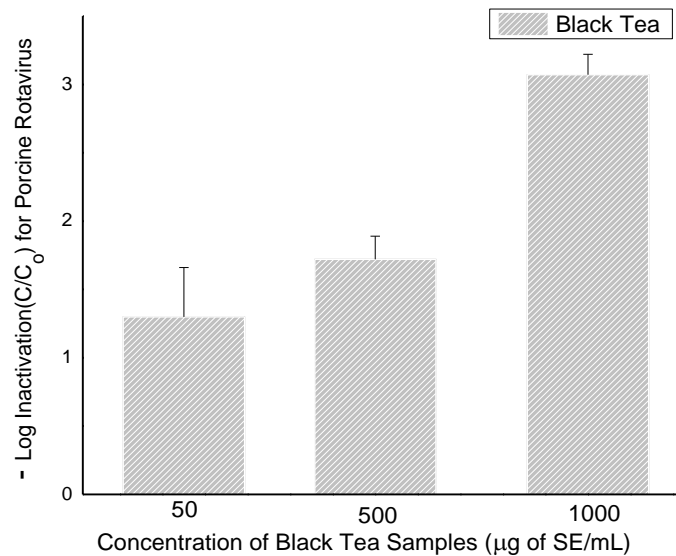
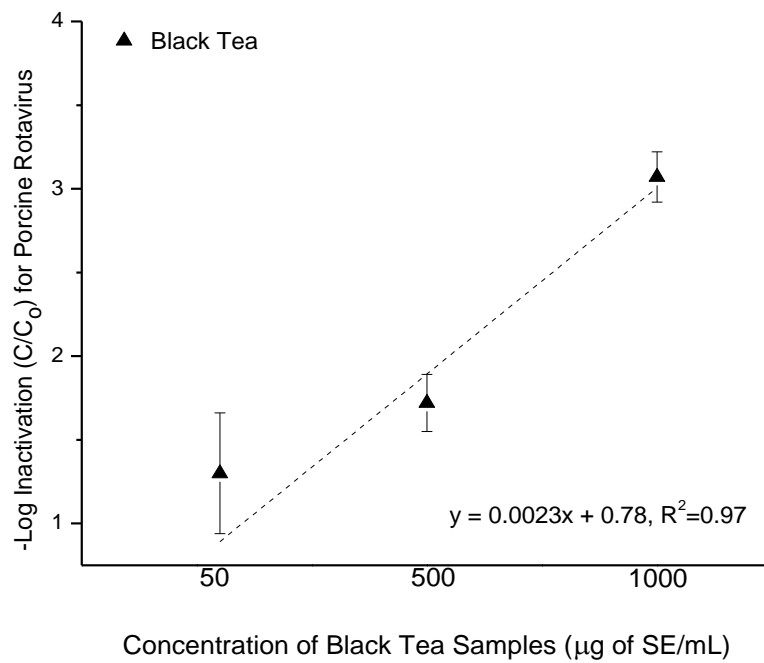
The log inactivation values are the average of fresh tea preparations in triplicates (N=3). Reported statistical comparison is reported in the above table. (ns) stands for not significant.

Figure 10-REMOVAL OF PORCINE ROTAVIRUS IN AQUEOUS WHITE TEA EXTRACT



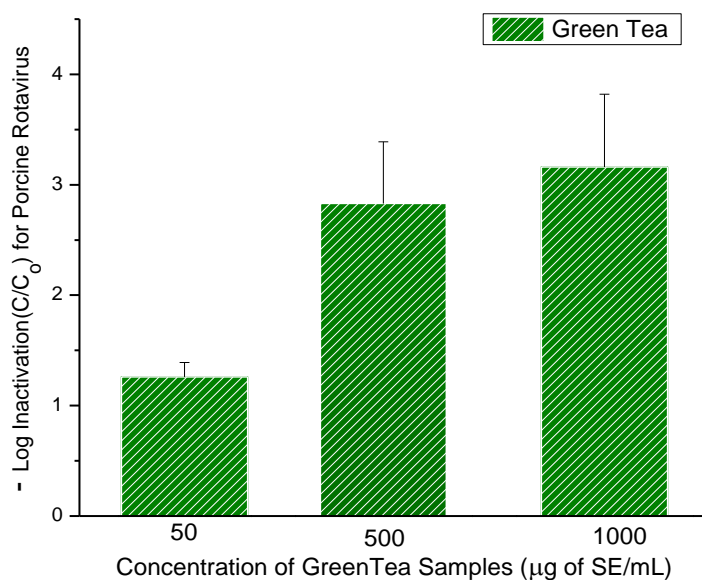
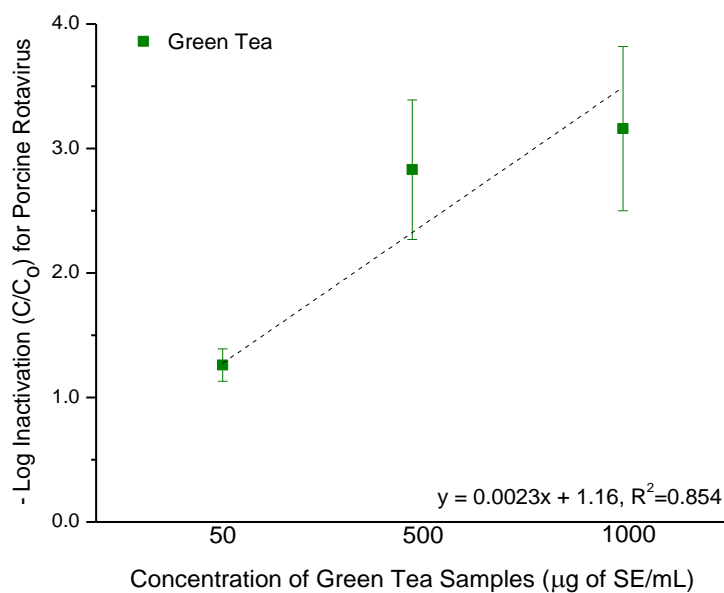
These are an average of (N=3) for each concentration
Values are not statistically different ($P > 0.001$).

Figure 11-REMOVAL OF PORCINE ROTAVIRUS IN AQUEOUS BLACK TEA EXTRACT



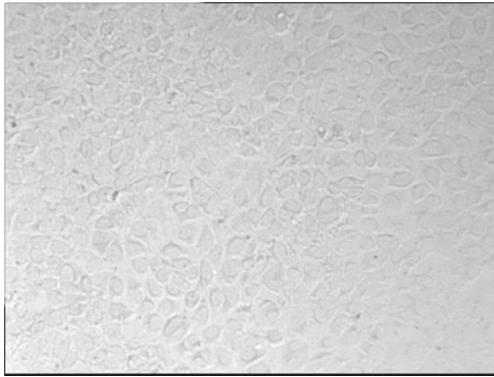
These are an average of (N=3) for each concentration.
 Statistical difference, only occurs at value 1000 μg SE/ mL
 Compared to the other values ($P < 0.001$).

Figure 12-REMOVAL OF PORCINE ROTAVIRUS IN AQUEOUS GREEN TEA EXTRACT

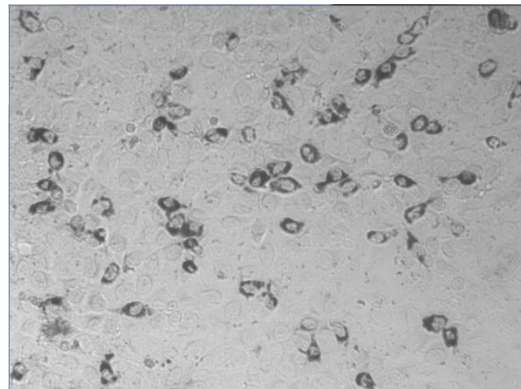


These are an average of (N=3) for each concentration.
 Statistical difference, occurs at 50 µg SE/ mL when compared to 500 and 1000 µg SE/ mL, ($P < 0.001$).
 No statistical difference between 500 and 1000 µg SE/ mL, ($P > 0.001$).

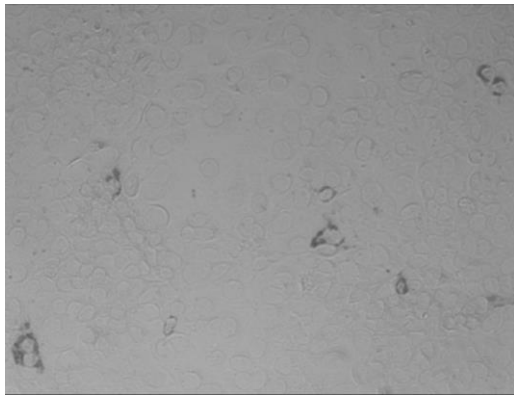
Figure 13-MA-104 Cells after FFU Experiments to detect rotavirus after tea treatment



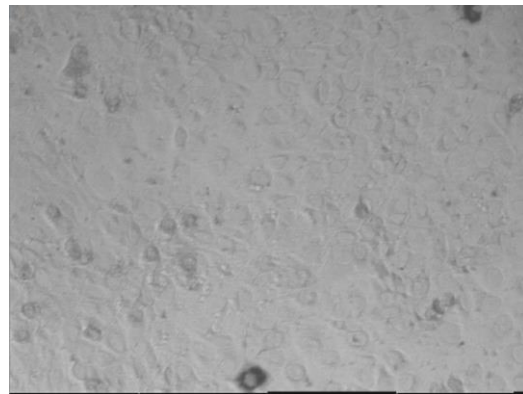
MA-104 Cells Only-PBS only-(20x)



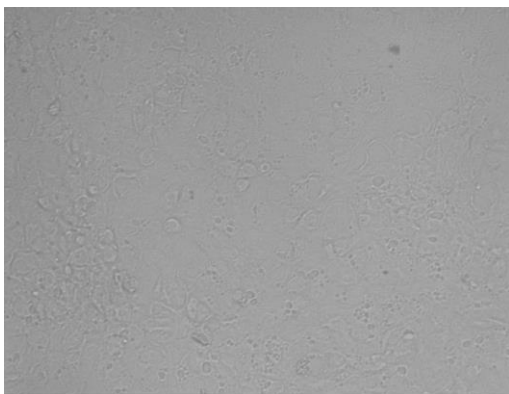
MA-104 cells infected with rotavirus-no tea-(20x)



MA-104 cells treated with 1000 µg SE/mL Black Tea (20x)



MA-104 cells treated with White Tea
1000 µg SE/ml (20x)



MA-104 cells treated with Green Tea
1000 µg SE/ml (20x)

CHAPTER 5

CONCLUSION

- a. The phenolic catechins in White, Black and Green Teas were GA, QT, CT, EC, EGC, GCG, EGCG.
- b. Quercetin, a flavonol, were also found in White, Black and Green Teas.
- c. Black, and Green Teas at confirmed concentrations 1000 µg of SE/mL, are effective doses for porcine rotavirus inhibition in African green (MA-104) monkey kidney cells.
- d. Quantification of tea polyphenol concentrations are associated with high effective inhibition dosages, shown in **Table 6**. This suggested the type of polyphenol and concentrations found in White, Black and Green Teas, play a role in inhibiting rotavirus survival in aqueous environments.
- e. White, Black and Green Teas phenolic composition may also inhibit oxidative stress and inflammation in the kidney as related to scavenging abilities of teas (Robards et al., 1999; Yoon et al., 2005).
- f. The chemical structure of tea catechins, found in **Figure 5**, donate OH radicals from the Aromatic B and C ring on the 3', 3 carbon position, respectively (Zhu et al., 1997). OH radicals are the most reactive of ROS and have been previously implicated with inactivation of pathogens (Romero et al., 2011; Juarez et al., 2010; Cho et al., 2004).
- g. The antioxidant capacity of tea extract and rotavirus inhibition may be associated with, presence of gallate groups and scavenging ability of polyphenols.
- h. Scavenging effects of catechin structures on ROS, were higher in more gallated catechins like EGC, GCG and EGCG (Nanjo et al., 1996). This may advocate that Green Tea, suggests a higher inhibition of porcine rotavirus, when compared to Black and White Tea.

- i. Further studies discerning inhibition of porcine rotavirus, in tea aqueous solutions; are needed to understand the inactivation mechanisms of polyphenols.
- j. Furthermore, additional research should be conducted on the use of tea polyphenols as inhibitors of kidney and gut inflammation, as a result of induced porcine rotavirus infection in humans and animals (Luce, 2011).

CHAPTER 6

REFERENCES

- Alejandro, M. B. and Domingo, J. D. et al. "Circovirus and impact of temporary withdrawal of rotavirus vaccines in Spain." *Human vaccines* 7.7 (2011):798-799.
- Alfajaro, Mia M., et al. "Anti-rotaviral effects of Glycyrrhiza uralensis extract in piglets with rotavirus diarrhea." *Virology journal* 9.1 (2012):310.
- Almajano, M. P., et al. "Antioxidant and antimicrobial activities of tea infusions." *Food chemistry* 108.1 (2008):55-63.
- Arnold, M. and McDonald, S. et al. (2009). "Culturing, Storage, and Quantification of Rotaviruses." *Current protocols in microbiology*.
- Baker, M.N. (1949). *The quest for pure water; the history of water purification from the earliest records to the twentieth century*. Public Domain, Google-digitized. p. 1864-1955.
- Bicudo, J. R.; Goyal, S. M. "Pathogens and manure management systems: A review." *Environmental technology*, 24.1 (2003):115-130.
- Carloni, P. et al. "Antioxidant activity of white, green and black tea obtained from the same tea cultivar." *Food research international* 53.2 (2013):900-908.
- Chandra, S.; Gonzalez de Mejia, E. "Polyphenolic Compounds, Antioxidant Capacity, and Quinone Reductase Activity of an Aqueous Extract of *Ardisia compressa* in Comparison to Mate (*Ilex paraguariensis*) and Green (*Camellia sinensis*) Teas." *Journal of agricultural and food chemistry* 52.11 (2004):3583-3589.
- Chen, Z. et al. "Microbial fermented tea – a potential source of natural food preservatives." *Trends in food science & technology* 19.3 (2008):124-130.

- Cho, Min, et al. "Linear correlation between inactivation of E. coli and OH radical concentration in TiO₂ photocatalytic disinfection." *Water research* 38.4 (2004):1069-1077.
- De Mejia et al. "Bioactive components of tea: Cancer, inflammation and behavior." *Brain, behavior, and immunity* 23.6 (2009):721-731.
- Del Rio, D. et al. "HPLC-MSn Analysis of Phenolic Compounds and Purine Alkaloids in Green and Black Tea." *Journal of agricultural and food chemistry* 52.10 (2004):2807-2815.
- Dufresne, C.; Farnworth, F. "Tea, Kombucha, and health: a review." *Food research international* 33.6 (2000):409-421.
- Ford, T. (1999). Microbial safety of drinking water: United States and global perspectives. *Environ. Health Perspect.* 107. (suppl.1):191-206.
- Gabbay, et al. (1999). "An outbreak of group C rotavirus gastroenteritis among children attending a day-care centre in Belém, Brazil." *Journal of diarrheal diseases research.* 17. (2). p. 69-74. <http://www.ncbi.nlm.nih.gov/pubmed/10897889>.
- Gillespie, J. "The isolation, propagation and characterization of tissue-cultured equine rotaviruses." *Veterinary microbiology* 9.1 (1984):1-14.
- Graham, H. N. "Green tea composition, consumption, and polyphenol chemistry." *Preventive medicine* 21.3 (1992):334-350.
- Gu, Y. et al. "Development of antirotavirus agents in Asia." *Pediatrics international* 42.4 (2000):440-447.

- Hong Anh, P. et al. "The prevalence and genetic diversity of group A rotaviruses on pig farms in the Mekong Delta region of Vietnam." *Veterinary microbiology* 170.3-4 (2014):258-265.
- Huang, D. et al. "The Chemistry behind Antioxidant Capacity Assays." *Journal of agricultural and food chemistry* 53.6 (2005):1841-1856.
- Juarez, N. et al. "Inactivation of MS2 coliphage in Fenton and Fenton-like systems: role of transition metals, hydrogen peroxide and sunlight." *Environmental science & technology* 44.9 (2010):3351-3356.
- Knipping, K. et al. "An evaluation of the inhibitory effects against rotavirus infection of edible plant extracts." *Virology journal* 9.1 (2012):137.
- Lin, L.; Harnly, J. et al. "New Phenolic Components and Chromatographic Profiles of Green and Fermented Teas." *Journal of agricultural and food chemistry* 56.17 (2008):8130-8140.
- Lopman, B. A., et al. "Infant Rotavirus Vaccination May Provide Indirect Protection to Older Children and Adults in the United States." *The Journal of infectious diseases* 204.7 (2011):980-986.
- Luce, N. (2011). "Proprietary green tea extract protects the kidneys." *Life Extension*, June, 2011, p. 66(9).
- Lynch, M., et al. "The Pathology of Rotavirus-Associated Deaths, Using New Molecular Diagnostics." *Clinical infectious diseases* 37.10 (2003):1327-1333.
- Matthijnssens, J. et al. "Full-length genomic analysis of porcine G9P[23] and G9P[7] rotavirus strains isolated from pigs with diarrhea in South Korea." *Infection, genetics and evolution* 12.7 (2012):1427-1435.

- Mawdsley, J. L. et al. "Pathogens in livestock waste, their potential for movement through soil and environmental pollution." *Applied soil ecology* 2.1 (1995):1-15.
- Morikawa, T. et al. "Flavonol glycosides with lipid accumulation inhibitory activity and simultaneous quantitative analysis of 15 polyphenols and caffeine in the flower buds of *Camellia sinensis* from different regions by LC-MS." *Food chemistry* 140.1-2 (2013):353-360.
- Mukoyama et al. "Inhibition of rotavirus and enterovirus infections by tea extracts." *Japanese Journal of Medical Science and Biology* 44.4 (1991):181-186.
- Nanjo, F. et al. "Scavenging effects of tea catechins and their derivatives on 1, 1-diphenyl-2-picrylhydrazyl radical." *Free radical biology & medicine* 21.6 (1996):895-902.
- Ndhlala et al, (2010). Natural antioxidants: fascinating or mythical biomolecules? *Molecules. (Basel, Switzerland)*. 15. (10) p. 6905-30.
<http://www.ncbi.nlm.nih.gov/pubmed/20938402>.
- Newell, A.M.B., et al. "Comparative in vitro bioactivities of tea extracts from six species of *Ardisia* and their effect on growth inhibition of HepG2 cells." *Journal of ethnopharmacology* 130.3 (2010):536-544.
- Pal, S. et al. "Studies on black tea (*Camellia sinensis*) extract as a potential antioxidant and a probable radioprotector." *Radiation and environmental biophysics* 52.2 (2013):269-278.
- Robards, K. et al. "Phenolic compounds and their role in oxidative processes in fruits." *Food chemistry* 66.4 (1999):401-436.

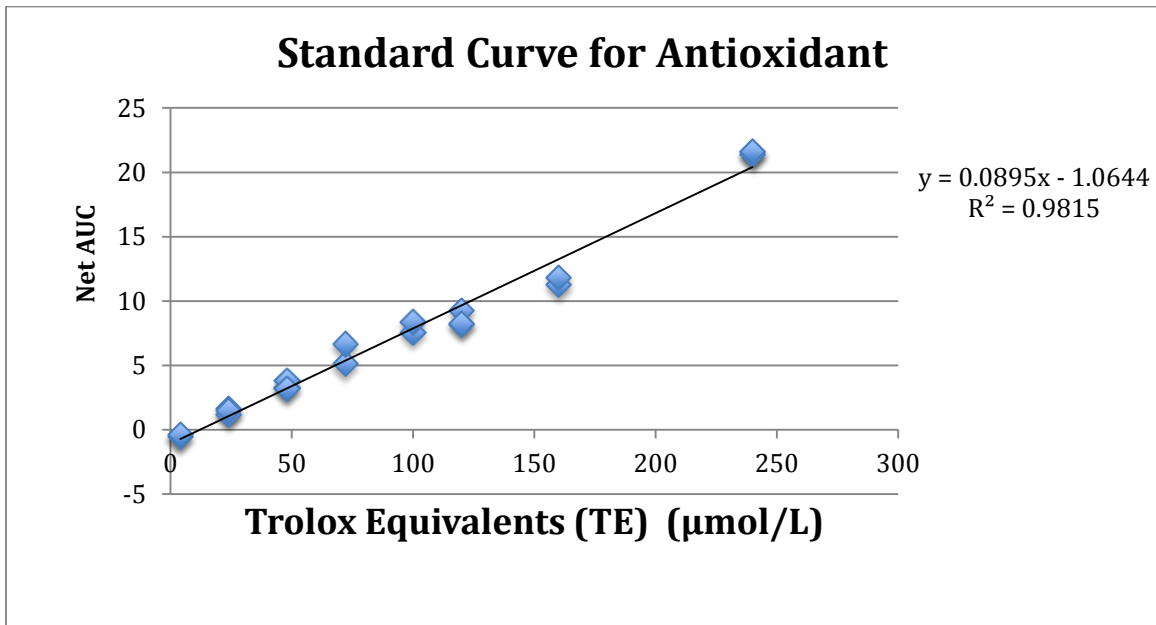
- Rolsma, M. et al. "Assay for evaluation of rotavirus-cell interactions: Identification of an enterocyte ganglioside fraction that mediates group A porcine rotavirus recognition." *Journal of virology* 68.1 (1994):258-268.
- Romero Maraccini, Ofelia C., et al. "Sunlight-Induced Inactivation of Human Wa and Porcine OSU Rotaviruses in the Presence of Exogenous Photosensitizers." *Environmental science & technology* 47.19 (2013):11004-11012.
- Romero, Ofelia C., et al. "Role of Temperature and Suwannee River Natural Organic Matter on Inactivation Kinetics of Rotavirus and Bacteriophage MS2 by Solar Irradiation." *Environmental science & technology* 45.24 (2011):10385-10393.
- Ruiz, et al, (1999). Characterization of a membrane calcium pathway induced by rotavirus infection in cultured cells. *Journal of virology*. 73. p. 2481-2490.
- Sánchez-Rangel, J. et al. (2013).The Folin–Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination. *Analytical Methods* 5. p. 5990.
- Song, J. et al. "Antiviral effect of catechins in green tea on influenza virus." *Antiviral research* 68.2 (2005):66-74.
- Tanaka, T. et al. "Oxidation of tea catechins: Chemical structures and reaction mechanism." *Food Science and Technology Research* 9.2 (2003):128-133.
- Theil, K. W.; Saif, Linda J., eds. *Viral Diarrheas Of Man And Animal*. Boca Raton, Fla.: CRC Press, 1990. Print.
- U.S. Department of Agriculture. 1992. *National engineering handbook: Agricultural waste management field handbook*. Part 651 (210-AWMFH, 4/92). Chapters 4 and 11. USDA, Washington, DC.

- U.S. Environmental Protection Agency. 1998. Environmental impacts of animal feeding operations. USEPA Office of Water, Standards and Applied Sci. Div., Washington, DC.
- Ueda, K. et al. "Inactivation of Pathogenic Viruses by Plant-Derived Tannins: Strong Effects of Extracts from Persimmon (*Diospyros kaki*) on a Broad Range of Viruses." PLoS ONE 8.1 (2013):e55343.
- Waddington, H. et al. (2009). Effectiveness and sustainability of water, sanitation, and hygiene interventions in combating diarrhea. *Journal of Development Effectiveness*. 1. (3). p. 295-335.
- WHO. "Global Use Of Rotavirus Vaccines Recommended." *Indian Journal Of Medical Sciences* 63.6 (2009): 272-273. *Academic Search Premier*. Web. 2 May 2014.
- World Bank. (2014). Poverty headcount ratio at \$1.25 a day (PPP) (% of population). <http://data.worldbank.org/indicator/SI.POV.DDAY>.
- World Health Organization (WHO). 1998. *The World Health Report 1998. Life in the 21st century. A vision for all*. Geneva: The Organization.
- World Health Organization. (2007). WHO. *Bulletin of the World Health Organization*. 85. (Past Issues) p.1.
- World Health Organization. (2009). Global use of rotavirus vaccines recommended. p.1.
- World Health Organization. <http://www.who.int/immunization/topics/rotavirus/en/>. Immunization, Vaccines and Biologicals: Rotavirus. (2004).
- Yoon, J. H. et al. "Molecular targets of dietary polyphenols with anti-inflammatory properties." *Yonsei Medical Journal* 46.5 (2005):585-596.
- Yu, Y. et al. "PLA2G4 A mutants modified protective effect of tea consumption against colorectal cancer." *International Journal of Colorectal Disease* 27.8 (2012):1005-1013.

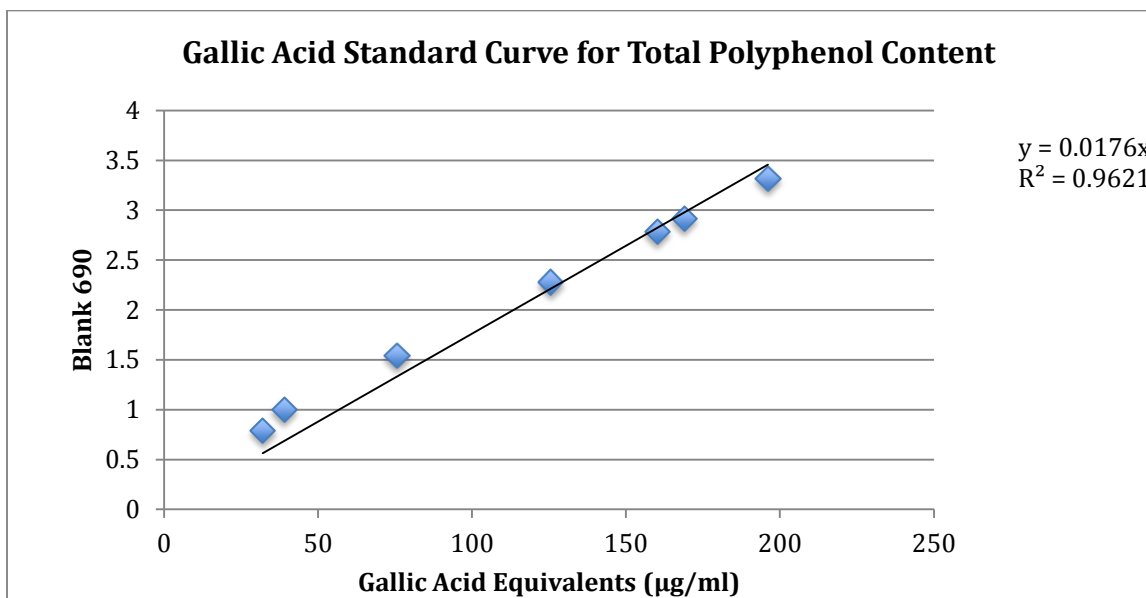
- Zhu, Qin Y., et al. "Stability of Green Tea Catechins." *Journal of agricultural and food chemistry* 45.12 (1997):4624-4628.
- Zimmerman, B. et al. (2006). 43. Diseases of Swine. *Blackwell Publishing*. p. 3-489.

APPENDICES

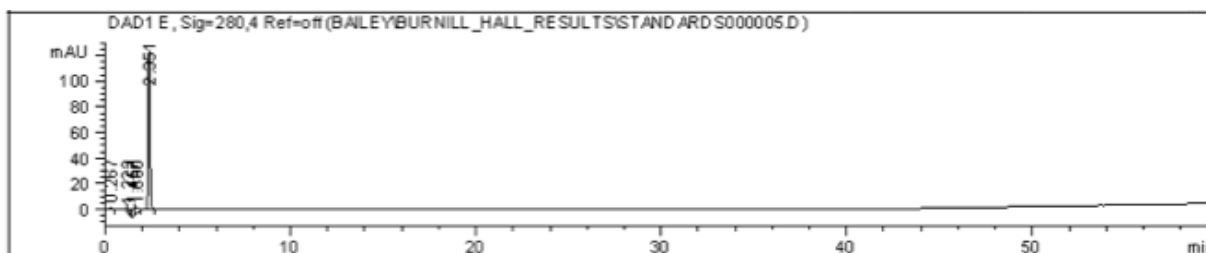
APPENDIX A. ANTIOXIDANT CAPACITY STANDARD CURVE



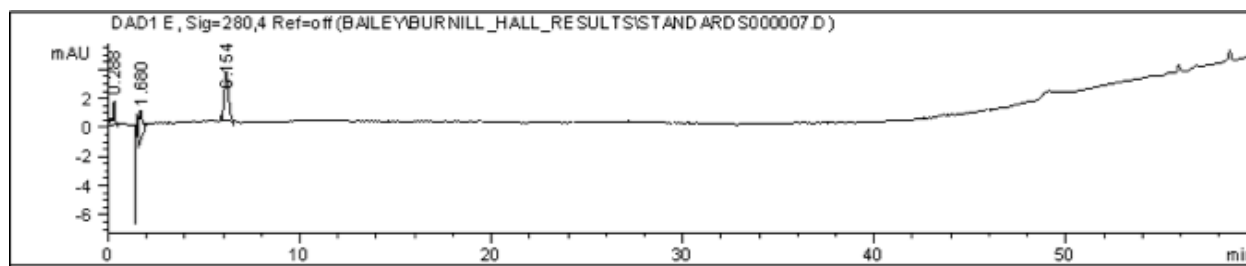
APPENDIX B.
TOTAL POLYPHENOL CONTENT STANDARD CURVE



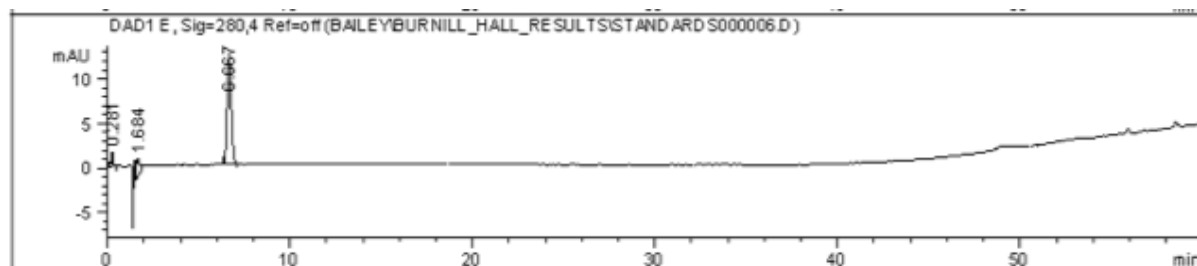
APPENDIX C. HPLC CHROMATOGRAM OF PURE STANDARDS



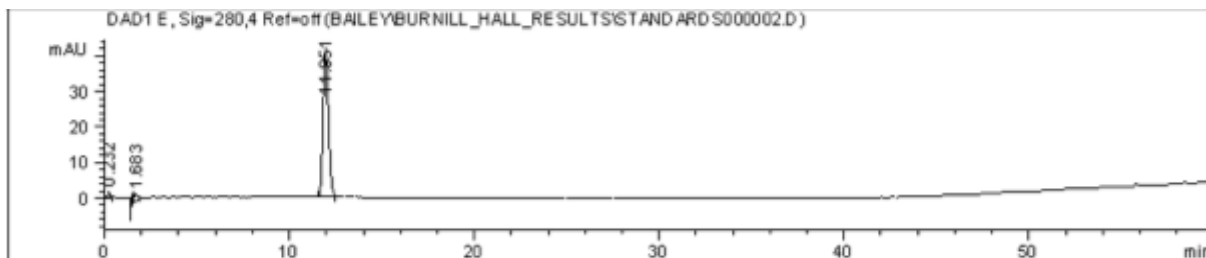
Gallic Acid (GA)-0.01mg/ml, RT~2.3



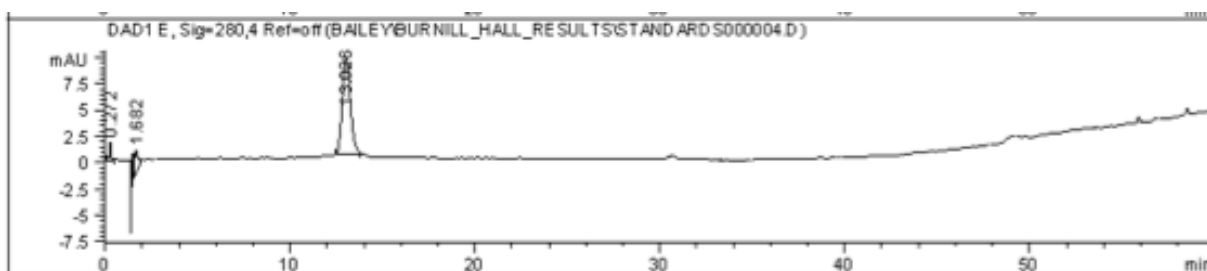
Epigallocatechin (EGC)-0.01mg/ml, RT~6.15



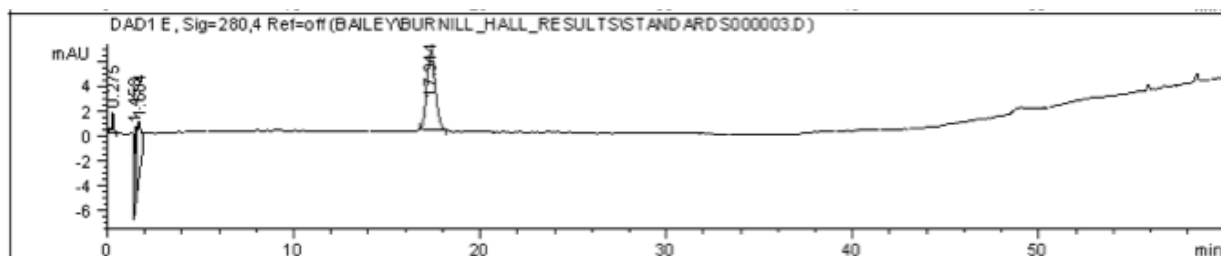
Catechin(CT)-0.01mg/ml, RT~6.6



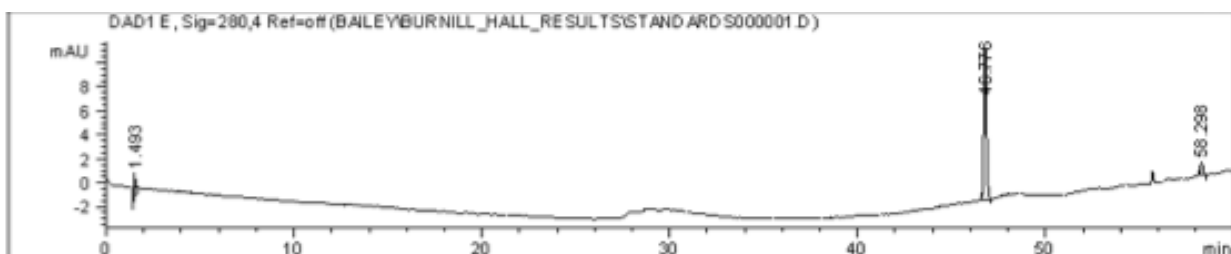
Caffeic Acid-0.01mg/ml, RT~12



Epigallocatechin gallate (EGCG)-0.01mg/ml, RT~13.00

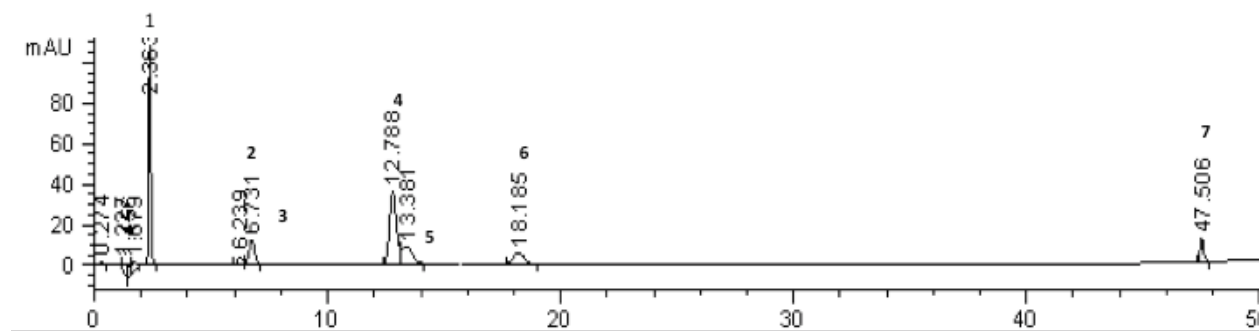


Epicatechin-0.01mg/ml, RT~17.3



Quercetin hydrate- 0.1mg/ml, RT~46.77-50

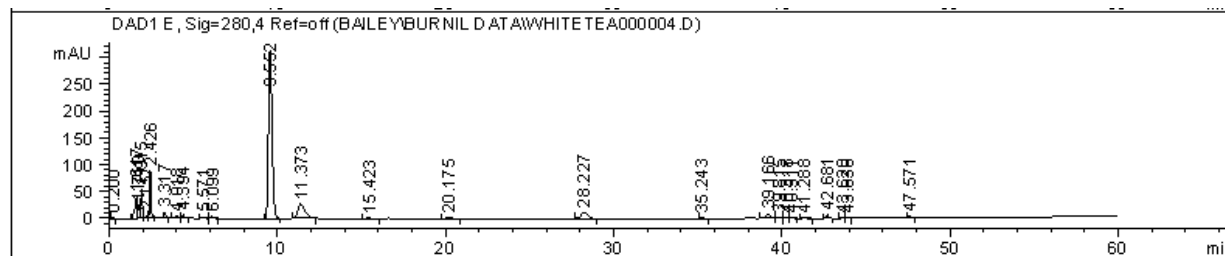
APPENDIX D. **CHROMATOGRAM OF MIXED PURE STANDARDS**



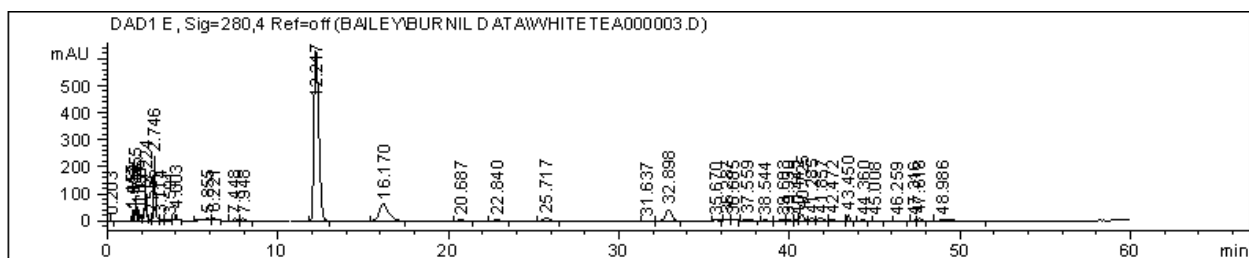
Tea Peak Number/ Compound	Standard	RT (min)	Molecular Weight
1	Gallic Acid Monohydrate	2.36	188.13
2	(-)-Epigallocatechin	6.24	306.27
3	(+)-Catechin hydrate	6.73	290.27
4	Caffeic Acid	12.75	180.16
5	(-)-Epigallocatechin gallate	13.81	458.37
6	Epicatechin	18.18	290.27
7	Quercetin hydrate	47.50	302.24

APPENDIX E. CHROMATOGRAMS OF WHITE TEA

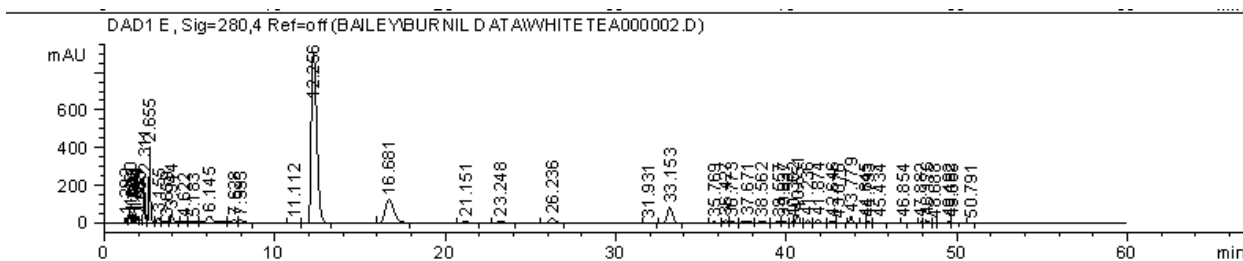
White Tea-Concentration 1 mg/ml at 280nm



White Tea-Concentration 3 mg/ml at 280nm

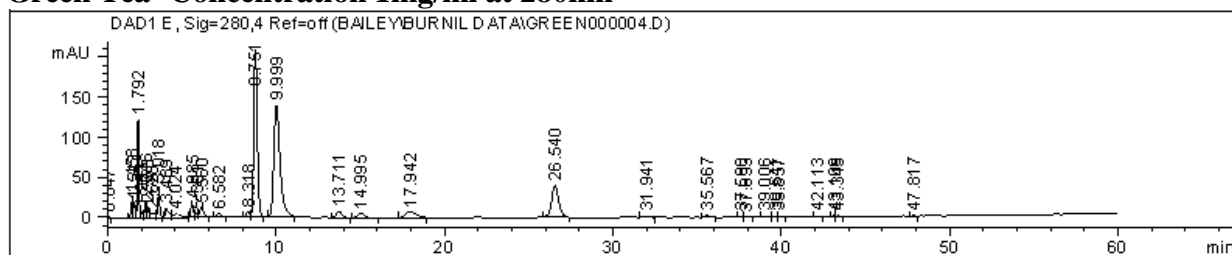


C) White Tea-Concentration 5 mg/ml at 280nm

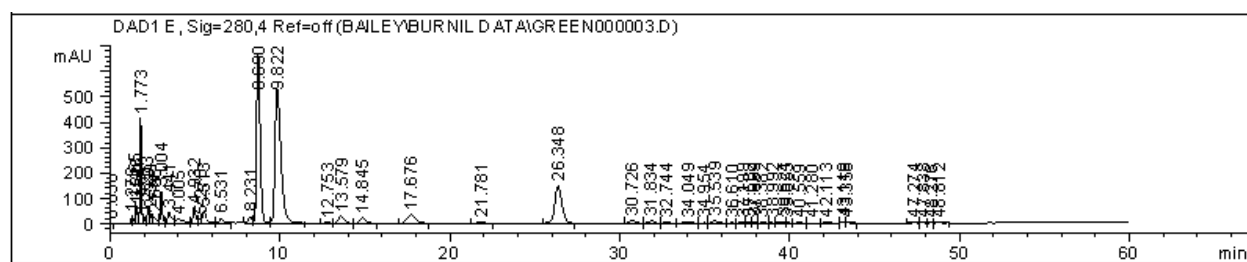


APPENDIX G. CHROMATOGRAMS OF GREEN TEA

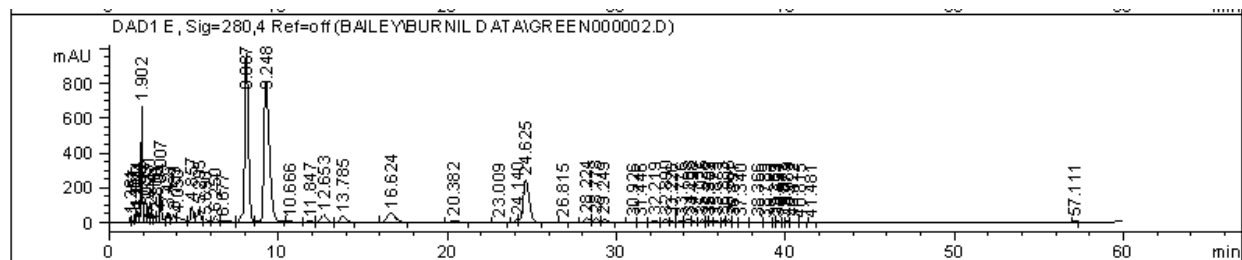
Green Tea- Concentration 1mg/ml at 280nm



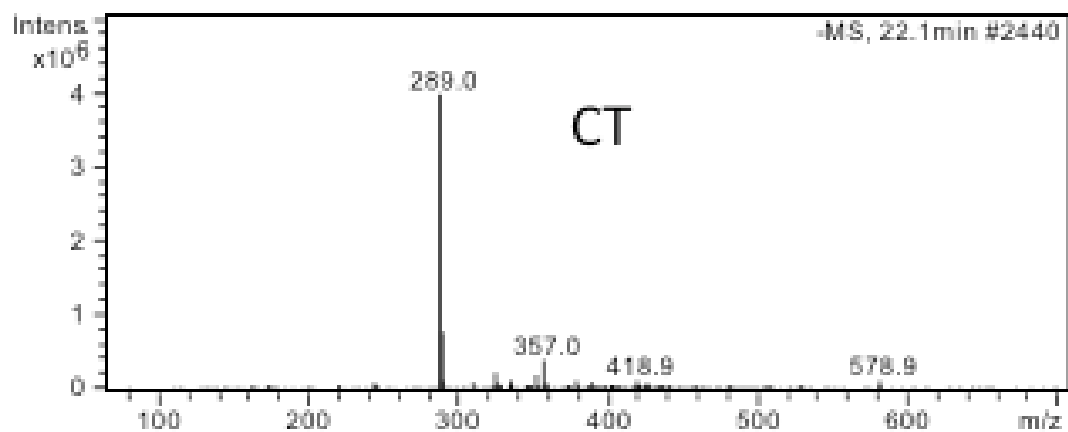
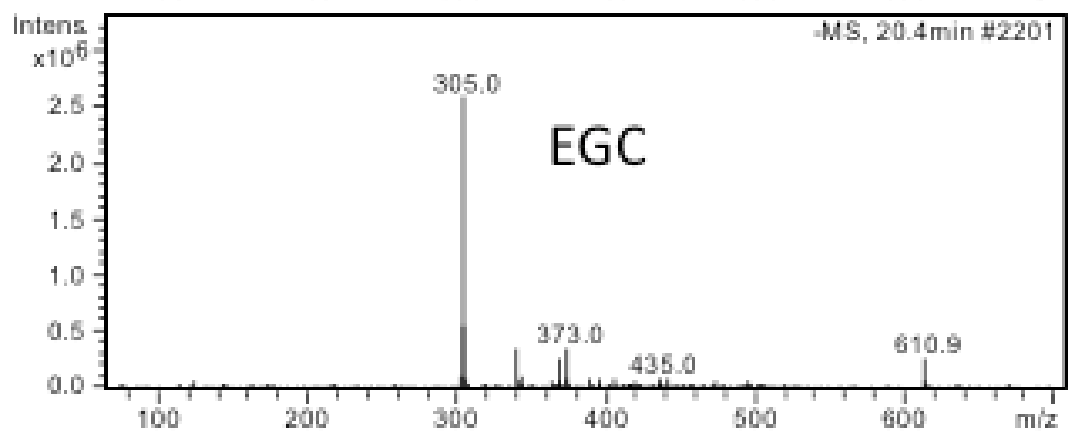
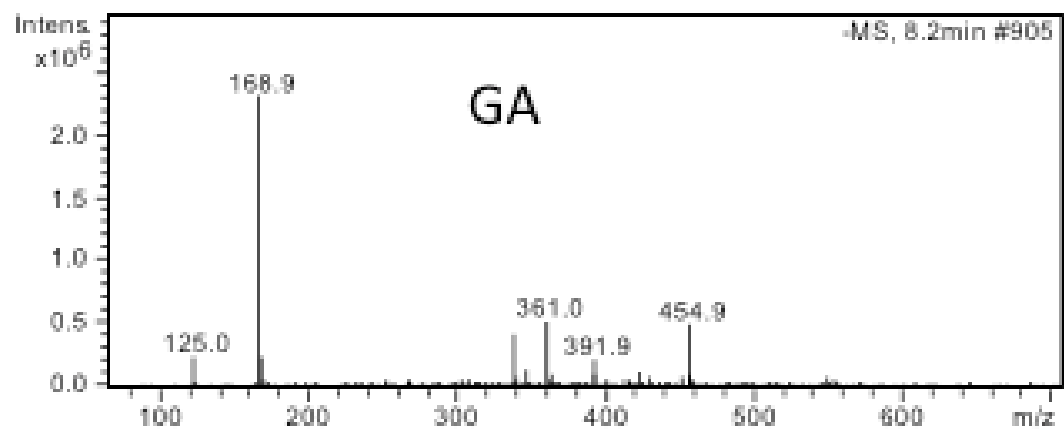
Green Tea- Concentration 3mg/ml at 280nm

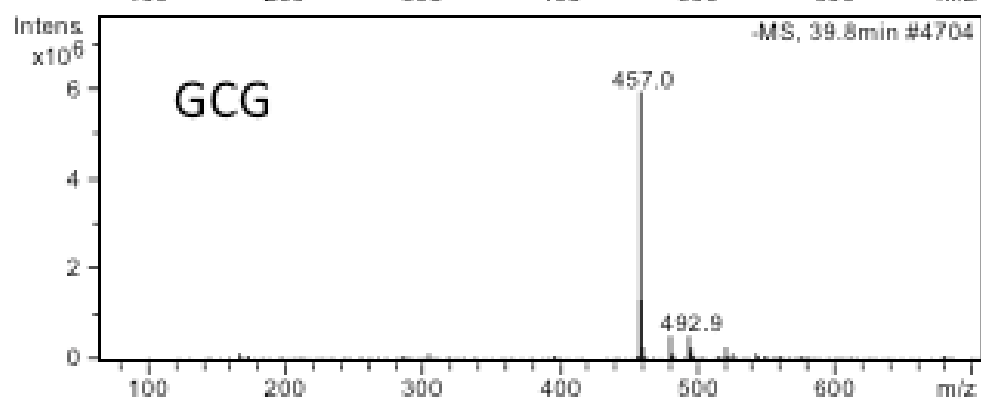
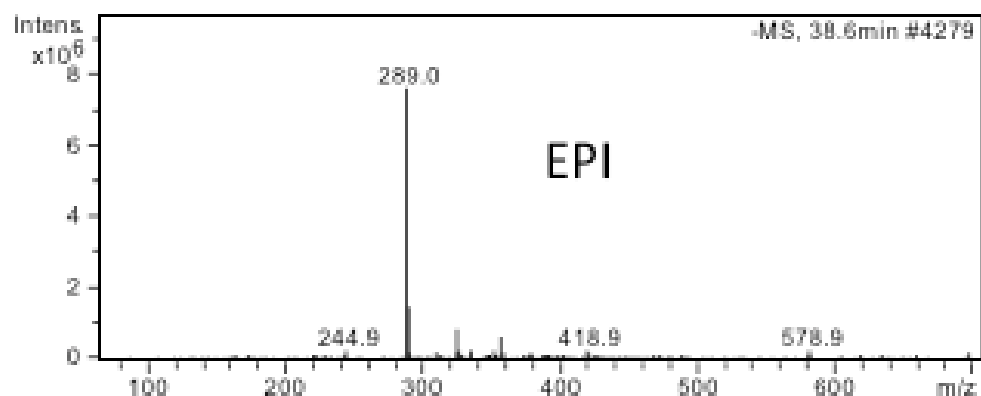
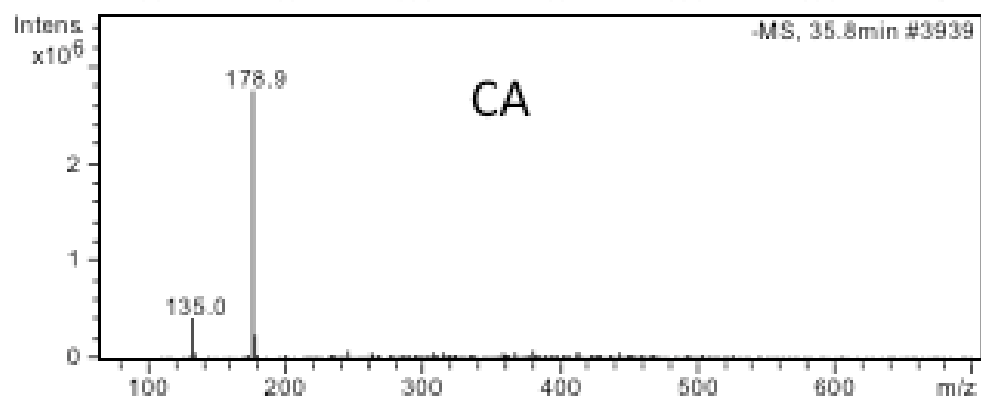
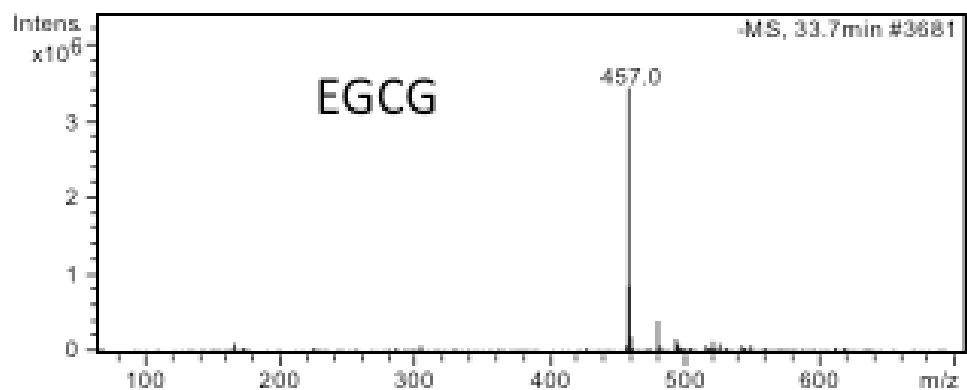


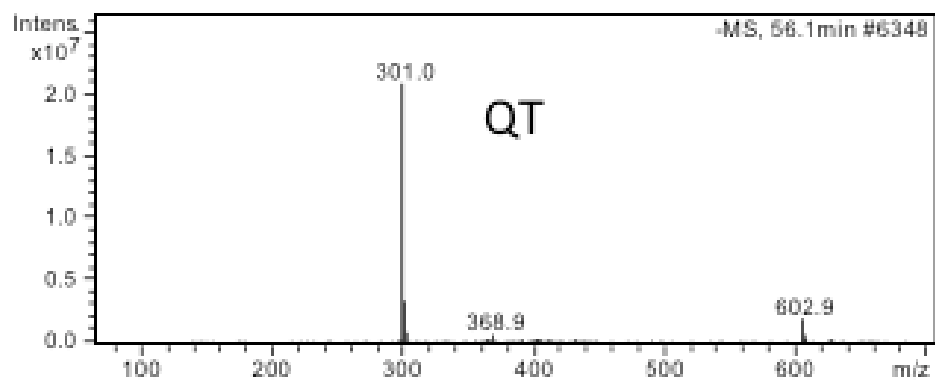
Green Tea- Concentration 5mg/ml at 280nm



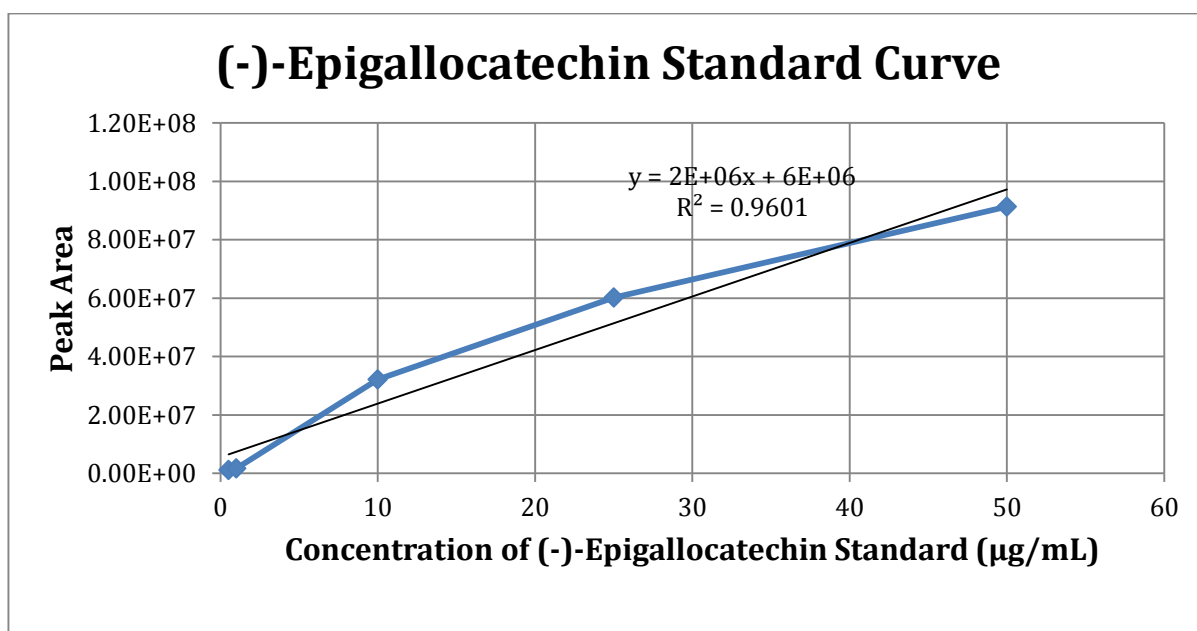
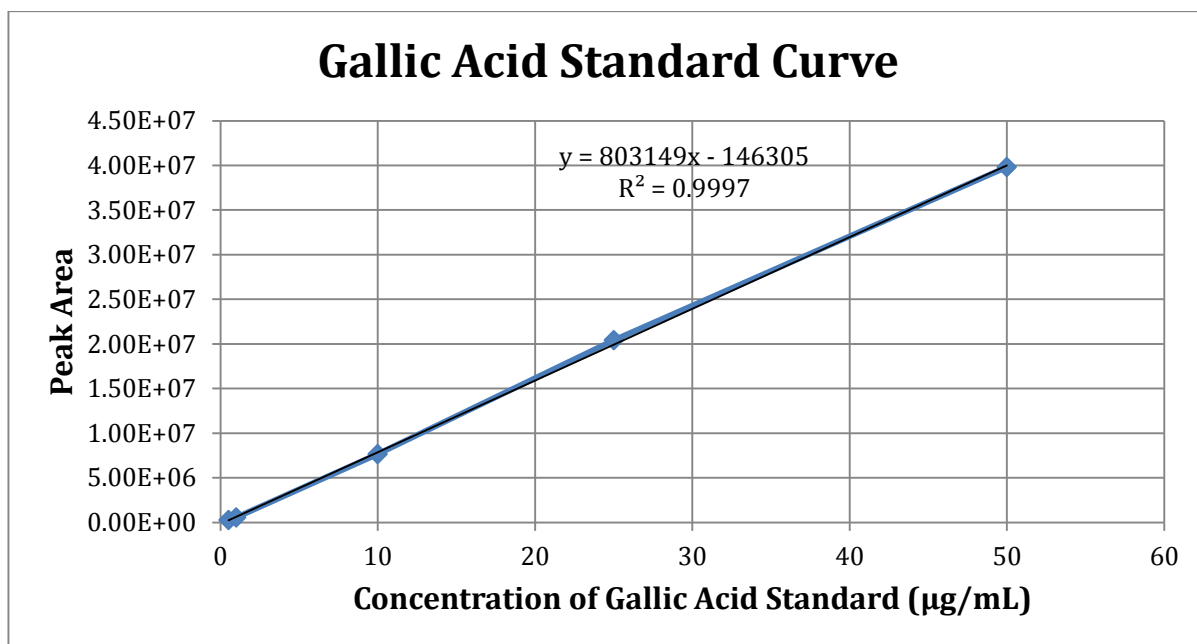
APPENDIX H.
LC-MS CHROMATOGRAMS OF PURE STANDARDS



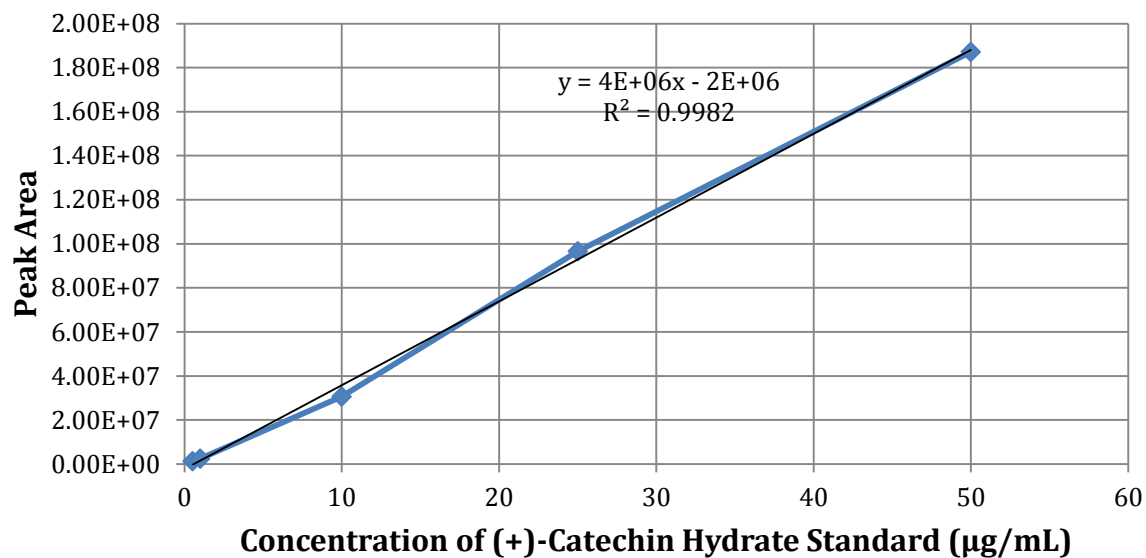




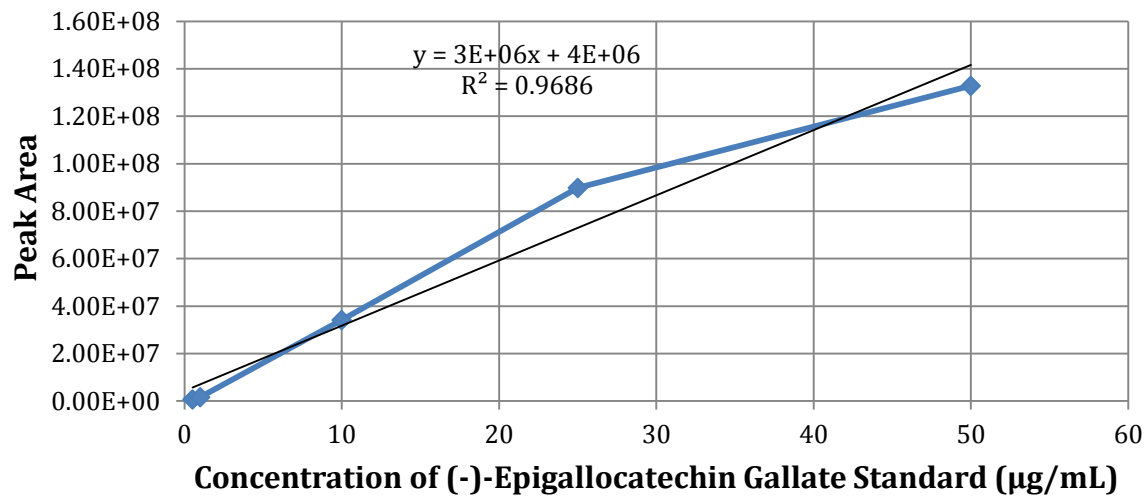
APPENDIX I.
STANDARD CURVES FOR PURE STANDARDS



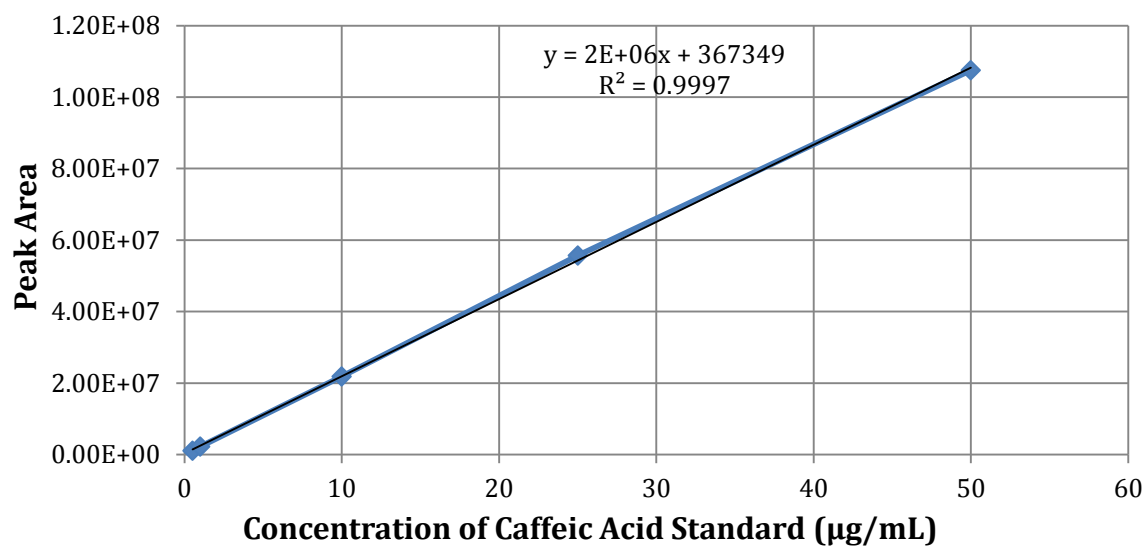
(+)-Catechin Hydrate Standard Curve



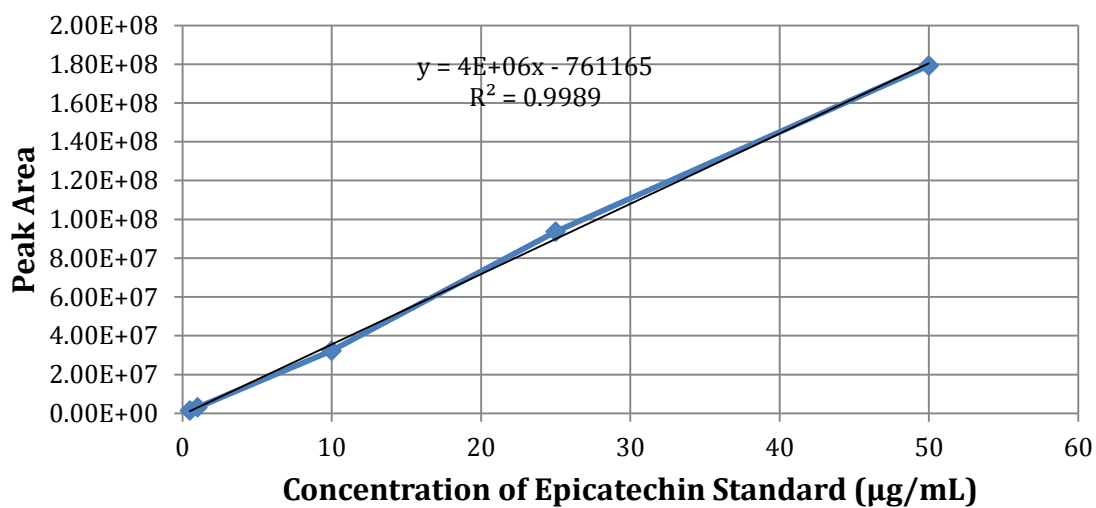
(-)-Epigallocatechin Gallate Standard Curve



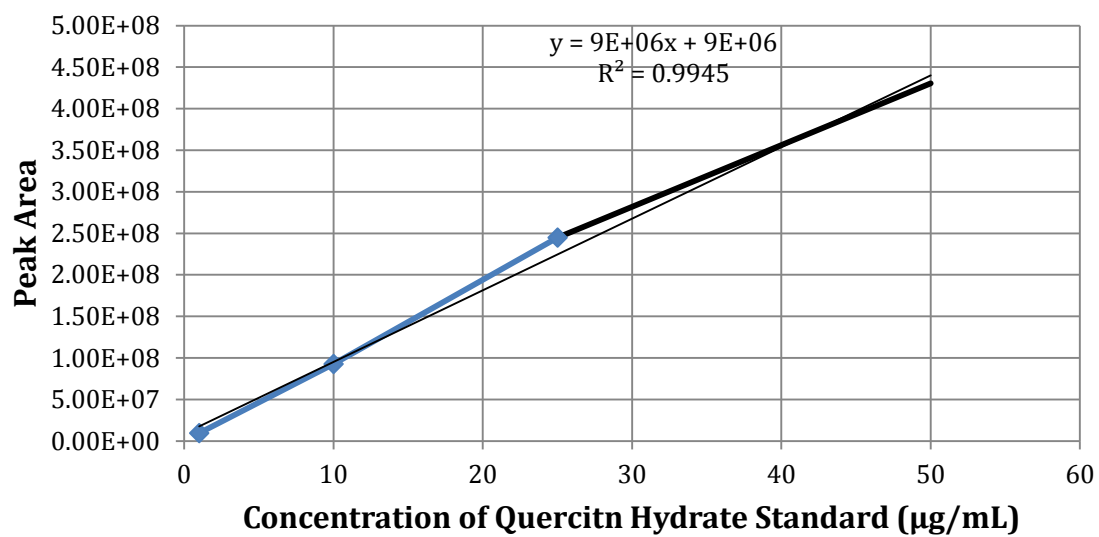
Caffeic Acid Standard Curve



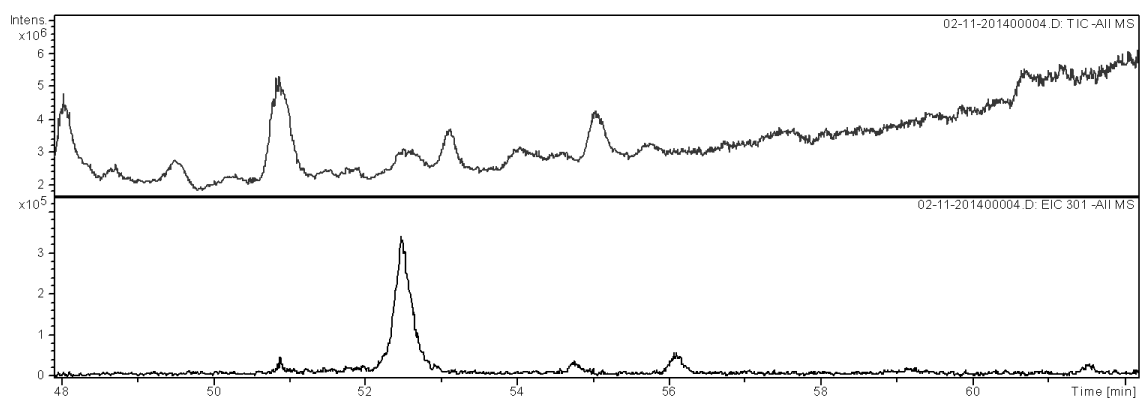
Epicatechin Standard Curve



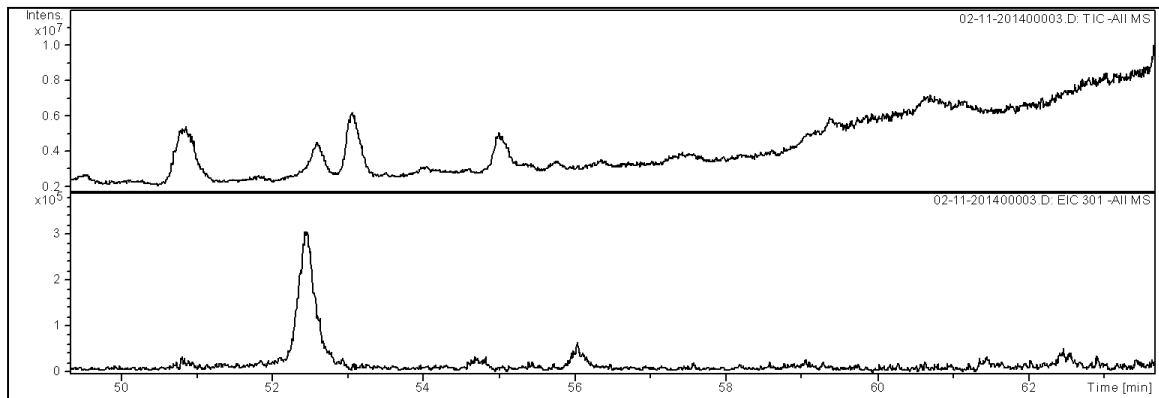
Quercetin Hydrate Standard Curve



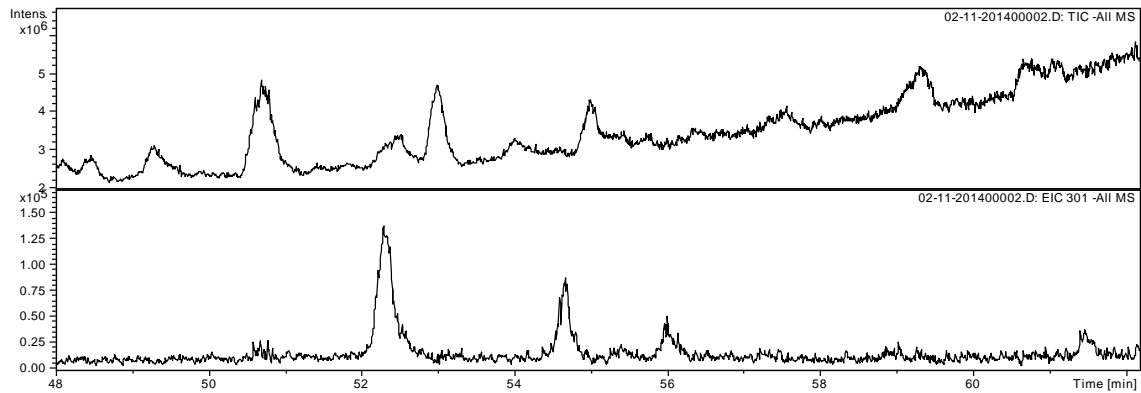
APPENDIX J.
QUERCETINE CHROMATOGRAM FOR WHITE TEA



APPENDIX K.
QUERCETINE CHROMATOGRAM FOR BLACK TEA



APPENDIX L.
QUERCETINE CHROMATOGRAM FOR GREEN TEA



APPENDIX M.
ACTUAL CONCENTRATIONS OF POLYPHENOLS IN TEAS

μmol/mL/mg SE	Green Tea	Black Tea	White Tea
GA	11.1	41.6	44.6
EGC	251.3	84.5	31.5
CT	16.5	13.1	3.4
EGCG	329.8	244.9	106.1
EPI	56.7	38.3	14.7
QT	2.5	2.9	2.3

μg/mg SE	Green Tea	Black Tea	White Tea
Gallic Acid	1.9	7.1	7.6
(-)Epigallocatechin	76.9	25.9	9.6
Catechin Hydrate	4.8	3.8	1.0
(-) Epigallocatechin gallate	151.0	112.1	48.6
Epicatechin	16.5	11.1	4.3
Quercetin hydrate	0.7	0.9	0.7

APPENDIX N. CYTOTOXICITY STATISTICS

Mean Rank

Comparison	Difference	P value
Black(100) vs. White(100)	4.250	ns P>0.05
Black(100) vs. Green(100)	1.375	ns P>0.05
White(100) vs. Green(100)	-2.875	ns P>0.05

Mean Rank

Comparison	Difference	P value
Black(150) vs. White(150)	-2.375	ns P>0.05
Black(150) vs. Green(150)	1.625	ns P>0.05
White(150) vs. Green(150)	4.000	ns P>0.05

Mean Rank

Comparison	Difference	P value
Black(200) vs. White(200)	-3.250	ns P>0.05
Black(200) vs. Green(200)	0.3750	ns P>0.05
White(200) vs. Green(200)	3.625	ns P>0.05

All P values for teas at 100ug/ml, 150ug/ml, 200ug/ml and 200ug/ml, 300ug/ml are not significant meaning they are more similar to each other than different.