Antioxidant activity and nitric oxide scavenging abilities of *Ilex guayusa* compared with *Camellia sinensis* and *Asphalathus linearis* teas

By

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Abstract

Guayusa tea, an extract of the *Ilex guayusa* plant, is ritualistically consumed by the Runa tribes of Ecuador. It is known for its healing abilities and for giving lucid dreams as well as being used by the Amaguajes Indians of South America to treat diabetes and has been shown to retard the development of steptozocin-induced diabetes in mice. Guayusa tea is now commercially available as Runa tea and in select areas of the USA as tea bags or iced tea. Guayusa tea has not been scientifically evaluated, or compared with more popular commercialised tea brands such as *Camellia sinensis* black tea (BT), green tea (GT) and *Aspalathus linearis* rooibos tea (RT), regarding possible health benefits. The aim of this study is to address this limitation in knowledge regarding the antioxidant and anti-inflammatory properties of this tea.

Chemical evaluation was performed according to the International Organisation of Standardisation (ISO) approved methods. Methanol extracts of commercially available guyasa tea and crushed factory leaves of fermented and unfermented guaysua tea was compared to nine brands of black and green *Camellia sinensis* tea brands. Antioxidant reducing capacity was observed with the use of an ISO-approved Folin-Ciocalteu (F-C) method. To elaborate on findings antioxidant activity were further evaluated using the Trolox equivalent antioxidant capacity (TEAC), 1,1-diphenyl-2-picrylhyrazyl (DPPH) and oxygen radical antioxidant capacity (ORAC) assays. The methods that involve an electron transfer mode of action such as the F-C, TEAC and DPPH showed antioxidant activity of all samples of guayusa tea to be significantly lower than all *Camellia sinensis* brands evaluated. In contrast the results obtained from the

ORAC assay, which involves a hydrogen atom transfer mode of action showed that guayusa tea had statistically similar antioxidant capacity to GT and BT *Camellia sinensis* brands.

To assess the possible health benefits associated with guayusa tea, water extracts of commercial and crushed black and green factory guaysa tea leaves were analysed and compared to BT, GT (*Camellia sinensis*) and RT (*Aspalathus linearis*) of the same brand with the F-C and ORAC assays. The health benefits were further evaluated in cellular models. The dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay was used to evaluate cellular antioxidant activity in the SC-1 and Caco-2 cell lines. LPS-stimulated RAW 267.9 cells were used to evaluate the nitric oxide (NO) scavenging properties of the tea extracts in a cellular environment. Cell viability was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

With the F-C reducing and the ORAC assay, the antioxidant activity of all guayusa tea samples were significantly lower than BT and GT, but similar to RT. Similar results were obtained for he NO scavenging activity. With regards to the cell culture-based assays, in both SC-1 and Caco-2 cell lines the DCFH-DA assay showed that guayusa had similar cellular protective effects to BT and GT, and higher than RT. For the NO suppressing assay in the RAW 264.7 cell line, guayusa tea showed similar results to BT, GT and RT.

In this study, *Ilex guaysa* extracts were shown to have antioxidant, cellular protective and anti-inflammatory properties. In some instances, such as with the ORAC assay conducted on alcohol extracts, guyausa tea showed antioxidant activity similar to that of BT and GT. Water extracts had antioxidant activity (F-C and ORAC assay) less than GT and BT but greater than RT. In contrast in cell model systems the antioxidant and anti-inflammatory properties of guayusa tea related to NO scavenging was similar to GT and BT. Overall, in a non-cellular setting, guayusa tea provides little antioxidant activity, especially when compared with *Camellia sinensis* tea, widely known for its antioxidant and health benefits. However, when exposed to a cellular environment, guayusa tea performs similarly to *Camellia sinensis* tea in terms of antioxidant acitivity which suggests that it may have health benefits in a biological system which can be more profoundly assessed and elaborated in future studies.

Declaration

I, Madelein Delgado Pardau, declare that this thesis entitled:

"Antioxidant activity and nitric oxide scavenging abilities of *Ilex guayusa* compared with *Camellia sinensis* and *Asphalathus linearis* teas"

Which I herewith submit to the University of Pretoria for the Degree Master of Science in Anatomy with specialization in Cell Biology and Histology, is my own original work and has never been submitted for any academic award to any other tertiary institution for any degree

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Presentations and publications in progress

The following presentations arose from this research:

Oral presentations:

M. Pardau, Z. Apostolides, J.C. Serem, M.J. Bester, Antioxidant content and activity and nitric oxide scavenging abilities of *Ilex guayusa* compared with *Camellia sinensis* and *Asphalathus linearis.* Anatomical Society of Southern Africa (ASSA), May 2015.

Postar Presentations:

M. Pardau, Z. Apostolides, J.C. Serem, M.J. Bester, Antioxidant content and activity of *Ilex guayusa* compared with *Camellia sinensis* and *Asphalathus linearis.* Anatomy Faculty day at University of Pretoria, August 2014 and DST-NRF Internship conference, January 2015.

The following manuscripts are in preparation for submission:

Pardau, M.D., Apostolides, Z., Bester M.J. Comparison of antioxidant capacity of *Ilex guayusa* and *Camellia sinensis* teas using DPPH, Folin-Ciocalteu, ORAC and TEAC assays. *Food Chemistry* (Ready for submision).

Pardau, M.D., Apostolides, Z., Bester M.J., Serem, J.C. Antioxidant and NO scavenging and suppressing capacity of *Ilex guayusa* compared with *Camellia sinensis* and *Aspalathus linearis* teas. *Food Chemistry* (In preparation for submission).

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LIST OF ABBREVIATIONS, SYMBOLS, CHEMICAL FORMULAE AND CELL LINES

AAPH	2,2′-Azobis (2-amidinopropane) dihydrochloride
Abs.	Absorption
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
Al_3Cl	Aluminium chloride
ANOVA	Analysis of variance
AUC	Area under curve
BT	Black tea
CAA	Cellular antioxidant assay
Caco-2	Transformed, adult, human colon epithelium cell line
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
DCF	Dichloro-fluorescein
DCFH	Dichloro-dihydro-fluorescein
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
dH_2O	Distilled water
DM	Dry mass
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-Diphenyl-2-picrylhyrazyl radical
EC	Epicatechin
ECG	Epicatechin-3-gallate
EDTA	Diaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallochatechin-3-gallate
ET	Electron transfer

EtOH	Ethanol
FC	Folin-Ciocalteu
FRAP	Ferric reducing ability of plasma
g	Gram
GA	Gallic acid
GAE	Gallic acid equivalents
GT	Green tea
GTC	Green tea catechins
GuT	Guayusa tea
Hrs	Hours
H_3PO_4	Phosphoric acid
HAT	Hydrogen atom transfer
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
HSB	Harney and sons' black tea
H_2O_2	Hydrogen peroxide
IC_{50}	Concentration that gives half-maximal response
IGB	*Ilex guayusa* black
IGBC	*Ilex guayusa* black (Commercially available)
IGG	*Ilex guayusa* green
INF-γ	Interferon-gamma
iNOS	Inducible nitric oxide synthase
ISO	International organization for standardization
$K_2S_2O_8$	Potassium persulphate
NF-κB	Nuclear factor kappa
LBT	Lipton's black tea
LDL	Low density lipoprotein
LGT	Lipton's green tea
LPS	Lipopolysaccharide

LRT	Lipton's rooibos tea
M	Molar
MDA	Malondialdehyde
MeOH	Methanol
Mg	Milligram
mg GAE/g	Milligrams gallic acid equivalents per gram
mg/ml	Milligram per millilitre
Min	Minutes
Ml	Millilitre
mM	Millimolar
mRNA	Mitochondrial ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	Sample size
$NaCO_3$	Sodium carbonate
$NaNO_2$	Sodium nitrite
NC	Negative control
NED	N-(1-naphthyl) ethylenediamine
nm	Nanometres
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOS	Nitric oxide synthase
O_2^-	Superoxide
ORAC	Oxidative radical absorbance capacity
p	Confidence interval
PBS	Phosphate buffered saline
PC	Positive control
pH	Measure of acidity and basicity
R^2	Correlation coefficient squared
RAW 264.7	Transformed, adult, mouse macrophage cell line

RFU	Relative fluorescence units
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
r/min	Revolution per minute
RNA	Ribonucleic acid
SA	Sulphanilimide
SC - 1	Normal, embryonic, mouse fibroblast-like cell line
Sec	Seconds
SEM	Standard error of mean
SNP	Sodium nitroprusside
SO^{-2}	Superoxide
TAB	Tazo awake black tea
TCG	Tazo China green tea
TE	Tolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TH	Theaflavins
TFC	Total flavonoid content
TPC	Total polyphenolic content
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TS	Tea sample
TwEB	Twinings English breakfast tea
TwG	Twining green tea
TZG	Tazo zen green tea
USA	United States of America
UV	Ultra violet
Vs.	Versus
v/v	Volume to volume
WBC	White blood cells
WE	Water extracts

w/v	Weight to volume
YG	Yogi green tea
µg	Microgram
µl	Microliter
µM	Micromolar
µmol TE/g	Micromole Trolox equivalents per gram
°C	Degrees Celsius
%	Percent
% P	Percent protection
±	Plus or minus

CHAPTER 1: INTRODUCTION

The aging process is an inevitable reality to all species on earth. As time passes the biological system starts to deteriorate into a state of senescence and subsequent death. The functional degeneration of somatic cells in the biological system has shown to go hand in hand with the onset of age related diseases, such as cancer, cardiovascular disease, immune system decline and brain dysfunction. This age-related decline is associated with formation of by-products of metabolism, known as oxidants or free radicals. These oxidants cause extensive damage to DNA, proteins and lipids and this damage accumulates with age and thus contributes to the development of various illnesses and diseases especially associated with old age (Ames *et al*., 1993; Shin *et al*., 2007).

Apart from the ability of metabolic systems to produce damaging free radicals, some lifestyle choices may add to oxidative stress. Smoking is a common lifestyle choice that contributes to oxidative stress, which in excess is implicated with serious illness and disease. It contributes to many deaths due to cancer, heart disease as well as cases of premature death. The body has an effective, but limited ability to combat oxidative damage such as numerous antioxidant enzymes that are able to neutralize the oxidants or free radicals generated by metabolism or other stressors like tobacco smoking. Some examples of these enzymes are superoxide dismutase, and glutathione peroxidase. In addition to the body's natural defenses, external dietary sources of antioxidants can further retard this process (Ames *et al*., 1993).

A variety of dietary sources have been shown to contain antioxidant molecules and this has fueled research related to the evaluation of the antioxidant properties of phytochemicals found in plant materials such as fruits, vegetables and teas.

Tea is generally a beverage made from water extracts of dried *Camellia sinensis* leaves and is the most widely consumed beverage in the world after water. The habit of consuming tea is an ancient one, and archaeological discoveries suggest that it may be traced back as far as 500 000 years ago (Gutman and Ryu, 1996). Different teas are consumed in different geographic locations and with varying degrees of popularity. Black teas (BT) make up 76 – 78% of consumption and are mainly consumed in Europe, North America and North Africa, with the exception of Morocco. Green teas (GT) make up 20-22% of consumption and are mainly

popular in China, Japan, Korea and Morocco. Oolong tea only constitutes 2% of tea consumption and it is mainly consumed in China and Taiwan (Wu and Wei, 2002).

Research has shown that tea is rich in polyphenolic compounds, particularly flavanols which are responsible for the antioxidant benefits presented by tea compsumption (Hertog *et al*., 1993; Gutman and Ryu, 1996). Studies have shown that when tea is ingested polyphenols are found to be present in the blood, urine and faeces. This is an indication that these polyphenols are absorbed and subsequently distribute throughout the body and exert their actions at a tissue and cellular level (He and Kies, 1994). Studies have shown that metabolism of compounds also result in the formation of metabolites which have unique antioxidant properties. Such an example is Chlorogenic acid, found in fruits, vegetables, coffee and tea, which is hydrolized by intestinal microflora into various aromatic acid metabolites including caffeic acid and quinic acid (Sato *et al*., 2011; Gonthier *et al*., 2003).

Tea types are not limited to GT and BT from *Camellia sinensis*, but include other tea types such as rooibos tea (RT), from dried leaf infusions of *Aspalathus linearis* which are generally referred to as “herbal teas”. RT has been shown to be effective against cardiovascular biomarkers, as well as having antioxidant and anti-inflammatory properties. Commercialization of such traditional herbal teas has increased interest in the beneficial effects of such teas. Another such tea is guayusa tea from dried leaf infusions of the *Ilex guayusa* plant. The tea is commercially available as Runa tea and health claims are often made by the producers that are not substantiated by scientific fact. In addition, direct comparisons between tea types using the available scientific literature is difficult due to different tea sources and cultivars as well as extraction methods and methodologies used for the evaluation of activity.

Related to the biochemical properties and health benefits guayusa tea, a relatively new commercialized product, several limitations of previous studies and lack thereof, need to be addressed. In this study, the antioxidant and anti-inflammatory activity of guayusa tea will be determined and findings compared with *Camellia sinensis* and *Aspalthus linearis* tea in order to address limitations in previous studies.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Functional degeneration of somatic cells in the biological system is associated with many age related diseases such as cancer, cardiovascular disease, immune system decline and brain dysfunction followed by inevitable death. Some by-products of metabolism, known as oxidants or free radicals, are unstable and react with DNA, proteins and lipids and thus causing cellular and/or tissue damage. In addition to normal biological systems that neutralize these free radicals, external sources such as antioxidants can be incorporated into the diet to limit degeneration by oxidative by-products of metabolism. A variety of dietary sources has shown to contain antioxidant molecules such as polyphenols and this has led to an increase in research associated with the structure, mechanism of action in chemical, cellular, tissue and animal models. Plant materials such as fruits, vegetables and teas are the best source of these dietary antioxidants. Tea consumption has increased in popularity as it has become known that tea possesses antimutagenic, anticarcinogenic, hypolipidemic and neuroprotective effects due to the presence of polyphenols (Shin *et al*., 2007).

2.2 Camellia sinensis

The term ‘tea’ generally refers to water extracts of the *Camellia sinensis* plant. There are two known varieties of tea plants namely *Camellia sinensis* var. *sinensis* and *Camellia sinensis* var. *assamica*. The *sinensis* variety of tea plants is characterized as a bush like plant with small leaves, known to grow in mild cold climates such as China and other Asian countries. The *assamica* variety is a large tree found in the Southwest region of China and India, known to grow in semitropical climates (Li *et al*., 2013). A variety of teas from the *Camellia sinensis* plant are commercially available and these include black (BT), green (GT), oolong, pu-erh and white tea. Differences are related to processing methods (Balentine *et al*., 1997). GT production is generally from the *sinensis* variety due to its unique flavour, whereas BT production is from the *assamica* variety because of its high tannin and catechin composition (Li *et al*., 2013).

To prepare GT, *Camellia sinensis* leaves are freshly harvested and enzymatic oxidation is inhibited by rolling and steaming the young leaves. Short enzymatic oxidation results in oolong

tea, which is considered “semi-fermented” and longer enzymatic oxidation results in BT which is fully “fermented”. “Fermentation”, although an inaccurate term, is the result of tea catechin conversion into complex condensation products, theaflavins and thearubigins (Balentine *et al*., 1997; Rusak *et al*., 2008). Pu-erh tea is fermented for long periods of time by the microorganism *Aspergillus niger* and as a result of this fermentation process, the most abundant polyphenol in puh-erh tea is gallic acid and it lacks GT and BT polyphenols (Duh *et al*., 2004; Li *et al*., 2013). White tea is quite special, considering its rarity. Only very young leaves, or buds with tiny white, silvery hairs are used for production. In addition, it is only harvested once a year in the spring and the leaves/buds are rolled and steamed and not allowed to oxidize (Rusak *et al*., 2008).

2.2.1 Polyphenols in Camellia sinensis

Different types of tea from the *Camellia sinensis* plant contain varying polyphenols due to different cultivation and processing methods. Tea types from the *Camellia sinensis* plant and the major polyphenols found in each are summarized in table 2.1. (Modified from Li *et al*., 2013).

Table 2.1: Tea types derived from *Camellia sinensis* derived and major polyphenols found in each type.

Tea	Major polyphenolic compounds
Green tea	Catechins
Black tea	Catechins, theaflavins, thearubigens
White tea	Catechins
Oolong tea	Catechins, theaflavins
Raw pu-erh tea	Catechin, minor theaflavins
Fermented pu-erh tea	Gallic acid

Polyphenol components of tea include flavanols, theaflavins, thearubigins and proanthocyanidins. Polyphenols are a diversified group of phytochemicals derived from phenylalanine and tyrosine and are secondary metabolites synthesized during normal plant development (Naczk and Shahidi, 2004). They occur widely in nature and can be divided into two major groups, the flavonoids and the phenolic acids which include coumarins, flavonoids,

stilbenes, hydrolysable and condensed tannins, lignans, and lignins (Gomez Caravaca *et al*., 2006; Blasa *et al*., 2011; Naczk and Shahidi, 2004).

Flavonoids can be divided into several classes and these classes as well as their dietary sources are presented in Table 2.4. The structure thereof is presented in Figure 2.2 (Rice-Evans *et al*., 1995).

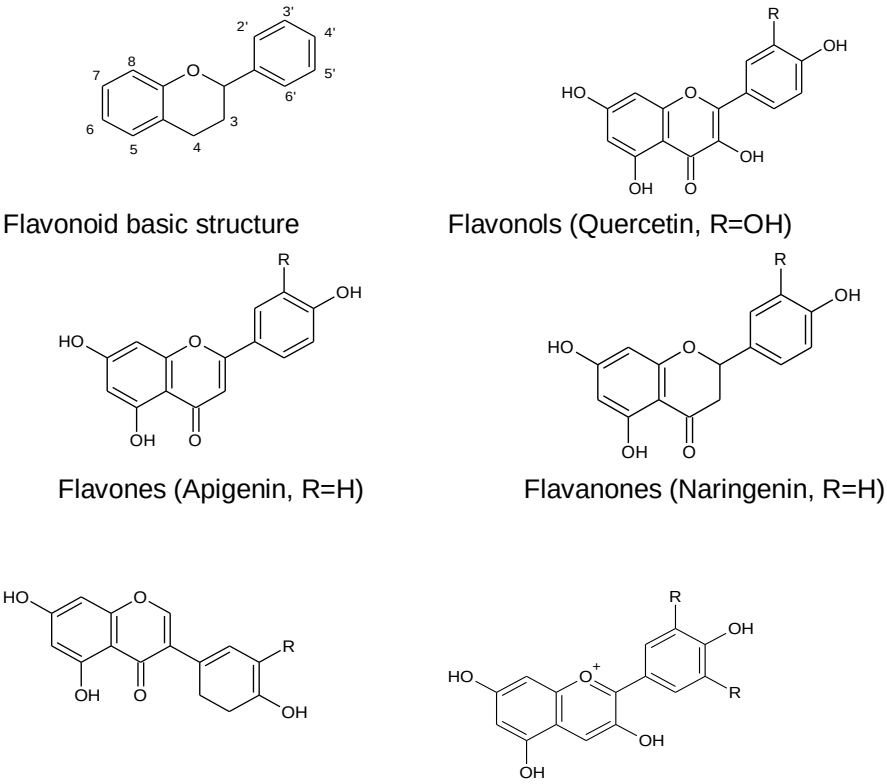
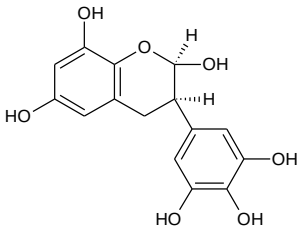
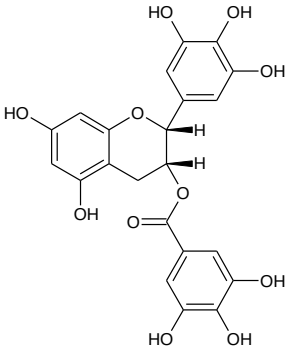


Figure 2.1: Basic structure of flavonoids and examples associated with each group.

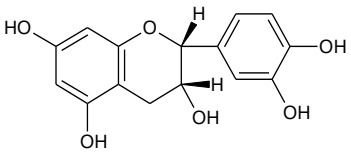
The major polyphenolics found in *Camellia sinensis* tea are (-) – epigallocatechin (EGC), (-) – epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin-3-gallate (ECG) which are simple catechins that when allowed to oxidze give rise to theaflavins and thearubigens, found mainly in BT. The chemical structures of these polyphenols are illustrated in Figure 2.2: (Anesini *et al*., 2008; Mukhtar and Ahmad, 2000).



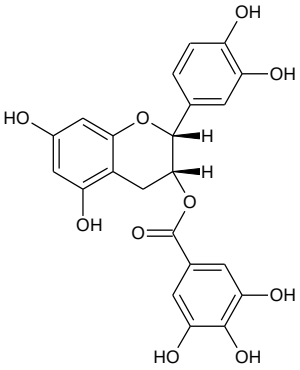
(-)- Epigallocatechin



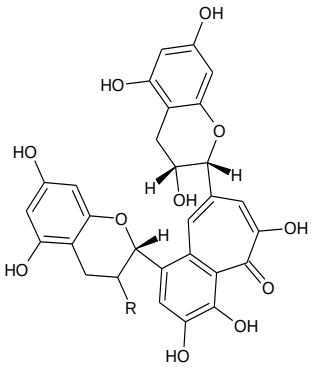
(-)- Epigallocatechin-3-gallate



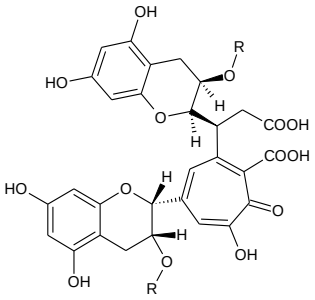
(-)- Epicatechin



(-)- Epicatechin-3-gallate



Theaflavin



Thearubigin

Figure 2.2: Basic structure of most abundant polyphenols found in GT and BT.

Other components in tea include proteins, chlorophyll, minerals, trace elements, volatile compounds, amino and organic acids, lignins, alkaloids (e.g. caffeine, theophylline and theobromine) (Cabrera *et al*., 2003).

Zuo *et al*. (2002) compared the catechin, caffeine and gallic acid content of GT, BT, oolong, and pu-erh teas with HPLC. GT contained higher levels of catechins than oolong tea. The catechin levels of BT and pu-erh teas were low and were explained to be due to the fermentaion process. In BT, fermentation converts catechins to theaflavins. GT contained the least amount of gallic acid and pu-erh teas the most. Astill *et al*. (2001) reported that the catechin content of GT infusions was 7.1-20.8% compared to 0.7-8.8% for BT infusions.

Liang *et al*. (2006) found that in both caffeinated and decaffeinated teas EGC and EGCG were the most abundant catechins. Shin *et al*. (2007) noted that in water extracts, EGC was most abundant followed by EGCG, EC and ECG. With ethanol extraction EGCG was most abundant followed by EGC, ECG and EC. In Jasmine GT, (GT that has been scented with Jasmine blossoms to produce an aromatic tea) Zang *et al*. (1997) investigated polyphenols in green tea and found that EGCG was the most prevelant polyphenol followed by ECG and EGC.

For water extracts Astill *et al.* (2001) reported that GT contains the highest percentage of polyphenols when compared to BT and the amount of polyphenolics extracted was dependent on extraction time. The catechin content of a variety of teas consumed in the United Kingdom was determined by Khokar and Magnusdotter, (2001) and catechin levels were higher in GT when compared to BT.

Henning *et al.* (2003) found that flavonol content of the GT was significantly less for decaffeinated GT. These levels were significantly greater than flavanol levels found in BT. In general it was found that decaffeinated teas have a lower flavonol and theaflavin content when compared to regular teas (Henning *et al*., 2003).

Besides fermentation and the processes used to decaffeinate tea, Astill *et al*. (2001) identified extraction time as an important factor that determines the polyphenolic and caffeine content of teas. In this study brewing times of 30 -300 seconds were evaluated and the authors found an increase in polyphenol and caffeine content with increased extraction time.

Rusak *et al*. (2008) reported that the polyphenolic content of 40% ethanol GT extracts is greater than white tea. Using HPLC the most effective method for polyphenolic extraction was with 40% ethanol. Extraction of loose GT compared to bagged GT was more effective. The differences between loose and bagged white tea was not significant.

Jayasekera *et al*. (2014) used HPLC analysis and determined total catechin content and individual catechins, flavonols and theaflavins of GT and BT leaves from different seasons and

plantations. Mean values for the constituents were significantly higher with solvent extraction as opposed to aqueous extraction. A significant interaction between plantation and season was observed for phenolic constituents of both GT and BT leaves. Ferric reducing antioxidant power was positively correlated with ECG and total phenolic content.

2.2.2. Health benefits

Oxidative stress is implicated in a series of diseases that are a result of tissue inflammation and damage. One such disease is cardiovascular disease, particularly the development of artherosclerosis due to excessive reactive oxygen species (ROS) formation. ROS can trigger various signaling pathways that initiate vascular inflammation. Oxidative stress has also been implicated in diseases such as heart failure, hypertension and stroke (Madamanchi *et al*., 2005). Antioxidants play a role in the neutralization of these reactive species and are therefore a preventative therapeutic strategy (Valko *et al*., 2006).

In an in depth review of the scientific literature, Rietveld and Wiseman, (2003) reported that the consumption of tea resulted in elevated plasma catechin levels as well as an improvement in the blood antioxidant capacity. This indicates that tea antioxidants are absorbed from the gut and have subsequent beneficial effects in cells, tissues and organ systems such as the cardiovascular system.

Flavonoids, which are prominent antioxidants in *Camellia sinensis* tea have strong antioxidant and metal chelating properties and are therefore able to protect cells against oxidative damage. (Rietveld and Wiseman, 2003). Additional benefits of tea flavonoids are cholesterol reduction, hypertension lowering and anti-microbial effects (Trevisanato and Young-In, 2000; Zuo *et al*., 2002).

Since the consumption of tea has been a topic of interest in its efficacy against oxidative stress, Coimbra *et al*. (2006) conducted a study to evaluate this efficacy. In 34 healthy individuals, 1 litre of daily GT consumption showed significant effects against products of oxidative stress. These results were shown by a decrease in malondialdehyde (MDA) and malondialdehyde-4-hydroxy-2(E)-nonenal, (products of lipid peroxidation) after 3 weeks of GT consumption. There was also a significant decrease in membrane-bound hemoglobin which accumulates in old or damaged red blood cells as hemoglobin within cells begins to denature as a result of oxidative stress.

Nantz *et al*. (2009) found that a standardized *Camellia sinensis* capsule product lowered the risk of cardiovascular disease. In a sample of healthy men, after a three week treatment period, it was found that the capsules lowered systolic and diastolic pressures. There were also reductions in amyloid-alpha and MDA, markers of inflammation and oxidative stress, respectively. LDL cholesterol levels were also reduced.

As oxidative stress has been implicated in the development of cancer, it has been a topic of interest in the field of tea research. Weisenburg *et al*. (1997) conducted an animal study on 42 day old rats, administering BT with or without milk instead of drinking water. It was found that the experimental rats showed a decrease in mammary gland tumour multiplicity and volume as well as a decrease in the production of crypts in the colon. It was also found that milk potentiated these effects. In a study by Caderni *et al*. (2000), the effect of GT and BT as well as wine polyphenols was tested on F344 rats with azoxymethane induced cancer. It was found that wine and BT polyphenols significantly reduced the number of colorectal tumours and adenomas and significantly increased the apoptotic index of cancer cells. GT polyphenols were found to be not as effective as BT polyphenols.

Thirty percent of men with high-grade prostate intra-epithelial neoplasia would develop prostate cancer within a year. Bettuzzi *et al*. (2006) conducted a double-blind, placebo controlled study and showed that a dosage of 600 mg of GT catechins in the non-placebo group, had a reduced prostate cancer incidence of 1 in 30 men as opposed to 9 in 30 men for the placebo group.

In a review article, Liu *et al*. (2008), reported that GT is effective against oral and oesophageal, stomach, pancreatic, liver, biliary, colorectal, breast, lung, prostate, urinary, endometrical and ovarian cancer as well as adult leukemia. The authors concluded that findings are not entirely sufficient to validate the significant effect of GT against cancer, however long term consumption of GT may reduce the risk of many cancers.

Mukhtar and Ahmad, (2000) identified EGCG is an important polyphenol that can inhibit urokinase by extracellular signal and cell proliferation suppression through epidermal growth factor receptor binding. EGCG can also down regulate NO-synthase by down regulating transcription factor nuclear factor kB. Together with ECGC, theaflavins also play a role in the inhibition of promoter-induced activator protein 1 activation and cell transformation. Another mechanism of action includes apoptosis induction and cell cycle arrest.

GT has also been suggested to have an impact on bone density, cognitive function, dental caries and kidney stones amongst other conditions (Standley *et al*., 2001; McKay and Blumberg, 1978).

Katiyar *et al*., (2007) found that GT polyphenol oral consumption and EGCG topical application prevents UVB-induced skin tumour development in mice. These polyphenols stimulate DNA repair, inhibit UV-induced immunosuppression and angiogenic factors while stimulating cytotoxic T cells in a tumour microenvironment. Katyar, (2011) further elaborated the benefits of GT topical use by showing that it is useful in the prevention of non-melanoma skin cancer by DNA repair enhancement. GT extract topical administration has also shown to be protective against UVB rays (Camousse *et al*., 2009), genital warts (Tatti *et al*., 2008; Meltzer *et al*., 2009) as well as having anti-wrinkle properties (Chuarienthong *et al*., 2010).

These health beneficial effects of BT and GT are largely due to the antioxidant and anti-inflammatory properties of the polyphenol constituents.

2.2.3. Antioxidant activity

Izzreen and Fadzelly. (2013) determined the total polyphenolic content (TPC) of different parts of the *Camellia sinensis* plant leaves. TPC values were highest for GT shoots compared to young and mature GT leaves. Likewise, the TPC of BT was also determined and was highest for BT shoots followed by young and mature BT leaves. In general, GT had a higher TPC than BT. Satoh *et al*. (2005) found that GT had the highest TPC followed by oolong tea, then BT and lastly roasted tea. However, Von Gadow *et al*. (1997) reported that oolong tea contained a significantly lower TPC than BT. Khokar and Magnusdotter, (2001) evaluated the TPC of tea types most commonly consumed in the United Kingdom, extracted with boiling water. The TPC of GTs was significantly lower than reported for BT which was attributed to high BT brand quality.

Satoh *et al*. (2005) reported that 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of the various teas tested were dose dependent. The scavenging activities decreased in the following order: GT > roasted tea > oolong tea > BT. These findings correlated strongly with the TPC which was in the order GT > oolong tea > BT > roasted tea with the exception of roasted tea which did not show a correlation. Roy *et al*. (2010) determined the TPC of five commercial tea brands. The TPC of these teas ranged from 25.8% to 98% and TPC correlated

with antioxidant activity measured with ORAC assay. Henning *et al*. (2003) showed that the antioxidant activity of GT, ORAC assay correlated with flavanol content. Caffeinated GT and BT had higher antioxidant activity compared to decaffeinated GT and BT.

Shin *et al*. (2007) determined the TPC of water and 75% ethanol extracts of GT. The percentage yield was lower for water extracts compared to ethanol extracts. Rusak *et al*. (2008) reported that GT is a richer source of polyphenol compounds compared to white tea, but extraction of polyphenolics from tea was dependent on the composition of the extraction solution, time and additives such as lemon juice. Shin *et al*. (2007) also evaluated the antioxidant activity, ORAC assay of water and 75% ethanol extracts of GT polyphenols. A dose-dependent increase in antioxidant activity was found and no differences in activity were found between the water and ethanol extracts.

Lin *et al*., (1996) with the ORAC assay evaluated the antioxidant activity of water extracts of different brands of GT and BT as well as EGCG. The order of antioxidant activity was Longjing tea GT polyphenols) > EGCG > Longjing tea water extracts > Assam GT water extracts > Assam BT water extracts. GT has a higher antioxidant activity than BT.

2.2.4. Cellular antioxidant activity

ROS, which are mainly products of metabolic reactions, are responsible for the oxidation and damage of essential biological membranes. Oxidation of erythrocytes is a popular model system (Kondo *et al*., 1997). ROS cause oxidation and damage to the lipids and proteins of the erythrocytes membrane. Oxidation of polyunsaturated fatty acids in biological membranes, leads to the lipid radical formation, increases oxygen uptake, causes double bond rearrangement in unsaturated lipids and even complete membrane lipid destruction (Zhu *et al*., 2002; Miki *et al*., 1978).

Erythrocytes are protected from oxidative damage by enzymatic and non-enzymatic antioxidant systems. Free radicals may be inhibited *in vivo* and *in vitro* by antioxidants like vitamin C and E and other blood constituents such as glutathione (GSH) and antioxidant enzymes such as superoxide dismutase as well as diet-derived antioxidants such as polyphenols found in fruits, vegetables, herbs and tea (Simon *et al*., 2000).

Satoh *et al*. (2005) evaluated the ability of several tea types to inhibit 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) -induced hemolysis. GT extract was the most effective against erythrocyte membrane oxidation compared to other teas such as BT, oolong tea and roasted tea. Zang *et al*. (1997) evaluated the ability of tea polyphenolics to inhibit haemolysis. EC, ECG, EGC and EGCG all inhibited AAPH induced hemolysis in a dose-dependent fashion. EGCG and ECG were the most effective inhibitors of oxidative damage.

Jiao *et al*. (2003) investigated the ability of GT polyphenols to protect human HepG2 cells against the effects of fenofibrate which is a lipid modifying drug. Studies in rodents have shown that long periods of exposure may lead to an increase in incidence of liver tumours. ROS production after a few minutes of fenfibrate exposure was lowered in cells that were pretreated with GT polyphenols. In a similar study by Liu *et al*. (2007) the protective effects of GT polyphenols on oxidative damage to FL (human amniotic) cells *in vitro* by tributylin (a paint ingredient) was investigated. Tributylin causes apoptosis by inducing oxidative stress. In cells pretreated with GT polyphenols, the ROS production was reduced to levels similar to the control.

Cellular antioxidant activity of tea polyphenols was further evaluated by Kelly and Owusu-Apenten (2015) in a resistant breast cancer cell line (MDA-MBB-231). Methotrexate-induced ROS was lowered by 28% and 16% in cells pre-treated with quercetin and EGCG, respectively. Intracellular ROS levels were also reduced.

2.2.5. Antioxidant activity of polyphenols in Camellia sinensis

Differences between GT and BT is that GT contains mainly simple catechins with low molecular weight and BT contains larger oxidized and condense versions of these catechins known as therubigins and theaflavins formed during fermentation. BT still contains some of its original simple catechins, about a third of that of GT (Yam *et al*., 1997).

A study conducted by Pulido *et al*. (2000) evaluated water and methanol extracts using the ferric reducing ability of plasma (FRAP) assay to compare the antioxidant activity of dietary polyphenols. The antioxidant activity of flavonoids (quercetin, rutin, and catechin), phenolic acids (tannic acid, gallic acid, caffeic acid and ferulic acid), carotenoids (β-carotene and zeaxanthine) as well as stilbene resveratrol, Trolox and ascorbic acid evaluated for a four min reaction time, the rankings from highest ferric reducing ability to lowest was as follows:

Quercetin > tannic acid > gallic acid > caffeic acid > BHA > rutin > Trolox > catechin > ferulic acid > ascorbic acid > resveratrol. The carotenoids showed no activity. The author also concluded that the efficiency of the phenolic as determined by the FRAP assay is dependent on the degree of hydroxylation and the extent of conjugation. Quercrtin has a high degree of hydroxylation and conjugation, which serves to explain its high ferric reducing abilities.

Leung *et al*. (2001) evaluated GT catechins EC, ECG, EGC and EGCG as well as BT theaflavins, theaflavin-3-gallate, theaflavin-3′-gallate and theaflavin-3,3′-digallate. All catechins and theaflavins inhibited Cu^{+2}-mediated LDL oxidation. It was found that theaflavins in BT have similar antioxidant potency as GT catechins and that oxidation during fermentation does not alter their free radical–scavenging activity significantly.

2.2.6. Anti-inflammatory activity

During inflammation or infection, nitric oxide (NO) is produced and it is one of the main markers of inflammation. NO reacts with oxygen to form a reactive nitrogen species (RNS) which has shown to contribute considerably to oxidative tissue and cellular damage. NO is synthesized from L-arginine by nitric oxide synthase (NOS) (Ghafourifa and Ritcher, 1997; Tsai *et al*., 2007). It is known to be an important mediator of vasodilation and neurotransmission. Inflammatory stimuli produce inducible nitric oxide synthase (iNOS) which produces NO for long periods of time (Nathan, 1992). This is an important process for immune defense against pathogens, but if an excess amount is produced, the production of RNS accelerates and thus the levels of cellular macromolecular damage (Mayer and Hemmens, 1997, Tsai *et al*., 2007).

Numerous diseases have been associated with excess NO production and the inhibition of NO by dietary molecules such as polyphenols is an important area of research (Tsai *et al*., 2007). Flavonoids such as found in tea, effectively reduce NO levels (Kim *et al*., 1999).

Tsai *et al*. (2007) investigated NO scavenging and NO suppressing activity of various herbal teas compared with GT in lipopolysaccharide - (LPS) activated RAW 264.7 cells. In this study, GT was found to be the strongest and jasmine the poorest NO scavenger. NO was inhibited in a dose dependent manner (Tsai *et al*., 2007).

Duh *et al.* (2004) observed a dose-dependent reduction of NO formation by tea extracts in LPS-activated RAW 264.7 cells. Hendricks and Pool, (2010) found that BT suppressed interleukin-6, interleukin-10 and INFγ production in LPS-stimulated whole blood cell cultures.

To further elaborate on the radical scavenging abilities of tea, Lin *et al*. (2003) showed the NO suppressing effects of *Camellia sinensis* flowers on RAW 264.7 cells compared to GT, BT, oolong and pu-erh tea extracted either with water or ethanol. NO production *in vitro* was strongly inhibited by GT, BT oolong and pu-erh tea. Extracts of *Camellia sinensis* flowers also inhibited NO formation.

Wang *et al*. (2008) demonstrated the effects pu-erh tea has on the oxidation of low-density lipoprotein (LDL) as well as its effect on NO production in macrophage cells. When inflammation occurs, macrophage cells rapidly produce NO and superoxide which react with each other to form peroxynitrite, known to oxidize LDL. Oxidation of LDL has been shown to be associated with the development of atherosclerosis (Pannala *et al*., 1997). A dosage dependent decrease in NO formation and LDL oxidation was reported. .

In a double-blind, placebo-controlled trial by Bogdanski *et al*. (2012), 56 obese, hypertensive subjects were randomized to receive a daily GT extract supplement for 3 months. Systolic and diastolic blood pressures, fasting serum glucose and insulin levels, LDL cholesterol and triglycerides were reduced. In addition, increasing HDL cholesterol, inflammatory biomarkers such as tumour necrosis factor – alpha (TNF- α) and C-reactive protein were significantly lower.

2.2.7. Anti-inflammatory activity of specific polyphenolics in Camellia sinensis

Flavonoids found in *Camellia sinensis* extracts have been shown to be efficient scavengers of the NO radical as well as peryoxynitrite. In GT, ECG and EGCG are the main contributors to NO and peryoxynitirite scavenging and only contribute 5.8% and 11.8% respectively of total scavenging activity. In BT, theaflavins (even though consisting of only 1.2% of total weight) is responsible for 25% while ECGC and EGC are responsible for 25% and 20% respectively of NO scavenging, respectively (Heijnen *et al*., 2000).

A study conducted by Paquay *et al*. (2000) indicated that GT polyphenols such as EC, ECG, EGC and EGCG as well as BT polyphenols such as theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate and theaflavin 3,3'-gallate are able to scavenge NO, as well as the peroxinitrite radical

which is a result of NO reacting with superoxide (O_2^-). Paquay *et al*. (2000) also showed that all the polyphenols also showed abilities to decrease iNOS production as well as nitrite/nitrate production in stimulated NR8383 cells (rat pulmonary macrophages).

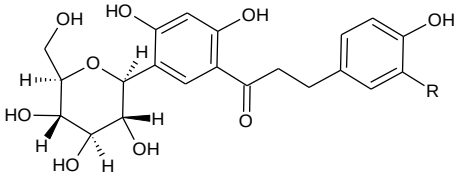
2.3. Aspalathus linearis

Rooibos tea (RT) is most commonly consumed in South Africa, where it grows indigenously; however, it has attracted consumers from all around the world with its increasing popularity (Standley *et al*., 2001). RT is a traditional herbal tea of the Khoi-San people of the Cederberg region of the Western Cape (van Wyk, 2008) and the only known source of the antioxidant compound, aspalathin (Joubert and Ferreira, 1996). Dutch settlers to the Cape consumed RT as an alternative to BT, which was expensive and not readily available to the settlers. RT is derived from a leguminous shrub indigenous to the mountainous areas of the north-western Cape in South Africa, *Aspalathus linearis*. In the 1930s, RT tea was cultivated and became a commercial crop (South African Rooibos Council, 2014). Today, RT is cultivated mainly in the Cederberg mountain area surrounding Citrusdal, Clanwilliam and Nieuwoudtville regions (Joubert *et al*., 2008). Mordern consumption of RT as an alternative to oriental brews such as *Camellia sinensis*, involves making a strong brew consumed with the addition of milk and sugar. Brewing involves boiling the water and keeping the tea on a low heat for extended periods of time with addition of more leaves after each brew. For convenience purposes, the popularity of tea bags has increased, where a 2g tea bag is added to a cup of boiling water for two to five minutes and consumed with or without milk or sugar (Joubert and Ferrerira, 1996). RT is marketed as a health beverage as it is naturally caffeine-free, additive-free, preservative-free, colourant-free, alkaloid-free and is low in tannin (Morton, 1983).

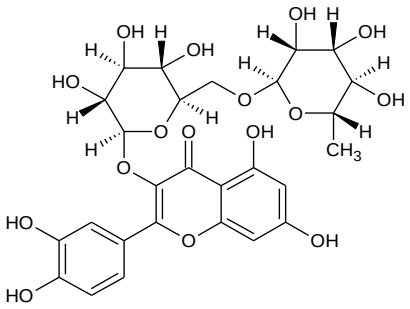
During the past 20 years, RT has gained much popularity, not only in South African markets, but also internationally. This increase in popularity may be attributed to the increasing awareness of its health promoting properties. RT is consumed in its fermented as well as unfermented form. However, fermented RT makes up the bulk of production and exports (> 95%) (Joubert *et al*., 2012) as the fermentation process gives the tea its characteristic rich red colour as well as a sweet taste (Joubert and de Villiers, 1997).

2.3.1 Polyphenols in *Aspalathus linearis*

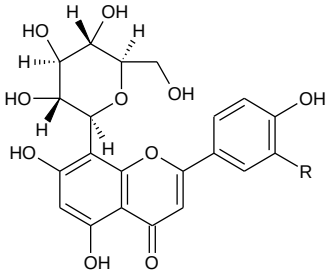
The major flavonoids found in *Aspalathus linearis* are asphalathin, rutin and orientin, The chemical structure of these flavonoids are presented in Figure 2.3. (Adapted from Joubert and Ferreira, 1996).



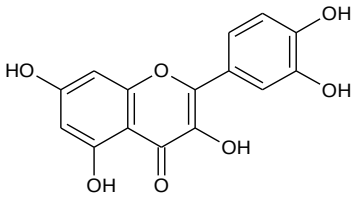
Aspalathin (R=OH), Nothofagin (R=H)



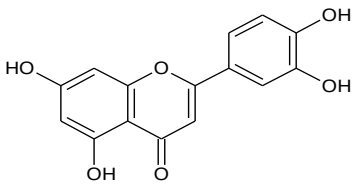
Rutin



Orientin (R = OH); Vetixin (R = H)



Quercetin



Luteolin

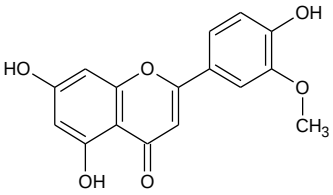


Figure 2.3: The basic structure of most abundant polyphenols found in *Aspalathus linearis*.

According to Joubert *et al*. (2012) the major phenolic components found in RT are isoorientin and orientin followed by quercetin-3-O-robinobioside, phenylpyruvic acid glucoside, aspalathin, isovitexin, vitexin, and hyperoside, rutin, ferulic acid, and iso-quercitrin present at lower concentrations. In addition to these findings phenolic compounds and antioxidant capacity was highly variable amongst various production grades as well as seasons. When the antioxidant capacity of various RT polyphenols was evaluated with the Trolox equivalent antioxidant capacity (TEAC) assay (Snijman *et al*., 2009), asphalathin was found to be the most potent

scavenger, followed by quercetin and nothofagin. The least effective radical scavengers were found to be isovitexin and vitexin.

2.3.2. Health benefits

Oxidative damage is implicated in the development of various diseases such as cardiovascular disease and cancer. Wanjiku, (2009) showed that water extracts of fermented and unfermented RT increased plasma antioxidant capacity and improved the GSH/GSSH ratio in erythrocytes. RT has also been identified to have antispasmodic, anti-inflammatory, antithrombotic, antiviral, antineoplastic, antimutagenic properties. In addition RT can inhibit skin tumour formation and lipoxygenase. These health benefits can be attributed to the flavonoids found in RT.

Marnewick *et al*. (2010) demonstrated the chemoprotective effects of RT compared with green and black *Camellia sinensis* as well as *Cyclopia intermedia* (Honeybush tea). Unfermented RT significantly reduced the total number of foci larger than 10 µm while all the teas reduced the relative amount of the larger foci. It was also found that fermentation reduced the protective effect of the herbal teas. Mazzio and Soliman, (2009) also identified possible tumouricidal effects of RT based on a study using a murine neuroblastoma cell line that forms spontaneous malignant tumours.

Presson *et al*. (2010) found that RT when orally ingested by healthy individuals caused significant angiotensin-converting enzyme inhibition and it can be concluded that RT may have a blood pressure lowering effect. Marnewick *et al*. (2010) also evaluated the effect of RT consumption on parameters of cardiovascular disease. Six cups of RT was consumed for two weeks by 40 healthy volunteers. Findings were that plasma polyphenol levels were significantly increased and markers of lipid peroxidation were significantly decreased. GSH and HDL-cholesterol levels were also increased and serum LDL-cholesterol was decreased. These results showed that RT consumption improves lipid profiles and redox status.

Antihemolytic effects of RT were evaluated by Simon *et al*. (2000) and it was found RT reduced peroxide-induced hemolysis of quail erythrocytes which was comparable to ascorbic acid. In addition topical applications of RT had anti-wrinkle properties (Chuarienthong *et al*., 2010) and promoted hair growth (Glynn, 2010).

2.3.3. Antioxidant activity

Standley *et al*. (2001) determined the TPC of RT. The TPC of unfermented (green) RT was greater than fermented (black) RT. Sun-dried RT had the least polyphenols while the TPC of steam pasteurized RT was between that of green and black RT, although differences were not significant. This study concluded that fermentation resulted in a decrease in polyphenols, which were further decreased by the process of sun-drying. During this process it was suggested that the polyphenols found in leave of rooibos such as aspalathin was converted to its corresponding flavones during the processes of fermentation and sun-drying. Yoo *et al*. (2008) found with the F-C method, the polyphenol content of RT was less than GT and BT while the TPC of GT was greater than BT and this was attributed to the fermentation process (Satoh *et al*., 2005; Standley *et al.*, 2001).

2.3.4. Cellular antioxidant activity

Yoo *et al*. (2008) evaluated the ability of GT, BT and RT to prevent H_2O_2 induced oxidative stress in V79-4 cells. It was found that GT, BT and RT all showed cellular protective effects, with the lowest effect reported for RT, although differences were not significant.

The ability of RT to inhibit ROS formation in the testicular tissue of oxidative stressed rats was evaluated (Awoniyi *et al*. 2012). Rats consuming black and green rooibos extract and a rooibos supplement showed a decrease in ROS formation which was the highest for the rooibos supplement. Awoniyi *et al*. (2012) also evaluated the effect of RT on ependymal sperm cells from oxidative stressed rats and found a significant decrease in ROS.

2.3.5. Antioxidant activity of polyphenols in Aspalathus linearis

Aspalathin is a polyphenol unique to RT. In a study conducted by Von Gadow *et al*. (1997)[2,] the DPPH assay and β-carotene bleaching method were used to compare the activitly of RT, GT, BT and oolong tea. These included α-tocopherol, BHT, BHA, the flavonoids vitexin, rutin, quericetin, lutelin, isoquercitin, (+)-catechin as well as the phenolic acids protocatechuic acid, caffeic acid, *p*-hyroxybenzoic acid, *p*-coumaric acid, ferulic acid, syringic acid and vanillic acid. All polyphenols had antioxidant activity which was the highest for aspalathin and caffeic acid determined with the DPPH radical scavenging assay, while with the β-carotene bleaching

method, and the automated Rancimat methods, α-tocopherol and BHT had the highest antioxidant activity.

In a study conducted by Von Gadow *et al*. (1997) used the DPPH assay and the β-carotene bleaching method. The scavenging activity of RT was compared with GT, BT and oolong tea. GT had the highest scavenging activity compared with the other teas.

In rooibos, aspalathin is the active scavenger of DPPH, however aspalathin, does not survive the fermentation process. Unfermented RT had the highest radical scavenging activity due to the presence of aspalathin. Semi-fermented rooibos had the least, due to the conversion of aspalathin into corresponding flavones, dihydro-orientin and dihydro-iso-orientin. Fermented RT fell in the middle, due to the presence of orientin and iso-orientin which are known to have a higher efficacy than the flavones of aspalathin. Overall the teas were placed in the following order from highest antioxidant activity to the lowest GT > unfermented RT > fermented RT > semi-fermented RT > BT > oolong tea. In this study RT showed higer activity than BT which may be due to the use of a high quality RT brand.

2.3.6. Anti-inflammatory activity

Antioxidants play a role in the reduction of mediators in damaging inflammatory pathways. Hendricks and Pool, (2010) determined, using whole blood cultures, the effects of RT and BT on the biomarkers of immune pathways. Both BT and RT were found to modulate immune function *in vitro.* In unstimulated cells, RT increased the interleukin-6, interleukin 10 and INF-γ levels. In LPS-stimulated cells interleukin-6 levels were increased while interleukin -10 and INF-γ levels were unchanged.

Other health benefits that have been associated with RT are anti-allergy and immune response reactions. This can be attributed to the presence of mangiferin, a bioactive xanthonoid that is a naturally occurring polyphenol (Sarwar and Lockwood, 2010). In an *in vivo* study by Baba *et al*. (2009), Wistar rats were administered RT for four weeks. Levels of the antioxidant enzyme, serum superoxide dismutase was measured compared to a control. Levels compared to the control were significantly increased in the RT group. Blood levels of the marker of DNA damage, 8-hydroxy-2'-deoxyguanosine were significantly lower in the experimental group. Furthermore, in rats with dextran sodium sulphate induced colitis associated decrease in haemoglobin levels

was prevented by RT. Low haemoglobin levels indicate erythrocyte damage that is associated with inflammation or oxidative stress.

2.3.7. Anti-inflammatory activity of polyphenols in Aspalathus linearis

Using LPS-activated human umbilical vein endothelial cells and mice, Lee and Bae, (2015) demonstrated that aspalathin and nothofagin, two of the major components of RT have anti-inflammatory activity. Each inhibited LPS-induced barrier disruption, expression of cell adhesion molecules, and adhesion/transendothelial migration of neutrophils to endothelial cells. Aspalathin and nothofagin also suppressed LPS-induced hyperpermeability and leukocyte migration *in vivo* as well as TNF-α, interleukin 6 and NF- κB activation by LPS. Lethal edotoxemia induced by LPS was also reduced, which confirmed the anti-inflammatory activity of aspalathin and nothofagin.

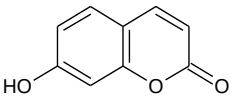
2.4. Ilex guayusa

Guayusa from the *Ilex guayusa* plant, a native holly tree, is grown exclusively in the Amazonian region of Ecuador. It is grown in what is known to be one of the most biodiverse regions in the world where the Amazon forest meets the Andes Mountains. Leaves are harvested from trees which are shaded by the canopy of the rainforest. It is marketed as source of sustainable energy, attributed to high caffeine content in addition to antioxidants, vitamins and amino acids. It is related to the more popular Yerba mate (*Ilex paraguariensis*) tea from the Atlantic rainforest in Argentina but has been claimed to have a smoother and less bitter taste. The commercialization of guayusa as Runa tea in the USA is linked to projects of agricultural assistance, deforestation and economic development as it is an incentive for reforestation and a source of income for indigenous farmers and communities of Ecuador (Runa LLC, 2014).

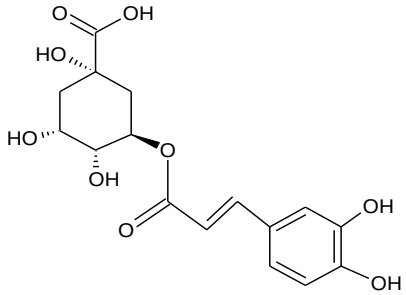
Traditionally, guayusa was consumed by Amazonian families around a fire in the early morning (Runa LLC, 2014). It is used as a balanced stimulant that has been claimed to energize the body and mind while promoting restful sleep with lucid dreams (Ferreria *et al*., 2012). This could be attributed to high caffeine content of the Guayusa plant, or a specific molecule/s that have not been identified.

2.4.1. Polyphenolics in *Ilex guayusa*

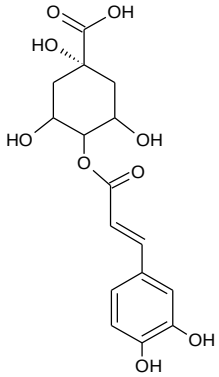
The polyphenols present in Runa tea (Figure 2.4) have been identified and are beta-umbelliferone, chlorogenic acid, 4-caffeoylquinic acid (cryptochlorgenic acid), neochlorogenic acid 1,5-dicaffeoylquinic acid (cynarine) and rutin (unpublished results, Department of Biochemistry, University of Pretoria). Chlorogenic acid was found to be the most abundant polyphenol in *Ilex guayusa* extracts. For this reason the antioxidant and anti-inflammatory effects of chlorogenic acid will be reviewed in greater detail.



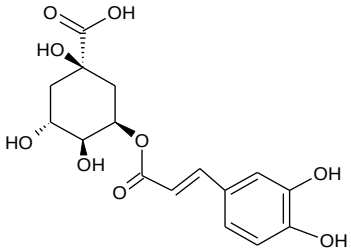
Beta-umbelliferone



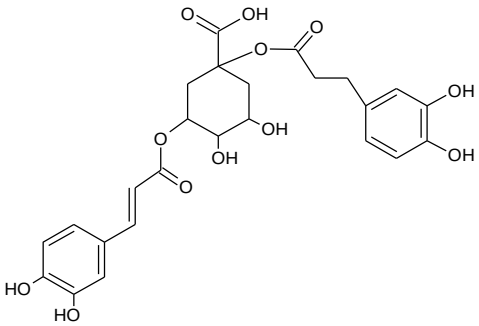
Chlorogenic acid



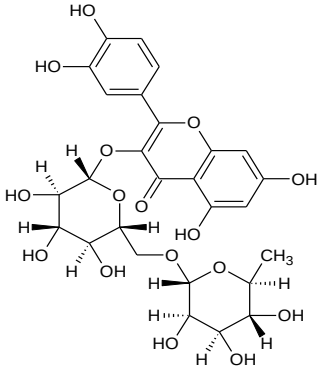
4-caffeoylquinic acid (Cryptochlorgenic acid)



Neochlorogenic acid



1,5-diCaffeoylquinic acid (Cynarine)



Rutin

Figure 2.4: Basic structure of most abundant polyphenols found in *Ilex guayusa* (Taken from unpublished HPLC-MS analysis)

Nishitani and Sagesaka, (2004) found that chlorogenic acid was only 0.01% 0.19%, 0.20%, 0.15% and 0.5% of EGCG levels found in two brands of Japanese GT, Chinese GT, oolong tea and BT, respectively. The chlorogenic acid found in *Ilex guayusa* may be more abundant than in *Camellia sinensis*, making it a unique tea type base on its polyphenol composition.

Chlorogenic acid, found in abundance in *Ilex guayusa* has been shown to be hydrolysed by intestinal microflora into various aromatic acid metabolites including caffeic acid and quinic acid (Sato *et al*., 2011; Gonthier *et al*., 2003).

2.4.2. Health benefits

Ilex guayusa which is used by the Amaguajes Indians of South America to treat diabetes has been shown to retard the development of steptozocin-induced diabetes in mice (Swanston-Flatt *et al*., 1989; Bailey and Day, 1989).

Cho *et al*., (2010) reported that chlorogenic acid and caffeic acid lowered anti-obesity properties in induced-obese mice. This included lowering of body weight and visceral fat mass, plasma leptin, insulin, triglyceride and cholesterol levels. Enzymes involved in lipid catabolism were also inhibited. Chlorogenic acid was found to be more potent then caffeic acid. Ong *et al*. (2013) further identified that the anti-diabetic and anti-lipidemic properties of chlorogenic acid are due to the activation of 5'AMP-activated protein kinase which is an enzyme that plays a role in cellular energy homeostasis. The chlorogenic acid, 5-caffeoylquinic acid was shown by Suzuki *et al*. (2006) to reduce blood pressure in spontaneously hypertensive rats. It was concluded that 5-caffeoylquinic acid reduced oxidative stress and also improved NO bioavailability.

2.4.3. Antioxidant activity

The manufacturers of guayusa tea have made claims that Runa tea contains twice the antioxidants, measured by the ORAC assay, of GT (Runa LLC, 2014). This has, however, not been subjected to scientific evaluation. Preliminary data (not published) has identified chlorogenic acid as the main polyphenol in Runa tea. According to Kondo *et al*. (1997), chlorogenic acid is which is a non-flavonoid polyphenol, is able to scavenge reactive species of oxygen and nitrogen.

2.4.4. Cellular antioxidant activity

Sato *et al*. (2011) investigated chlorogenic acid uptake by Caco-2 cells and found that pH did not affect chlorogenic acid uptake. Some polyphenols such as catechins and theaflavins have been reported to be unstable and undergo auto-oxidation with increased pH (Sun *et al*., 2003). and as a consequence with duodenal digestion all antioxidant activity is lost. The stability of chlorogenic acid implies that this polyphenol can protect the mucosa of the gut against oxidative damage. Furthermore, vascular permeability increased in rats with ischemia/ reperfusion damage (a model of oxidative stress), treatment with chlorogenic and caffeic acid was found to decrease vascular permeability. (Sato *et al*., 2011).

2.4.5. Antioxidant activity of chlorogenic acid

Sato *et al*. (2011) reported that chlorogenic acid and caffeic acid had similar O_2^- scavenging activity almost similar to that of allopurinol, which is a xanthine oxidase inhibitor, prescribed for gout. The relevance of these findings is due to the fact that hypoxanthine–xanthine oxidase system is an important source of free radical production after hypoxia and reperfusion of the gut. Chlorogenic acid is hydrolyzed into caffeic acid in the intestine and therefore protective effects of chlorogenic acid are indirect and the observed protective effects are due to caffeic acid. Chlorogenic acid uptake by Caco-2 cells was more efficient than caffeic acid, which is a metabolite of chlorogenic acid. This study emphasised the importance of metabolism in a cellular environment, as caffeic and chlorogenic acid have similar scavenging abilities, but chlorogenic acid shows more efficient uptake and would therefore exert a more favourable result in a cellular environment.

Yun *et al*. (2011) examined the MDA (the end-product of lipid peroxidation) and GSH levels in blood of rats with induced liver ischemia and reperfusion and subsequent chlorogenic acid administration. MDA levels were increased after ischemia and reperfusion and were decreased after chlorogenic acid administration. GSH levels were decreased after ischemia and reperfusion induction but were restored after chlorogenic acid administration.

2.4.6 Anti-inflammatory activity of chlorogenic acid

Shan *et al*. (2009) showed that chlorogenic acid inhibits a LPS-induced inflammatory response in RAW 264.7 cells. A decrease in cyclooxygenase (COX-2), an important enzyme in the inflammatory pathway was found. Nuclear factor-kappaB (NF-κB) and c-Jun N-terminal kinase-c-Jun-activator protein pathways were also significantly suppressed by chlorogenic acid.

In Sprague-Dawley rats with liver ischemia and reperfusion Yun *et al*. (2011) using histological analysis of livers showed that hepatic necrosis and Kupffer cell hyperplasia were decreased in chlorogenic acid-treated groups. Cytokine levels were increased after reperfusion and attenuated significantly by chlorogenic acid administration. mRNA levels for the cytokines were also significantly decreased.

In conclusion, all tea types, *Camellia sinensis, Aspalathus linearis* and *Ilex guayusa* have shown to contain antioxidant and anti-inflammatory activity which is generally attributed to their polyphenolic constituents.

2.5. Aim

The purpose of this study is to evaluate, using internationally standardized methodologies, the antioxidant activity of alcoholic extracts from *Ilex guayusa* compared with a variety of commercially *Camellia sinensis* teas. Then to evaluate possible health benefits related to antioxidant and anti-inflammatory activity of each type of tea including *Asphalathus linearis* tea as conventionally consumed.

2.6. Objectives

The following objectives are related to internationally standardized methodologies:

1) To determine the moisture content of each tea sample.
2) Prepare alcohol extracts of each tea type.
3) For each extract determine the polyphenolic content.
4) To further evaluate antioxidant activity using several different assays.
5) To determine whether the antioxidant content activity of guayusa (*Ilex guayusa*) tea is significantly different from black and green (*Camellia sinensis*) tea.

The following objectives related to possible health benefits are to:

1. Prepare 2-60 minutes (min) water extracts of each tea type.
2. For each time interval of each extract determine the total polyphenol content.
3. For each time interval for each extract determine the antioxidant activity.
4. To confirm antioxidant activity using a cell based model.
5. To determine whether the antioxidant content and activity of guayusa tea (*Ilex guayusa*) tea is significantly different from black, green (*Camellia sinensis*) and rooibos (*Asphalathus linearis*) tea.
6. For each time interval for each extract determine the NO scavenging activity.
7. To confirm anti-inflammatory activity related to NO scavenging in a cell based model.
8. To determine whether the anti-inflammatory activity of guayusa tea (*Ilex guayusa*) tea is significantly different from black, green (*Camellia sinensis*) and rooibos (*Asphalathus linearis*) tea.

2.7 Hypotheses:

Hypothesis 1: Methonol extracts of *Ilex guayusa* have higher antioxidant capacity compared with *Camellia sinensis* measured with F-C, TEAC, DPPH and ORAC assays.

Hypothesis 2: Water extracts of *Ilex guayusa* have higher antioxidant capacity compared with *Camellia sinensis* and *Aspalathus linearis* teas measured with F-C, ORAC assays as well as the DCFH-DA assy using the SC-1 and Caco-2 cell lines.

Hypothesis 3: Water extracts of *Ilex guayusa* has higher NO scavenging activity and NO suppressing activity in the RAW 264.7 cell line when compared with *Camellia sinensis* and *Aspalathus linearis* teas, measured with the NO scavening and NO suppressing assays, respectiviely.

CHAPTER 3: CHEMICAL ANTIOXIDANT EVALUATION OF *ILEX GUAYUSA* COMPARED WITH *CAMELLIA SINENSIS*

3.1. Introduction

Antioxidant research focuses on the evaluation of a wide range of plant products for activity. A major limitation is related to differences in the methodologies and strategies used between studies. These include differences in sample preparation, extraction procedures and the methods used for analysis. A typical example of such limitations is in the field of tea research and as a result the International Organisation for Standardization (ISO) that develops and publishes international standards has published standardised methods for tea research, especially with regards to extraction methods (solvent, extraction time and temperature), moisture content and total polyphenolic content (TPC) determination (Ref. No. ISO 14502-1:2005/Cor-1:2006(E); 2006).

Other factors that can also contribute to differences in antioxidant activity are related to geographical region where the tea is collected, processing method, seasons, harvesting time and plant age. For this reason in this study the antioxidant activity of guayusa tea was compared with several *Camelllia sinensis* tea types and brands. The standardised ISO method will used be to determine dry mass, prepare the extracts and determine reducing activity with the Folin-Ciocalteu (F-C) method. In addition two other commonly used electron transfer (ET) methodologies will be used to determine antioxidant activity and these are the DPPH and TEAC assays (Huang *et al*., 2005; Prior *et al*., 2005). The ORAC assay which is a hydrogen atom transfer (HAT) based system is generally considered a more physiologically relevant antioxidant assay as it provides a direct measure of the hydrophilic and lipophilic chain-breaking antioxidant capacity (Huang *et al*., 2005). In addition, this method also measures antioxidant activity over time due to the possible presence of slow-reacting antioxidants found in extracts which are heterogeneous in composition.

The aim of this study is to evaluate, using internationally standardized ISO methods and additional antioxidant assays, the antioxidant activity of *Ilex guayusa* compared with a variety of commercial *Camellia sinensis* teas.

3.2. Materials and methods

3.2.1. Materials

3.2.1.1. Reagents, equipment and disposable plasticware

Folin-Ciocalteu's (F-C) reagent, sodium carbonate anhydrous (Na_2CO_3), gallic acid (GA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and potassium persulphate ($K_2S_2O_8$), 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH), 2,2-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azo-bis (3-ethylbenzothiazoline-6-sulfonic acid) diamonium salt (ABTS) and flourescein were obtained from Sigma-Aldrich Company, Atlasville, SA. The organic solvent methanol was of analytic grade and was purchased from Merck, Johannesburg, SA.

FLUOstar OPTIMA plate reader from BMG lab technologies, Offenburg, German, a BioTek plate reader purchased from Analytical and Diagnostic Products, Johannesburg, South Africa was used. A Hermle Z300 centrifuge, a Crison GLP 21 pH Meter and Eppendorf pipettes from Eppendorf AG Hamburg, Germany were all supplied by the Scientific Laboratory Equipment Company (LASEC), Cape Town, UNICO 1100 spectrophotometer by Monitoring and Control Laboratories, Durban, South Africa. Water was distilled (dH_2O) with a Continental water system.

Disposable plasticware included: Opaque and clear 96-well plates, 50 ml, 15 ml tubes and pipette tips (10, 25, 100, 200, and 1000 µl) which were also supplied by Greiner Bio-one also supplied by LASEC.

3.2.1.2. Laboratory facilities

All research was conducted in the research facilities of the Departments of Anatomy of the Faculty of Health Sciences and Department of Biochemistry of the Faculty of Natural and Agricultural Sciences, University of Pretoria.

3.2.1.3. Samples

Ilex guayusa and *Camellia sinensis* samples were purchased from supermarkets in New York, USA. Three batches were obtained for each brand, each with different lot numbers. All tea purchases were made during 2013-2014. In addition, two different batches of non-commercial black and green *Ilex guayusa* factory samples were obtained from the Runa tea factory, Ecuador. To evaluate the tea samples in a standardised manner, extracts were performed in

accordance with the method approved by the ISO (Ref. No. ISO 14502-1:2005/Cor-1:2006(E); 2006).

Each tea type and the abbreviations used throughout this study is presented in Table 3.1.

Table 3.1: List of samples and abbreviations of each used throughout the study

Camellia sinensis	
Green	**Black**
LGT - Lipton's green tea	**LBT -** Lipton's black tea
TCG - Tazo China green tea	**TAB -** Tazo Awake black tea
TwG - Twining's green tea	**TwEB -** Twinings English breakfast tea
YGT - Yogi green tea	**HSB -** Harney and sons black tea
TZG - Tazo zen green tea	
Ilex guayusa	
Green	**Black**
IGG* - Green *Ilex guayusa* (factory)	**IGB* -** Black *Ilex guayusa* (factory)
	IGBC - Black commercial *Ilex guayusa*

***** *Indicates non-commercial factory Ilex guayusa samples, as leaf form, from Runa tea factory, Ecuador*

To evaluate the tea samples in a standardized manner extracts was performed according to the method approved by the ISO (Ref. No. ISO 14502-1:2005/Cor-1:2006(E); 2006) and the strategy and methodologies used are presented in Figure 3.1 and sections 3.2.1, 3.2.2 and 3.3. In addition the antioxidant activity of each extract was also determined using the TEAC, DPPH and ORAC assays.

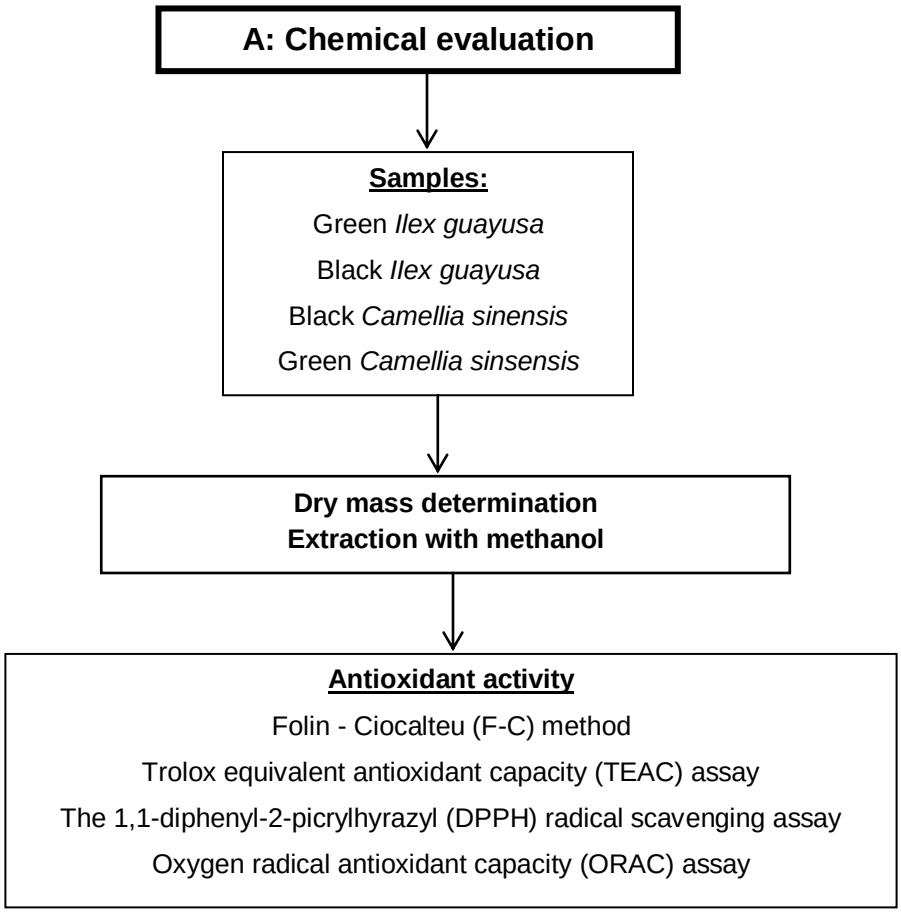


Figure 3.1: Experimental strategy for the evaluation of the antioxidant properties of tea.

3.2.2. Methods

3.2.2.1 Moisture content

Dry leaf matter takes up moisture while in storage. The level of moisture absorbed is dependant of many factors such as composition of plant material like stalks or leaves as well as the age of the plant material. In this study various types of teas, different brands and processing methods may cause variation in moisture uptake during storage.

To determine the dry mass of the tea material crucibles were dried overnight in a drying oven at 103°C and then were cooled in a desiccator and weighed to determine the dry mass of the crucible. From each sample 2 g of ground leaves were placed in the dried crucibles. The tea and crucibles were then placed overnight in an oven at 103°C for drying. The next morning the crucibles containing the tea were weighed and dry mass of the crucible and the dried tea was calculated using the following equation:

$$\text{Dry mass (DM)} = \frac{\text{(Dry mass of crucible + tea) – (Dry crucible mass) x 100}}{\text{Wet mass of tea (2g)}}$$

All data generated in this study is expressed as units per g (DM) tea.

3.2.2.2. Sample extraction

Each tea sample was extracted and prepared in accordance with the ISO approved method (Ref. No. ISO 14502-1:2005/Cor-1:2006(E); 2006). Each sample was ground to a fine powder, using a conventional coffee grinder. A 0.200g ± 0.001 g teas (wet mass) was weighed out and placed in a test tube for extraction. A 5.0 ml volume of extraction solution (70:30 methanol:water) was dispensed into each test tube, in a hot water bath at 70^{o}C. A stopper was inserted and each tube was mixed well before heating for 10 min. Each sample was mixed well at five min intervals. The test tubes were then removed and allowed to cool to room temperature before centrifugation for 10 minutes at 3 500 rpm (2054 x g). The supernatant was then decanted into graduated tubes. The extraction process was repeated using the remaining tea pellet. Both supernatants were combined and made up to 10 ml with cooled extraction solution.

3.2.2.3. Folin - Ciocalteu (F-C) method

In the F-C method electrons in an alkaline medium are transferred from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, found in the F-C reagent. A blue coloured product is formed that can be quantified spectrometrically. Everette *et al*. 2010 found that the F-C reagent is reactive towards other compounds besides phenols; it would be wise to consider the method as quantitative measure of total antioxidant capacity, especially by the mechanism of action of electron transfer (Singleton and Rossi, 1965; Prior *et al*., 2005, Huang *et al*., 2005; Ainsworth and Gillespie, 2007). Although this method is used to determine TPC, it is also a measure of electron transfer or reducing ability of ROS by antioxidants (Huang et al, 2005).

The F-C method is an ISO-approved method to quantify TPC in tea extracts and was conducted according to the ISO methodology (Ref. No. ISO 14502-1:2005/Cor-1:2006(E); 2006). A 10% (v/v) F-C reagent solution in distilled water and a 7.5% (w/v) sodium carbonate solution was prepared. A 1mg/ml gallic acid (GA) standard solution was prepared and diluted to prepare a

0 – 0.05 mg/ml concentration range. A 1% (v/v) tea solution was also prepared. Of the diluted standard or tea extract 200 µl was pipette into centrifuge tubes, before adding 1ml F-C solution. The mixture was allowed to stand at room temperature for 10 min and then 800 µl 7.5% sodium carbonate solution was added. After incubating for a further 60 min at room temperature, absorbance was measured at 765 nm using a UNICO 1100 spectrophotometer and the antioxidant reducing capacity was expressed as mg GA equivalents (GAE)/g DM tea.

3.2.2.4. Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay is an ET assay that works on the principle of the oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{2-}$) to $ABTS^{+}$ by $K_2S_2O_8$ (Figure 3.2) (Huang *et al*., 2005). The generated $ABTS^{+}$ radical cation, a bluish-green compound, is quenched or reduced by antioxidants to a colourless product. The TEAC assay is generally easy, rapid and inexpensive to use and is stable to pH variations (Zulueta *et al*., 2009). Antioxidants such as the polyphenols in tea can quench oxidation the extent of which can be quantified colorimetrically (Re *et al*., 1999) and the reaction is presented in Figure 3.2 (Adapted from Zulueta *et al*., 2009).

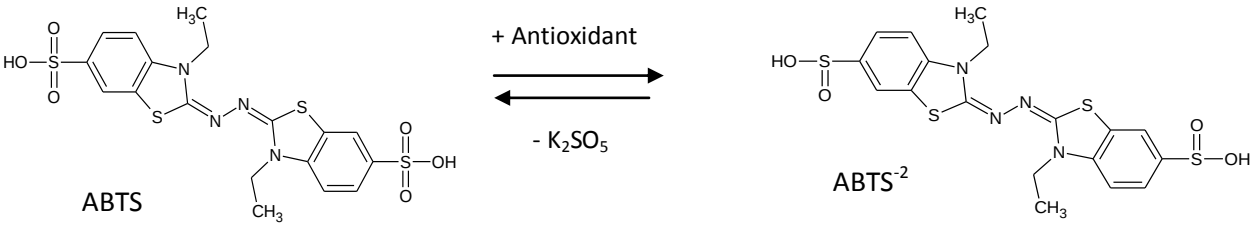


Figure 3.2: Reaction of the ABTS radical in the presence of an antioxidant compound during the TEAC reaction.

The TEAC assay was conducted according to the method by Awika *et al.*, 2003 and adapted to a 96-well format according to Serem and Bester, 2012. Three millimolar $K_2S_2O_8$ was added to 8 mM ABTS, to prepare the $ABTS^{2-}$ solution. The mixture was then left in the dark at room temperature for 12 hours. An ABTS working solution was prepared by further diluting the $ABTS^{2-}$ 30x with PBS (0.2 M Na_2HPO_4, 0.2 M $NaH_2PO_4.H_2O$, 0.15 M NaCl, pH = 7.4). A 1 mM Trolox solution was diluted to a concentration series of 0 – 1 mM. For the standard curve 10 µl Trolox standard or 1% (v/v) tea solution was added to the wells of a 96-well plate. A volume of 290 µl of the ABTS working solution was then added. The plate was left to incubate at room temperature, in the dark, for 15 min for the standards and 30 min for the tea samples. The

Trolox standard was incubated for a shorter time interval as Trolox is a homogenous solutrion that rapidly reacts to reach the endpoint. The tea samples are a heterogenous mixture that takes longer to reach endpoint. The absorbance was then measured at 570 nm with a BioTek plate reader and all data was reported as µmol Trolox equivalents (TE)/g DM tea.

3.2.2.5. 1,1-Diphenyl-2-picrylhyrazyl (DPPH) radical scavenging assay

DPPH is a stable nitrogen free radical with an absorption maximum of 515 nm (Huang *et al*., 2005). When DPPH is scavenged in a strong hydrogen bond accepting solvent, solution colour will change from deep purple to yellow due to an ET reaction from the phenolic compound to the radical as shown in Figure 3.3 (Foti *et al*., 2004). In contrast to the TEAC assay that quantifies antioxidant activity in an aqueous environment the preferred solvent for the DPPH assay is methanol or buffered methanol. The DPPH reaction has been shown to be sensitive to light and pH (Sharma and Bhat, 2009) and is presented in Figure 3.3 (Adapted from Foti *et al*., 2004).

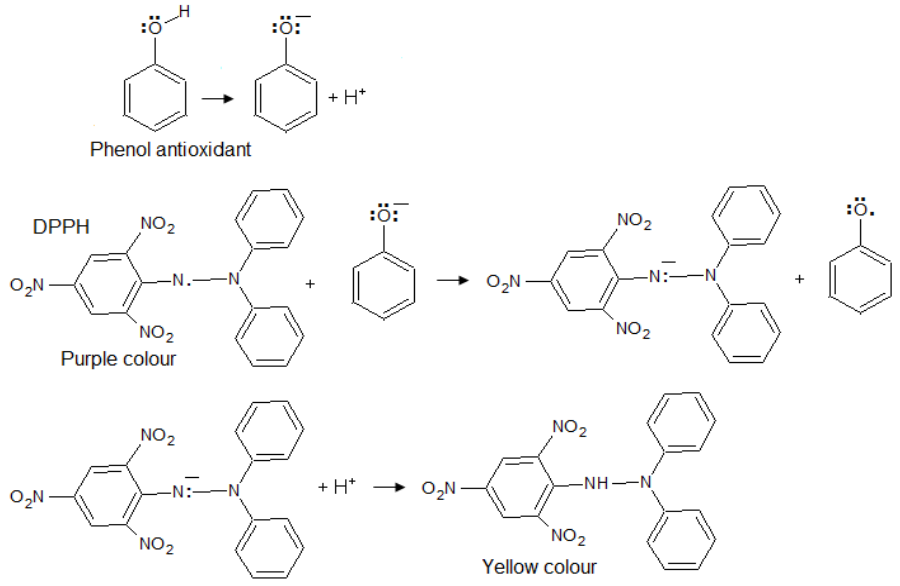


Figure 3.3: Reaction of a phenolic antioxidant with DPPH radicals.

The DPPH assay was performed according to Awika *et al.* (2003), with some modifications specified by Serem and Bester, (2012). A DPPH stock solution was prepared by mixing 24 mg DPPH with 100 ml of methanol. The solution was sonicated for 20 min and covered to avoid any evaporation. Once dissolved, a 10x diluted working solution was prepared. A 1 mM Trolox

solution was diluted to a concentration series of 0 – 0.8 mM. A 15 µl volume of either the Trolox concentration series or 1% (v/v) diluted tea solution in water was added to the wells of a 96-well plate. A volume of 285 µl DPPH solution was then added to each well. The plate was then incubated at room temperature, in the dark, for 15 min. The absorbency was read at 570 nm and all data was reported as µmol TE/g DM tea.

3.2.2.6. Oxygen radical antioxidant capacity (ORAC) assay

The antioxidant activity of the tea samples was evaluated with the ORAC assay as this assay, compared with the ET assays, is more biologically relevant (Prior *et al*., 2005). The ORAC assay provides a direct measure of the hydrophilic and lipophilic chain-breaking antioxidant capacity versus peroxyl radicals and is therefore known as a HAT-based system (Huang *et al*., 2005). This assay consists of three components, a synthetic free radical generator, in the case of this study, and most studies is AAPH, an oxidizable molecular probe, fluorescein and an antioxidant, the tea (Huang *et al*., 2005). AAPH is a stable free radical and when added to the solution acts as a peroxyl generator. The peroxyl radical abstracts a hydrogen atom from fluorescein, which is the fluorescent probe, and over time the fluorescence intensity starts to decrease (Ou *et al*., 2001). In the presence of an antioxidant, the decay of fluorescence is inhibited or delayed. Essentially a higher concentration of an antioxidant or a more potent antioxidant will inhibit fluoresce decay to a greater extent. The reaction is represented in a schematic diagram, Figure 3.3. Huang *et al*. (2005) also identified that an advantage of such a method is that it measures antioxidant activity over time and also takes into consideration antioxidants that have a lag to their efficacy. The area under the fluorescence decay curve (Figure 3.4) is then used to quantify antioxidant activity (Huang *et al*., 2005).

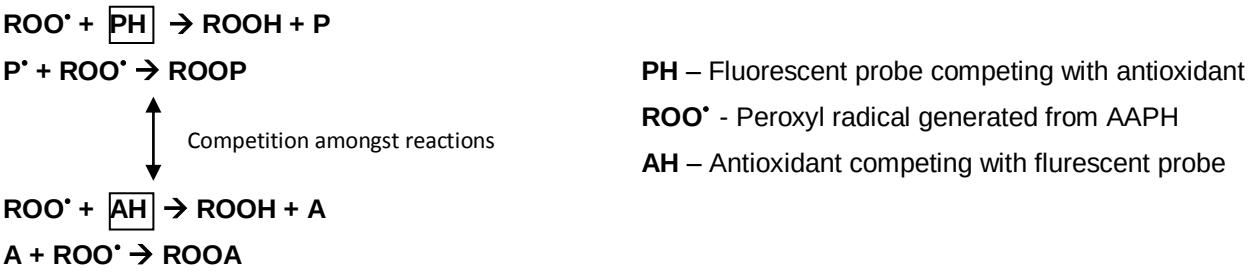


Figure 3.4: Schematic diagram of the principle of the ORAC assay (Adapted from Huang *et al.,* 2005).

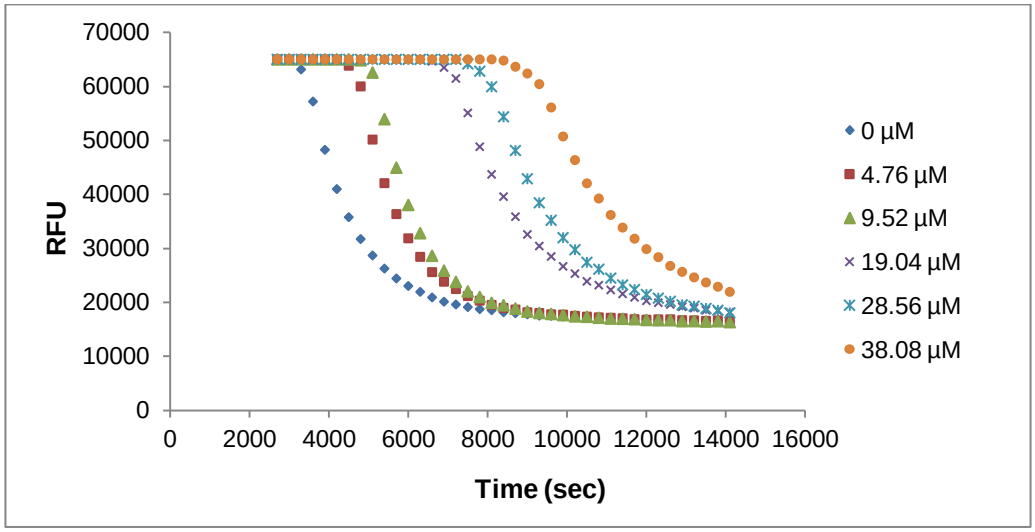


Figure 3.5: A typical ORAC fluorescence decay curve for 0 – 38.08 µM Trolox. Curve shows relative fluorescence units (RFU) vs. time (sec).

Procedures used were based on a modified method by Ou *et al.*, (2001). AAPH was used as a peroxyl radical generator and Trolox as standard (0 - 1000µM) and fluorescein as a fluorescent probe. A 200 µM fluorescein stock solution was prepared (3.76 mg fluorescein disodium in 50 ml PBS. This stock solution was diluted to a final concentration of 558 nM (140 µl stock solution in 5 ml PBS and 45 ml dH_20) to prepare a fluorescein working solution. A final experimental solution was then prepared by rapidly mixing 16 ml fluorescein working solution with 4 ml of a 74 µM AAPH solution. A concentration series of 0 – 1 mM was prepared from a 1 mM Trolox stock solution. A 10 µl volume of the Trolox standard or 1% (v/v) tea extract was added to each well of a Greiner Bio-one 96-well opaque plate. A 200 µl volume of the experimental solution was added and samples were mixed well. The microplate was placed into the plate reader and incubated at 37^0C. Fluorescence was measured at five min intervals for four hours using a FLUOstar OPTIMA plate reader. The assay protocol was as follows: 0.0s measurement start time, 10 flashes per cycle, 300 seconds cycle time the excitation (Ex) and emission wavelengths (Em) were 485 and 520 nm respectively. The final sample ORAC values were calculated from the net area under the decay curves (AUC). The following equation was used:

AUC = Sample AUC – Control AUC

Net AUC to represent antioxidant capacity was expressed in µmol TE/g DM tea.

3.3. Data management and statistics

For each commercial tea type, three different lot numbers were used. Only two factory guayasa batches were available for the green and black guayasa tea. Each sample was analysed three times in triplicate. The results are expressed as mean ± standard error of the mean (SEM). Data was statistically evaluated using analysis of variance (ANOVA) with samples as independent variables and the values determined as dependent variables. Fisher's least significant difference (LSD) test was used for mean comparison using software Version 9.0 (StatSoft, Tulsa, OK). R^2 correlations between the different assays were determined (Microsoft Exel 2007).

3.4. Results and discussion

The aim of this study was to evaluate, using internationally standardized methodologies, the antioxidant activity of methanol extracts of *Ilex guayusa* tea. Then to compare this activity with a variety of commercially available *Camellia sinensis* teas. In addition, the antioxidant activity of each tea was also evaluated using DPPH, TEAC and ORAC assays.

Ilex guayusa tea is marketed as Runa tea (Runa LLC, 2016) and is commercially available in the USA. Therefore for comparative purposes black and green *Camellia sinensis* brands (Table 3.2) were also obtained from New York, USA supermarkets at the same site where commercial Runa was purchased. In addition, two different batches of green and black *Ilex guayusa* tea was obtained from the Runa tea factory, Ecuador and was included. The percentage moisture content of each tea was determined and is presented in Table 3.2. The average moisture content for green and black *Camellia sinensis* tea was 6.57 and 6.87% respectively. Green and black Guayusa tea had an average moisture content of 6.35 and 6.09% respectively.

4.4.1. Moisture content

Table 3.2: Moisture content (%) of each tea type

Camellia sinensis			
Green		**Black**	
LGT	5.72	**HSB**	7.39
TCG	6.30	**LBT**	5.14
TwG	6.82	**TAB**	7.39
TZG	7.06	**TwEB**	7.54
YGT	6.95		
Average	**6.57 ± 0.12**		**6.87 ± 0.18**
Ilex guayusa			
Green		**Black**	
IGG	6.35	**IGB**	5.93
		IGBC	6.25
Average	**6.35 ± 0.19**		**6.09 ± 0.16**

Due to possible variation between the tea samples all data was expressed as units per gram DM. According to the ISO method methanol extracts were prepared and the antioxidant reducing capacity was determined with the F-C method.

3.4.2. Antioxidant reducing activity - F-C method

The F-C method was used due to its simplicity, repeatability and popularity in literature and is the ISO method for quantifying antioxidant capacity in tea extracts (Prior *et al.*, 2005). Results acquired for the F-C method are presented in Figures 3.6 and table 3.3 and summarized in table 3.3.

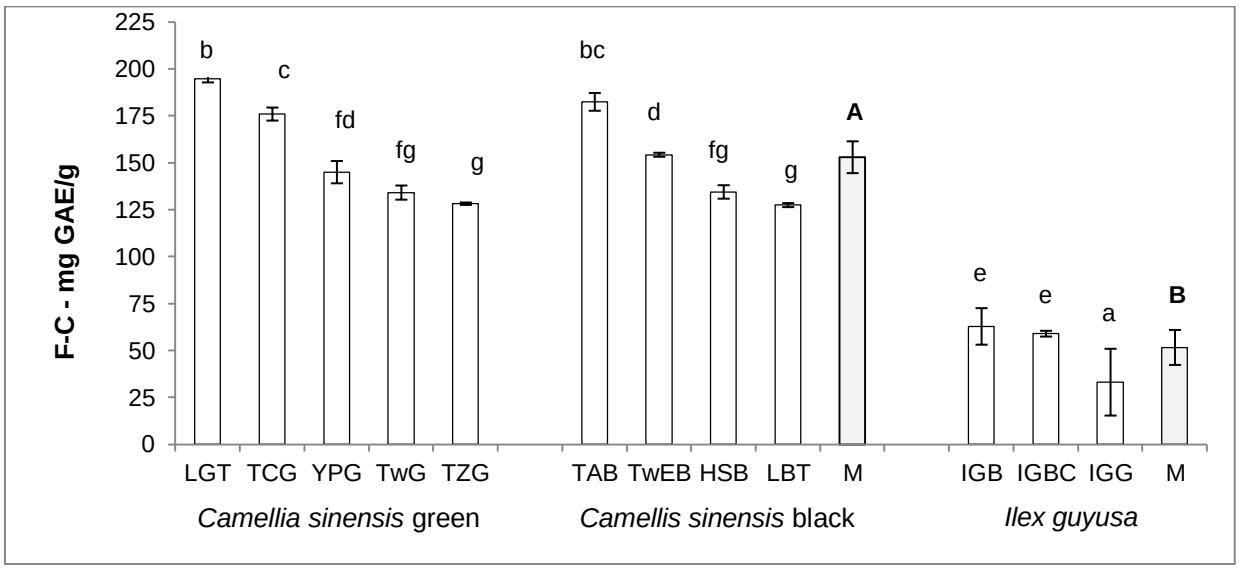


Figure 3.6: Antioxidant reducing capacity determined with the F-C method of green and black *Camellia sinensis and Illex guayusa* tea. Error bars indicating SEM three independent experiments for commercial tea and two independent experiments for factory teas (IGB and IGG). Tea brands indicated with different lower case letters (a-g) and means of tea types, indicated with different upper case letters (A-B) are statistically different from each other at the 95% level of confidence. M = mean.

Green *Camellia sinensis* antioxidant capacity measured with the F-C method was 128.2 ± 0.649 -194.6 ± 1.867 mg GAE/g tea with a mean value of 155.55 ± 3.14 mg GAE/g tea. The antioxidant reducing capacity of GT was variable with, LGT > TCG > YG > TwG > TZG and LGT and TCG having significantly higher activity than the other GT brands.

For black *Camellia sinensis* the range was 127.4 ± 1.065 - 182.4 ± 4.721 - mg GAE/g tea with a mean value of 149. 62 ± 2.61 mg GAE/g tea. Differences between GT and BT were not statistically significant. The antioxidant reducing capacity for individual BT samples was the highest for TAB with TAB > TwEB > HSB > LBT with HSB similar to LBT. For the same GT and BT brands the antioxidant reducing activity was TwEB > TwG, LGT > LBT and TAB = TCG > TZG. This highlights the importance of using several tea brands or sources in studies where antioxidant activity is compared.

The lowest range for antioxidant reducing capacity was for guayusa tea (*Ilex guayusa*) extracts and was 54.92 ± 16.30 62.81 ± 11.92 mg GAE/g tea with a mean value of 59.29 ± 9.93 mg GAE/g tea. *Ilex guayusa* antioxidant reducing capacity was significantly lower than both GT and BT. The average for black and green guayusa tea was only 26.76% and 39.63% of the GT and BT extracts, respectively.

Table 3.3: Summary and statistical evaluation of antioxidant reducing activity (F-C method) (mg GAE/g) of different tea types

	Green *Camellia sinensis*		Black *Camellia sinensis*		*Ilex guayusa*	
	LGT	194.62 ± 1.87^{b*}	**TAB**	182.39 ± 4.72^{bc}	**IGB**	62.81 ± 11.92^{e}
	TCG	175.91 ± 3.47^{c}	**TwEB**	154.24 ± 1.07^{d}	**IGBC**	60.13 ± 1.559^{e}
	YG	144.97 ± 5.94^{fd}	**HSB**	134.43 ± 3.58^{fg}	**IGG**	54.92 ± 16.30^{a}
	TwG	134.09 ± 3.76^{fg}	**LBT**	127.42 ± 1.07^{g}		
	TZG	128.16 ± 0.65^{g}				
Minimum	128.16 ± 0.65		127.42 ± 1.07		54.92 ± 16.30	
Maximum	194.6 ± 1.87		182.4 ± 4.72		62.81 ± 11.92	
Mean	155.55 ± 3.14^{A}		149.62 ± 2.61^{A}		59.29 ± 9.93^{B}	
% of GT			96.19		26.76	
% of BT					39.63	

**Different lower case letters (a-g) down columns shows statistical significance between tea samples, different upper case letters across rows (A-B) indicate statistical significance between tea types (ANOVA, p = 0.05).*

Using ISO methodology Zhao *et al*., (2014) reported the antioxidant reducing capacity determined with the F-C method was 108.8 ± 0.6 – 323.6 ± 9.3 mg GAE/g tea and 115.3 ± 0.4 – 181.7 ± 1.2 mg GAE/g tea for 5 brands of Chinese GT and BT, tea respectively. Differences were significant and the total polyphenol content and the catechin content decreased in the following order: green tea > oolong tea > black tea > dark tea.

In a study conducted by Anesini *et al*., (2008) to compare commercial Argentinian GT and BT brands using ISO methodology, the antioxidant reducing capacity for three GT brands was 14.32 ± 0.45 – 21.02 ± 1.54% GAE (equivalent to 143.2 – 210.2 mg GAE/g tea) and for six BT was 8.42 ± 0.55 – 17.62 ± 0.42% GAE (equivalent to 84.2 – 176.2 mg GAE/g tea). The ranges for measured antioxidant reducing activity, reported in both studies, were similar to the findings of the present study. Astill *et al*. (2001), using the packaging instructions to infuse the tea samples, reported that GT contains the highest percentage of polyphenols (11.9-25.2%) when compared with BT (7.3-21.9%).

Izreen and Fadzelly, (2013) evaluated GT and BT shoots, young leaves and mature leaves extracted with 50% ethanol. It was found that all GT plant samples (shoots and leaves) had higher F-C reducing capacity compared with BT, although not significant. It was also found that shoots had highest F-C reducing capacity, followed by young leaves and then mature leaves. This study elaborates that any differences found amongst samples and differences in studies are attributed to differences in brand quality which is dependent on several factors. These

include the inherent chemical composition of the tea leaves such as the type and concentration of flavonols found in the vacuoles of tea leaves as well as the oxidized black tea pigments such as theaflavins and thearubigens that form with fermentation (Senthil Kumar *et al*., 2011; Bhuyan *et al*., 2013). The theaflavin and thearubigen content of BT is largely dependent on factors such as the genetic variation of the particular cultivar, geographic location and environmental conditions surrounding the cultivar as well as factory factors associated with quality control (McDowell *et al*., 1991). These factors may account for the lack of statistical differences found between GT and BT.

3.4.3. Antioxidant activity - TEAC assay

The TEAC assay is an antioxidant assay that involves of ET and has been used to measure the antioxidant capacity of various food samples (Huang *et al*., 2005). The antioxidant activity determined using this method is presented in Figure 3.7 and summarised in Table 3.4.

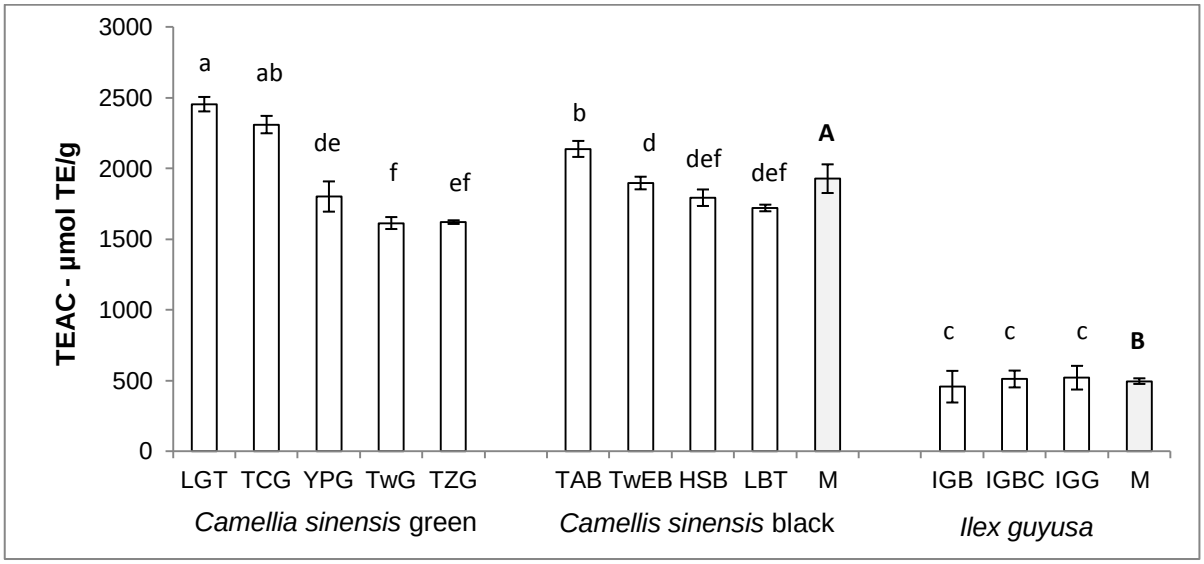


Figure 3.7: Antioxidant activity measured with the TEAC assay of green and black *Camellia sinensis* and *Illex guayusa* tea. Error bars indicating SEM of three independent experiments for commercial teas and two independent experiments for factory teas (IGB, IGG). Tea brands indicated with different lower case letters (a-f) and means of tea types indicated with different uppercase letters (A-B) are statistically different from eachother at a 95% confidence interval. M = mean.

Green *Camellia sinensis* antioxidant activity measured with the TEAC assay was 1619.41 ± 12.90 – 2453.35 ± 51.08 µmol TE/g with a mean value of 959.01 ± 54.91 µmol TE/g. The highest antioxidant activity was found for LG and TCG which was greater than YGT, TwG, TZG (TwG and TZG are similar). For black *Camellia sinensis* the range was 1719.36 ± 21.73 - 2136.41 ± 52.18 with a mean value of 1886.03 ± 45.73 µmol TE/g. For BT antioxidant activity for

TAB was significantly higher than that found for TwEB, HSB and LBT (all similar). Differences between GT and BT were not significant. Average BT antioxidant activity was 96.27% of GT brands. *A*ntioxidant reducing activity for the same brands was TwEB > TwG, LGT > LBT and TAB similar to TCG but higher than TZG. *Ilex guayusa* antioxidant activity was significantly lower than GT and BT with a 455.65 ± 111.74 -519.19 ± 83.70 µmol TE/g range and a mean value of 4.96 ± 84.97 µmol TE/g. *Ilex guayusa* tea was found to have the lowest TEAC values within the range of 590.75 ± 100.65 - 485.71 ± 100.22 µmol TE/g and a mean value of 455.65 ± 111.74 µmol TE/g. The average antioxidant antioxidant activity was 25.27% and 26.24% of measured GT and BT activity respectively. Correlation between the F-C and TEAC assays was evaluated (Figure 3.8) was 0.978.

Table 3.4: Summary and statistical evaluation of TEAC values (µmol TE/g) of different tea types

	Green *Camellia sinensis*		Black *Camellia sinensis*		*Ilex guayusa*	
	LGT	2453.35 ± 51.08^{a*}	**TAB**	2136.95 ± 56.60^{b}	**IGB**	455.65 ± 111.74^{c}
	TCG	2309.06 ± 61.50^{ab}	**TwEB**	1895.83 ± 44.53^{d}	**IGBC**	510.05 ± 59.47^{c}
	YG	1800.30 ± 106.85^{de}	**HSB**	1791.97 ± 58.40^{def}	**IGG**	519.19 ± 83.70^{c}
	TwG	1612.93 ± 42.23^{f}	**LBT**	1719.36 ± 23.40^{def}		
	TZG	1619.41 ± 12.90^{ef}				
Minimum	1619.41 ± 12.90		1719.36 ± 21.73		455.65 ± 111.74	
Maximum	2453.35 ± 51.08		2136.41 ± 52.18		519.19 ± 83.70	
Average	1959.01 ± 54.91^{A}		1886.03 ± 45.73^{A}		494.96 ± 84.97^{B}	
% of GT			96.27		25.27	
% of BT					26.24	

**Different lowercase letters (a-f) down columns shows statistical significance between tea samples, different uppercase letters across rows (A-B) indicate statistical significance between tea types (ANOVA, p = 0.05).*

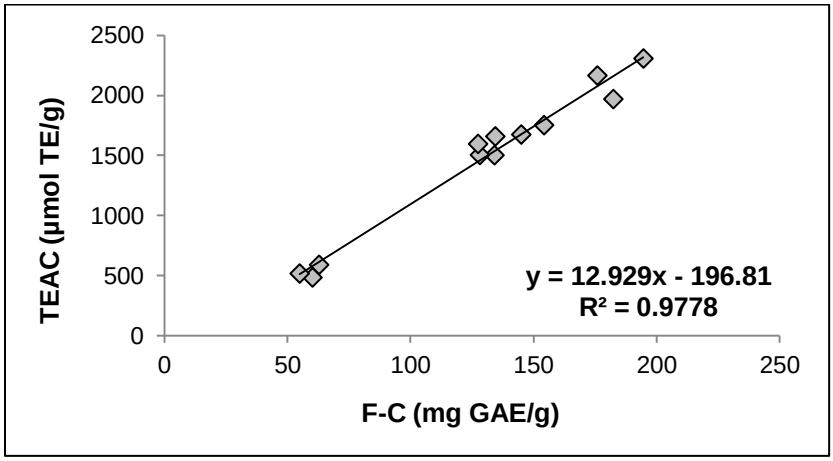


Figure 3.8: Correlations between reducing capacity according to the F-C method vs. TEAC assay.

Rusaczonek *et al*. (2010) determined the antioxidant properties of water infusions from several brands of BT and GT with the F-C method and TEAC assay. The antioxidant reducing activity (F-C assay) was 120 ± 1.7 – 185 ± 5.8 mg GAE/g and 112 ± 2.5 – 151 ± 4.3 mg GAE/g for green and black *Camellia sinensis* tea, respectively. Antioxidant activity, TEAC assay was 1418 ± 27.4 – 2191 ± 136.4 µmol TE/ g and 1149 ± 47.8 – 1703 ± 68.7 µmol TE/g tea for GT and BT, respectively. The measured antioxidant activity, TEAC assay was similar to the findings of the present study. Rusaczonek *et al*. (2010) reported that the antioxidant activity of GT was statistically higher than BT. Similar levels between this and the present study indicates that the amount of antioxidant molecules extract with methanol and water are similar. Studies have shown that antioxidant activity of GT determined with the TEAC assay was significantly higher than that of BT (Pellegrini *et al*., 2003; Almajano *et al*., 2008) which is different from the findings of this study.

This may be due to differences in factors such as the extraction efficiency in different solvents, differences between brands, quality control, harvesting conditions and geographic origin of the teas. Pellegrini *et al*. (2003) reviewed a database including juices, oils and beverages and the antioxidant activity of only two tea samples was determined. Alamajano *et al*. (2008) compared 13 different types of tea but only one brand of each was evaluated.

A high degree of correlations between antioxidant activities, measured with the TEAC assay and F-C method was found and this is similar to the findings of Rusaczonek *et al*. (2010) for *Camellia sinensis* tea with a R^2 correlation of 0.985.

3.4.4. Antioxidant activity – DPPH assay

The antioxidant activity of the tea extracts was further evaluated with the DPPH radical scavenging assay. The antioxidant activity determined using the DPPH antioxidant method is presented in Figure 3.9 and summarised in table 3.5.

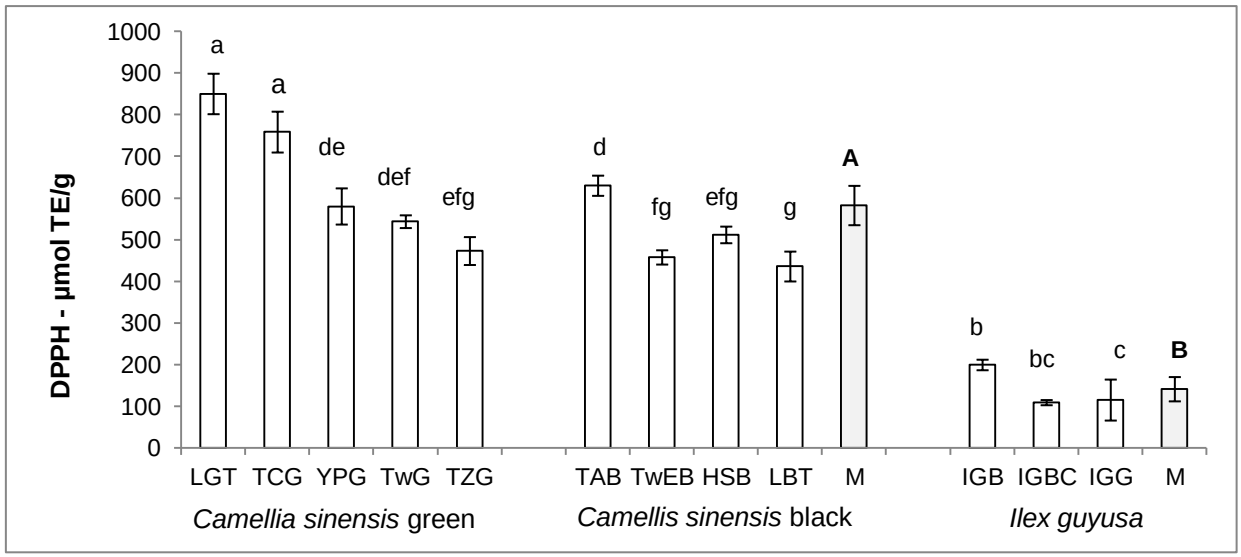


Figure 3.9: Antioxidant activity measured with the DPPH assay of green and black *Camellia sinensis* and *Ilex guayusa* tea. Error bars indicating SEM three independent experiments for commercial tea and two independent experiments for factory teas (IGB and IGG). Tea brands indicated with different lower case letters (a-g) and means of tea types, indicated with different upper case letters (A-B) are statistically different from each other at the 95% level of confidence. M = mean.

Green *Camellia sinensis* antioxidant activity, DPPH assay was 472.93 ± 33.48 – 850.05 ± 48.58 µmol TE/g with a mean value of 640.95 ± 37.96 µmol TE/g. The highest antioxidant activity was found for LGT and TCG which was greater than YGT, TwG, TZG (YGT, TwG, TZG with similar antioxidant activity). For black *Camellia sinensis* the range was 435.75 ± 35.85 - 629.67 ± 24 µmol TE/g with a mean value of 508.65 ± 22.48 µmol TE/g. The antioxidant activity for TAB was significantly higher than that found for TwEB, HSB and LBT (all similar). Differences between green and black *Camellia sinensis tea* were not statistically significant.

Average black *Camellia sinensis* antioxidant activity was 79.36% of GT brands. Antioxidant activity between the same brands showed that TwEB and TwG was similar, LGT > LBT and TCG > TAB > TZG.

The antioxidant activity for *Ilex guayusa* measured with the DPPH assay was significantly lower than GT and BT with a 108.96 ± 12.06 - 199.38 ± 12.63 µmol TE/g range and a mean value of

141.16 ± 22.68 µmol TE/g. Average antioxidant activity was 22.02% and 27.75% of measured GT and BT activity respectively.

Table 3.5: Summary and statistical evaluation of DPPH values (µmol TE/g) of different tea types

	Green *Camellia sinensis*		Black *Camellia sinensis*		*Ilex guayusa*	
	LGT	850.05 ± 48.58^{a*}	**TAB**	580.51 ± 22.34^{b}	**IGB**	199.38 ± 12.62^{b}
	TCG	758.47 ± 49.06^{a}	**TwEB**	423.06 ± 15.83^{fg}	**IGBC**	108.96 ± 6.23^{bc}
	YG	579.94 ± 43.41^{de}	**HSB**	473.84 ± 18.47^{efg}	**IGG**	115.15 ± 49.20^{c}
	TwG	543.38 ± 15.30^{def}	**LBT**	404.72 ± 33.29^{g}		
	TZG	472.93 ± 33.48^{efg}				
Minimum	472.93 ± 33.48		435.75 ± 35.85		108.96 ± 12.06	
Maximum	850.05 ± 48.58		629.67 ± 24.23		199.38 ± 12.63	
Average	640.95 ± 70.36^{A}		508.65 ± 22.48^{A}		141.16 ± 22.68^{B}	
% GT			79.36		22.02	
% BT					27.75	

**Different lowercase letters (a-g) down columns shows statistical significance between tea samples, different uppercase letters across rows (A-B) indicate statistical significance between tea types (ANOVA, p = 0.05).*

The correlations achieved for DPPH with F-C and TEAC assays are shown in Figure 3.10. Correlations between antioxidant activity measured with DPPH assay and the F-C method and TEAC assay was 0.928 and 0.941 respectively. Similar findings were reported by Anesini *et al*. (2008) who found a r^2 value of 0.9141 between TPC (F-C method) and DPPH values.

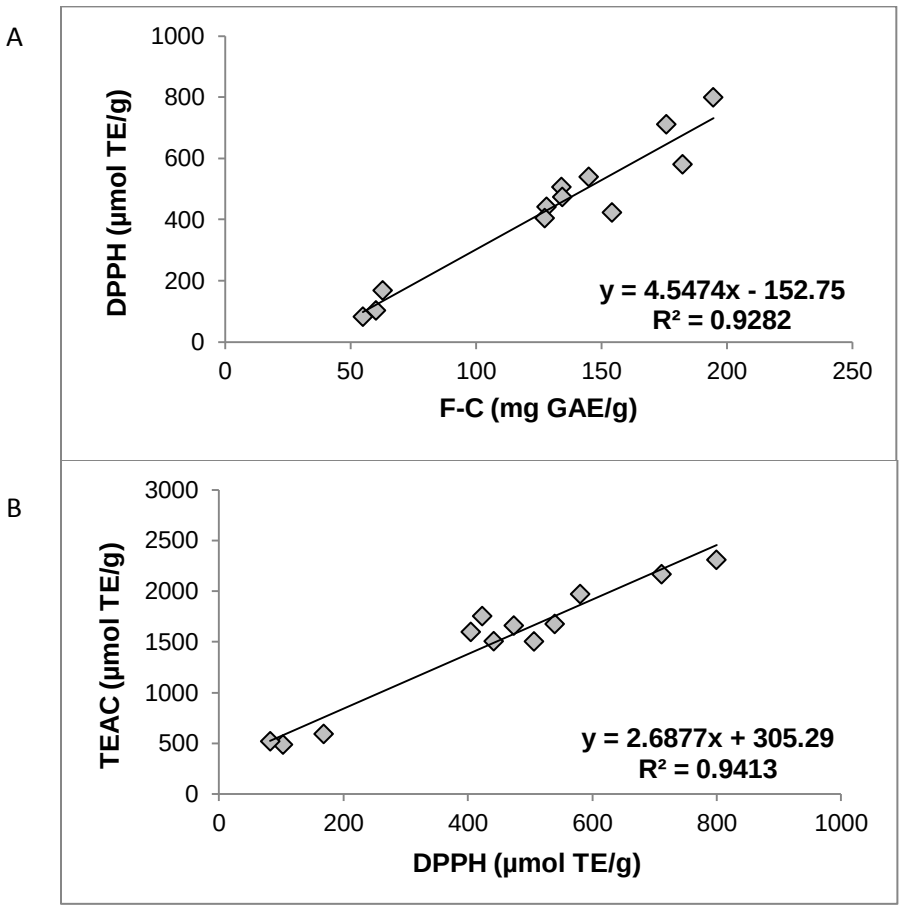


Figure 3.10: Correlation between DPPH assay and A) F-C method and B) TEAC assays.

Similar to the TEAC assay, the DPPH is also an ET assay. A limitation of the DPPH assay is that data is often expressed as IC_{50}, % inhibition or as µmol TE/g. In addition Sharma and Bhat, (2009) point out that many research groups use various versions of the method and this also makes comparisons between studies difficult. Differences are related to differences in DPPH concentration, incubation time, reaction solvent, and the pH of the reaction mixture.

Zhao *et al*. (2014) determined the antioxidant activity of water extracts of BT, GT and oolong tea brands with the DPPH assay. In this study these researchers reported EC_{50} values acquired for GT ranging from 0.142 – 0.246 mg tea/ml and for BT 0.187 – 0.365 mg tea/ml. Since the lower the EC_{50} value, the higher the antioxidant capacity, these results indicate that GT has higher antioxidant capacity than BT. For all ET methods, no literature could be found about the antioxidant activity of *Ilex guayusa* tea.

3.4.5. Antioxidant activity – ORAC assay

The antioxidant activity of the tea samples was further evaluated with the ORAC assay which is considered to be more biologically relevant as a biologically relevant radical is used in this assay (Prior *et al*., 2005). Unlike the F-C, TEAC and DPPH assays, the ORAC assay is a HAT method. The results obtained for the ORAC assay are presented in Figures 3.11 and summarized in table 3.6.

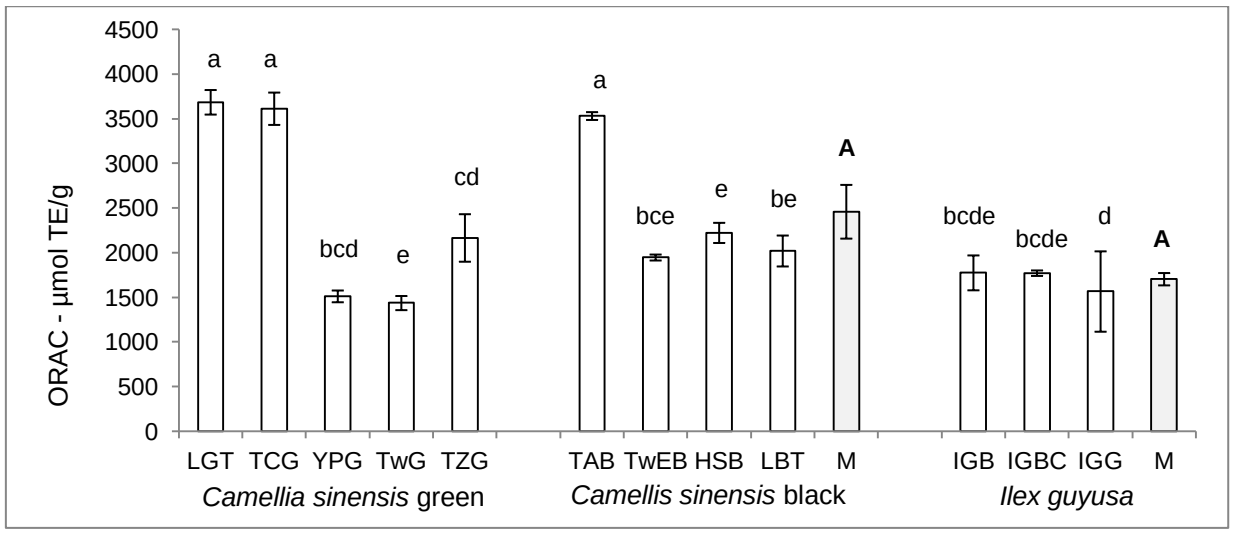


Figure 3.11: Antioxidant activity measured with the ORAC assay of green and black *Camellia sinensis* and *Illex guayusa* tea. Error bars indicating SEM three independent experiments for commercial tea and two independent experiments for factory teas (IGB and IGG). Tea brands indicated with different lower case letters (a-e) are statistically different from each other at the 95% level of confidence. The means of tea types, indicated with the same upper case letter (A) are not statistically different from each other at the 95% level of confidence. M = mean.

Green *Camellia sinensis* ORAC values ranged 2167.52 ± 266.19 - 3687.22 ± 137.51 µmol TE/g with a mean value of 2484.2 ± 146.00 µmol TE/g. LG and TCG had the highest antioxidant activity compared with YGT and TwG (YGT and TwG, similar ORAC values) and TZG. Black *Camellia sinensis* had lower ORAC values with a 2031.37 ± 173.28 - 3533.14 ± 43.81 µmol TE/g range and 2431.75 ± 90.57 mean value. Average BT values was 97.89% of GT and differences were not significant. TAB antioxidant activity was greater than TwEB, HSB and LBT (all similar). LG > LB, TwG is similar to TwEB and TAB is also similar to TCG > TZG when taking similar brands into consideration.

Antioxidant activity measured with the ORAC assay for *Ilex guayusa tea* was statistically similar GT and BT with a 1567.15 ± 450.17 - 1776.34 ± 195.41 µmol TE/g range and 1705.42 ± 225.42 µmol TE/g mean value. The average antioxidant activity was 68.65% and 70.13% of measured

GT and BT antioxidant activity respectively. This comparison is higher than the 26.76%, 25.27% and 22.02% measured with the F-C, TEAC and DPPH assays, respectively. Statistical differences between all tea types were not significant.

Table 3.6: Summary and statistical evaluation of ORAC values (µmol TE/g) of different tea types

	Green *Camellia sinensis*		Black *Camellia sinensis*		*Ilex guayusa*	
	LGT	3687.22 ± 137.51^{a}	**TAB**	3533.14 ± 43.81^{a}	**IGB**	1776.34 ± 195.41^{bcde}
	TCG	3614.92 ± 181.22^{a}	**TwEB**	1948.42 ± 33.32^{bce}	**IGBC**	1772.76 ± 30.69^{bcde}
	YGT	1513.13 ± 65.88^{bcd}	**HSB**	2224.08 ± 112.85^{e}	**IGG**	1567.15 ± 450.17^{d}
	TwG	1438.24 ± 79.20^{e}	**LBT**	2021.37 ± 172.28^{be}		
	TZG	2167.52 ± 266.19^{cd}				
Minimum	2167.52 ± 266.19		2031.37 ± 173.28		1567.15 ± 450.17	
Maximum	3687.22 ± 137.51		3533.14 ± 43.81		1776.34 ± 195.41	
Average	2484.2 ± 146.00^{A}		2431.75 ± 90.57^{A}		1705.42 ± 225.42^{A}	
% of GT			97.89		68.65	
% of BT					70.13	

**Different lower case letters (a-e) down columns shows statistical significance between tea samples, similar upper case letters across rows (A) indicate no statistical significance between tea types (ANOVA, p = 0.05).*

The correlations achieved for ORAC with the F-C, TEAC and DPPH methods are shown in Figure 3.12. Correlations between the ORAC assay and F-C, TEAC and DPPH assays for all teas was poor with values of 0.433, 0.399 and 0.441 respectively. When only *Camellia sinensis teas* were compared (*Ilex guayusa excluded)* the generated r^2 correlations of 0.739, 0.792 and 0.622 for F-C, TEAC and DPPH, respectively where achieved which is similar to the findings of Zhao *et al*. (2014) where a correlation of $r^2 = 0.77$ was reported for *Camellia sinensis* extracts. Roy *et al*. (2010) also reported a similar correlation between F-C and ORAC assays of 0.7323. This poor correlation is based on the differences between the assays and the polyphenols in *Ilex guayusa* tea have very specific type of activity related to the scavenging of the hydroxyl radical.

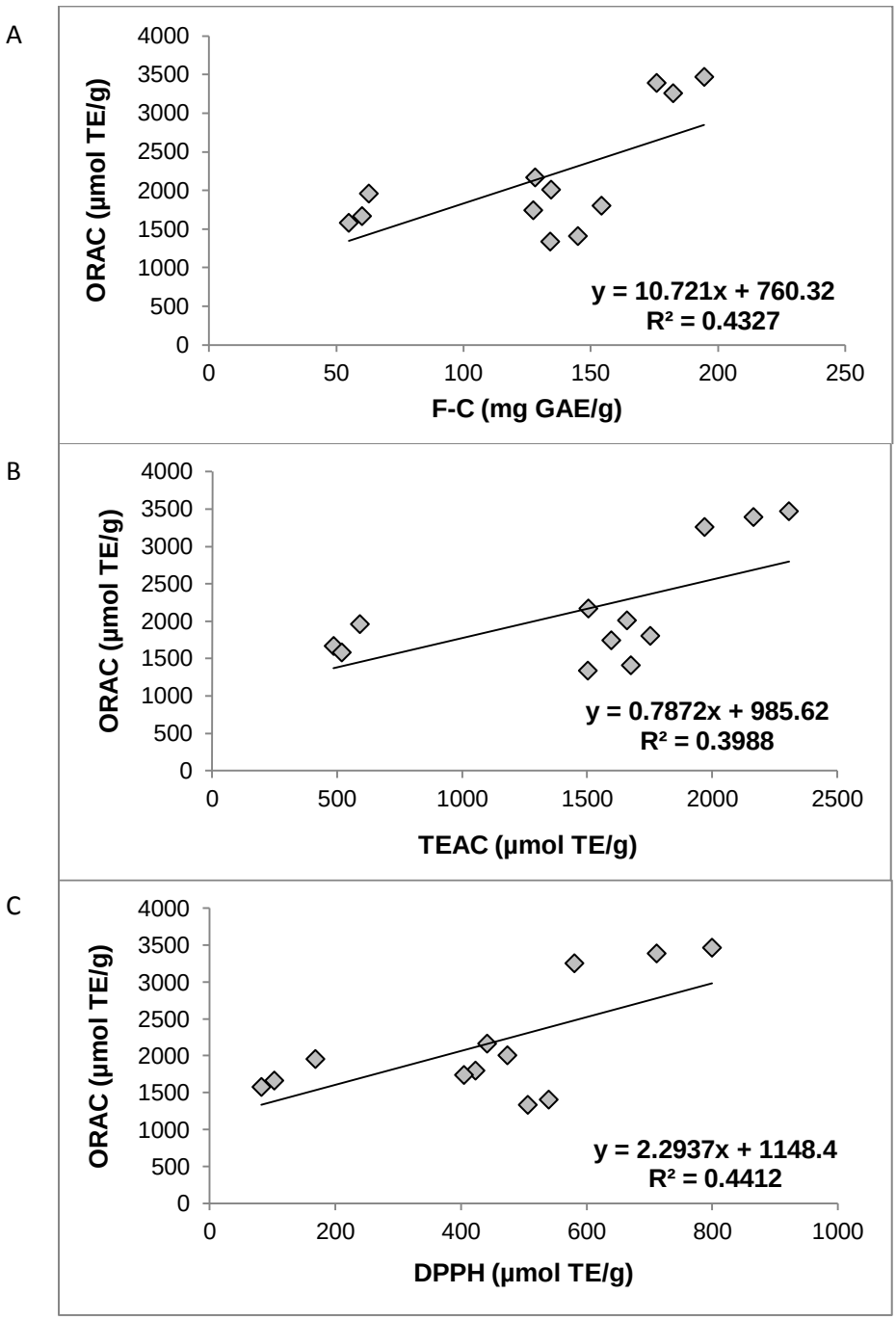


Figure 3.12: Correlation for ORAC and A) F-C method B) TEAC assay C) DPPH assay.

In a study conducted by Zhao *et al*. (2014) using the ISO extraction method, similar to this study, the ORAC values of various GT, BT and oolong tea extracts were evaluated and for these teas the antioxidant activity was 909.28 – 3092.51 µmol TE/g which is similar to the range found in the present study. Even though the author does not specify the ranges for GT, BT and oolong tea specifically, it is stated that the rankings of antioxidant capacity, measured by ORAC

were as follows: green tea > oolong tea > black tea. For this study a positive correlation of R^2 = 0.77 was found between the TPC (F-C method) and ORAC values.

Roy *et al*. (2010) evaluated five brands of GT water infusions and reported ORAC values ranged from 10 888.6 ± 159.4 µmol TE/g - 4 513.3 ± 219.8 µmol TE/g which also similar to the findings of the present study.

Tabart *et al*. (2009) and Kevers *et al*. (2007) found higher correlations between TEAC and DPPH than with the ORAC assays. Cao and Prior, (1998) compared the ORAC, FRAP and TEAC assays in blood serum samples and a poor correlation was found between ORAC and FRAP assays and no correlation was found between the between ORAC and TEAC assays. These authors speculated that this was due to the ORAC assay being capable of responding to a wider range of antioxidant types compared with the other two methods. Zulueta *et al*. (2009) also reported a poorer correlation between the ORAC and TEAC and this was also attributed to the complexity of tea samples. Differences were attributed to differences in reaction mechanisms and kinetics as well as the type of antioxidants present in the samples. A strong correlation between the ET methods, the F-C method, TEAC and DPPH assays indicates that differences between the reaction mechanisms may account for the poor correlation between these assays and the ORAC assay which is an HAT method that also measures the total sum of time dependent, antioxidant activity.

In the present study, with all assays, there were no significant differences between BT and GT brands. Leung *et al.* (2001) also found that the antioxidant effects of catechins and thearubigens predominantly found and GT and BT respectively on Cu^{2+}-mediated LDL oxidation were similar. It was concluded that the conversion of catechins to thearubigens during fermentation does not significantly reduce the antioxidant capacity of the thearubigens in BT.

Evaluation of antioxidant properties with ET based methodologies showed that the *Ilex guayusa* had significantly lower antioxidant properties than GT and BT. In contrast with the ORAC assay antioxidant activity of *Ilex guayusa* tea was statistically similar to BT and GT brands. Differences may be related to the type of antioxidants present in this tea as well as the reaction mechanism and kinetics. For example, measured antioxidant activity compared with Trolox as a reference antioxidant, activity of flavonols, anthocyanins, and flavonons measured with the ORAC assay were considerably higher than when measured with other assays (particularly the TEAC and DPPH assays) (Tabart *et al*., 2009).

Chlorogenic acid is an abundant polyphenol found in *Ilex guayusa* and is formed by the esterification of caffeic and quinic acids (Suzuki *et al*., 2006), which have also been found to be

an abundant polyphenol in *Ilex guayusa*. There are a number of health benefits associated with chlorogenic acid (Swanston-Flatt *et al*., 1989; Bailey and Day, 1989; Shan *et al*., 2009; Sato *et al*., 2011; Yun *et al*., 2011). Kondo *et al*. (1997) showed that chlorogenic acid was able to scavenge reactive species of oxygen and nitrogen. To further elaborate on the health properties of chlorgenic acid, Suzuki *et al*. (2006) showed that the chlorogenic acid, 5-caffeoylquinic acid, reduced blood pressure and oxidative stress in spontaneously hypertensive rats. Inhibition of excessive ROS improved endothelial function and reduced vascular hypertrophy, and hypertension. Besides antioxidant activity related to HAT, the main polyphenol found in *Ilex guayusa* tea also has reported health benefits. Therefore, it is important to further evaluate the antioxidant properties of water extracts of *Ilex guayusa,* as consumed in a relevant cell model system.

3.5. Conclusion

All tea samples evaluated showed antioxidant activity although the antioxidant activity of *Ilex guayusa tea is* significantly less than green and black *Camellia sinensis* tea with regards to ET mechanisms and similar to GT and BT with regards to HAT mechanisms. As the latter is physiologically relevant the health benefits of *Ilex guayusa* was further evaluated in Chapter 4.

CHAPTER 4: HEALTH BENEFITS OF *ILEX GUAYUSA* COMPARED WITH *CAMELLIA SINENSIS* AND *ASPALATHUS LINEARIS*

4.1 Introduction

Tea water extracts are universally prepared for consumption purposes. In China, GT leaves are steeped in water at 70-80°C, oolong tea leaves at 80-90°C and BT leaves at 100°C for 20 to 40 seconds. This process is then usually repeated seven times. The Japanese have a different method of tea preparation where tea leaves are steeped in hot water for two minutes and the leaves are reused for 2 or 3 infusions. In countries such as Canada, Ireland, and the United Kingdom, black tea is most popularly consumed and is often had with milk and sugar, whereas in America, iced tea has become a popular beverage which involves cooling hot tea with ice. Another method of extraction includes cold water steeping, where leaves are left to steep in water at 4 or 25°C for 2 hours or longer. This method has increased in popularity due to the notion that cold water steeping allows for lower amounts of caffeine and bitterness and higher levels of aroma (Venditti *et al*., 2010; Yang *et al*., 2007). Further variation is due to individual preference (Peterson *et al*., 2004). Guayusa *(Ilex guayusa)* tea is steeped for 1-5 min in freshly boiled water. The herbal Rooibos tea (*Aspalathus linearis*) is steeped in freshly boiled water for 2-4 minutes, but may also be prepared as a strong brew left on heat for long periods of time and consumed with or without milk and sugar or even cold with the addition of lemon juice. The Japanese, however, consume rooibos in a very diluted form, usually adding 3.5-4.5 g of per 2 litres of water for 20 minutes (Joubert *et al*., 2008). Traditionally both types of tea are steeped for several hours in either close to a fire or on a stove top. Differences in the methods of preparation makes it difficult to evaluate the health benefits of different types of tea.

Green *Camellia sinensis* tea is known to be rich in flavonoid antioxidants such as catechins. Theaflavins and thearubigens are unique to black tea and form during fermentation (Balentine *et al*., 1997; Li *et al*., 2013; Rusak *et al*., 2008). Rooibos tea as commonly consumed is a fermented product derived from the *Aspalathus linearis* plant (Joubert *et al*., 2012). Rooibos tea is rich in flavonoids and these are mainly aspalathin, rutin and orientin (Joubert and Ferreira, 1996). Many studies using chemical based assays have identified the antioxidant properties of *Camelllia sinensis* (Izreen and Bhat, 2013; Shin *et al*., 2007; Rusak *et al*., 2008; Astill *et al*., 2001; Khokar and Magnusdotter, 2001; Roy *et al*., 2010; Zuo *et al*., 2002; Henning *et al*., 2003;

Liang *et al*., 2006; Lin *et al*., 1996) and *Aspalathus linearis (*Standley *et al*., 2001 and Von Gadow *et al*., 1997). All studies are in agreement that tea from *Camellia sinensis* and *Aspalathus linearis* are excellent sources of polyphenols with antioxidant activity however differences between studies are due to differences between cultivars, season of collection, sample preparation, extraction solvent and the extraction time used. In addition to having chemical antioxidant activity, both *Camellia sinensis* (Satoh *et al*., 2005; Zhu *et al*., 2002; Zang *et al*., 1997; Jiao *et al*., 2003 and Liu *et al*., 2007) and *Aspalathus linearis* (Yoo *et al*., 2006; Awoniyi *et al*., 2012) have been shown to exert antioxidant protective and anti-inflammatory effects in cellular, animal and clinical studies (Baba *et al*., 2009; Hendricks and Pool, 2010).

These antioxidant and anti-inflammatory properties are responsible for various health benefits associated with the consumption of *Camellia sinensis* and *Aspalthus linearis* tea. *Camellia sinensis* has been shown to reduce oxidative stress (Madamanchi *et al*., 2005; Rietveld and Wiseman, 2003; Trevisanato and Young-In, 2000; Coimbra *et al*., 2006) which is implicated in various diseases such as cardiovascular disease (Nantz *et al*., 2009; Mukmal *et al*., 2007) and cancer (Weisenburger *et al*., 1997; Betuzzi *et al*., 2006; Liu *et al*., 2008; Mukhtar and Ahmad, 2000). The consumption of *Camellia sinensis* tea has also been shown to have an impact on bone density, cognitive function, kidney stones (McKay and Blumberg*, 1978*; Sandley *et al*., 2001) and aids in the eradication of the influenza virus (Song et al., 2005). Flavonoid rich *Aspalathus linearis* has also been shown to have chemopreventive effects (Marnewick *et al*., 2009).

Claimed health benefits of *Ilex guayusa* tea includes the energizing of the body and mind (Ferreira *et al*., 2012). Studies have shown that *Ilex guayusa* tea reduces steptozocin-induced diabetes in mice and this has been attributed to the presence guadine (Swanston-Flatt *et al*., 1989; Bailey and day, 1989). No scientific information is available regarding the antioxidant and anti-inflammatory properties of *Ilex guayusa* tea.

The aim of this study is to evaluate the health benefits of *Ilex guayusa* tea compared to *Camellia sinensis* and *Aspalathus linearis* tea related to antioxidant and anti-inflammatory properties. This study will address previous limitations of other comparative studies related to brewing time and the type of cellular models used to evaluate activity.

4.2. Materials and methods

4.2.1. Materials

4.2.1.1 Reagents, equipment and cell lines

Mouse fibroblasts (SC-1) were obtained from Highveld Biological Company, Johannesburg, South Africa. Human colon cancer cells (Caco-2) were obtained from American type culture collection (ATCC) and transformed. Adult, mouse macrophage cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC).

The media and supplements used were: Eagles Minimum Essential Medium (EMEM) powder, foetal calf serum (FCS) and antibiotic solution (containing, streptomycin, penicillin and fungicide) were obtained from Highveld Biological Company, Johannesburg, South Africa. Dulbecco's Modified Essential Medium (DMEM), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Crystal Violet (CV), sodium nitroprusside (SNP), PBS (Sodium phosphate dibasic dihydrate ($Na_2HPO_4.2H_2O$), sodium phosphate (NaH_2PO_4) and sodium chloride (NaCl), dichloro-dihydro-fluorescein diacetate (DCFH-DA), lipopolysaccharide (LPS) and interferon-gamma (INF-γ) were obtained from Sigma-Aldrich, Atlasville, South Africa. Fixatives, acids, salts and organic solvents such as: acetic acid, were analytic grade and together with ethylene diaminetetraacetic acid (EDTA) ($C_{10}H_{16}N_2O_8$) and dimethyl sulphoxide (DMSO) were obtained from Merck, South Africa. Trypsin was obtained from Life Technologies Laboratories and was supplied by Gibco BRL Products, Johannesburg, South Africa. Sartorius cellulose acetate membrane filters 0.22 µm were obtained from National Separations, Johannesburg, South Africa. Water was double distilled and de-ionised (ddH_2O) with continental water system and all media, enzyme solutions and buffers were sterilized by filtration through a Millex 0.2 µm filter. Glassware was sterilized at 121°C for 20 minutes in a Prestige Medical Autoclave (series 2100).

Disposable plasticware included: 96 and 24 well plates, 25 cm^2 and 75 cm^2 tissue culture flasks, 50 ml and 15 ml centrifuge tubes, 10 and 5 ml pipettes and pipette tips (10, 25, 100, 200, and 1000 µl) and were obtained from either Greiner Bio-one supplied by LASEC, Cape Town, South Africa or NUNC™ supplied by AEC-Amersham, Johannesburg, South Africa.

4.2.1.2. Samples

Commercial *Ilex guayusa* (Runa tea) samples were obtained from New York in a local supermarket. Non-commercial, factory samples were obtained from the Runa tea factory in Ecuador. Lipton's black, green and rooibos tea were purchased from various local South African supermarkets. Three different batches, each batch with a different lot number were purchased, in order to achieve variety of samples. All purchases were made during 2013-2014. Lipton's GT was chosen as in Chapter 3 it was found to have the highest antioxidant activity when compared to various *Camellia sinensis* brands. For this reason, Lipton's tea was used throughout this study as all teas undergo the same quality control. Lipton's RT was included in this study as it is a herbal tea commonly consumed in South Africa and has various health benefits. The experimental strategy that was used is presented in Figure 4.1.

Table 4.1: List of tea samples and abbreviations

Camellia sinensis	
Green	**Black**
LGT - Lipton's green tea	**LBT** - Lipton's black tea
Aspalathus linearis	
	Black
	LRT - Lipton's rooibos tea
Ilex guayusa	
Green	**Black**
IGG* - Green *Ilex guayusa* (factory)	**IGB*** - Black *Ilex guayusa* (factory)
	I**GBC** - Black commercial *Ilex guayusa*

** Indicates non-commercial brands, attained as leaf form directly from factory*

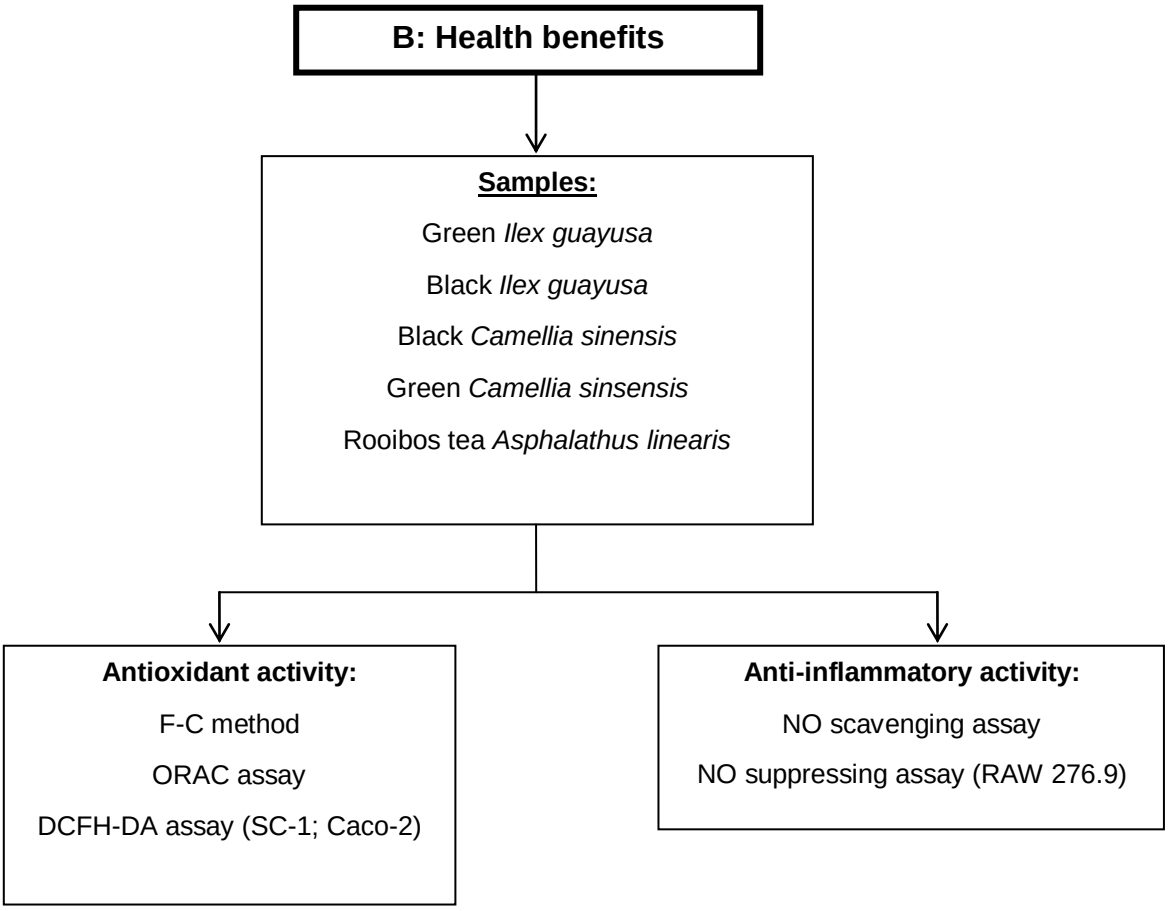


Figure 4.1: Experimental strategy for evaluation of antioxidant and NO scavenging/ suppressing properties of tea samples.

4.2.2. Methods

4.2.2.1. Moisture content

Moisture content was determined as described in 3.4.2

4.2.2.2. Sample extraction

Water extractions were prepared, as this is the conventional way in which tea is consumed. Extracts of 0-60 minutes were prepared from each tea type and included most steeping times commonly used for tea preparation. Water was added to flasks and allowed to reach 90°C, in a

water bath, before each tea bag was added at concentration of 1g/100 ml H_2O to prepare a final 10% tea solution. A sample of tea was then removed at 2, 5, 10, 20, 30, 40, 50 and 60 minutes time intervals. Each sample was evaluated for antioxidant and anti-inflammatory activity as shown in Figure 4.1.

4.2.2.3. F-C method

Due to the large number samples the antioxidant reducing activity was determined with the F-C method in a microplate format as described by Serem and Bester (2012). To a 96-well plate, 10 µl of a 10% (v/v) tea solution of each sample was added to each well. This was followed by 50 µl of a 1:15 FC reagent solution and 50 µl of a 7.5% (w/v) Na_2CO_3 solution. The samples were well mixed and the absorption determined at 630 nm using a plate reader. A 1 mg/ml gallic acid solution was diluted to 0 - 0.4 mg/ml to prepare a standard curve and calculate antioxidant reducing activity, expressed as mg GAE/g DM tea.

4.2.2.4. ORAC assay

The ORAC assay was performed as described in section 3.2.2.5. Due to the large number of samples, time intervals 0, 2, 10, 30 and 60 minutes were selected and evaluated. All data was expressed in µmol TE/g DM tea.

4.2.2.5. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay

The DCFH-DA assay is a cellular antioxidant assay that is useful for the measurement of antioxidant capacity *in vitro.* In this assay (Figure 4.2) non-fluorescent, DCFH-DA, is added to the cell culture medium. The DCHF-DA diffuses into the cell (Kalyanaraman *et al*., 2012) where it is hydrolysed into nonfluorescent, DCFH by intercellular esterases. In the presence of ROS, DCFH is converted to fluorescent DCF. Antioxidants added added to the cell culture will result in lower levels of DCF production (Wang and Joseph, 1999; Blasa *et al*., 2011). The reaction that takes place is presented in Figure 4.2 (Adapted from Afri *et al*., 2004). In this study the cellular antioxidant activity (CAA) was evaluated in the SC-1 and Caco-2 cell lines.

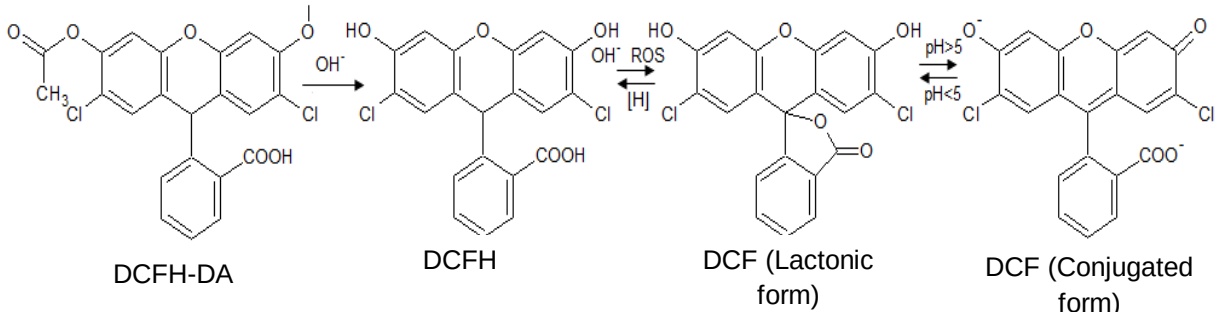


Figure 4.2: Conversion of colourless DCFH-DA to fluorescent DCF within cells.

In a 96-well plate, SC-1 and Caco-2 cells were plated at a 2 x 10^4 cells/ml concentration and the cells were cultured for a further 24 h period at 37°C at 5% CO_2. For the evaluation of cellular protection against oxidative damage 50 µl of a 75 µM solution of DCHF-DA was added to each well and incubated for 45 minutes at 37 °C. Following incubation, the medium was removed and the wells washed once with PBS to remove excess DCHF-DA. To each well 50 µl of a 1% or 10% (v/v) tea solution was then added, followed by 50 µl of a 0.02 M AAPH solution. The samples were mixed well and the fluorescence was measured every two minutes for 60 minutes at Ex_{485} and Em_{520} using a FLUOstar OPTIMA fluorescence plate reader. The gradient of the change of fluorescence was measured and the final results were reported as percentage protection (%P) relative to the control (no tea added) and was calculated as follows:

$$\%P = 1 - [(gradient_{AAPH} - gradient_{AAPH + tea})] \times 100$$

4.2.2.6. NO scavenging activity assay

The excessive production of NO by iNOS during inflammation has potential damaging effects on cellular and tissue structure. Natural products, such as flavonoids, also found in tea have shown to have an inhibitory effect on NO formation.

In this study, NO inhibition is firstly examined in a non-cellular environment with the use of sodium nitroprusside (SNP) which spontaneously produces NO at physiological pH (Marcocci *et al*., 1994). The NO is then oxidized by hydrogen to produce nitrites which can then be detected with the Griess reagent (Arnold *et al*., 1984). The detection of these nitrites involves the deionization of sulfanilimide (SA) in an acid medium to a diazonium salt which is then coupled with N-1-naphthylethylenediamine dihydrochloride (NED) to produce a purple product (Sun *et al*., 2003). NO scavenging results in a decrease in the formation of the coloured product. The

scavenging of NO by phenolic compounds is shown in Figure 4.3 (Adapted from Yenes and Messeguer, 1999).

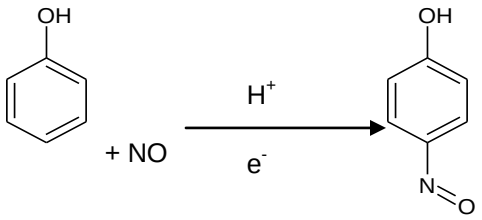


Figure 4.3: Scavenging reaction of phenolic compounds with NO radicals.

A modified method of Kim *et al.* (1999) was used to determine NO-scavenging abilities. A 5 mM SNP solution was prepared in PBS. To quantify the amount of NO formed a standard solution of 0.1 mM $NaNO_3$ was used. A concentration series of 0 - 0.01mM of $NaNO_3$ was used to prepare a standard curve. To 20 µl of a 10% tea solution 80 µl of the 5mM SNP solution was added.

The Trolox standard and the tea samples were incubated with the SNP for 1 hour at room temperature in the dark. The levels of NO were determined with the Griess reagent as follows: 50 µl of a 1% SA prepared in 20% glacial acetic solution was added to each well. After 10 minutes, 50 µl of a 0.1% NED solution prepared in 2.5% phosophoric acid was added. The samples were mixed well and the absorbance was measured at 570 nm and NO scavenging activity was expressed as µmol TE/mg DM tea.

4.2.2.7. NO suppressing assay

Lipopolysaccharides (LPS) are a major component of the outer surface of Gram-negative bacteria and are known to strongly stimulate the innate immunity in a very diverse range of species from insects to humans (Alexander and Rietschel, 2001). Interferon- gamma (INF-γ) is a cytokine that, as part of the innate immune response, is produced mainly by natural killer and natural killer T cells and is a crucial factor in the process of immunity against intracellular pathogens (Schoenborn and Wilson, 2007). In immunity, NO is synthesized from L-arginine by nitric oxide synthase (NOS) (Ghafourifa and Richter, 1997; Tsai *et al*, 2007). Inflammatory stimuli induce inducible nitric oxide synthase (iNOS) which produces NO (Nathan, 1992) which is also involved in the immune defence against pathogens. However if excessive amounts of NO are formed, the production of RNS is accelerated (Mayer and Hemmens, 1997, Tsai *et al*., 2007). In macrophages such as RAW 264.7 cells, in which an inflammatory response has been

elicited with the use of LPS and INF-γ, the ability of molecules such as polyphenols to scavenge or inhibit NO formation can be quantified. This is done by measuring the decrease in NO formation and suppression with the Griess reagent.

RAW 264.7 cells (derived from murine macrophages) were plated in a 96-well plate at a cell density of 2x 10^4 cells per well. After 24 hours the cells were stimulated by adding to the media, 10 µl of 10 µg/ml LPS solution prepared in cell culture media and 10 µl of a 200 U/ml of IFN-γ diluted in PBS. To each well, 10 µl of the 10% tea samples was added. To the positive control wells (100% NO production) (PC), dH_2O was added instead of sample while the negative controls (NC) contained no sample, LPS and IFN-γ. The ability of each tea sample to either scavenge and/or reduce NO formation was determined after 24 hours. To 50 µl of the medium removed from the well, 100 µl of Griess reagent prepared as described (1% SA and 0.1% NED in 2.5% phosphoric acid was added). The absorbance was measured at 570 nm and the percentage of NO inhibition was calculated as follows:

$$\textbf{\% Change in NO levels} = 100 \times [(Abs_{TS} - Abs_{NC})/(Abs_{PC} - Abs_{NC})]$$

4.2.2.8. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT assay is widely used to assess cell viability and gives a measure of viable living cells *in vitro*, especially after exposure to drugs or therapies. The mitochondrial reductase found in the mitrochondira of living cells reduces MTT into formazan crystals which can then be solublized and quantified spectrometrically, represented in Figure 4.4. Production of formazan crystals is directly proportional to the viability of cells *in vitro* (Fotakis and Timbrell, 2006). This assay was applicable to the current study as it will give an indication if lower NO levels production was due to the cytotoxicity of the tea samples, in the RAW 264.7 cell line.

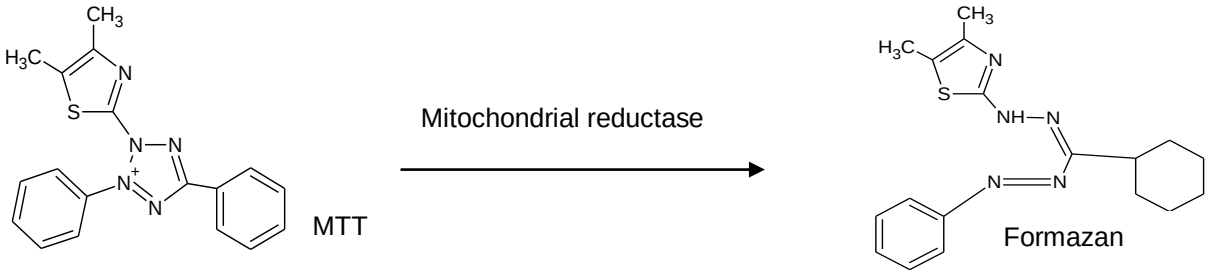


Figure 4.4: Conversion of MTT into soluble formazan crystals by mitochondrial reductase found in mitochondria of living cells.

Following sampling of the cell culture media for NO determination, 5 µl of a 1mg/ml MTT solution was added. After 3 hours incubation at 37 °C the media was removed and the plates were allowed to dry before the formazan that had formed was extracted with shaking for 10 minutes with 100 µl of a 25% DMSO solution. The absorbance was then measured at 570 nm and viability was expressed as percentage of control.

4.3. Data management and statistics

For each commercial tea type, three different lot numbers were used. Only two factory guayasa batches were available for the green and black guayasa tea. Each sample was analysed three times in triplicate. The results are expressed as mean ± standard error of the mean (SEM). Data was statistically evaluated using analysis of variance (ANOVA) with samples as independent variables and the values determined as dependent variables. Fisher's least significant difference (LSD) test was used for mean comparison using software Version 9.0 (StatSoft, Tulsa, OK). R^2 correlations between the different assays were determined.

4.4. Results and discussion

To evaluate health benefits of tea as consumed, water extracts were prepared. The amount of polyphenolic compounds extracted from the dry leaves is dependent on extraction time (Astil *et al*., 2001). To account for the different brewing times used in tea preparation and times used in different studies, sampling was done at intervals between 2 and 60 minutes.

4.4.1. Antioxidant reducing activity - F-C method

The F-C method has been established as the standardized method in the process of quantifying polyphenol content and executing quality control in food products and dietary supplements (Prior *et al*., 2005; Ainsworth and Gillespie, 2007; Singleton and Rossi, 1965) and is the ISO approved method for quantifying polyphenols in tea. The tea samples were evaluated at time intervals from 0-60 minutes at constant temperature of 90^0C using the F-C method to determine antioxidant reducing capacity. Although often data that is generated from this assay is reported as TPC, it is essentially a reducing reaction based on ET. For all teas there was a rapid

extraction of polyphenols from 0-10 minutes and later from 10 - 60 minutes the reducing capacity was constant (Figure 4.5 and Table 4.2).

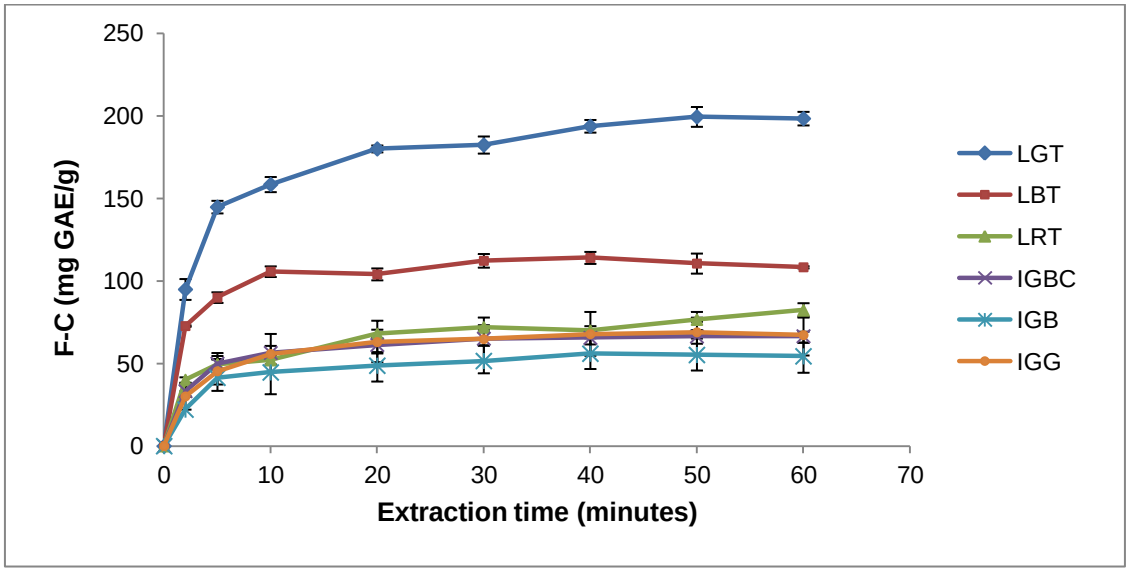


Figure 4.5: F-C values of all tea samples at 2, 5, 10, 20, 30, 40, 50 and 60 minutes extraction time. All data is an average of three independent experiments ± SEM.

LGT was found to contain the highest reducing capacity with a range of 94.9 ± 6.3 - 198.4 ± 4.1 mg GAE/g for water extraction times of 2 – 60 minutes. This was followed by LBT with a range of 72.71 – 108.33 mg GAE/g, then LRT with 40.06 – 82.23 mg GAE/g and guayusa tea with 22.12 – 67.23 mg GAE/g for the same time intervals.

For all subsequent assays (ORAC, DCFH-DA, NO scavenging and NO suppressing) 2, 10, 30 and 60 minutes extraction times were used, therefore, for comparative purposes for the F-C method, these time intervals were considered for statistical evaluation. All tea types showed a statistical difference in F-C reducing capacity between the 2 and 60 minutes extraction time.

Table 4.2: Statistical evaluation of tea F-C method at extraction times of 2, 10, 30 and 60 minutes (Figure 4.5)

	Extraction time (minutes)			
	2	**10**	**30**	**60**
LGT	94.90 ± 6.32[b]	158.43± 4.58[a]	182.38 ± 5.18[c]	198.36 ± 4.11[c]
LBT	72.71 ± 0.45[a]	105.67 ± 3.22[b]	112.25 ± 4.14[b]	108.33 ± 0.68[b]
LRT	40.06 ± 1.63[a]	52.08 ± 2.53[b]	71.91 ± 1.35[c]	82.23 ± 4.35[d]
IGBC	33.30 ± 3.43[a]	56.45 ± 4.28[b]	65.09 ± 3.93[b]	66.55 ± 2.76[b]
IGB	22.13 ± 7.94[a]	44.64 ± 9.56[a]	51.56 ± 9.21[a]	54.39 ± 8.25[a]
IGG	30.10 ± 8.01[a]	55.79 ± 12.17[a]	64.82 ± 13.07[a]	67.23 ± 11.15[a]

**Data is an average of three independent experiments ± SEM. Different letters across rows (a-d) indicates statistical significance between extraction times (ANOVA, p = 0.05).*

Compared to the control, LRT showed a significant increase in F-C reducing capacity at all time intervals. In contrast the F-C reducing capacity of IGB and IGG was unchanged. Variable effects were observed for LGT, LBT and IGBC.

Statistical evaluation of IGB and IGG extraction times indicate that there was no statistical significance in reducing capacity between 2, 10, 30 and 60 minutes extraction times which is due to the presence of large error bars. These samples were obtained directly from the factory and were not yet processed for packaging. The leaves were crushed in the laboratory prior to extraction. This highlights the importance of quality control which is present in the processing parameters of commercial sample brands, as seen from small error bars due to similar composition amongst batches.

Tea types differ in the rate at which polyphenols are extracted. For this reason the teas were ranked from high to low F-C reducing capacity for each extraction time (Figure 4.6).

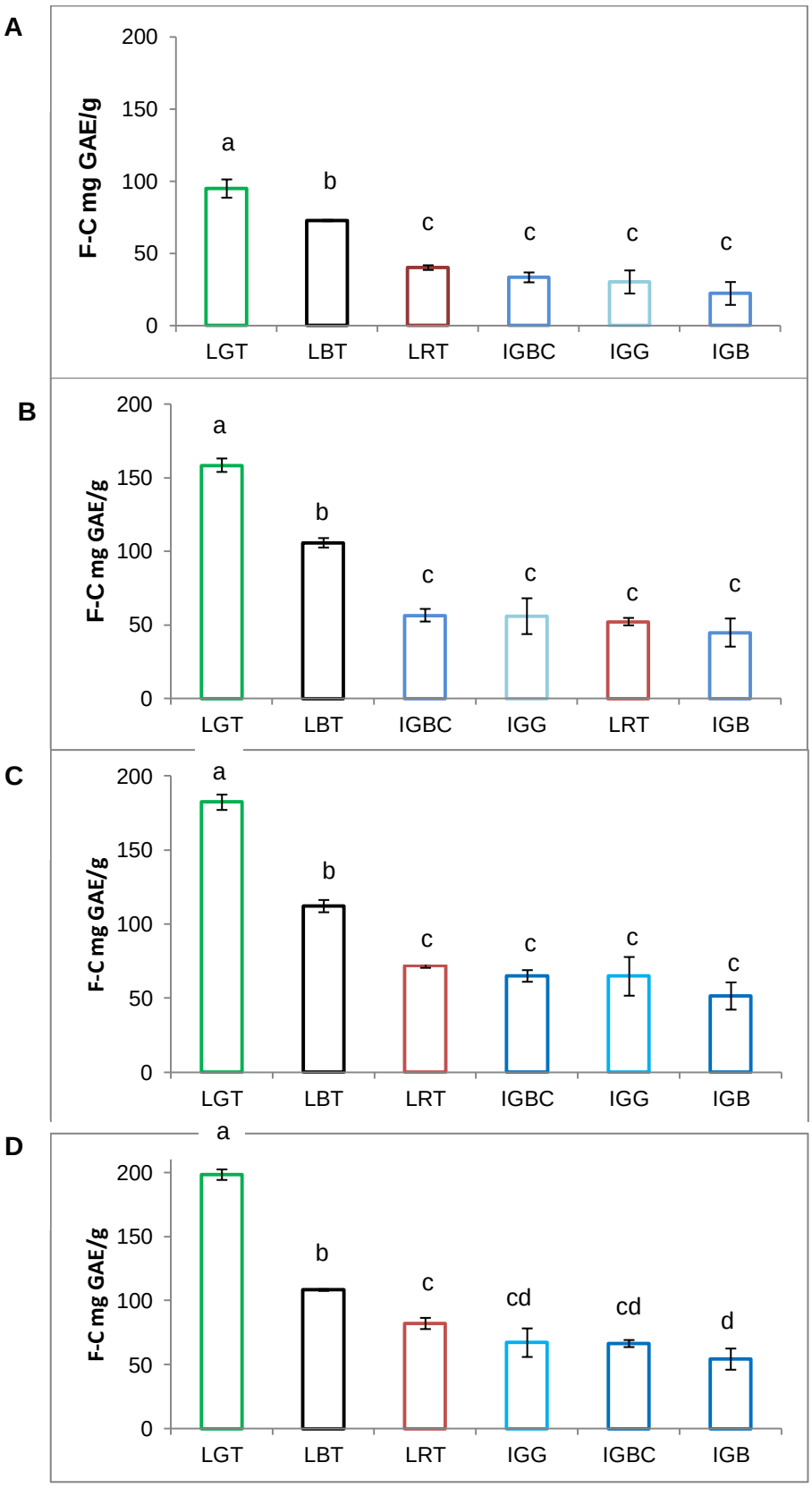


Figure 4.6: Ranking high to low of F-C values of tea samples for extraction times A) 2 B) 10 C) 30 and D) 60 minutes. All data is an average of three independent experiments ± SEM. Different letters indicate statistical significance amongst tea samples (ANOVA, $p < 0.05$).

Throughout all time intervals, LGT had the highest F-C reducing capacity. This was followed by LBT and then by LRT. For all extraction times, all *Ilex guayusa* samples were similar to LRT, and lower than LGT and LBT, except for the 60 minutes extraction time where IGG was lower than LRT, LBT and LGT.

The results of the current study are consistent with the findings of Anesini *et al*., (2008), that reported a 14.32 ± 0.45- 21.02 ± 1.54% GAE for GT and 17.62 ± 0.42% - 8.42 ± 0.55% GAE for BT. When converted to mg GAE/ g, these concentrations are 143.2 – 210.2 mg GAE/g and 176.2 – 84.2 mg GAE/g respectively.

Liebert *et al*., (1999), poured boiling water over GT leaves and after 10 minutes brewing reported a F-C reducing capacity of 137.3 mg GAE/g. This is slightly lower than the findings of this study where a 10 minutes brewing time resulted in a F-C reducing capacity of 158.4 ± 4.6 mg GAE/g. This slight difference could be attributed to difference in brand or water temperature where in the present study the temperature was kept constantly at 90^0C. Chan *et al*., (2007) determined the F-C reducing capacity of methanol and water extracts of Malaysia tea collected at different geographical regions. Hot water extracted BT (extracted for 1 hour in boiling water, allowed to cool) was 84.94 mg GAE/g tea similar to the findings of the present study.

Chan *et al*. (2007) also concluded that the GT brands explored in their study had a higher F-C reducing capacity than BT tea (191.26 mg GAE/g and 113.67 mg GAE/g). Satoh *et al*. (2005) performed a study that compared the F-C reducing capacity of water extracts of GT, BT, oolong tea and roasted tea. GT had the highest F-C reducing capacity (62.5 ± 0.3 µg GAE/200µg), followed by oolong tea (50.2 ± 0.3 µg GAE/200µg), BT (44.2 ± 0.3 µg GAE/200µg) and then roasted tea (38.1 ± 0.9 µg GAE/200µg). GT was found to be significantly higher than BT, which is similar to the current study although only one brand of GT and BT was evaluated.

LRT (*Asphalathus linearis*) was found to contain the lower F-C reducing capacity when compared with GT and BT (*Camellia sinensis)*. In a study by Standley *et al*. (2001), the F-C reducing capacity of RT tea was examined in its unfermented, fermented, sun-dried and steam pasteurised forms boiled for 30 minutes. Results were 40.99, 34.95, 33.91 and 35.30 mg GAE/g, respectively, which correspond with the two minute extraction time of the current study. Differences may be attributed to differences sample process and the temperature used. For LRT the F-C reducing capacity at 30 minutes was greater than reported by Standley *et al*. (2001).

Yoo *et al*. (2008) found with the F-C method in teas extracted by 20 minute sonication in 70% methanol that GT had the highest polyphenol content (769.8 ± 07mg GAE/100g), followed by BT (745.5 ± 09 mg GAE/100g) and then RT (659.2 ± 2.1 mg GAE/100g) which is a result consistent with the rankings of teas in this study.

The F-C reducing capacity for all *Ilex guayusa* extracts were significantly lower than both LGT and LBT but not different from LRT (ANOVA, $P < 0.05$). No previous studies could be found to compare the F-C reducing capacity of *Ilex guayusa* with *Camellia sinensis*.

4.4.2. Antioxidant activity - ORAC assay

The ORAC assay is the direct measurement of the hydrophilic and lipophilic chain-breaking antioxidant capacity against peroxyl radicals, generated by AAPH (Huang *et al*., 2005). Since the ORAC assay measures change in fluorescence over time as opposed to an end-point reading such as for the F-C method, the contribution of slow acting antioxidants on measured antioxidant activity is taken into account. This effect was observed for *Ilex guayusa* methanol extracts in chapter 3, where antioxidant activity was lower than *Camellia sinensis* brands when measured with end-point ET assays, but similar to *Camellia sinensis* with the ORAC assay. For the ORAC and all subsequent experiments, activity was evaluated at 2, 10, 30 and 60 minutes which represents short and longer extraction times (Figure 4.7 and Table 4.3).

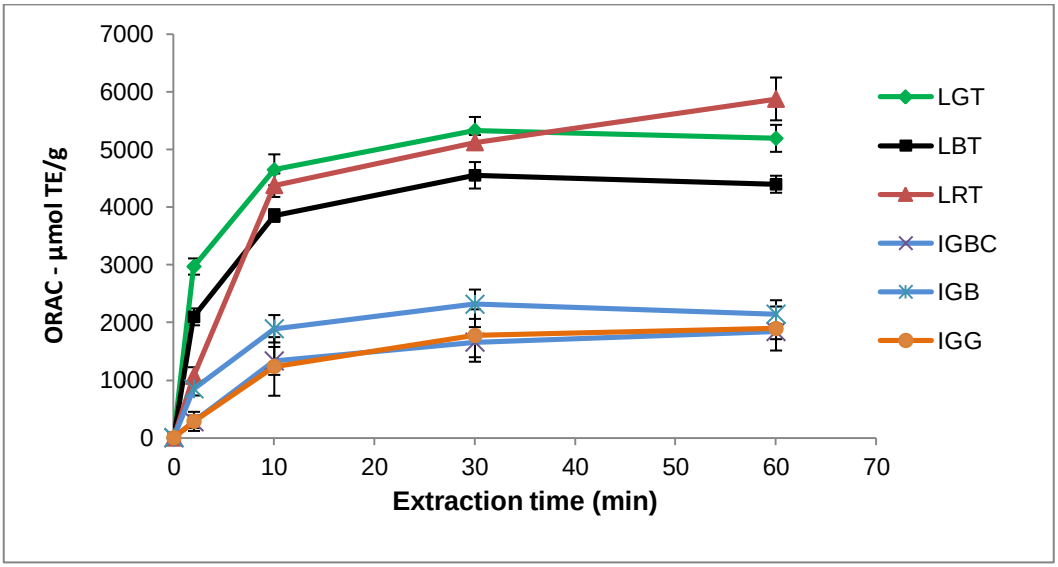


Figure 4.7: ORAC values of all tea samples at 2, 10, 30 and 60 minutes extraction time. All data is an average of three independent experiments ± SEM.

LGT had the highest antioxidant activity measured with the ORAC assay for 2-60 minutes. LRT showed the lowest activity after 2 minutes extraction time but similar to LGT after 10-60 minutes. For all extraction times, all guayusa tea samples had lower ORAC values compared with LGT, LBT and LRT (ANOVA, $p < 0.05$).

Table 4.3: Statistical evaluation of tea ORAC values at 2, 10, 30 and 60 minutes extraction times (Figure 4.7)

	Extraction time (minutes)			
	2	**10**	**30**	**60**
LGT	2800.59 ± 132.55^{a}	4383.54 ± 252.73^{b}	5029.16 ± 218.51^{b}	4897.92 ± 220.88^{b}
LBT	1941.44 ± 97.02^{b}	3585.7 ± 83.65^{a}	4319.49 ± 217.79^{c}	4056.72 ± 217.87^{c}
LRT	890.47 ± 46.45^{a}	3747.67 ± 61.65^{c}	4411.24 ± 203.88^{bc}	5244.04 ± 624.31^{b}
IGBC	259.11 ± 101.65^{a}	1250.24 ± 227.59^{b}	1555.94 ± 245.01^{b}	1728.55 ± 123.74^{b}
IGB	798.08 ± 109.85^{b}	1779.84 ± 228.15^{a}	2180.44 ± 244.40^{c}	2019 ± 229.11^{c}
IGG	264.96 ± 155.01^{a}	1161.43 479.28^{b}	1659.63 ± 425.45^{b}	1773.41 ± 357.31^{b}

**Data is an average of three independent experiments ± SEM. Different letters across rows (a-c) indicates statistical significance between extraction times (ANOVA, p = 0.05).*

For all tea samples, the two minutes extraction time yielded a significantly lower ORAC value when compared with other extraction times. For all teas the ORAC values were similar for 30 and 60 minutes extraction with no statistical variation between them (ANOVA, $p < 0.05$). As seen with the F-C reducing capacity, different tea types differed in the rate at which polyphenols were extracted. Therefore the teas were ranked from high to low ORAC values for each extraction time (Figure 4.8).

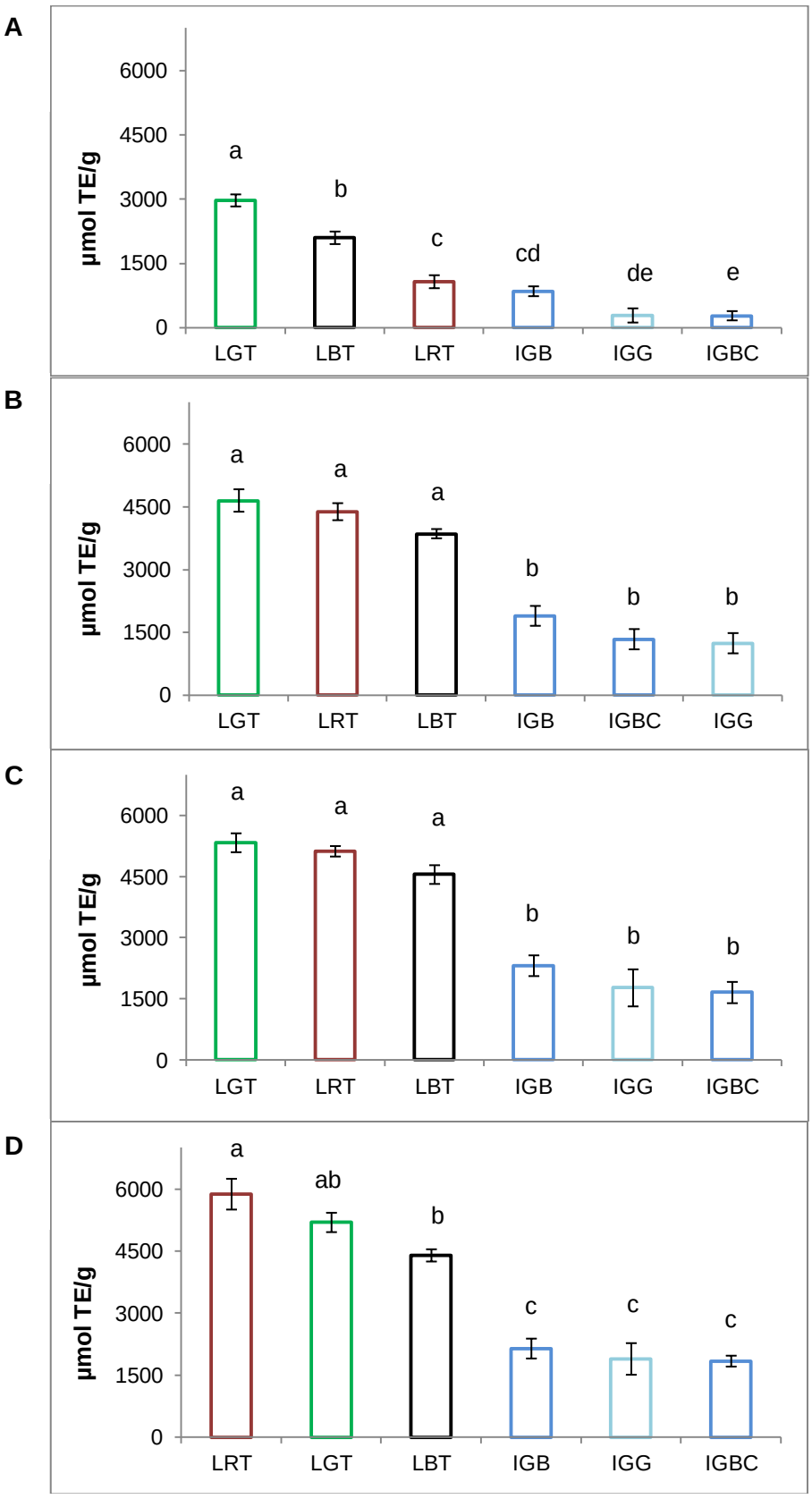


Figure 4.8: Ranking high to low of ORAC values of tea samples for extraction times A) 2 B) 10 C) 30 and D) 60 minutes. All data is an average of three independent experiments for ± SEM. Different letters indicate statistical significance amongst tea samples (ANOVA, $p < 0.05$).

LGT (2970.41 ± 140.59 µmol TE/g) and LBT (2096.81 ± 146.54 µmol TE/g) had higher ORAC values compared with LRT (1071.176 ± 151.77 µmol TE/g) for the 2 minutes extraction time. However, for the 10 and 30 minutes extraction time LRT had higher ORAC values (4411.24 ± 203.88 and 5244.04 ± 624.31 µmol TE/g) when compared with LBT (4319.49 ± 217.79 and 4056.72 ± 217.87 µmol TE/g). At the 60 minutes extraction time LRT had ORAC values even higher than that of LGT and LBT although it was not statistically higher than LGT. As with the findings of the F-C method, guayusa tea had the lowest antioxidant capacity at all time points evaluated.

Statistically, for all the extraction times, all guayusa tea samples were found to have significantly lower ORAC values than LGT, LBT and LRT (ANOVA, $p < 0.05$) except for the 2 minutes extraction where IGB was statistically similar to LRT. With regard to all extraction times, there was no statistical difference between the ORAC values of LGT, LBT and LRT, with the exception of the 60 minutes extraction time where LRT had an ORAC value significantly higher than that of LBT.

GT has been reported to have a higher antioxidant capacity when compared to BT using the ORAC assay (Henning *et al*., 2003 and Roy *et al*., 2010). Alarcon *et al*. (2008) concluded using two different ORAC methodologies that BT had higher antioxidant capacity compared with GT. In the current study there was a statistical difference in ORAC values between LGT and LBT at 2 minutes extraction however with longer extraction times no differences were found.

Awoniyi *et al*. (2012) concluded that GT had significantly higher ORAC values when compared with RT when left in boiling water for 30 minutes. In the present study it was only at 2 minutes extraction that the antioxidant activity of LGT was greater than LRT. At all other extraction times no differences were found. Von Gadow *et al*. (1997) ranked radical scavenging capacity, DPPH assay of various teas as follows: GT > unfermented RT > fermented RT > semi-feremented RT > BT > oolong tea, which follows the same ranking found in the present study at 10 and 30 minutes extraction times. No studies could be found comparing ORAC values of *Ilex guayusa* to *Camellia sinensis.*

Past studies have shown that correlations between F-C and ORAC assays are good (Zhao *et al*., 2014; Roy *et al*., 2010). Correlation analysis was conducted to compare F-C and ORAC results across extraction times and for rankings of tea samples (Tables 4.4 and 4.5).

Table 4.4: Correlation of all tea samples for F-C vs ORAC

Tea	LGT	LBT	LRT	IGBC	IGB	IGG
R^2	0.957	0.979	0.824	0.995	0.965	0.989

Table 4.5: R^2 correlation of tea samples for each extraction time for F-C vs ORAC

Extraction time (minutes)	2	10	30	60
R^2	0.897	0.439	0.496	0.391
Without LRT	0.900	0.889	0.848	0.832

Correlation analysis of F-C method vs. ORAC assay each tea type is presented in Tables 4.3. A strong correlation of 0.824 – 0.995 was found for all teas. Roy *et al*. (2010) found similar r^2 correlation TPC vs. ORAC values of 0.7323 for methanolic extracts of tea.

At each time interval, the F-C of each tea was correlated with the ORAC value. A good correlation was obtained for 2 minutes but a poor correlation of 0.391 – 0.496 was obtained for 10 - 60 minutes. Removal of LRT improved correlation of 0.832 – 0.900. This reflects the change in ranking of LRT (Figure 4.4) relative to other teas. The correlation values of ORAC with F-C are in agreement with Henning *et al*. (2003) who found a linear correlation between ORAC and F-C of eighteen GT and BT brands, extracted for three minutes with boiling water.

The F-C and ORAC assays were also conducted in chapter 3 with ISO methanol extracted batches of LGT, LBT, IGBC, IGB and IGG. Comparisons were made for both extraction methods by evaluating the water extract data as a percentage of the methanol extracted data.

Table 4.6: A) F-C and B) ORAC water extracts as a % of methanol extracts

A	Extraction time (minutes)			
	2	10	30	60
LGT	48.76	81.40	93.71	101.92
LBT	57.06	82.93	88.09	85.01
IGBC	55.37	93.88	108.24	110.68
IGB	35.23	71.08	82.09	86.60
IGG	54.81	101.58	118.03	122.42

B	Extraction time (minutes)			
	2	10	30	60
LGT	85.64	134.05	153.79	149.78
LBT	120.36	221.32	261.37	252.46
IGBC	16.57	79.94	99.49	110.53
IGB	43.29	96.52	118.26	109.54
IGG	17.92	78.28	112.24	119.94

These results indicate that after a 10 minute extraction with dH_2O at 90^0C, a large percentage of antioxidants were extracted when compared with the ISO method using 70% methanol at 70^0C. With regards to the F-C method, after a 10 minutes extraction time, activity was 71.08 – 101.58% of methanol extraction methods and with the ORAC assay 78.28 – 96.52% of alcohol extracts. For the ORAC assay LGT and LBT water extracts showed much higher antioxidant capcity compared with methanol extracts as at the 2 minutes extraction time capacity was already 85.64 – 120.36% of the ethanol extracts. Rusak *et al.* (2008) did an extensive study on extraction times and solvents used for green and white tea. Even though overall averages showed that 40% ethanol showed the most efficient extraction time, for the ABTS method of antioxidant capacity measurement, water extraction showed to be more effective and yielded higher antioxidant capacities then 70% ethanol solvent for a 15 and 30 minute extraction time. Variation in the current study between ORAC results for water and ethanol extracts may be a result of geographic variation between the teas used for dH_2O and methanol extracts.

4.4.3. Cellular antioxidant activity - DCFH-DA assay

Chemical assays, such as the ORAC, DPPH and TEAC assay fail to take into consideration processes such as cellular absorption, distribution, metabolism and excretion. *In vitro* assays using a cell line will include some of these parameters and therefore studies on cell lines provide some physiologically relevant information.

The DCFH-DA assay is simple and has been widely used, so comparisons can be made with the available literature (Kalyanaraman *et al*., 2012). In this study two cell lines were used and these were the SC-1 mouse fibroblasts and the physiologically relevant Caco-2 cell line. Prior to the evaluation of cellular antioxidant activity each sample in the absence of AAPH was evaluated for possible oxidative effects and no oxidative effects were observed (data not shown).

4.4.3.1. SC-1 cell line

SC-1 cells are an adherent mouse fibroblast cell-line that was originally attained from a feral mouse embryo (ATCC, 2014). Fibroblasts are the most common cell type in connective tissue which is located throughout the body and act as supporting tissues in organs and form organ capsules, tendons and ligaments (Kerr, 2010). Fibroblasts are responsible for collagen

production which is an important function when taking into consideration products that have topical use applications, such as wound healing.

Oxidative stress plays a large role in the pathogenesis of fibrosis which is a chronic progressive disorder where excessive deposition of extracellular matrix by fibroblasts leads to irreversible scarring. In conditions such as idiopathic pulmonary fibrosis (Walters *et al*., 2008) and hepatic fibrogenesis (Poli, 2000), fibroblasts are central mediators of pathological fibrotic accumulation of the extracellular matrix such as collagen. Increased fibroblast proliferation and differentiation occurs with prolonged tissue injury and chronic inflammation (Kendall and Feghali-Bostwick, 2014). This chronic inflammation and injury is attributed to the direct damaging effects of ROS and/or the indirect role of ROS, where ROS act as a signalling molecule for collagen synthesis, fibroblast proliferation and differentiation.

Fibroblasts play an important role in wound healing. Some tea based products are available for topical use, to reduce inflammation associated with wounds which further supports the relevance of the SC-1 cell line used in this study.

The cellular antioxidant activity of all tea samples at time intervals of 0, 2, 10, 30 and 60 minutes was evaluated (Figure 4.9 and Table 4.7).

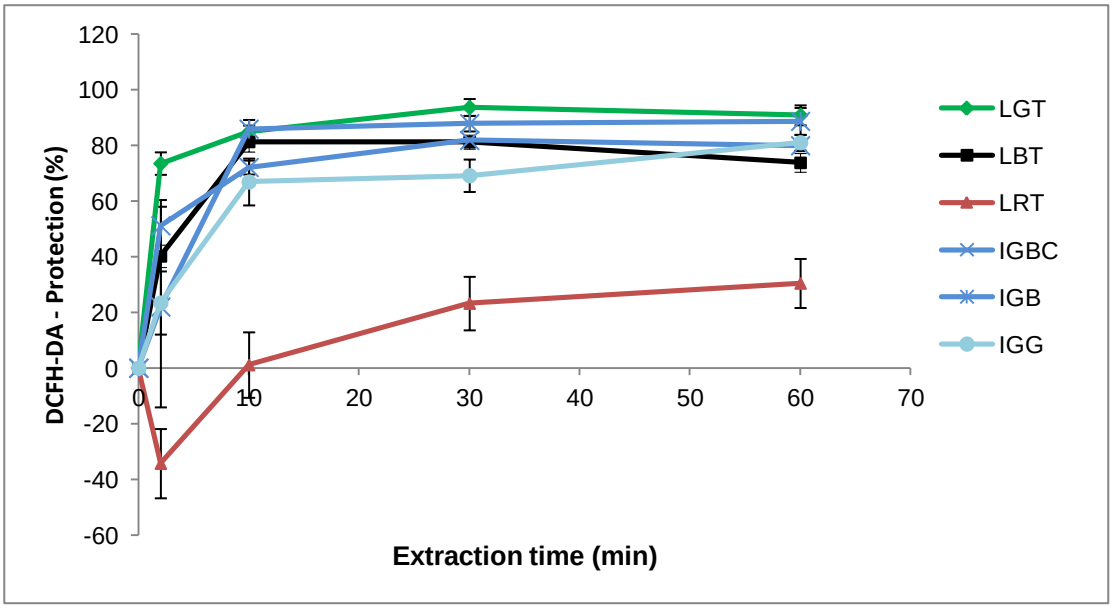


Figure 4.9: SC-1: Percentage protection against AAPH induced oxidative damage (100%) for all tea samples at 2, 10, 30, and 60 minute extraction time. All data is an average of three independent experiments ± SEM.

All teas except LRT followed the same trend, namely an increase in cellular antioxidant activity from 0-10 minutes reaching a plateau after 10 minutes with 80-90% cellular protection. For LRT a pro-oxidant effect was observed at 2 minutes and after 10 minutes reached a plateau of 30% cellular protection.

Table 4.7: SC-1: Statistical evaluation of the % protection of SC-1 cells at 2, 10, 30 and 60 minutes extraction times (Figure 4.9)

	Extraction time (minutes)			
Tea	**2**	**10**	**30**	**60**
LGT	73.59 ± 4.04^{c}	84.97 ± 2.29^{a}	93.77 ± 3.04^{b}	90.94 ± 3.59^{ab}
LBT	40.24 ± 4.04^{b}	81.37 ± 3.63^{a}	81.3 ± 2.52^{a}	73.85 ± 3.35^{c}
LRT	-34.3 ± 12.45^{c}	1.15 ± 11.77^{a}	23.26 ± 9.62^{ab}	30.49 ± 8.81^{b}
IGBC	49.94 ± 9.28^{b}	72.17 ± 2.50^{a}	81.91 ± 1.49^{a}	80.02 ± 1.94^{a}
IGB	22.01 ± 36.07^{a}	85.94 ± 3.34^{b}	87.95 ± 2.79^{b}	88.76 ± 4.83^{b}
IGG	23.46 ± 11.35^{c}	66.97 ± 8.41^{a}	69.22 ± 5.83^{ab}	81.07 ± 2.81^{b}

Data is an average of independent experiments ± SEM. Different letters across rows (a-c) shows statistical differences between extraction times (ANOVA, $p < 0.05$)

The two minutes extraction time showed statistically lower % cellular protection against AAPH-induced oxidative damage when compared to the longer extraction times (ANOVA, $p < 0.05$). LGT, LBT and IGBC showed a decrease in cellular protection at the 60 minutes extraction time, although this was only statistically significant for LBT. This can be attributed to the degradation of theaflavins and thearubigens found in BT following extended exposure to high temperatures. Lun Su *et al*. (2003) investigated the stability of GT catechins and theaflavins and found when teas were heated at 100°C, for three hours there was a 25% degradation of catechins while the theaflavins were completely degraded. At 70°C for three hours 29% of catechins had degraded while 56% of theaflavins had degraded.

For all extraction times LGT had the highest cellular antioxidant activity. Interestingly the cellular antioxidant activity of *Ilex guayusa* tea was similar to LBT and LGT for 10-60 minutes extraction times. Each individual extraction time, the teas are ranked from high to low (Figure 4.10).

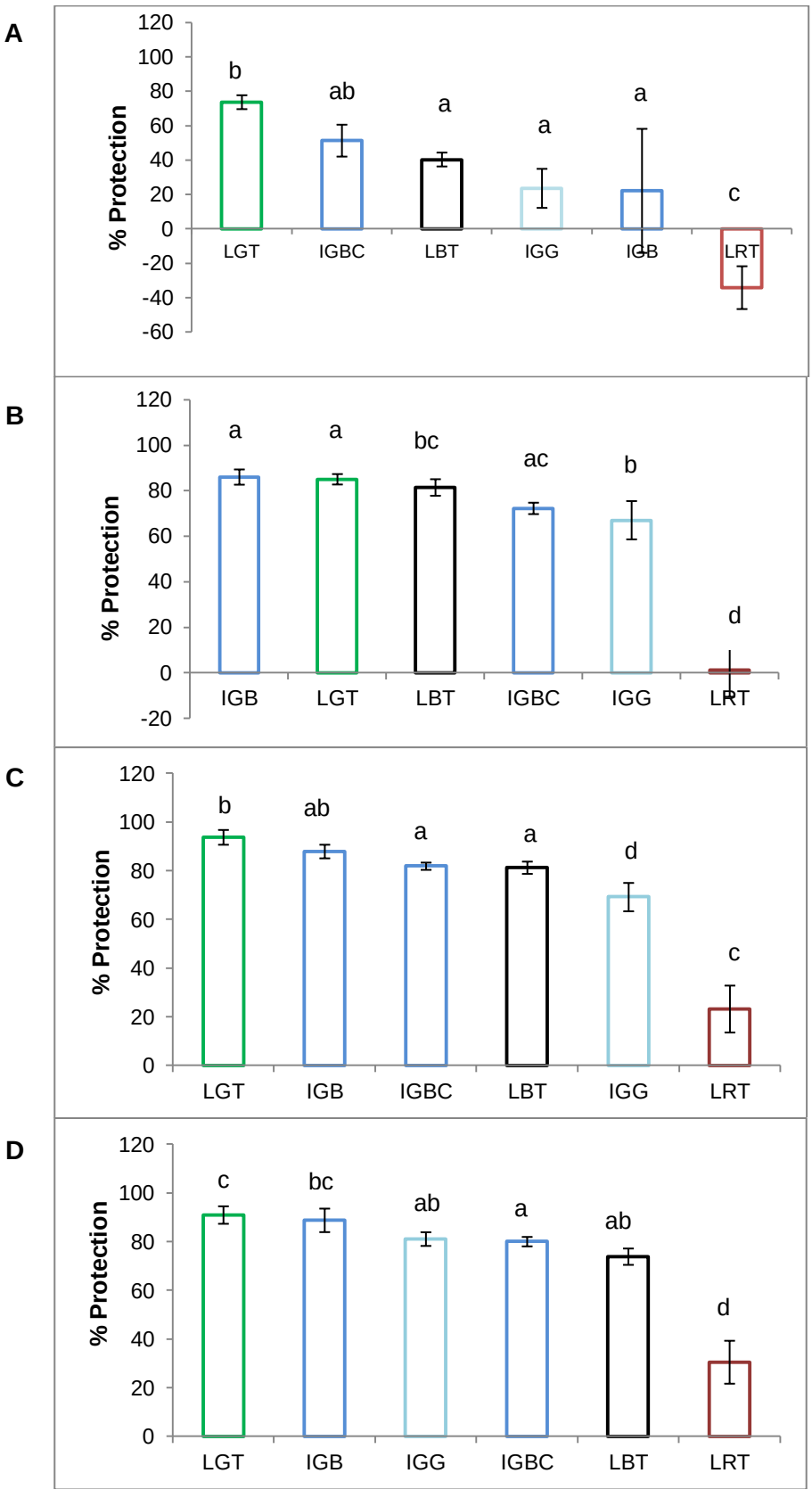


Figure 4.10: SC-1: Ranking high to low of % protection of tea samples for extraction times of A) 2 B) 10 C) 30 and D) 60 minutes. All data is an average of three independent experiments ± SEM. Different letters indicate statistical significance amongst tea samples (ANOVA, $p < 0.05$).

For all extraction times IGB was statistically similar to LGT. All samples of *Ilex guayusa* showed significantly higher cellular protective abilities compared with LRT. At the 2 minutes extraction time all guayusa samples were similar to LBT and IGB was also similar to LGT. At the 10 minutes extraction time IGB had the highest cellular protection, although not significant. At the 30 minutes extraction time IGB was similar to LGT and LBT and IGBC was similar to LBT. At 60 minutes, all guayusa samples were similar to LBT with IGB similar to LGT.

With regard to the *Camellia sinensis* teas, LGT had higher cellular protective abilities than LBT (significant at all extraction times). In contrast *Ilex guayusa* tea that had a statistically lower F-C and ORAC than *Camellia sinensis* teas were found to have a similar ability to protect SC-1 cells against oxidative damage.

ORAC and DCFH-DA data was correlated as the principles of both assays are similar i.e. Both the ORAC and DCFH-DA assays use AAPH as a source of peroxyl radicals and fluorescein as probe (Tables 4.8 and 4.9).

Table 4.8: SC-1: R^2 correlation of all tea samples for ORAC vs. DCFH-DA

Tea	LGT	LBT	LRT	IGBC	IGB	IGG
R^2	0.977	0.871	0.967	0.975	0.942	0.930

Table 4.9: SC-1: Correlation of tea samples for each extraction time for ORAC vs. DCFH-DA

Extraction time (minutes)	2	10	30	60
R^2	0.183	0.098	0.077	0.331

Correlation between both assays was high for each tea type (Table 4.7). At each time interval, ORAC values for all teas were correlated with cellular antioxidant activity (Table 4.8). A poor correlation implies that the each tea differed in the amounts of antioxidants extracted and the bioactivity of these molecules. Examples of these differences were the differences between LRT and other teas and the higher cellular antioxidant activity observed for *Ilex guayusa* tea compared to the data generated with the ORAC assay.

4.4.3.2. Caco-2 cell line

Each tea extract was further evaluated in the Caco-2 cell line. These cells are a semi-adherent cell line originally derived from a human colon adenocarcinoma. This cell line is widely used as an intestinal barrier model. Upon differentiation Caco-2 cells express morphological biochemical characteristics of small intestine enterocytes found in the intestinal barrier (Sambuy *et al*., 2005; ATCC, 2014). Enterocytes are the most abundant cell type along the barrier of the small intestine, known as the crypt-villus axis. The absorptive function of the small intestine is further facilitated by the entrecote secretion of fluid that creates a more fluid environment for mixing of food with digestive enzymes (Kerr, 2010). Furthermore, the gastrointestinal tract is the main source of ROS. The epithelial layer creates a limited protection barrier against ingested materials and pathogens which activate cells in the gut to produce inflammatory responsers which further lead to oxidative stress. A variety of diseases such as gastroduodenal ulcers, gastrointestinal malignancies, and inflammatory bowel disease are partly as a result of oxidative stress (Bhattacharyya *et al*., 2014).

The gastrointestinal tract is therefore a very important site when taking into consideration antioxidant properties and therefore the Caco-2 cell line is a relevant cell line, to examine the cellular antioxidant activity. Caco-2 cells are constantly exposed to any consumed food or beverage, such as tea. The use of two different cell lines will confirm observed effects. Comparisons can be made between cell line sensitivity and correlations can be made between cell lines (Figure 4.11 and table 4.10).

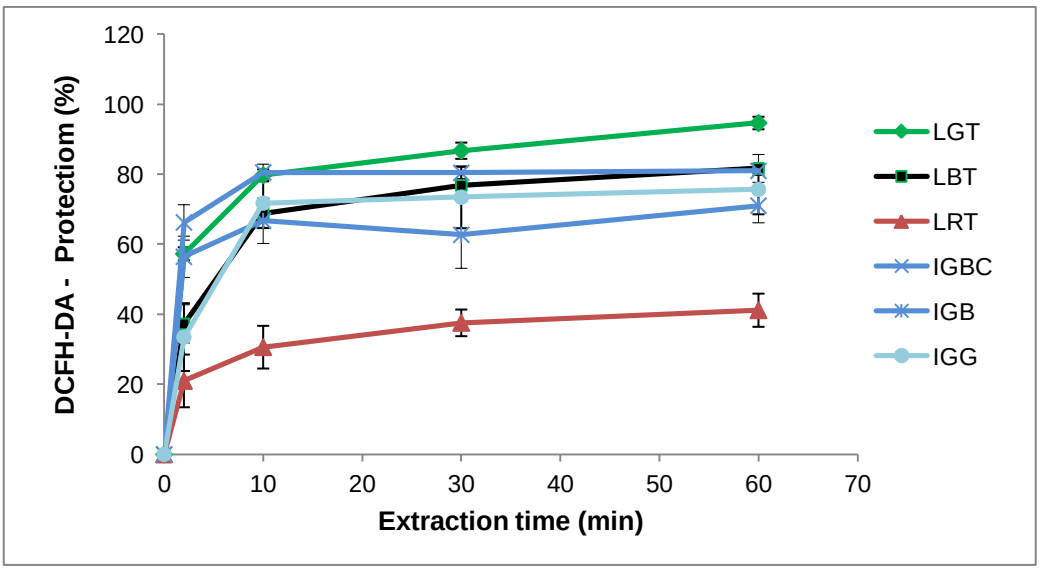


Figure 4.11: Caco-2: % Protection against AAPH induced oxidative damage (100%) for all tea samples at 2, 10, 30, and 60 minute extraction time. All data is an average of three independent experiments ± SEM.

For all extraction times LRT, as with the SC-1 cell line showed the lowest cellular protective effects. The cellular effect of IGBC was statistically similar to LGT for extraction times of 2, 10 and 30 minutes extraction times (ANOVA $p < 0.05$).

Table 4.10: Caco-2: Statistical evaluation of % protection at 2, 10, 30 and 60 minutes extraction times (Figure 4.11)

	Extraction times (minutes)			
Teas	**2**	**10**	**30**	**60**
LGT	57.28 ± 1.86^{a}	79.70 ± 1.72^{b}	86.73 ± 2.32^{c}	94.63 ± 1.79^{d}
LBT	37.28 ± 5.45^{a}	68.74 ± 4.25^{c}	76.84 ± 3.93^{bc}	81.67 ± 3.97^{b}
LRT	20.99 ± 7.52^{a}	30.63 ± 6.09^{ab}	37.57 ± 3.80^{b}	41.18 ± 4.73^{b}
IGBC	66.26 ± 7.13^{a}	80.51 ± 3.36^{b}	80.39 ± 5.08^{b}	80.96 ± 2.74^{b}
IGB	56.43 ± 5.90^{a}	66.74 ± 6.49^{a}	62.71 ± 9.56^{ab}	71.04 ± 4.90^{b}
IGG	32.35 ± 9.69^{a}	71.74 ± 7.04^{b}	73.43 ± 8.83^{b}	75.67 ± 7.09^{b}

Data is an average of three independent experiments ± SEM. Different letters across rows (a-d) shows statistical differences between extraction times (ANOVA, $p < 0.05$).

In the Caco-2 cell line, each tea sample showed increased cellular protection with increased extraction time. A statistical difference in cellular protection between the 2 minutes and all the other extraction times for IGBC, LGT, LBT and IGG was observed. For LGT, with time a significant continuous increase in cellular protection was found. Generally there were no statistical differences between 30 and 60 minutes extraction times, except for LGT. As shown for SC-1 cells extraction time is a major factor in the measured amount of cellular antioxidant activity (Figure 4.12).

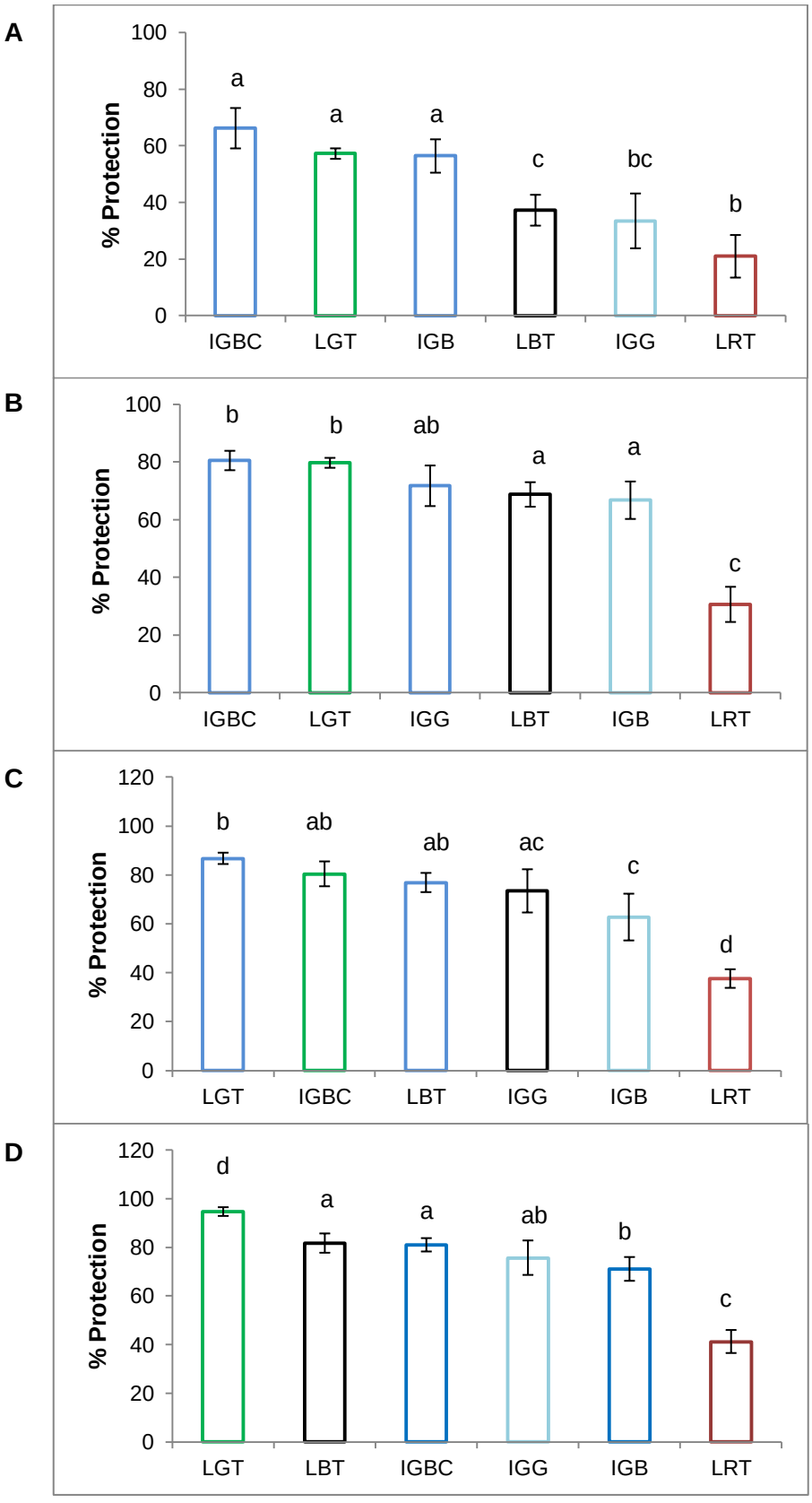


Figure 4.12: Caco-2: Ranking high to low of % protection of tea samples for extraction times of A) 2 B) 10 C) 30 and D) 60 minutes. All data is an average of three independent experiments ± SEM. Different letters indicate statistical significance amongst tea samples (ANOVA, $p < 0.05$).

After 2 minutes extraction time, the cellular protective rankings of tea samples were as follows: IGBC > LGT > IGB > LBT > IGG > LRT. IGBC, LGT and IGB were found to be statistically similar and had significantly higher cellular protective effects compared with other tea types. At 10 and 30 minutes extraction time, the rankings remained the same. LRT was found to have significantly the lowest cellular protective effects compared with all other tea samples. At the 60 minutes extraction time, LGT had significantly higher cellular protective effects than all other tea types followed by LBT which was similar to IGBC and IGG. The cellular protective effects observed in the Caco-2 cell line was similar to that observed in the SC-1 cell line as indicated correlations presented in Table 4.10.

Yoo *et al.*, (2008) evaluated the antioxidant and cytoprotective activities of alcohol extracts of 17 herbs including GT, BT and RT. Using H_2O_2 as oxidant and Lucifer yellow for detection, GT was found to strongly protect V79-4 cells against oxidative damage. BT had a lower cellular protective effect while RT had the least. This was similar to the findings of this study where LGT effectively protected SC-1 and Caco-2 cells against oxidative damage.

The principle of the ORAC assay is similar to the DCHF-DA assay in that both assays use AAPH as a source of peroxyl radicals and also involves time dependent measurement of change in fluorescence. Correlation between the ORAC and the DCHF DA assay showed a $R^2 \geq 0.843$ (Table 4.11).

Table 4.11. Caco-2: R^2 correlation of all tea samples for ORAC vs DCFH-DA

Tea	LGT	LBT	LRT	IGBC	IGB	IGG
R^2	0.933	0.976	0.947	0.921	0.899	0.461

Table 4.12. Caco-2: R^2 correlation of tea samples for each extraction time for ORAC vs DCFH-DA

Extraction time (minutes)	2	10	30	60
R^2	0.0003	0.165	0.043	0.106

In contrast evaluation of cellular antioxidant activity at each time interval showed a poor correlation (Table 4.12) and this is possibly due to differences in the extraction rate, polyphenol types and cellular mechanism of action. The cellular antioxidative effects of *Ilex guayusa* was significantly greater than other tea types, which is in contrast to the antioxidant activity measured with the F-C and ORAC assays and this discrepancy will account for the low

correlation found between the antioxidant assays and measured cellular protective effects at each time interval. Correlations between cell lines are shown in tables 4.13 and 4.14.

Table 4.13. R^2 correlation between extraction times for DCFH-DA - SC-1 vs Caco-2 cell lines

Tea	LGT	LBT	LRT	IGBC	IGB	IGG
R^2	0.890	0.828	0.993	0.912	0.565	0.973

Table 4.14. R^2 correlation between all tea samples for DCFH-DA: SC-1 vs Caco-2 cell lines

Extraction time (minutes)	2	10	30	60
R^2	0.609	0.828	0.774	0.859

Correlation between results found in both cell lines demonstrated was good (Table 4.13) especially for 10, 30 and 60 minutes extraction times which indicates a similar mechanism of antioxidant activity.

In both cell line there was a protective effect exerted by all tea types which can be attributed to the antioxidant constituents of the tea. Kelly and Owusu-Apenten (2015) demonstrated that EGCG and quercetin have a cellular protective effect of breast cancer cells and anti-ROS properties. Sato *et al*. (2011) investigated the cellular antioxidant effects of chlorogenic acid, which has been found to be an abundant antioxidant in *Ilex guayusa* (unpublished data). Polyphenols such as gallic acid undergo rapid auto-oxidation in an alkaline environment such as found in the small intestine. Sato *et al*. (2011) reported that in the Caco-2 cell line pH did not affect the activity or uptake of chlorogenic acid and therefore this phenolic acid plays an important role in the protection of the intestine against injury associated with oxidative damage.

4.4.4. Anti-inflammatory activity - NO-scavenging assay

Excessive inflammation can lead to tissue damage and during this process, excessive amounts of NO are produced which can react with oxygen to form reactive nitrogen species (RNS) or O_2^- to form a peroxynitrite ion (Xu *et al*., 2011). RNS can cause oxidative tissue and cellular damage.

Peroxynitrite has also been shown to initiate various toxic processes such as LDL oxidation a main cause of atherosclerosis (Graham *et al*. 1993). Besides having antioxidant activity,

flavonoids such as those found in tea have also been found to have direct NO scavenging and inhibiting activity (Kim *et al*., 1999).

A further aim of this study was to evaluate the direct NO scavenging and inhibiting activity of different tea types. To determine the direct NO scavenging activity, the SNP assay with the quantification of NO as nitrite with the Griess reagent was used. The NO scavenging activity was determined for each extract (Figure 4.13 and Table 4.13).

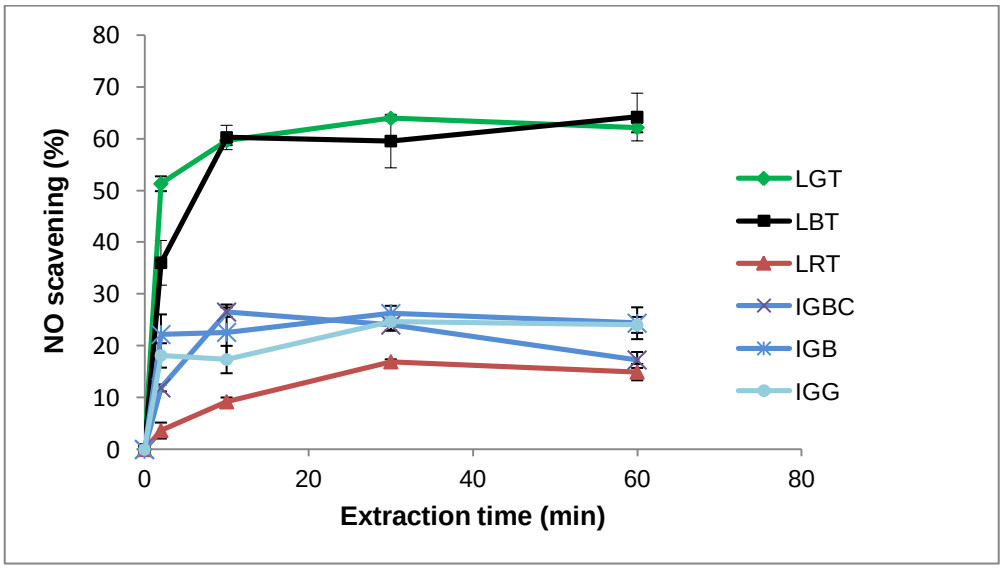


Figure 4.13: Percentage NO scavenging ability of all tea samples at 2, 10, 30 and 60 minutes extraction time. All data is an average of three independent experiments ± SEM.

Table 4.15: Statistical evaluation of tea NO scavenging ability at 2, 10, 30 and 60 minutes extraction times (Figure 4.13)

	Extraction time (minutes)			
Tea	**2**	**10**	**30**	**60**
LGT	51.31 ± 1.43^{c}	59.65 ± 0.90^{a}	63.95 ± 0.41^{b}	62.16 ± 0.91^{ab}
LBT	36.05 ± 4.31^{a}	60.25 ± 2.36^{b}	59.55 ± 5.17^{b}	64.19 ± 4.61^{b}
LRT	3.65 ± 1.54^{b}	9.21 ± 0.83^{c}	16.93 ± 0.48^{a}	14.94 ± 1.58^{a}
IGBC	11.89 ± 0.67^{b}	26.52 ± 0.90^{a}	24.03 ± 1.10^{a}	17.28 ± 1.54^{c}
IGB	22.19 ± 3.89^{a}	22.61 ± 5.39^{a}	26.25 ± 1.50^{a}	24.38 ± 3.07^{a}
IGG	18.16 ± 2.35^{a}	17.37 ± 2.64^{a}	24.66 ± 1.64^{b}	24.06 ± 1.53^{b}

Data is an average of three independent experiments ± SEM. Different letters across rows (a-c) shows statistical significance between extraction times (ANOVA, p = 0.05).

With increasing extraction time there was an increase in NO scavenging activity for all tea types. After 10 minutes both LGT and LBT had 62.16 ± 0.91% and 64.19 ± 4.61% NO scavenging activity (no significant differences). For IGB, IGG, IGBC and LRT this was reduced to an average of 20.17 ± 4.78% which was significantly less than LGT and LBT.

There was an increase in NO-scavenging ability of each tea with increased extraction time. In general there was a significant difference in NO-scavenging ability between the 2 minutes extraction times and all other times except for IGG which showed similar results for the 2 and 10 minutes and IGB which had no significant difference in any of the extraction times. Except for IGBC, there was no significant difference in NO scavenging ability with 30 and 60 minute extraction times.

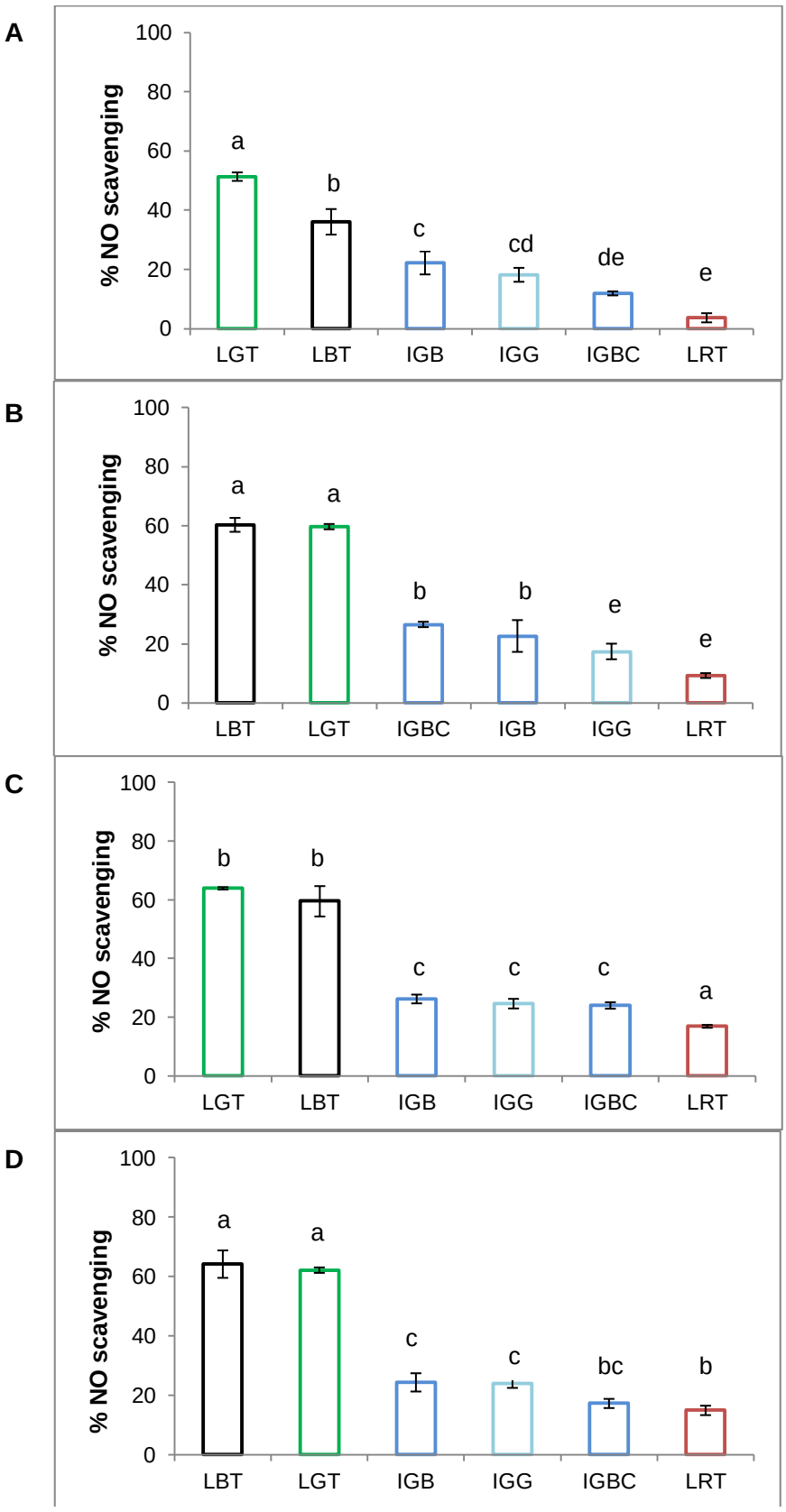


Figure 4.14: Ranking high to low of % NO scavenging ability of tea samples for extraction times A) 2 B) 10 C) 30 and D) 60 minutes. All data is an average of three independent ± SEM. Different letters indicate statistical significance amongst tea samples (ANOVA, $p < 0.05$).

LRT had the lowest NO-scavenging abilities for all extraction times and was significantly lower than all other tea samples for the 30 minutes extraction time. All *Ilex guayusa* samples (IGB, IGG and IGBC) had similar NO scavenging abilities for all extraction times. At a two minutes extraction time IGBG had significantly lower activity compared with IGB while at a 10 minutes extraction time, IGG showed statistically lower results compared with IGB and IGBC.

For all extraction times assessed, *Camellia sinensis* teas had significantly higher NO scavenging abilities when compared with *Ilex guayusa* and *Aspalathus linearis* samples. LGT is generally had the highest NO scavenging ability when compared with LBT, however, not significantly (ANOVA, $p < 0.05$).

Xu *et al*. (2011) and Heijnen *et al*. (2000) evaluated the NO-scavenging abilities of GT and BT water extracts. Xu *et al*. (2011) using SNP as a NO generator and Heijnen *et al*. (2000) using a method in which water is purged with NO gas, both studies found that GT was more effective at scavenging NO than BT, although these differences were found not to be significant as with the current study.

Wang *et al*., (2008) investigated the NO-scavenging abilities of water extracts of puh-erh tea to scavenge NO produced by SNP using the Griess reagent. Water extracts of puh-erh tea (0-1.0 mg/ml) were shown to have good NO scavenging abilities. At a concentration of 0.1mg/ml, puh-erh tea and Trolox scavenged NO by 56% and 90% respectively (Wang *et al*., 2008). This study identified the potential therapeutic properties of tea consumption related to anti-inflammatory activity.

A study conducted by Tsai *et al*. (2007) examined the NO-scavenging abilities of green tea extracts compared with herbal tea extracts. SNP was used as a NO generator and Griess reagent as a nitrite detector. All the extracts demonstrated NO-scavenging effects and green tea had the highest NO scavenging effects compared to two other tea leaf extracts (rosemary and lemongrass) as well as 5 flower tea extracts (daisy, jasmine, lavender, rose and sweet osmanthus). Similarly in the present study LGT and LBT had the highest NO scavenging activity which greater than *Illex guayusa* and *Aspalathus linearis* tea which were considered to be herbal teas.

4.4.5. Cellular anti-inflammatory activity - NO-suppressing assay (RAW 264.7)

In a cellular environment the ability of tea extracts to supress or scavenge NO can be determined using the RAW 264.7 cell line. This is an adherent transformed monocyte/macrophage cell line, derived from a tumour which was induced by an Abelson murine leukemia virus. RAW 264.7 cells are from the BALB/c mouse strain (ATCC, 2014). Monocytes are found in blood tissue and then enter tissues and serous cavities where these cells are transformed into macrophages. Macrophages are found in all tissue, but mostly connective tissue, lungs, liver (Kuppfer cells), bone (oestoclasts) lymphoid organs, kidneys (mesengial cells) and the brain (microglial cells). Macrophages are phagocytes that act as-antigen presenting cells and form part of the immune surveillance system. As part of the innate immune system, macrophages are programmed to detect and recognise threats by structures known as pathogen-associated molecular patterns, which include bacterial cell walls, viral nucleic acids and bacterial endotoxins such as LPS. The immune response is subsequently initiated to destroy any threat by this response (Kerr, 2010).

In this study RAW 264.7 cells are exposed *in vitro* to LPS and INF-γ which induces NO formation. LPS, generally found on the outer membrane of *E.coli* bacteria, is known to elicit an immune response in animals and one of the consequences of this response is an increase in NO production. Following 24 hours exposure Kim *et al*. (1999) reported a dramatic increase in NO production following exposure of RAW 264.7 from a basal level of 2.86 ± 0.1 mM to 58.0 ± 4.5 mM nitrite. Flavones/isoflavones/flavonols which included apigenin, wogonin, luteolin, tectorigenin, iristectorigenin A, quercetin, and myricetin were found to have a strong dose dependent direct inhibition of NO production (Kim *et al*., 1999). Balentine *et al*. (1997) reported that flavonols and flavonol glycosides make up 3% of the weight of dry plant leaf, which is an indication of the possible therapeutic benefits of consuming tea for the purpose of NO inhibition. In this study the ability of the tea types to inhibit or suppress NO formation was evaluated.

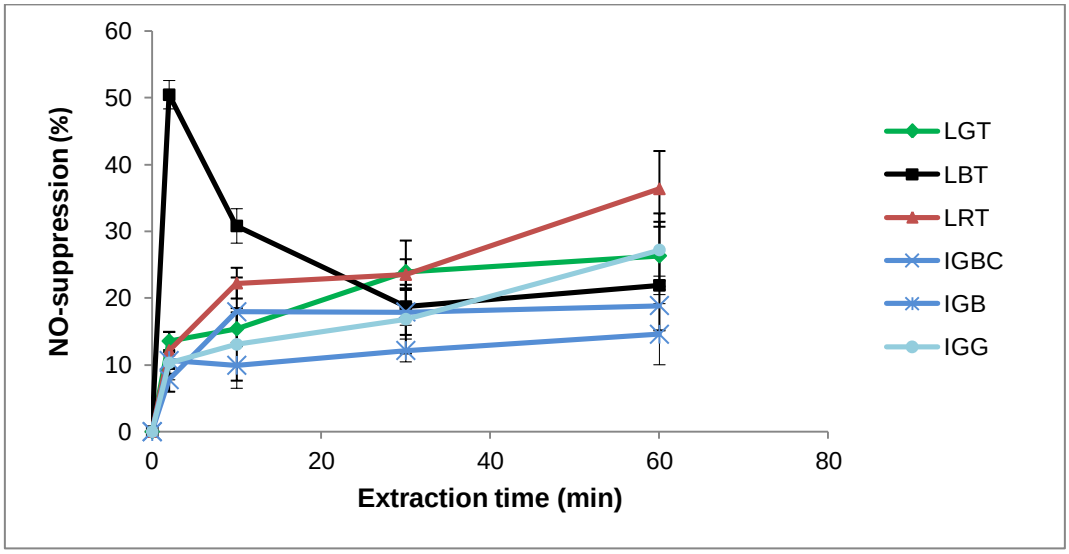


Figure 4.15: RAW 264.7: % NO suppressing ability of all tea samples at 2, 10, 30 and 60 minutes extraction time. All data is an average of three independent experiments for three commercial and two factory *Ilex guayusa* batches ± SEM.

All tea samples, except for LBT, showed an increase in NO-suppressing abilities with increased extraction time (Figure 4.5 and Table 4.4). Interestingly LBT showed an increase in % NO scavenging at 2 and 10 minutes of 50.46% and 30.81% respectively. NO scavenging activity then decreased to 18.73% and 21.91% at 30 and 60 minutes, respectively. This can be explained by research of Lun Su *et al*. (2003) on the stability of catechins and theaflavins. Catechins found predominantly in GT and theaflavins a product of fermentation are present in high levels in BT. Lun Su *et al*. (2003) reported that when tea was heated at 100°C, at 3 hours there was a 25% degradation of catechins whereas the theaflavins were completely degraded. At a lower temperature of 70°C for 3 hours 29% of the catechins and 56% of the theaflavins had degraded. Although longer extraction times result in the extraction of more polyphenols, the extracted polyphenols such as the theaflavins may undergo degradation. In addition, NO inhibition effects may be polyphenol specific.

Table 4.16: RAW 264.7: Statistical evaluation of tea NO scavenging ability at 2, 10, 30 and 60 minutes extraction times (Figure 4.15)

	Extraction time (minutes)			
	2	**10**	**30**	**60**
LBT	50.46 ± 2.13^{a}	30.81 ± 2.57^{b}	18.73 ± 4.65^{c}	21.91 ± 5.09^{c}
LGT	13.57 ± 1.35^{a}	15.41 ± 2.45^{ab}	23.97 ± 3.29^{bc}	22.40 ± 0.84^{c}
LRT	12.17 ± 2.78^{b}	22.24 ± 2.30^{a}	23.52 ± 2.30^{a}	36.36 ± 5.67^{c}
IGBC	7.79 ± 1.81^{a}	17.95 ± 5.14^{b}	17.93 ± 3.44^{b}	18.89 ± 3.72^{b}
IGB	10.65 ± 2.89^{ab}	9.94 ± 3.44^{a}	12.16 ± 1.71^{ab}	14.59 ± 5.48^{b}
IGG	10.30 ± 1.63^{a}	13.07 ± 5.43^{a}	16.85 ± 5.14^{a}	27.21 ± 3.16^{b}

Data shows averages of three independent repeats for commercial samples and two independent repeats for factory samples (IGB, IGG) ± SEM. Different letters across rows shows statistical differences between extraction times (ANOVA, $p < 0.05$).

The NO scavenging ability of IGB, IGG, IGBC was only significantly less than LBT at 2 and 10 minutes. At 30 and 60 minutes the NO scavenging activity of all teas were relatively similar. Interestingly the LRT showed a significant increase in NO scavenging activity from 2-60 minutes and at 60 minutes showed the highest NO scavenging activity of all teas.

Correlation between the chemical and cellular assays for the evaluation of NO scavenging was high with $R^2 \geq 0.736$. As for antioxidant activity a poor correlation was found for each extraction time which indicates differences in extraction of NO scavenging molecules or mechanism of action. The NO suppressing activity of IGBC, IGB and IGG was significantly lower than LBT at 2 and 10 minutes but similar to all tea types at 30 and 60 minutes.

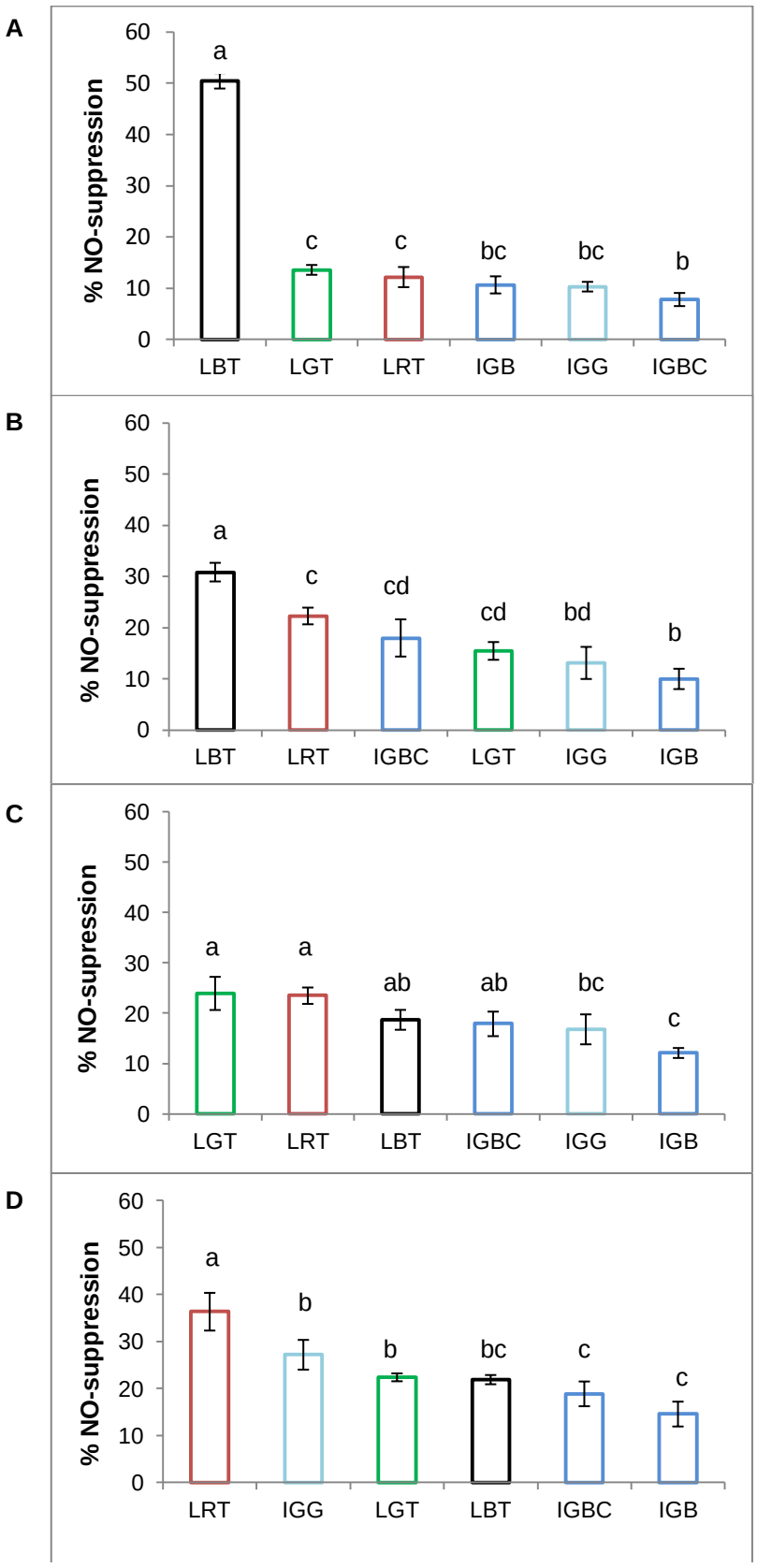


Figure 4.16: RAW 264.7: Ranking high to low NO suppressing abilities of tea samples for extraction times A) 2 B) 10 C) 30 and D) 60 minutes. All data is an average of three independent experiments ± SEM. Different letters indicate significance amongst tea samples (ANOVA, $p < 0.05$).

In addition to directly scavenging NO, Kim *et al*. (1999) demonstrated that increasing concentrations of flavonoids inhibited iNOS expression. Wang *et al*. (2008) reported that puh-erh tea showed a concentration dependent inhibition of iNOS in LPS activated RAW 264.7 cells. This effect elicited by puh-erh tea could be as a result of the flavonoids present in the tea (Wang *et al*., 2008). Paquay *et al*. (2000) found in a study that compared black and green tea, that black tea (IC_{50}, 0.24 ± 0.07 mg/ml) was twice as effective as green tea (0.47 ± 0.04 mg/ml) at inhibiting iNOS. Xu *et al*. (2011) and Heijnen *et al*. (2000) showed that due to the high catechin content of green tea, green tea has a better NO scavenging activity. In contrast in a cellular environment using LPS stimulated RAW 264.7 BT had a higher potential to suppress NO production. According to Pan *et al*. (2000), theaflavins, particularly theaflavin-3,3'-digallate from black tea shows the highest ability to suppress NO production when compared to other polyphenols and involves the down-regulation of iKB kinase activity. This may account for the higher cellular NO scavenging activity observed in the present study at 2 and 10 minutes extraction times, preceding the degradation of the theflavins and consequent decrease in NO suppression with increased extraction time.

Lin *et al*. (2006), found that two species of BT extracts (95^0C for 5 minutes) inhibited the NO production, iNOS catalytic activity and iNOS protein expression in LPS-activated cells, more weakly than did green and paochong teas. The NO suppressing activity was highly correlated with the total phenolics content. In contrast, Xu *et al*. (2011) reported that the ability of BT and puh-erh tea to inhibit NO levels was greater than GT. All teas were extracted for 10 minutes in boiling water. This is similar to the findings of the present study in which after 10 minutes the NO activity of LBT was greater than LGT. Hendricks and Pool, (2010) showed that BT extracts added to LPS-stimulated white blood cells and significantly reduced INF-γ production compared to the control. In the same study rooibos tea extracts were found not to reduce the INF-γ production indicating a different mechanism of NO suppression (Hedricks and Pool, 2010).

The effects attributed to the NO-suppressing abilities of guayusa tea may be due to the presence of polyphenol compounds particularly chlorogenic acid. The findings of Shan *et al*. (2009) show that with the use of western blotting, chlorogenic exhibits anti-inflammatory properties by inhibiting a LPS-induced inflammatory response in RAW 264.7 cells. COX-2, an important enzyme in the inflammatory pathway, was decreased at protein level as well as its transcription at mRNA levels. This further resulted in the inhibition of prostaglandin E2 release. In addition, NF-κB and c-Jun N-terminal kinase-c-jun-activator protein pathways were significantly suppressed by chlorogenic acid.

The anti-inflammatory properties of chlorogenic acid were also investigated by Yun *et al*. (2011), in Sprague-Dawley rats with liver ischemia and reperfusion, known to initiate damaging inflammatory responses. On histological analysis of livers, it was found that hepatic necrosis and Kupffer cell hyperplasia were decreased in chlorogenic acid-treated groups. Furthermore, the examination of blood cytokine levels such as TNF-α, iNOS, COX-2 showed that after reperfusion, these levels were increased and were attenuated significantly by chlorogenic acid administration.

Carcinogenesis has been shown to be a result of excessive or chronic inflammation which has been shown to be the driving forces in the premalignant and malignant transformation of cells. The intake of polyphenols in tea has shown to reduce cancer risk (Mukhtar and Ahmad, 2000; Bettuzzi *et al*., 2006; Liu *et al*., 2008; Mazzio and Soliman, 2009; Wanjiku, 2009). This can be due to an increase in pro-inflammatory enzymes such as COX-2 and iNOS which are prevalent in chronic inflammation that creates a microenvironment that contributes to carcinogenesis. Inhibition of COX-2 and iNOS has shown to be protective against development of tumours especially in the colon. NF-κB, a redox sensitive transcription factor that regulates COX-2 and iNOS, has been implicated in inflammation-induced colon carcinogenesis, so inhibition is a crucial factor in the prevention of cancer progression (Rodríguez-Ramiro *et al*., 2013). Correlations between extraction times and tea samples for NO scavenging and NO suppressing assays are presented in tables 4.15 and 4.16.

Table 4.17: RAW 267.9: R^2 correlation of all tea samples for NO scavenging vs. NO suppressing assay

Tea	LGT	LBT	LRT	IGBC	IGB	IGG
R^2	0.736	0.840	0.758	0.975	0.839	0.741

Table 4.18: RAW 267.9: R^2 correlation of rankings of tea samples for each extraction time for NO scavenging assay vs. NO suppressing assay

Extraction time (minutes)	2	10	30	60
R^2	0.165	0.173	0.112	0.011

Good correlations of 0.736 – 0.975 were obtained between NO scavenging activity and NO suppressing activity which is an indication that activity observed *in vitro* is a direct consequence of NO scavenging. Poor correlations of 0.011 – 0.173 existed between NO scavenging and suppressing assays when taking into consideration tea rankings indicating a change of tea

rankings and therefore different mechanisms of actions. The manner in which NO is scavenged may be due to the targeting of different sites, for example the direct scavenging of NO, binding LPS or the inhibition of iNOS. Further studies should focus on the identification of these specific targets.

4.4.6. Cell viability – MTT assay

The viability of SC-1 and Caco-2 cells following the evaluation of NO suppression activity was evaluated. The effects of tea extracts on RAW 264.7 cell viability were determined after 24 hours exposure. Following exposure there was no toxicity as a result of tea exposure (Figure 4.17A) or after LPS and INF-γ exposure (Figure 4.17B). These results indicate that the tea samples used in the study were not toxic to the RAW 264.7 cell line and that any reduction in NO production was not as a result of toxicity and/or cell death.

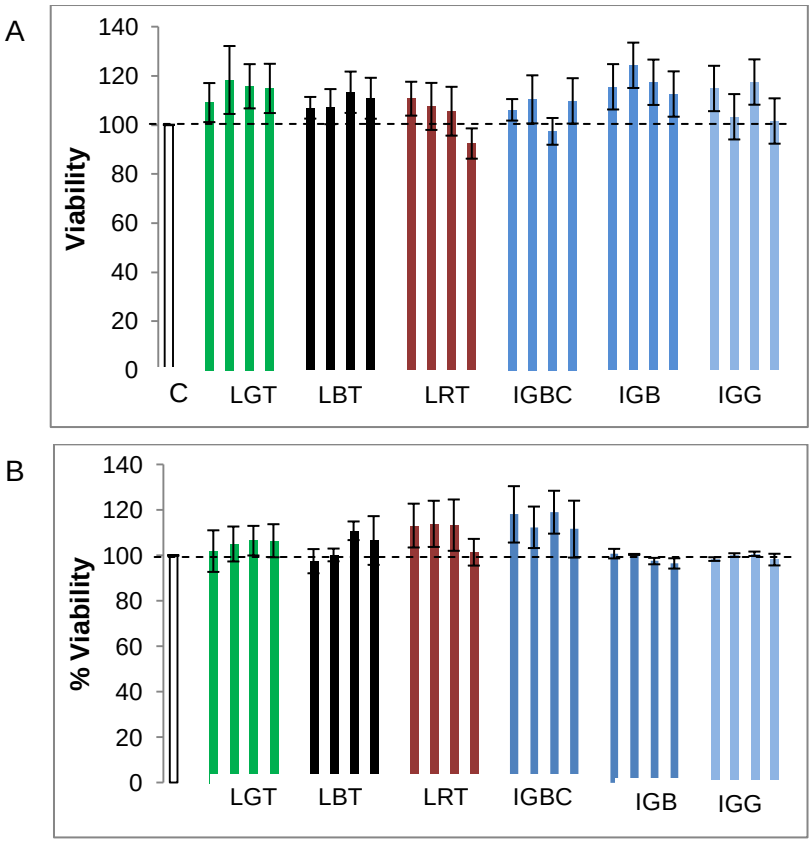


Figure 4.17: Percentage cell viability after 24 hr exposure to tea samples A) without and B) with LPS and INF-γ for extraction times 2, 10, 30 and 60 minutes. All data is an average of three independent ± SEM.

The importance of these results is due to the fact that bicarbonate ions are a component of the buffering system in cell culture media. When polyphenols are added to cell culture media, especially in cytotoxicity tests, toxicity against several cell lines is observed. The toxicity is attributed to the formation of H_2O_2 by polyphenols in the presence of bicarbonate ions (Halliwell *et al*., 2000, Odiatou *et al*., 2013). This reaction involves the reaction of hydroxides with phenolic hydrogen to form phenoxide anions; followed by the phenoxide anion reacting with oxygen to form phenoxyl radicals and finally the phenoxyl radical forms H_2O_2 and o-benzoquinone. The RAW 264.7 cell line was incubated in the presence of polyphenols for 24 hours and the MTT assay confirmed that no toxicity was observed.

4.5. Conclusion

All water extracts (2, 10, 30 and 0 minutes) of *Camellia sinensis, Aspalathus linearis* and *Ilex guayusa*, extracted at 90°C were shown to possess anti-oxidant and anti-inflammatory properties. In chemical based assays *Ilex guayusa* tea had low antioxidant and NO scavenging properties which were in contrast to findings in cellular based assays where *Ilex guayusa* had good cellular antioxidant and NO suppression properties. This measured activity was similar to *Camellia sinensis tea* but less than that found for *Aspalathus linearis.*

CHAPTER 5: CONCLUDING DISCUSSION

5.1 Summary of main findings

Tea is a widely consumed beverage all over the world and is generally *Camellia sinensis* plant dried leaf water infusions. Popularity of tea has increased due to many health benefits attributed to its consumption and this has been followed by extensive research on the properties and mechanisms involved. Teas that are not of the *Camellia sinensis* plant have also been commercialised, such as rooibos tea from *Aspalathus linearis* and guayusa tea from *Ilex guayusa*. With regards to *Ilex guayusa*, there is very little knowledge on the beneficial health properties of this product, especially with regards to its antioxidant and anti-inflammatory properties, in both a chemical and cellular environment.

The aim of this study was to evaluate, using internationally standardized methodologies, the antioxidant activity of methanol extracts of *Ilex guayusa* tea compared with a variety of commercially available *Camellia sinensis* teas. The second aim was to further evaluate the antioxidant and anti-inflammatory properties related to NO scavenging/suppression by water extracts of *Ilex guayusa* as representative of tea as it is commonly consumed, compared with *Camellia sinensis* and *Aspalathus linearis* tea.

5.1.1. Chemical evaluation

Various brands of black and green *Camellia sinensis* teas were selected to be compared with the available brand of *Ilex guayusa* tea in both its commercial and factory form. The antioxidant activity of methanol extracts of each tea type was evaluated using the ISO method for dry mass determination, sample preparation and determination of antioxidant activity (F-C). In addition, antioxidant activity was further evaluated with the TEAC, DPPH and ORAC assays. These antioxidant capacity assays encompass two types of mechanism of action, the F-C, TEAC and DPPH assays involve single ET whereas the ORAC assay involves transfer of a hydrogen atom. The latter is considered to be a physiologically relevant measure of antioxidant activity.

Findings of this aspect of this study showed that with the ET based antioxidant methodologies, guayusa tea had a lower antioxidant capacity when compared with several brands of green and black *Camellia sinensis* tea. In contrast the antioxidant activity measured with the ORAC assay

was similar to green and black *Camellia sinensis* tea. A summary of findings is presented in table 5.1.

Table 5.1. Summary of the antioxidant properties of *Ilex guayusa* compared to *Camellia sinensis* tea

	GT	BT	
		ET methods	
F-C	Lower	Lower	
TEAC	Lower	Lower	
DPPH	Lower	Lower*	*IGB comparable to TAB
		HAT method	
ORAC	Similar	Similar	

Due to the ORAC assay showing antioxidant activity similar to various brands of black and green *Camellia sinensis* tea, the health benefits related to antioxidant and anti-inflammatory properties using water extracts were further evaluated.

5.1.2. Health benefits

Throughout the world tea is prepared in different ways and this includes differences in extraction time. A major limitation in tea research related to antioxidant activity is that only one extraction time is used. To address this limitation, *Camellia sinensis, Aspalathus linearis* and *Ilex guayusa* were extracted with water for time intervals from 2 to 60 minutes. The antioxidant activity of these samples were determined with the F-C and ORAC assays and the cellular antioxidant activity was determined in the SC-1 and Caco-2 cell lines. Likewise NO scavenging activity was evaluated using a chemical assay and then the anti-inflammatory properties related to NO scavenging/suppressing activity was further evaluated in the RAW 264.7 cell line. A summary of the findings is presented in table 5.2.

Table 5.2. Summary of the health properties of *Ilex guayusa* compared to *Camellia sinensis* and *Aspalathus linearis* tea

ANTIOXIDANT CAPACITY				
	GT	**BT**	**RT**	
Chemical assays				
F-C	Less	Less	Similar	
ORAC	Less	Less	Less*	**2 minutes. IGB=LRT*
Cellular assays				
DCFH-DA (SC-1)	Similar	Similar	Higher	
DCFH-DA (Caco-2)	Similar	Similar	Higher	

ANTI-INFLAMMATORY – NO SCAVENGING/SUPPRESSING				
	GT	**BT**	**RT**	
Chemical assay				
NO scavenging	Less	Less	Similar	
Cellular assay				
NO suppressing (RAW 249.7)	Similar	Similar*	*Similar***	*2 minutes. < LBT ** 2 minutes. < LRT, 60 minutes > LRT

Generally, in a chemical environment assay, such as the F-C, ORAC and NO scavenging assays, guayusa tea had antioxidant and anti-inflammatory activity lower than that of *Camellia sinensis* and generally similar to *Aspalathus linearis*. In contrast in a cellular environment, the cellular antioxidant activity and NO suppressing activity was comparable to or higher than that of *Camellia sinensis* and *Aspalathus linearis* tea respectively.

Hypothesis 1: Alcohol extracts of *Ilex guayusa* had lower antioxidant capacity compared with *Camellia sinensis* according to the F-C, TEAC and DPPH assay, but similar antioxidant capcity according to the ORAC assay.

Hypothesis 2: Water extracts of *Ilex guayusa* had lower antioxidant capacity compared with *Camellia sinensis* according to the F-C and ORAC assays, but similar to *Aspalathus linearis* according to the F-C assay. In the SC-1 and Caco-2 cell lines *Ilex guayusa* had lower antioxidant capacity compared with *Camellia sinensis* but higher than *Aspalathus linearis* according to the DCFH-DA assay.

Hypothesis 3: Water extracts of *Ilex guayusa* had lower NO scavenging abilities compared with *Camellia sinensis,* but was similar to *Aspalathus linearis*. In the RAW 264.7 cell line, *Ilex guayusa* had similar NO suppressing abilities compared with *Camellia sinensis* and *Aspalathus linearis*

5.2. Limitations

5.2.1. Chemical analysis

Evaluation of the chemical antioxidant activity of each tea with ET based assays involved the measurement of specific endpoints. This fails to take into consideration slow-acting antioxidants that may have a lag period in their mechanism of action.

The polyphenol composition of each tea sample was unknown. Even although the polyphenol content of *Camellia sinensis* and *Aspalathus linearis* is known, differences between brands may account for the lack of difference in antioxidant activity between GT and BT.

5.2.2. Health benefits

A limitation was that only one brand of tea was evaluated for activity. Another limitation is the lack of methodologies that specifically identify the cellular mechanism/s of action related to antioxidant activity and the suppression of NO formation. Cellular models do not take into account the contribution of absorption, distribution, metabolism and excretion (ADME) to measured activity and possible health benefits and recent studies have shown that the metabolism of polyphenols results in the formation of certain metabolites that also may have unique antioxidant properties.

5.3. Future perspectives

Future research should involve the identification of polyphenols in *Ilex guayusa* tea. This can be achieved with HPLC and mass spectrometry (MS) methodologies, similar to the studies of Nishitani and Sagesaka, (2004), where simultaneous determination of catechin, caffeine and other phenolic compounds in green, oolong and black tea samples was undertaken. A similar

study can be undertaken to compare *Ilex guayusa* to *Camellia sinesis* and *Aspalathus linearis* and identify the unique polyphenols associated with *Ilex guayusa*.

Each identified polyphenol must be evaluated individually for activity as well as in combination in order to determine any synergistic effects between polyphenols.

Several pathways are associated with NO formation; polyphenols can bind to specific receptors and/or suppress gene expression. These include binding to LPS, inhibition of iNOS and/or inhibition of INF-γ and other cytokines which mediate the inflammatory response. Techniques that can be used are the *Limulus amebocyte* lysate (LAL) assay for determination LPS binding activity and Western blotting for the determination iNOS and cytokine expression. These parameters can be evaluated in different cellular models.

Antioxidant activity and NO reducing effects can be evaluated in a single animal model. For example in LPS-induced sepsis in Sprague-Dawley rats. A hallmark of sepsis is increased inflammation and oxidative damage eventually leading to organ failure. In a study by Molinett *et al*. (2015) the effect of Chilean strawberry consumption was evaluated in Sprague-Dawley rats. Such a model takes into account ADME. The effect of tea extracts on blood markers of oxidative damage and inflammation can be evaluated. Furthermore histological evaluation of the organ systems such as the liver and kidneys will provide important information regarding the protective effect of *Ilex guayusa* against LPS induced oxidative damage and inflammation.

This information is important to provide the public, data that has been subjected to sound scientific evaluation and that has been peer reviewed by the scientific community regarding the health benefits of *Ilex guayusa* tea.

5.4. Conclusions

Ilex guaysa extracts have cellular antioxidant activity protective and anti-inflammatory properties which is comparable to *Camellia sinesis* tea. It would therefore be of benefit to further investigate the health benefits of Guayusa tea to identify specific cellular mechanisms of action.

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