The Antibacterial Activity of Tea Infusions and Their Effect Against The Hospital Pathogen *Clostridium difficile*

A thesis submitted for the degree of

Doctor of Philosophy (Ph.D)

by

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“Drinking a daily cup of tea will surely starve the apothecary“

- Old Chinese Proverb

Summary

Clostridium difficile is one of the UK's most common hospital acquired infections and there is anecdotal evidence to suggest that the bacteria are sensitive to the antibacterial properties of tea. Surprisingly, little research has been undertaken to characterise the inhibitory activity of aqueous tea infusions that are representative of traditional drinking habits. The antibacterial properties of tea are thought to be due to a group of polyphenols called catechins. However, their contribution to the inhibitory activity of tea infusions and their mechanism of action is still subject to debate.

An antimicrobial assay, developed using *Staphylococcus aureus* as a model organism, was used to determine the antibacterial activity of a range of tea infusions against 75 clinical isolates of *C. difficile* that represented all the major strain ribotypes over 11 years.

Green teas demonstrated more potent antibacterial activity than black teas and their activity was positively correlated with antioxidant power, hydrogen peroxide production, and catechin content. Furthermore, the country of origin of the tea affected the catechin content and subsequent antimicrobial activity of the infusion.

Detailed chemical analysis using high performance liquid chromatography and counter current chromatography suggests that the antibacterial activity of tea is probably the result of synergistic interactions between a number of catechins rather than the activity of an individual compound. With regards to the mode of action by which tea inhibits *C. difficile*, electron microscopy studies of the bacterium treated with green tea revealed distinct changes to the outer cell structures of the bacteria. These changes were indicative of cell membrane blebbing, thus supporting the theory that tea compounds interact with the bacterial membrane and/or cell wall.

Overall, this investigation concluded that tea infusions have inhibitory activity against *C. difficile in vitro* and may be useful in the treatment or prevention of *C. difficile* infections *in vivo*.

Abbreviations

$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BC-*x*	Black commercial tea (*x* = associated tea sample number)
BHI	Brain heart infusion
BP-*x*	Black single plantation tea (*x* = associated tea sample number)
C	Catechin
CDI	*C. difficile* infection
CDT	Binary toxin
CE	Catechin equivalent
CFU	Colony forming units
CI	Confidence interval
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
Cu	Copper
Da	Dalton
diH_2O	Deionised water
DNA	Deoxyribonucleic acid
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FDA	Food and Drug Administration
Fe	Iron
FIC	Fractional Inhibitory Concentration
FOX	Ferrous oxidation-xylenol orange
FRAP	Ferric reducing antioxidant power
g	gram
× *g*	Gravitational force (Relative centrifugal force)
GA	Gallic acid
GAE	Gallic acid equivalent

GC	Gallocatechin
GC-*x*	Green commercial tea (*x* = associated tea sample number)
GCG	Gallocatechin gallate
GP-*x*	Green single plantation tea (*x* = associated tea sample number)
GTP	Guanosine-5'-triphosphate
h	hour
H_2	Hydrogen
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPCCC	High performance counter current chromatography
HPLC	High performance liquid chromatography
L	litre
LB	Lysogeny broth
M	Molar
Md	Median
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
min	minute
mL	millilitre
MRSA	Methicillin-resistant *Staphylococcus aureus*
n	Number of replicates
N. Ireland	Northern Ireland
N_2	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NB	Nutrient Broth
NCIMB	National Collection of Industrial, Food and Marine Bacteria
NCTC	National Collection of Type Cultures
nm	nanometre
NMR	Nuclear magnetic resonance
O_2	Oxygen
°C	Degrees Celsius
OD	Optical density

p	Value of significance
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ppb	Parts per billion
Q-Q	Quantile-quantile
r	r-value (Pearson product-moment correlation coefficient)
r^2	Coefficient of determination
RNA	Ribonucleic acid
SD	Standard deviation
SE	Standard error of the mean
sec	second
SEM	Scanning electron microscope
t	t-test value
TcdA	Toxin A
TcdB	Toxin B
TE	Trolox® equivalent
TEM	Transmission electron microscope
TFC	Total flavonoid content
TPC	Total polyphenol content
TPTZ	2,4,6-tris(2-pyridyl)-1,3,5-triazine
TSA	Tryptone soya agar
TSB	Tryptone soya broth
U	Mann-Whitney U test value
UHW	University Hospital of Wales
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
WR	Working reagent
z	z-score
η^2	eta squared (ratio of variance)

Contents

CHAPTER 1
GENERAL INTRODUCTION

1. General Introduction

1.1 Natural Therapeutics Derived from Plants

Natural products have provided the foundation for many of today's longstanding medicinal treatments. A natural product is defined as a compound or substance produced by a living organism, such as plants, bacteriophages and bacteria (G & C. Merriam Co., 1913). An example of an important plant derived natural product is morphine, a powerful opioid analgesic used worldwide for treating pain. It was isolated from the dried sap of un-ripened seedpods of the poppy plant (*Papaver rhoeas*) in 1804 by Friedrich Sertürne (Lockermann, 1951). Another good example of a plant derived natural product is the cardiac glycoside digoxin, it is used for a variety of heart conditions such as atrial fibrillation (Martin, 2013) and was isolated from the foxglove plant (from the *Digitalis* family) (Krikler, 1985). On a global scale, approximately 75% of drugs used in infectious diseases and 60% of drugs used in anticancer treatments are directly from or derived from natural products (Newman *et al*., 2003). Furthermore, approximately 60% of the world's population relies almost entirely on plants for medication (Harvey, 2000). However, considering the importance of natural products to drug discovery, in the last decade major drug companies have reduced the amount of capital they invest in natural product research (Beutler, 2009). The extent of this trend was evident when the number of approved drugs derived from natural products reached a 20-year low in 2002 (Ortholand and Ganesan, 2004). The main reasons for this shift in drug discovery was the perception that identifying new clinically-relevant drugs from natural products, particularly plants, is a slow process and one that often yields complex molecules that are very difficult to synthesise or purify in significant quantities (Beutler, 2009). When this is combined with the challenge of physically obtaining the biomass needed to provide a workable amount of the isolated compound and the tedious pathway to take a drug to market, investing in natural product research becomes a difficult financial decision. Therefore, pharmaceutical companies have opted for the synthetic approach to finding novel drug entities, such as high throughput screening and synthetic chemistry to generate 'hits' for clinical targets (Koehn and Carter, 2005). These techniques are very quick and inexpensive methods of generating large numbers of novel compounds. The promise of a seemingly inexhaustible supply of compound

libraries precipitated a further shift away from natural products (Ortholand and Ganesan, 2004).

Combinatorial chemistry is good example of a high throughput technique that is used extensively in drug discovery (Myers, 1997). This technique identifies points of diversity in a starting hit compound or pharmacophore. Each point of diversity in the molecule is then varied based on an inventory of starting materials and mathematical models of drug-target interactions. This will then generate huge libraries of similar but structurally different compounds (Furka, 2002). When used alongside automated combinational synthesis, thousands of compounds can be physically generated for biological screening. However, the initial drawbacks of these methods were small yields, poor solubility and low purity of the generated compounds (Beutler 2009). Progress has been made to improve on these limitations, but the success rate of producing clinically effective novel therapeutics using these techniques has been lower than expected (Newman and Cragg, 2007).

After the implementation of this technology, the number of new chemical entities reached a 24 year low of 25 in 2004 (Newman and Cragg, 2007). In the United States of America (USA), 487 new drugs were approved by the Food and Drug Administration (FDA) from 1998 through to 2003, 378 (78 %) of which were classed by the FDA as appearing to have therapeutic qualities similar to those of one or more drugs already in the market (Kumar and Singh, 2008). Furthermore, 333 (68 %) of these drugs were not new molecular entities, but re-formulated versions of current drugs (Kumar and Singh, 2008). The expectations of high throughput screening and combinational chemistry in its purest form had clearly not come to fruition, as between 1981 and 2006 there was only one *de novo* drug, the multikinase inhibitor sorafenib, approved by the FDA (Newman and Cragg, 2007). Although these high throughput techniques have not yielded as many new drugs as once thought, they are still important tools in drug discovery and seem better placed to expand on existing lead compounds rather than inventing new ones (Beutler, 2009).

It is evident that initial efforts to produce new therapeutics using these techniques were not as successful as anticipated, however progress has been made to combine high throughput technology with natural product screening (Li and Vederas, 2009) and there

has been a slow resurgence of drugs approved by the FDA derived from natural products (Newman and Cragg, 2007). This is not surprising, considering that many of the novel chemicals isolated from nature are often unique and stereo-chemically complex structures (McChesney *et al*., 2007).

Generally, natural products are chemically different in their composition in comparison with synthetic drugs. They tend to be more oxygen rich, contain less nitrogen atoms and contain more chiral centres (Ortholand and Ganesan 2004). This is partially due to the complex biosynthetic pathways from which they are derived. These pathways are often more efficient than their synthetic counterparts and are usually extremely difficult to emulate due to the complexity of the chemistry and the lack of suitable starting materials (Ortholand and Ganesan 2004). Although this would prove to be a disadvantage to a drug company seeking to mass produce/purify a hit compound, it may not pose a problem if a crude extract of the natural product was clinically effective and could be produced on a large scale. With this in mind, it raises the question whether food grade plants that contain high concentrations of the desired compounds as a result of environmental conditions, genetic manipulation or species could be specifically selected for clinical application. This would eliminate the need for chemical synthesis and complicated extraction techniques. It is a very exciting possibility, especially with regard to plants such as *Camellia sinensis*, a plant that is well accepted as a common foodstuff and the leaves of which are infused in water by millions of people worldwide (Fernandez-Caceres *et al*., 2001).

1.2 *Camellia sinensis*

Camellia sinensis (fig 1.1) is an evergreen shrub belonging to the *Theaceae* plant family. There are two major varieties of *Camellia sinensis*: Chinese *Camellia sinensis* var. *sinensis* and *Camellia sinensis* var. *assamica* (Integrated Taxonomic Information System, 2013). *Camellia sinensis* var. *sinensis* is hardier than Assam tea and has relatively small and narrow leaves (Kew Royal Botanical Gardens, 2013; table 1.1). The leaves from this variety are used to produce Chinese green and black tea (Kew Royal Botanical Gardens, 2013). The *C. sinensis var. assamica* variety has larger leaves, which have a droopy and leather like appearance. This variety of tea bush is predominantly grown to produce Assam and Indian black tea (Kew Royal Botanical

Gardens, 2013). Both types of tea bush are cultivated across the world, but mainly in tropical and subtropical regions, primarily to be harvested for tea.

Table 1.1. *The taxonomy of Camellia sinensis (adapted from Kew Royal Botanical Gardens, 2013).*

Camellia sinensis	
Kingdom	*Plantae*
Sub Kingdom	*Tracheobionta*
Division	*Magnoliopsida*
Order	*Theales*
Family	*Theaceae*
Genus	*Camellia* (L.)
Species	*Camellia sinensis* (L.) Kuntze
Main varieties	*Camellia sinensis var. assamica*
	Camellia sinensis var. sinensis

Figure 1.1. *Artist representation of Camellia sinensis (taken from Koehler's Medicinal Plants, 2013).*

The history of infusing *Camellia sinensis* leaves in water can be traced back to China in 2700 BC (Weisburger, 1997). According to legend, Chinese Emperor Shen Nung discovered the drink when a few *Camellia sinensis* leaves accidentally fell into a pot of boiling drinking water (Wheeler and Wheeler, 2004). When the Emperor tasted the infusion, he was pleasantly surprised by the taste and from then onward the infusion became known as the popular drink tea (Wheeler and Wheeler, 2004). Around the 17th century, tea was imported into the United Kingdom (UK) from India and became an important part of the British culture (Weisburger, 1997). Britain played an important role in popularising the drink and during the late 17th Century, it became so popular it had value in currency and international trade (Hara, 2001).

Today tea continues to be one of the most popular drinks worldwide, second only to water (Kuhnert, 2010; Park *et al.,* 2012). This is probably due to many different varieties of tea and also the versatility of creating different flavoured drinks by altering the infusion time (Stahl, 1962). It is estimated that 20 billion cups are consumed daily (Fernandez-Caceres *et al*. 2001). The UK accounts for 165 million of these cups making it one of the biggest global consumers of tea worldwide (UK Tea Council, 2013, 2009; fig. 1.2).

Black tea, the most popular variety in the UK is usually consumed with milk, while in China and Japan green tea is the most popular tea and is usually consumed neat (Stewart, 2012). Cold tea drinks are now becoming more popular, especially in the US and Japan. In the US nearly 75 % of tea drinks are consumed cold (Weisburger, 1997). Tea extracts are also being added to many foodstuffs, such as biscuits, cakes and other confectionary, a trend that is prominent in Japan but is slowly moving into other countries around the world (Wang *et al*., 2003).

With a huge demand for tea worldwide, it is estimated that the annual global production is 2.9 million tons (World Green Tea Organisation, 2010), with the biggest producers being China, India and Sri Lanka (fig. 1.3).

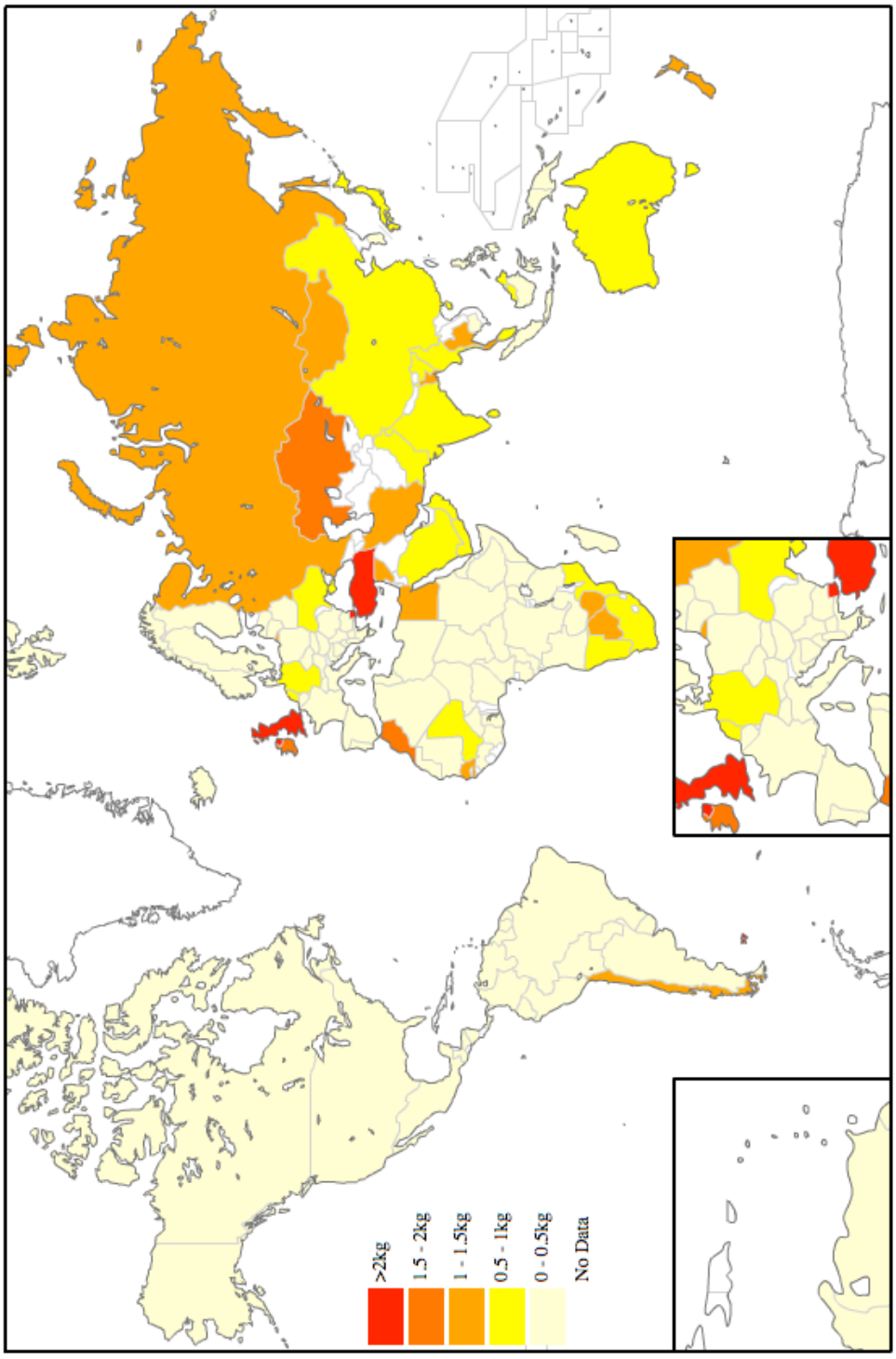


Figure 1.2. *The amount of tea (kilograms) consumed per head globally in 2009 (data obtained from Food and Agriculture Organisation of the United Nations, FAOSTAT, 2009)*

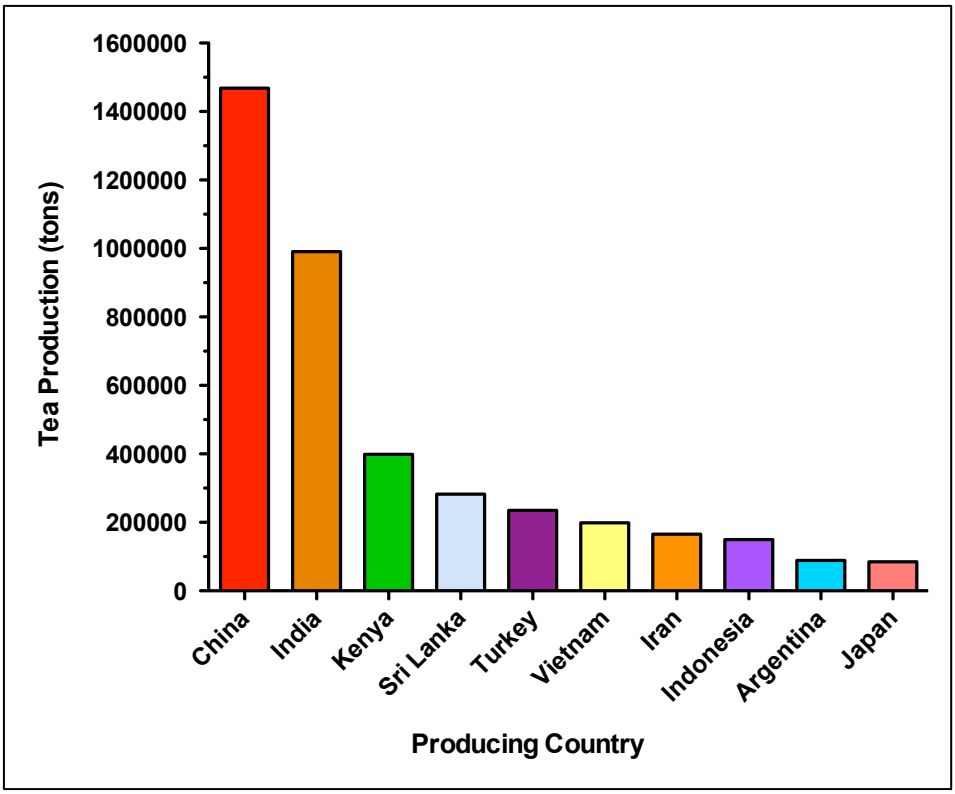


Figure 1.3. *Amount of tea (tons) produced by country in 2010 (data obtained from Food and Agriculture Organisation of the United Nations, FAOSTAT, 2009).*

1.3. Tea Leaf Processing

There are many types and variations of tea available, though the four main types are white, green, oolong and black. Teas are primarily differentiated by level of fermentation they have undergone, but can also be distinguished by the grade of leaf, season of picking, province of tea plant and the position of where the leaves are picked from the plant (Hara, 2001). There are four main processing steps involved in producing tea, the duration and combination of which will determine the type of tea obtained. An overview of the processing steps for the main types of tea can be seen in figure 1.4.

1.3.1 Withering or Wilting

Once a leaf is plucked from the tea plant, a small amount of enzymatic oxidation will take place and the tea leaf will start to wilt (Roberts, 1958). This is usually done in sunlight or a cool room with plenty of air movement to remove water from the leaf. The removal of water enhances the breakdown of leaf proteins by peptidases (approximately

1.2%) (Hampton, 1992) to free amino acids, which is important in the first stage of fermentation (Roberts, 1958). High levels of caffeine are released in this stage and polyphenol oxidase is released as the leaf cells become more permeable (Bondarovich *et al*., 1967).

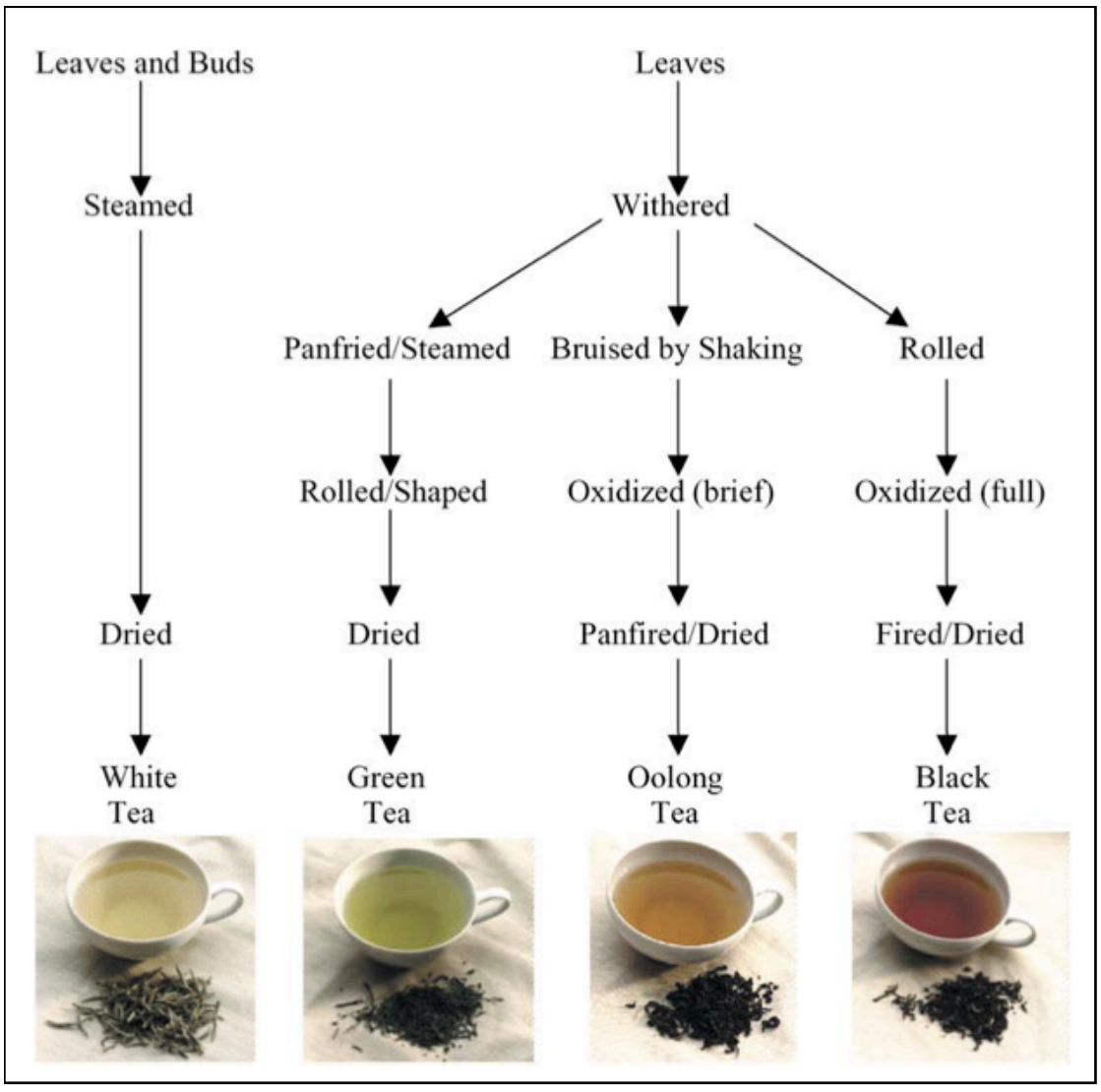


Figure 1.4. *Processing of different types of common teas (taken from Santana-Rios et al., 2001).*

1.3.2 Leaf Bruising

After the leaves have dried from wilting, they are bruised to break down the leaf structure, which allows the mixing of enzymes and polyphenols promoting oxidation (Bondarovich *et al*., 1967). This is one of the most diverse steps in tea leaf processing as the degree leaves are bruised can range from gentle bruising such as shaking and tossing to more extreme bruising like rolling, crushing and tearing of the leaf (Varnam and Sutherland, 1994). For effective bruising to take place, it is essential that most of the moisture from the leaf has been lost during the wilting stage (Hampton, 1992).

1.3.3 Oxidation (Fermentation)

In this step the tea leaves are left in a climate controlled room to oxidise or ferment for the desired amount of time. During this process, the leaves will darken and the degree of bruising and tearing will determine how quickly this process takes place (Bondarovich *et al.*, 1967). This step results in many of the major changes in chemical content of the tea leaf (Roberts, 1958).

1.3.4 Drying

The drying stage is the final step in tea production and takes place immediately after oxidation. The drying process can be carried out in many ways, some traditional tea producers pan fry the leaves while others use techniques such as sunning and oven drying (Graham, 1992). The application of heat in this stage deactivates the oxidising enzymes and prevents any further fermentation of the tea leaf (Bondarovich *et al.,* 1967). It also removes any remaining moisture in the leaf (Bondarovich *et al.,* 1967). To produce green tea, this step is carried out immediately after wilting, whereas in black tea the leaf is allowed to ferment completely before drying. Oolong teas are partially oxidised before drying, and depending on the type of Oolong tea required, the range of oxidation is usually between 5-70% (Bhattacharyya *et al.,* 2007).

1.4 Chemical Composition of Tea Leaves

Tea leaves are rich with many different compounds, a summary of their typical composition is detailed in table 1.2.

Table 1.2. *A summary of the main components of dried tea leaves and tea infusions (Graham, 1992; Hara, 2001).*

Class	Compounds	Average weight in dried tea leaf % (w/w)	Average weight in tea infusions % (w/v)
Protein	-	15 - 20	Trace
Amino acids	Theanine, glutamic acid, trytophan, glycine, serine, aspartic acid, tyrosine, valine, leucine, threonine, arginine and lysine	1 - 4	1 - 3.5
Fibre	Cellulose	26	Trace
Carbohydrates	Glucose, sucrose, fructose and pectins	7	4
Lipids	Linoleic acids and sterols	7	Trace
Pigments	Chlorophylls and carotenoids	2	Trace
Minerals	Calcium, magnesium, chromium, maganese, iron, zinc, copper, selenium, molybedenum, sodium, phosphorus, cobalt, strontium, nickel, potassium, fluorine and aluminium	5	4.5
Alkaloids	Methylxanthines (caffeine, theobromine, theophylline)	3 - 4	7 - 9
Phenolics[1]	Flavonoids (flavones, flavonols, flavones, flavanones, isoflavones and anthocyanins), theaflavins, thearubigins, theasinensins and phenolic acids	25 - 36	45 - 65

[1]*Phenolic content largely determined by degree of fermentation (adapted from Graham, 1992 and Zhang, 2012)*

1.4.1 Polyphenols

For many years, tea consumption has been assigned various health benefits. Scientific research has shown that the compounds found in tea possess anticancer (Link *et al*., 2010), antimicrobial (Ikigai *et al*., 1993) and antiviral properties (de Siqueira *et al*., 2006). It has also been reported to be cardio-protective (Dreger *et al*., 2008) and have anti-diabetic properties (Chen *et al.,* 2010a). Evidence has indicated that these effects may be due to of a group of compounds in tea called polyphenols.

Polyphenol is a generic term to describe any chemical that is built around two or more phenol groups and tea, like most plants, contains many different types of polyphenols (Bradfield and Bate-Smith, 1950). The largest group of tea polyphenols by weight is the flavonoids (~30 %(w/w)) (Chou *et al*., 1999). The composition of flavonoids in tea leaves varies with the type of processing the tea has undergone. Fresh tea leaves consist mainly of monomeric catechins (a subgroup of flavonoids) (Chou *et al*., 1999). When the leaf is picked from the plant, enzymatic oxidation starts to take place (fermentation), which converts monomeric catechins into simple theaflavins and multimeric thearubigins (Wheeler and Wheeler, 2004). This oxidation process can be stopped by heat inactivation of the enzymes (polyphenol oxidases) as described earlier in the processing methods (Weisburger, 1997).

1.4.1.1 Flavonoids

As discussed, tea is abundant in flavonoids; the types of which are dependent on the degree of fermentation. Flavonoids are divided into 7 main classes: flavones, flavanones, isoflavones, flavonols, flavanols, chalcones and anthocyanins, based on the structure of the heterocyclic oxygen ring (Wang *et al*., 2000) (fig. 1.5). The main groups found in tea are flavonols and flavanols (Wang *et al*., 2000). The flavonoids are secondary metabolites in tea plants, which means they are not known to participate directly in the plants growth and development (Croteau *et al*., 2000). To date their exact function within the plant remains unknown. Although their biosynthesis from phenylalanine derivatives and malonyl-coenzyme A in tea leaves is well documented (fig. 1.6).

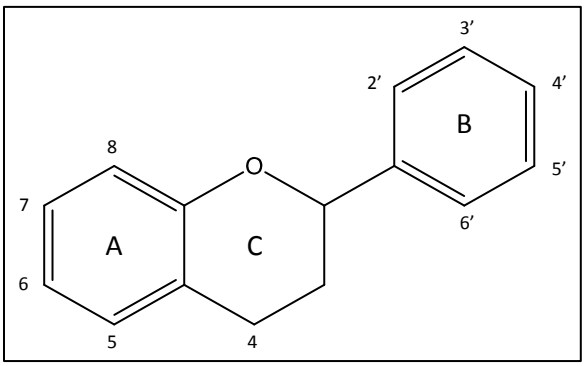


Figure 1.5. *The chemical backbone of flavonoids (adapted from Wang et al. 2000).*

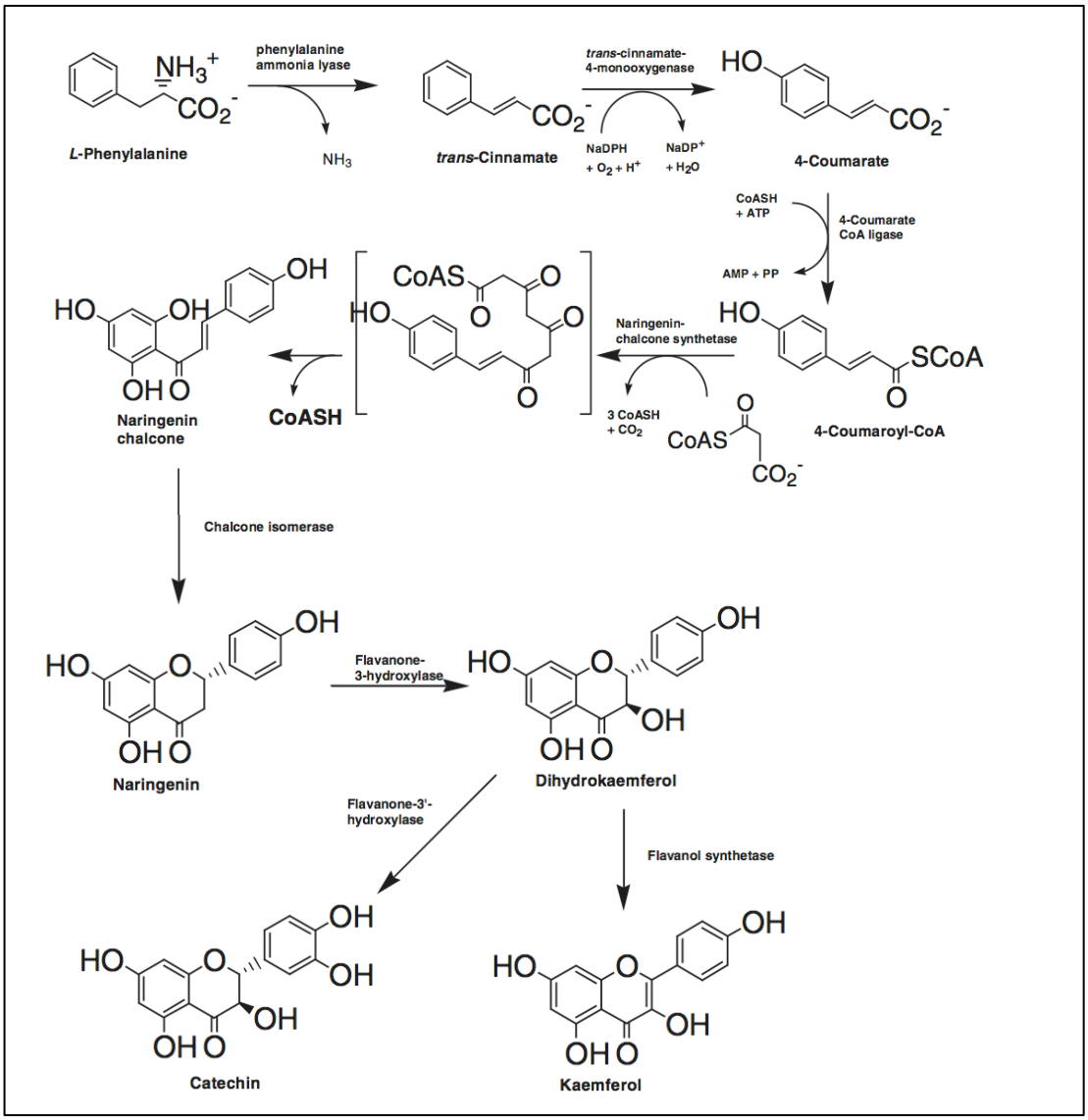


Figure 1.6. *The biosynthesis of flavonoids from malonyl-coenzyme A (taken from Wheeler and Wheeler, 2004).*

Despite the uncertainty surrounding the function of flavonoids, a few studies have hypothesised that they may be produced to prevent infections (antimicrobials), plant signalling or as a general stress response (Winkel-Shirley, 2001). They may also be produced to prevent the plant being eaten by predators as some polyphenols, particularly catechins and proanthocyanidins, give tea a very bitter taste (Croteau *et al.,* 2000).

Another plausible role of these secondary metabolites is to act as a 'sunscreen' for the leaves of the plant, which was proposed in a study that looked at the evolution of land based plants in response to changes in sunlight (Stafford, 1991). This idea is further supported by a study that reported that keeping tea plants in the dark inhibited polyphenol production whilst exposing them to excess light increased their concentrations (Forrest, 1969). This may be explained by the conjugated electron dense ring systems found in flavonoids, which may allow the leaves to absorb and displace the energy of excessive Ultraviolet (UV) B radiation (Croteau *et al.,* 2000). One flavonoid that has been identified as having this role is the flavonol kaempferol, which is present in tea leaves and tea infusions (Croteau *et al.,* 2000).

1.4.1.2 Catechins

The major group of flavanols in tea are catechins (fig 1.6). The catechins are synthesised within the tea leaf during the daylight hours and then stored in the leaf vacuoles (Hara, 2001). Catechins are present in all teas but are at their highest concentration in green teas where they form 20 – 30 % of the dry weight of tea leaf (Balentine *et al*., 1997; table 1.3). In black tea leaves, the presence of catechins is much lower because of the enzymatic oxidation that takes place during fermentation. Therefore, catechins only contribute 3 – 10 % of the total dry weight of black tea leaves (Graham, 1992).

Catechins give rise to the astringency and aroma of tea (Wang *et al.,* 2000). Almost all of the characteristics of manufactured tea, including its taste, colour and aroma are associated directly or indirectly from modifications to the catechins (Wang *et al.,* 2000). An example of this is the de-galloation from ester catechins to non-ester catechins, which results in a decrease in bitterness and astringency (Wang *et al*., 2000). Epigallocatechin gallate (EGCG) is one of the most abundant catechins in green tea and has been the most widely studied catechin (Schramm, 2013) (fig. 1.7). The amount of EGCG present in an average cup of green tea has been shown to be around 90 - 100 mg/ cup (Henning *et al*., 2013).

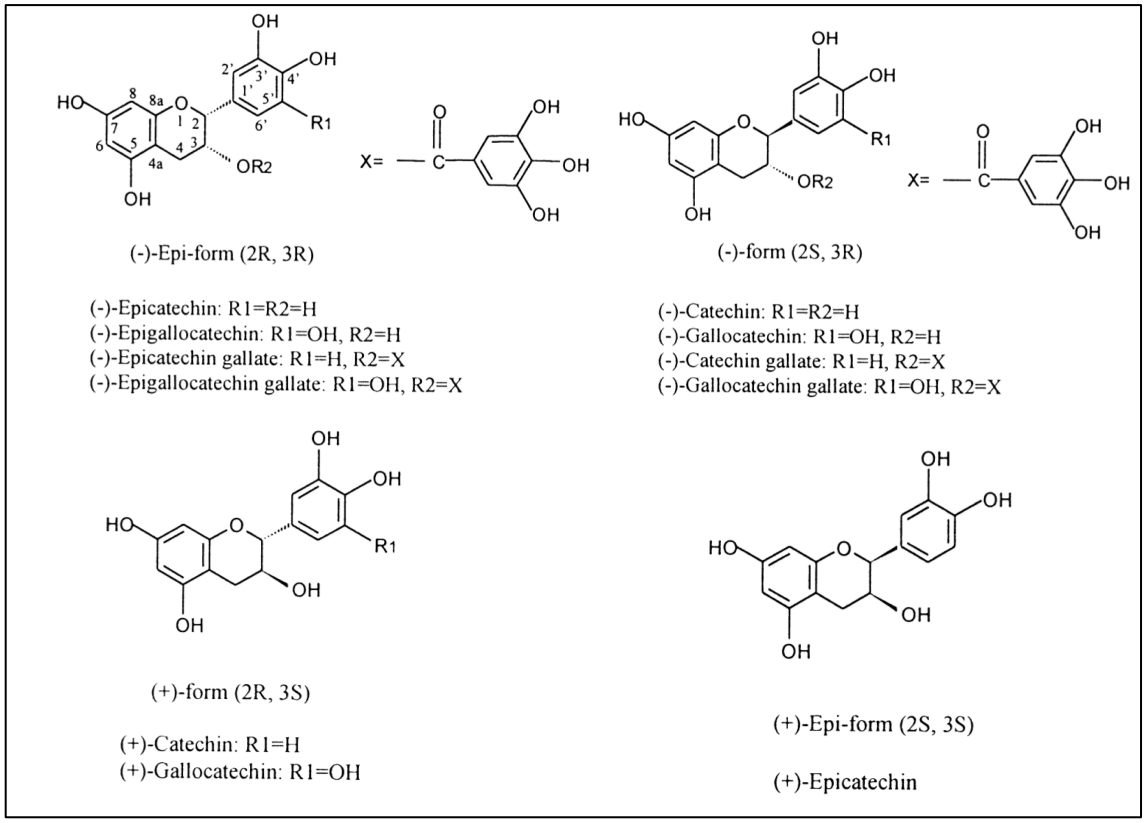


Figure 1.7. *The catechins in fresh tea leaves (taken from Wang et al., 2000).*

Table 1.3. *Average weight of catechins in fresh tea leaves. (adapted from Graham, 1992).*

Catechin	Acronym	Average dry weight in fresh tea leaf %(w/w)
Catechin	C	1 - 2
Epicatechin	EC	1 - 3
Epicatechin gallate	ECG	3 - 6
Gallocatechin	GC	1 - 3
Epigallocatechin	EGC	3 - 6
Epigallocatechin gallate	EGCG	7 - 13

1.4.1.3 Flavonols

Flavonols make up 2 – 3 % of the aqueous extract of tea (Balentine *et al*., 1997). They differ from catechins predominantly by the addition of a ketone group on the C ring (fig 1.7). The three most abundant flavanols in tea are quercetin, kaempferol and myricetin (Wang *et al.,* 2000). The primary structure of non-glycosylated flavonols (aglycones) is shown in figure 1.8. However, they are dominantly present as glycosides with sugars such as glucose, rhamnose and fructose attached (Wang *et al*., 2000). The water solubility of aglycones is very poor and therefore they are only present in low levels in prepared tea drinks compared to glycosylated state (Finger *et al.,* 1991).

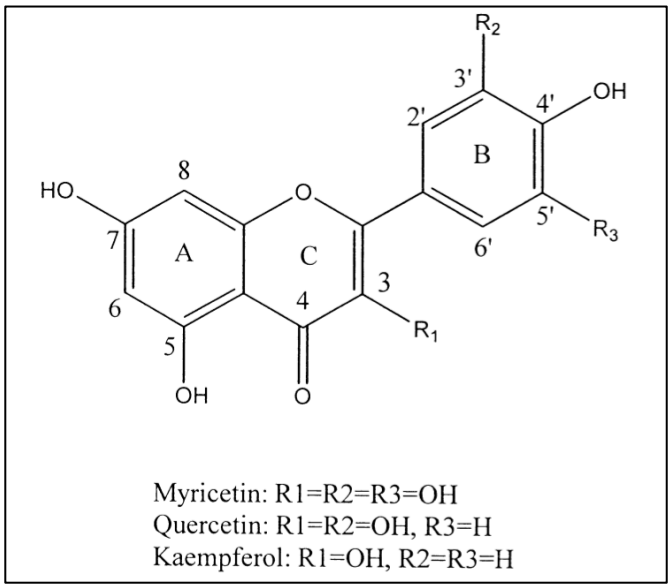


Figure 1.8. *The chemical structure of the flavonols (taken from Wang et al., 2000).*

1.4.1.4 Theaflavins, Thearubigins and Theasinensins

The presence of theaflavins and thearubigins in tea leaves is the result of dimerisation and oxidation of monomeric catechins during the fermentation process (Roberts, 1958; fig. 1.9). Their concentrations are highest in black teas and are responsible for the dark colours in fermented teas (Roberts, 1958). Theaflavins give rise to the orange colours while the thearubigins are responsible for the brown pigments (Wang *et al*., 2000).

Thearubigins are heterogeneous polymers of catechins and catechin gallates (Wheeler and Wheeler, 2004). Theaflavins possess a characteristic benzotropolone moiety and are further oxidised in black tea infusions to bistheaflavins and theanapthoquinone (Tanaka *et al*., 2009a; fig. 1.10).

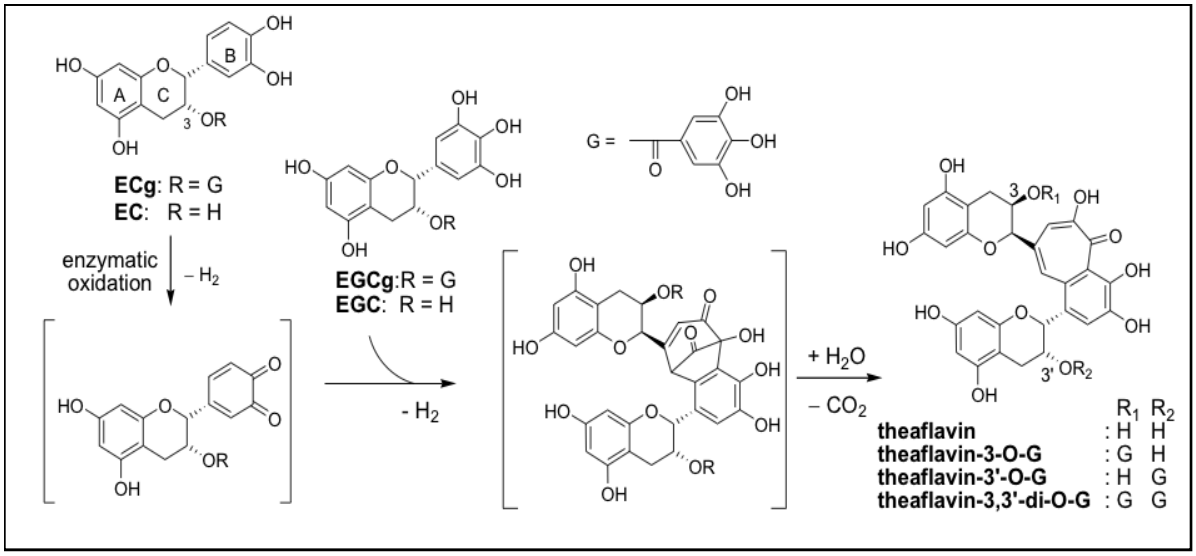


Figure 1.9. *Oxidation of catechins to theaflavins (taken from Tanaka et al., 2009a).*

The most abundant black tea pigments are the thearubigins, which make up an estimated 60 % of the solids in a black tea infusion (Kuhnert, 2010). They are extremely complex molecules and since their discovery 50 years ago, efforts to clarify their definite structure have been in vain (Kuhnert, 2010). Nevertheless, evidence has shown that they range from dimeric catechins to large polymeric structures with molecular weights ranging from 700 to 40,000 Daltons. It is hypothesised that the likely precursors to thearubigins are ECG and EGCG (Haslam, 2003). Investigations using high performance liquid chromatography (HPLC) and C^{13} nuclear magnetic resonance spectra have failed to provide any clarity on the possible structures of thearubigins due to the poor separation and low signal to noise ratios (Haslam, 2003). The only signal on the chromatograph that characterises the thearubigins is a broad peak that is referred to as the 'thearubigin hump' (Bailey *et al*., 1991) (fig. 1.10 (B)).

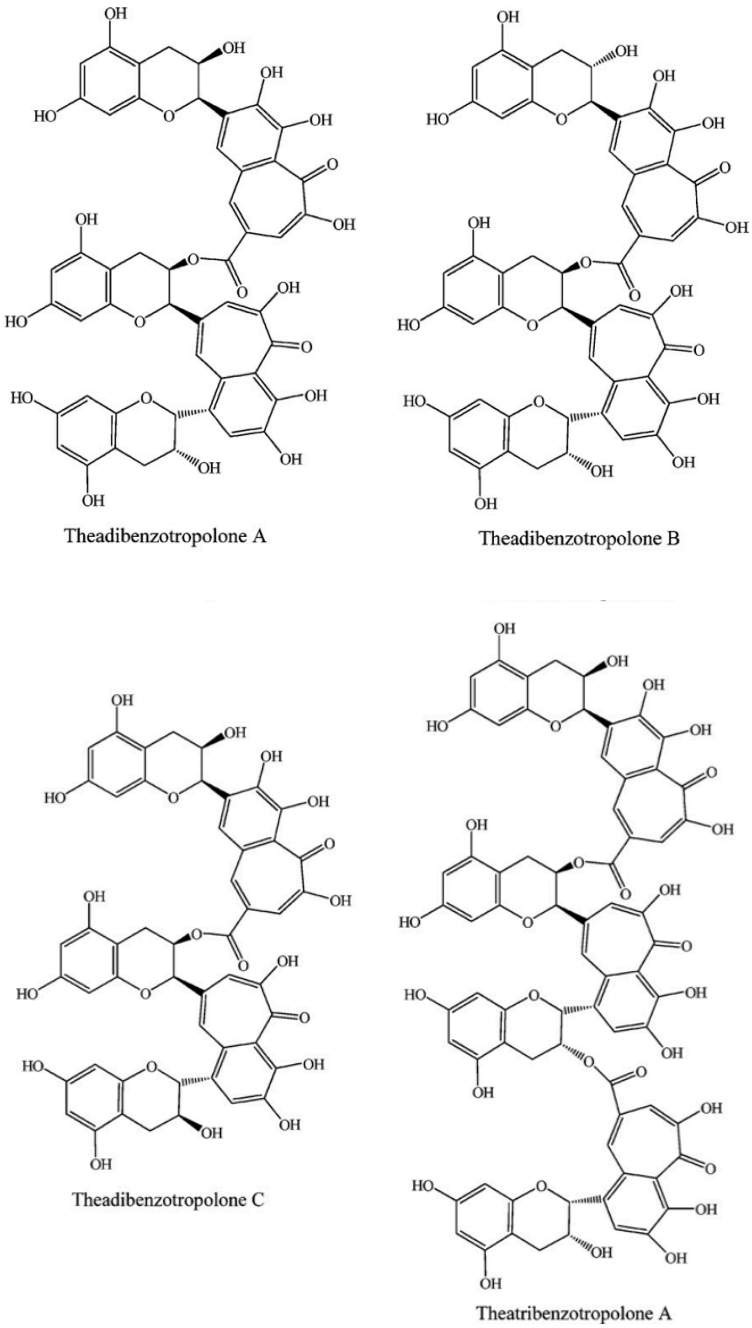


Figure 1.10. *Chemical structures of the theaflavins in black tea (taken from Sang et al., 2011).*

A minor group of polyphenols found in tea are the theasinensins (Tanaka *et al*., 2009b) (fig. 1.11 (A)). Theasinensins are colourless compounds formed from the dimerisation of two catechins via a carbon-carbon bond on the B rings (Tanaka *et al*., 2009b). They are thought to be the intermediates to some theaflavins and thearubigins. As a result, they are found mainly in semi-fermented oolong teas (Hashimoto *et al*., 1988).

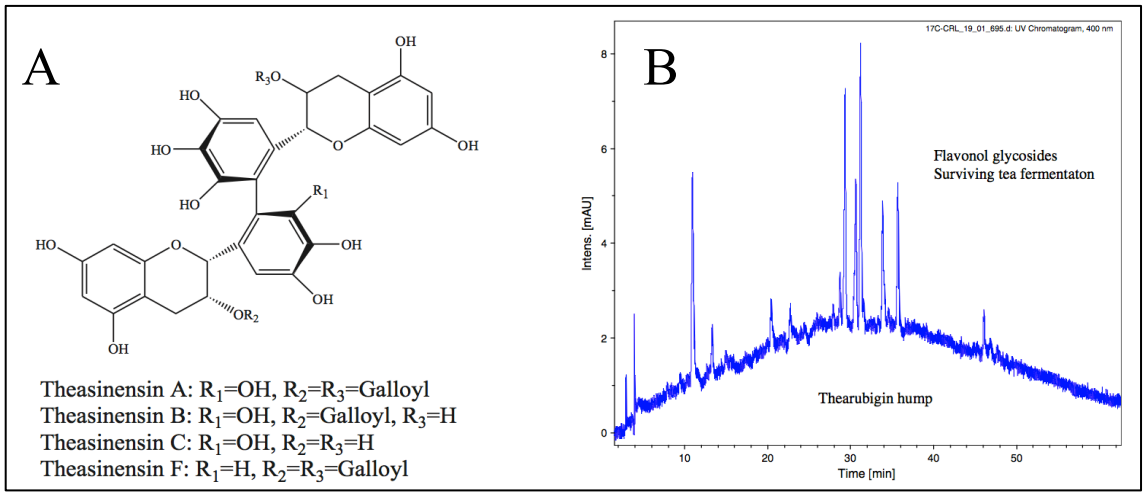


Figure 1.11. *(A) The structure of the theasinensins (taken from Sang et al., 2011). (B) An HPLC plot showing the classic 'thearubigin hump' (taken from Kuhnert, 2010).*

1.4.2 Chlorophylls and Carotenoids

Organic tea pigments, chlorophylls (fig. 1.12) and carotenoids (fig. 1.13), are found in both fresh and processed tea leaves. They are poorly soluble in polar solvents, and when in isolation they are immiscible with water (Suzuki and Shioi, 2003). However, small quantities have been found in hot water tea infusions. This is thought to be due to the action of saponins in the tea leaf. Saponins are mild surfactants, which in aqueous solution make chlorophylls and carotenoids more miscible (Suzuki and Shioi, 2003).

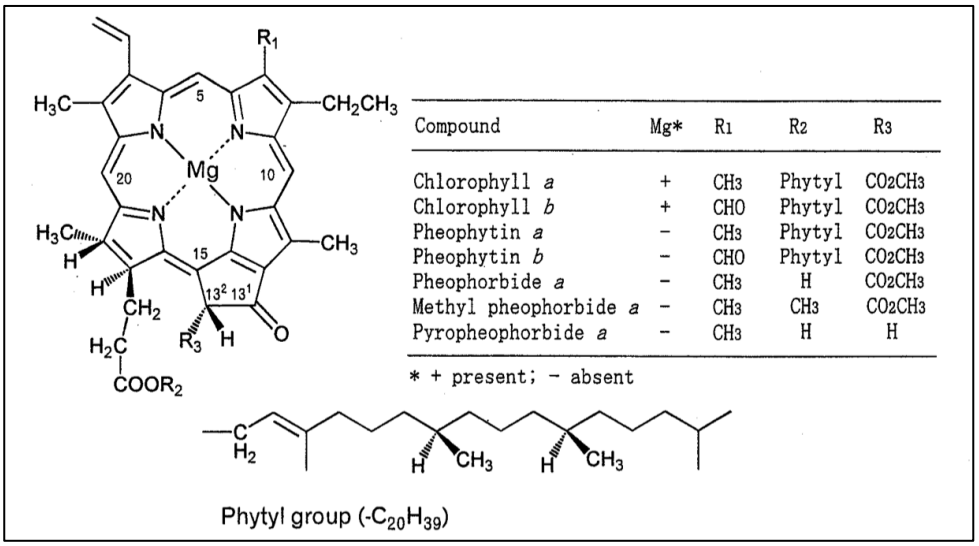


Figure 1.12. *The structures of different chlorophylls found in fresh tea leaves (taken from Kohata et al., 1998).*

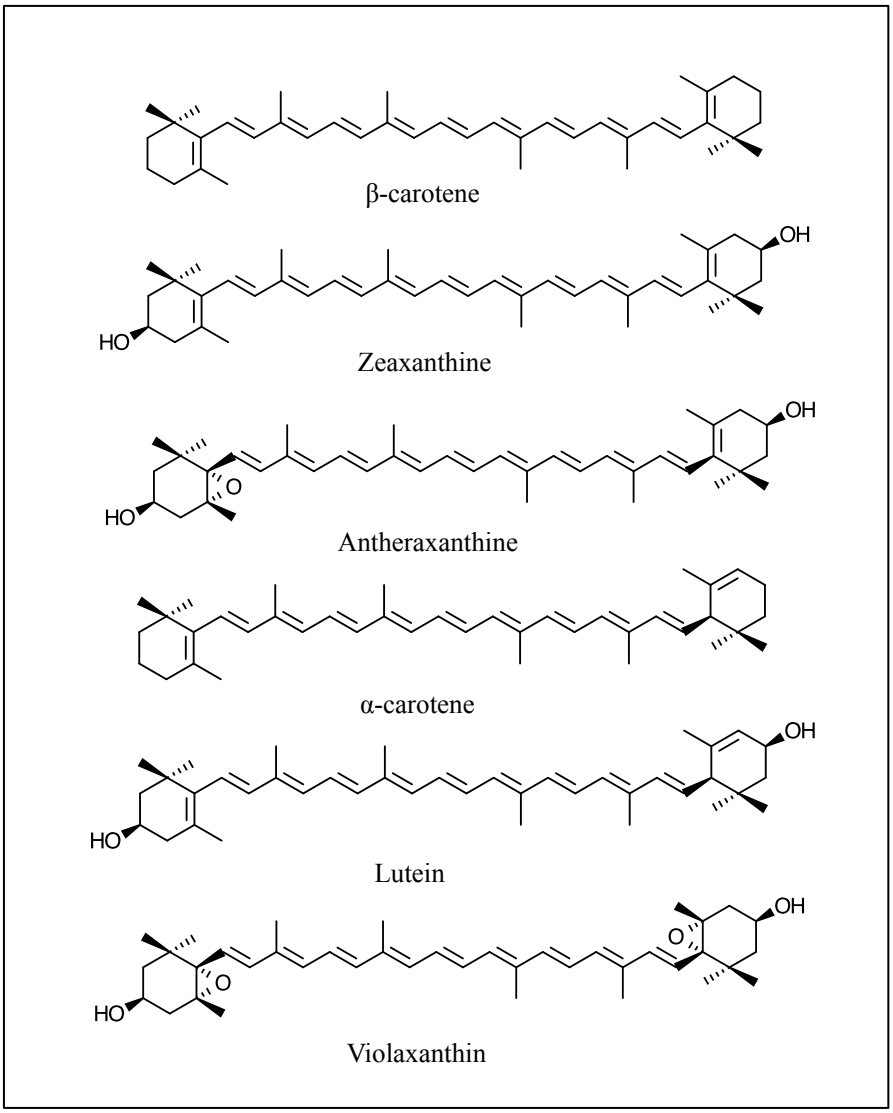


Figure 1.13. *The structures of different carotenoids found in fresh tea leaves (adapted from Suzuki and Shioi, 2003).*

In an average green tea infusion (2.5 g of tea brewed for 5 min at 100 °C), the amount of chlorophylls in the infusion will be in the range of 1-11 mmols/g whilst the amount of carotenoids is approximately between 1-5 mmols/g (Suzuki and Shioi, 2003). Chlorophylls are also present in black tea infusions but at lower levels 0-6 mmols/g, while carotenoids are barely detectable (0-1 mmol/g) (Suzuki and Shioi, 2003).

The main organic pigments found in tea leaves are chlorophyll a, chlorophyll b, pheophytin a, pheophytin b, β-carotene and lutein (Higashi-Okai *et al*., 2001). Like monomeric polyphenols, chlorophyll undergoes chemical changes during the tea leaf fermentation process. During fermentation, chlorophyll is degraded by chlorophyllase

into pheophorbide and other minor degradation products (Suzuki and Shioi, 2003). Unlike polyphenol oxidase, it has been shown that chlorophyllase is not completely inactivated by heating, or the firing process in green and black tea manufacture (Kohata *et al*., 1998). Therefore after processing, enzymic degradation of chlorophylls may still take place long after the tea leaf has been processed (Kohata *et al*., 1998).

1.4.3 Methylxanthines

Methylxanthines are methylated purine derivatives, which are a subgroup of the alkaloid family of compounds (Stavric, 1988). Their presence in nature is limited to only a few plants species and can be found in foods such as tea, coffee and chocolate (cocoa) (Ashihara *et al.,* 2011). The primary methylxanthines in tea are caffeine, theobromine and theophylline (fig 1.14).

The average tea brew is reported to contain between 50 – 90 mg of caffeine (Astill *et al*., 2001). It is the most abundant methylxanthine in tea and is well known for its stimulant activity on the central nervous system and the cardiovascular system (Talik *et al*., 2012). Theobromine and theophylline also have similar effects on the body but are found at much lower concentrations in tea infusions, 4 mg and 0.3 mg respectively (Stavric, 1988; UK Food Standards Agency, 2004).

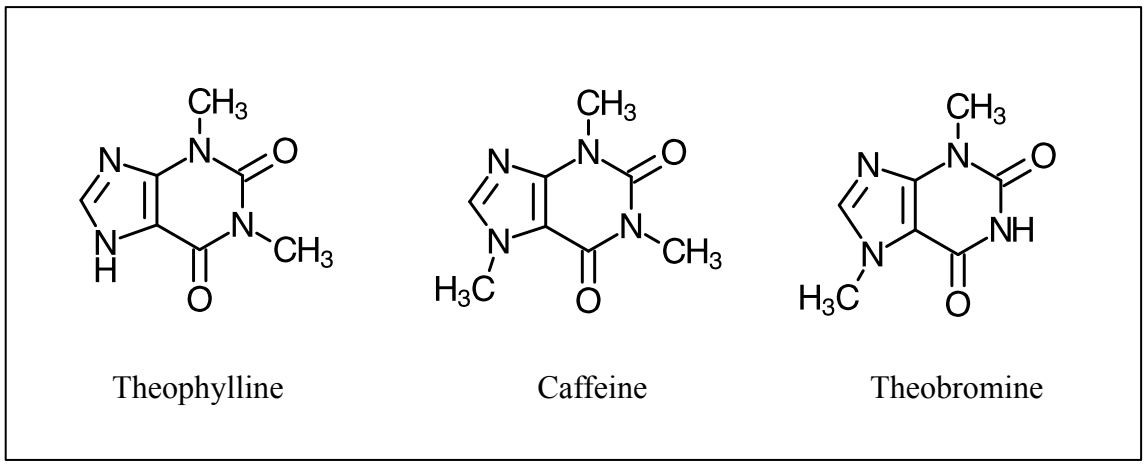


Figure 1.14. *The structures of the methylxanthines theophylline, caffeine and theobromine (adapted from Stavric, 1988).*

1.5 Environmental Factors Affecting the Polyphenol Content of *C. sinensis*

As discussed previously, tea is produced in many countries and as a result the tea plants would be subjected to different environmental and cultivation conditions. Some studies that will be discussed have shown that these factors can affect the levels of polyphenols in the tea leaf.

1.5.1 Seasonal Variation

The purpose of polyphenol production remains unknown, but it has been shown that seasonal differences and the conditions under which the plants grow can alter the concentrations of polyphenols they produce (Lin *et al*., 2003b; Yao *et al*., 2005). An investigation conducted in Australia found that the levels of the principle tea catechins, epigallocatechin gallate (EGCG), epigallocatechin (EGC), catechin (C) and combined total polyphenols varied greatly between the different seasons (Yao *et al*., 2005). In the warmer months (April and May), there were higher levels of all catechins, while in the colder months (July, August and September); the levels were at their lowest (Yao *et al*., 2005). This trend was also observed in Phoenix Mountain Provence, China. Chen *et al*. (2010b) reported that levels of EGC, C, gallocatechin (GC) and simple catechins in the tea leaves were significantly higher in the spring months compared to the colder autumn months. The seasonal effects observed in these studies imply that changes in temperature, humidity and sunlight hours may all contribute to the level of polyphenol produced in tea plants.

1.5.2 Position of Tea Leaves

The position of the leaf on the tea plant has also been shown to affect the levels of polyphenols. The best tea is said be produced using the young leaves (apical bud) or the 'flush', which is made up of the tips of the plant (Wickremasinghe and Perera, 1973; fig 1.15). A study has shown that the young leaves at the apical bud and the two youngest leaves were 2.7 fold more concentrated in polyphenolic compounds than the older leaves (Lin *et al*., 1996). Levels of caffeine and amino acids were also higher in the younger leaves (40-60 %) (Wickremasinghe and Perera, 1973). Although unproven, it is hypothesised that like un-ripened fruits, the higher levels of polyphenols in younger

shoots increase their bitterness, providing protection from predators (Mayer and Harel, 1979).

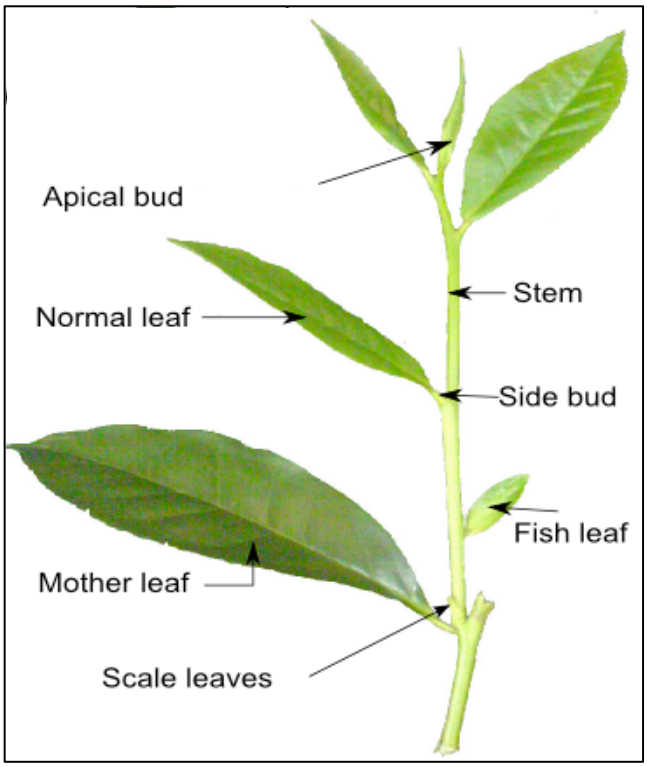


Figure 1.15. *Diagram of the stem and leaves of the Camellia sinensis plant (taken from Tea Plantation Agronomy, 2013).*

1.5.3 Light

It has been hypothesised that flavonoids, as well as other components in leaves, protect the plant against excess UV light by mopping up free radicals produced by UV light and the excess production of nicotinamide adenine dinucleotide phosphate (NADPH) (Edreva, 2005). It is also thought that the aromatic rings of the flavonoids absorb some light of the UV spectra and help dissipate the energy throughout the leaf (Dixon and Paiva, 1995). In support of these theories, it has been reported that exposure of tea plants to low influence short burst UV-B radiation increases the accumulation of catechins in the leaf, particularly EGCG (Zheng *et al*., 2010). Conversely, exposing the leaves to prolonged high influence long burst UV-B radiation suppressed the levels of total catechins (Zheng *et al*., 2010).

Shading has also been reported to affect the levels of polyphenols in tea leaves (Ku *et al*., 2010) It as found that shading tea bushes for 7-21 days, a common practice in Japanese cultivars for the production of sencha, reduced the levels of total catechins but increased the levels of chlorophylls and amino acids (Ku *et al*., 2010).

1.5.4 Soil Composition

Studies looking specifically at the levels of tea polyphenols in relation to soil composition are sparse but one study has shown that soil composition can influence the presence of polyphenols in tea leaves (Ruan *et al*., 2009). Soil provides the plants with many key nutrients and is a vital source of carbon and nitrogen. In a recent study, tea plants were grown hydroponically in a selection of rich nutrient media, which varied in their nitrogen content (Ruan *et al*., 2009). It was found that high concentrations of nitrogen in the growth medium (4.5 mmol/L) reduced the amount of polyphenols in the younger shoots (where they are greatest), increased the production of amino acids and the growth rate of the tea plant (Ruan *et al*., 2009).

1.5.5 Water

Water is another key component vital for plant growth. It is essential for photosynthesis and the subsequent production of primary and secondary metabolites in plants (Bray, 1997). A study conducted by Cheruiyot *et al*. (2007) reported that reducing the soil water content of potted tea plants reduced the growth of new shoots and total polyphenols. Interestingly, some clones in the study were found to be drought-tolerant variants and thus maintained their polyphenol levels with fluctuating soil water content (Cheruiyot *et al*., 2007).

1.5.6 Cultivating Altitude

In many parts of China and India, tea is grown at high altitude (> 500 m above sea level). This is believed to enhance the flavour of teas, in particular, oolong and black teas (The Art of Tea, 2013). An example of this is Huang Zhi Xiang oolong tea, which is grown at high altitude regions of northern China. A study conducted on this tea showed that the plants growing at higher altitude had significantly (p<0.05) higher levels of EGCG (26 % increase), catechin gallate (CG) (22 % increase) and total catechins (TC) (11 % increase) than those grown at lower levels (≤ 300 m above sea

level) (Chen *et al*., 2010b). However, levels of simple catechins such as GC and C increased (2 %) in tea leaves grown at the lower altitude (Chen *et al*., 2010b). It is thought that the combination of higher air temperature, reduced humidity and increased exposure to sunlight at low altitudes accelerated growth of the tea plant (Hilton and Palmer-Jones, 1973), which consequently reduced the amount of secondary metabolites (Chen *et al*., 2010b). This is because fast growing tea plants use higher levels of amino acids to sustain growth, which causes a reduction in the production of secondary metabolites such as polyphenols (Ruan *et al*., 2009).

1.6 The Health Benefits of Tea

Since its discovery, tea has been associated with many health benefits (Sato and Miyata, 2000; Weisburger, 2000; Hara, 2001). The most studied health benefits of tea are its antibacterial (Ikigai *et al*., 1993), antiviral (Nakayama *et al*., 1993), anticancer (Suganuma *et al*., 2010) and cardiovascular effects (Peters *et al*., 2001). Other studies have shown that tea may possess anti-inflammatory (Sueoka *et al*., 2006) and neuro-protective effects (Hong *et al*., 2000). All these health benefits are thought to be associated either directly or indirectly with the presence of polyphenols in tea infusions.

1.6.1 Cardiovascular Disease

There have been several observational studies and meta-analyses of the cardiovascular benefits of both green and black tea (Deka and Vita, 2011). However, confounding variables such as lifestyle and diet make the data difficult to interpret. An early study in 1989 of the consumption of green tea in Japan followed a cohort of 5910 women over 4 years. It was found that the risk of cerebral haemorrhage and stroke was two times higher in women who drank less than 5 cups of green tea a day (Sato *et al*., 1989).

More recently, a study was carried out on a cohort of 40,530 Japanese adults over a period of 11 years. An inverse dose dependant relationship was observed between tea consumption and cardiovascular mortality. This relationship was strongest in women who consumed more than 5 cups per day (Kuriyama *et al*., 2006). Of the different types of cardiovascular disease, the inverse relationship was strongest between tea consumption and stroke mortality (Kuriyama *et al*., 2006). The most recent and largest study to date was conducted by Mineharu *et al*., (2011), which enrolled 76,979

participants to determine the relationship between the consumption of coffee, green tea, black tea, oolong tea and the risk of cardiovascular mortality. A strong inverse relationship was found between people who drank more than 6 cups of green tea a day and their risk of cardiovascular mortality. It was also found that drinking more than one cup of oolong tea a day reduced the risk of cardiovascular disease (Mineharu *et al*., 2010).

Other studies in Asia have also shown a relationship between the consumption of green tea and a risk reduction in cardiovascular disease (Liang *et al*., 2009). However in the US and Europe, the majority of people consume black tea, which is the most consumed tea worldwide (World Green Tea Organisation, 2010). In light of this, a study investigating the trend in black tea consumption and cardiovascular disease was conducted in the Netherlands by de Koning Gans *et al*. (2010). This study followed the black tea consumptions habits of 37,514 participants over 13 years. It was found that drinking more than 6 cups of black tea a day was inversely associated with coronary heart disease (de Koning Gans *et al*., 2010). However, black tea consumption, unlike previous studies with green tea, was not shown to have an inverse relationship with the incidence of stroke (de Koning Gans *et al*., 2010).

Two major risk factors of cardiovascular disease are increased blood pressure and dyslipidaemia. Tea consumption has been shown to have beneficial effects on these risk factors (Liang *et al*., 2009), which is likely to explain the beneficial effects of tea consumption against cardiovascular disease.

It is also worth emphasising that there are several studies that have not been able to show a beneficial relationship between black tea and cardiovascular disease (Brown *et al*., 1993; Hertog *et al*., 1997). In Wales, a study involving 1900 men, showed that men who drank 8 or more cups of tea a day were at higher risk of ischemic heart disease than men who drank less than 2 cups per day (Hertog *et al*., 1997). As previously discussed, the chemical constituents of black tea are very different to that of green tea and this may be a factor in the results found in these studies. However, it must be noted that the authors were aware that most people in the UK consumed black tea with milk and since it is thought that milk binds to tea polyphenols, it may have affected the results (Hertog *et al*., 1997).

1.6.2 Antiviral Properties

Tea is known to have a broad range of antiviral activity. Table 1.4 shows a list of some viruses that have been reported to be sensitive to tea and its polyphenols.

Table 1.4. *A summary of studies investigating the anti-viral properties of tea and tea polyphenols.*

Virus	Tea/tea compoud	Literature cited
Adenovirus	Catechins EGCG and theaflavin	Webber and Piddock, 2003
Bacteriophages	Green and black tea	de Siqueira *et al.,* 2006
Bovine coronavirus (BCV)	EGCG	Clark *et al.,* 1998
Human Rotavirus	EGCG and theaflavin	Mukoyama *et al.,* 1991
Epstein-Barr	EGCG	Chang *et al.,* 2003
Herpes Simplex	Catechins	Savi *et al.*, 2006
HIV	Catechins	Liu *et al.*, 2005
Influenza	Catechins	Nakayama *et al.,* 1993
Enterovirus	EGCG and theaflavin	Mukoyama *et al.,* 1991
Hepatitis B	Pu-erh tea extract	Pei *et al.,* 2011
Human papillomavirus (HPV)	Green tea extract	Tzellos *et al.,* 2010

The reported mechanisms of the antiviral action of tea include the ability of phenolic agents to act as antioxidants (Tzellos *et al*., 2010), inhibit adsorption (Mukoyama *et al*., 1991; Song *et al*., 2005), disrupt cell membranes (Ikigai *et al*., 1993), prevent viral binding and penetration into cells (Imanishi *et al*., 2002) and trigger host defence mechanisms (Friedman, 2007).

EGCG, the most abundant green tea polyphenol, has been of particular interest in antiviral screening. In a study by Mukoyama *et al*. (1991) EGCG was found to have anti-viral activity against rotavirus and enterovirus when added directly to the virus suggesting interference to virus adsorption. This observation was also seen observed theaflavin indicating both green and black tea may be effective antiviral agents against enterovirus and rotavirus (Mukoyama *et al*., 1991).

Another example of the antiviral activity of tea polyphenols was demonstrated in a study by Ho *et al*. (2009). They found that the tea polyphenols EGCG and gallocatechin gallate (GCG) inhibited the activity of enterovirus 71, a virus that can clinically manifest itself in a variety of debilitating diseases such as hand, foot and mouth disease (Shih *et al*., 2004). In combination with reducing the infectivity of the virus, the polyphenols also increased cell viability of infected cells 5-fold and suppressed replication of viral genomic RNA (Ho *et al*., 2009).

Interestingly, the antiviral activity of tea has been exploited as a potential replacement of pomegranate rind as a virucidal agent in the phage amplification assay (de Siqueira *et al*., 2006). In this study, it was found that that a range of commercial black and green teas had the ability to inactivate Felix 01 and P22 bacteriophages. Furthermore, they found that in comparison to pomegranate rind, prepared tea solutions were more stable and demonstrated less batch-to-batch variation in virucidal activity (de Siqueira *et al*., 2006). Another key advantage of using tea in the phage amplification assay is its broad range of activity against several bacteriophages, allowing the potential for extending the assay to other host organisms (de Siqueira *et al*., 2006).

In 2006 the FDA has approved a green tea polyphenol extract incorporated into an ointment (Polyphenon E) for use as an antiviral agent against external anogenital warts (FDA, 2006). A supporting meta-analysis concluded that this preparation was clinically effective in treating the condition, showed low recurrence rates and had a favourable safety and tolerability profile (Tzellos *et al*., 2010).

1.6.3 Anticancer Properties

The anticancer properties of tea have been widely studied and research has shown that tea and some of its polyphenols can induce apoptosis in cancer cell lines of the prostate, colon and breast (Suganuma *et al*., 1999). Furthermore, epidemiological studies have reported that green tea has inhibitory and protective effects against esophageal (Wang *et al*., 2002), stomach (Gao *et al*., 2002), colon (Schut and Yao, 2000) and bladder (Bu-Abbas *et al*., 1994) cancer. Of note, one study conducted by Wang *et al*. (2002) observed that the highest incidence of esophageal cancer in China was in the northern regions of the country, an area where tea is not produced or regularly consumed. These results are further supported by an earlier study by Gao *et al*., 1994, who reported that

daily consumption of tea reduced the incidence of esophageal cancer by up to 50% (Gao *et al*., 1994).

Another anticancer mechanism that the tea polyphenols may exert is their free radical scavenging activity (antioxidant activity) of potential carcinogens such as oxygen radicals that may be involved in DNA damage and the promotion of tumours (Suganuma *et al*., 1999). In combination with this radical scavenging activity, it is thought that tea polyphenols may prevent the bio-activation of carcinogens by interacting with p450 enzymes, as these phase 1 metabolism enzymes make the carcinogen more reactive and consequently more likely to bind to DNA (Suganuma *et al*., 1999). This suggests that tea may have cancer preventative as well as inhibitory properties.

The antioxidant property of tea has been well characterised *in vitro* (Yang *et al*., 2002), but *in vivo*, it was observed that this effect only takes place when the subject is under oxidative stress (Yang *et al*., 2011). For example, a decrease in lipid peroxidation and protein carbonylation was observed after the administration of EGCG in old rats (Kumaran *et al*., 2008). However, in young rats no significant change was found (Kumaran *et al*., 2008). In human studies, the antioxidant effect of tea was demonstrated when smokers treated with green tea over a four week period showed a 50% decrease in the amount of cellular 8-hydroxydeoxyguanosine, a major DNA oxidation product and marker of oxidative stress, compared to the pre-treatment level (Schwartz *et al*., 2005).

1.6.4 Antibacterial Properties

The antibacterial activities of tea have been evident for some time and research is continually revealing new bacterial species that are sensitive to its effects. A summary of a few important bacteria that are known to be sensitive to tea and its polyphenols is shown in table 1.5.

Some tea extracts and compounds have also been shown to inactivate bacterial toxins such as anthrax toxin, botulinum neurotoxins and pertussis toxin (Satoh *et al*., 2002; Deli'Aica *et al*., 2004). Looking at the cumulative evidence, there would appear to be an inverse relationship between antibacterial activity and the degree of tea fermentation suggesting that the catechins rather than their multimeric counterparts are likely to be

pivotal in the antibacterial activity of tea. However, some bacteria like *B. cereus* and *C. tetani* were shown to be sensitive to black teas and the direct components of catechin fermentation (Satoh *et al*., 2002; Friedman *et al*., 2006).

Table 1.5. *A list of some common bacteria sensitive to the effects of tea and its components.*

Bacteria	Tea/tea compoud	Literature cited
Bacillus cereus	Green and black tea	Friedman *et al*., 2006
Campylobacter jejuni	Green and black tea	Hamilton-Miller and Shah, 1999
Escherichia coli	Green tea extract	Chou *et al*., 1999
Mycobacterium tuberculosis	EGCG	Yam *et al*., 1997
Staphylococcus aureus	Green tea and EGCG	Taguri *et al*., 2004
Bacillus anthracis	Green and black tea	Dell'Aica *et al*., 2004
Clostridum tetani	Thearubigin	Satoh *et al*., 2002
Helicobacter pylori	EGCG	Mabe *et al*., 1999

The mechanism by which polyphenols exert their antibacterial activity is not well understood, but one group has demonstrated that catechins may damage the lipid bi-layer of bacteria (Ikigai *et al*., 1993). They found that EGCG and EC (to a lesser extent) caused leakage of fluorescent markers from phosphatidylserine liposomes and aggregation of liposomes in solution. Furthermore, they observed that this activity was reduced when liposomes were negativity charged and remained the same when liposomes had positive or no charge (Ikigai *et al*., 1993). Another study revealed that the presence of EC increased the uptake of EGCG into the lipid bi-layer (Kajiya *et al*., 2002). Interestingly, a separate group observed the same relationship in an anticancer assay. They found that combining EC with EGCG greatly enhanced the effect of apoptosis (7.2 fold) compared to EGCG alone (EC had no direct effect on apoptosis) suggesting synergy between the catechin polyphenols (Suganuma *et al*., 1999).

It has been reported that bacterial DNA gyrase, an enzyme involved in DNA unwinding, may also be possible target for tea polyphenols (Gradisar *et al*., 2007). A study by Gradisar *et al*. (2007) observed that tea catechins inhibit bacterial DNA gyrase by interaction with its ATP binding site. They also found that EGCG had the largest

inhibitory activity followed by ECG and EGC. EC was shown to have no inhibitory activity against bacterial DNA gyrase (Gradisar *et al*., 2007). They concluded that EGCG and ECG had the highest inhibitory activity due to the ability of the galloyl moiety to anchor the benzopyran ring into the active site of the enzyme (Gradisar *et al*., 2007).

In addition to affecting vegetative bacteria, one study observed a reduction in the heat resistance of *B. stearothermophilus* and *C. thermoaceticum* spores when treated with EGCG and a mixture of tea polyphenols (Sakanaka *et al*., 2000). *C. thermoaceticum* was the most vulnerable of the two bacteria. In the presence of 500 μg/mL of green tea and heating the spores at 120 °C for 50 min, a 6 log drop in CFU/mL was observed compared to the control (Sakanaka *et al*., 2000).

Overall, it may be a combination of factors that contribute to antibacterial activity of tea and the studies conducted by Suganuma *et al*. (1999) and Kajiya *et al*. (2002) have demonstrated how important the synergistic effects of the tea 'cocktail' are.

1.7 *Clostridium difficile*

Clostridium difficile (*C. difficile*) is a Gram-positive spore forming bacteria that has been subjected to intense media interest of late (fig. 1.16 and 1.17). Hall and O'Toole first discovered the bacteria in 1935 when they were investigating the intestinal flora of newborn infants. It was identified as a strict anaerobic bacterium with sub terminal, elongate non-bulging spores. Due to these characteristics and the difficulty experienced in culturing the bacteria, it was named *Bacillus difficilis* (Hall and O'Toole, 1935). It was also found to be a highly pathogenic organism that produced soluble exotoxins causing convulsions in young infants (Hall and O'Toole, 1935). The group hypothesised that the symptoms of the infection occurred due to the disruption of commensal flora allowing the proliferation of *Bacillus difficilis* (Hall and O'Toole, 1935).

Bacillus difficilis was later identified as belonging to the *Clostridia* family of bacteria and was subsequently named *Clostridium difficile* (Voth and Ballard, 2005). Since its discovery, reports of *C. difficile* isolation were infrequent until 1978 when it was identified as the primary cause of pseudomembranous colitis (Voth and Ballard, 2005).

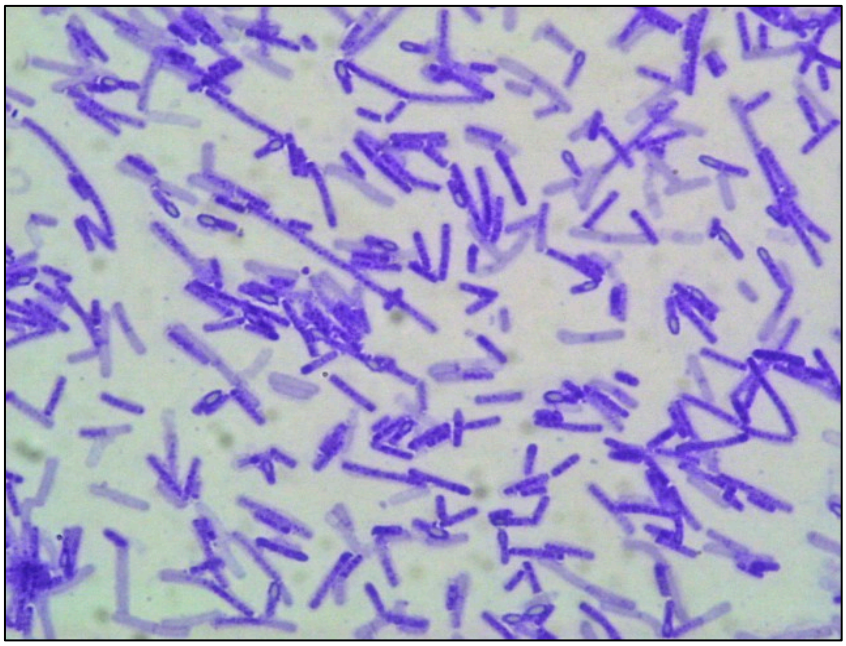


Figure 1.16. *Gram stain of C. difficile under a light microscope.*

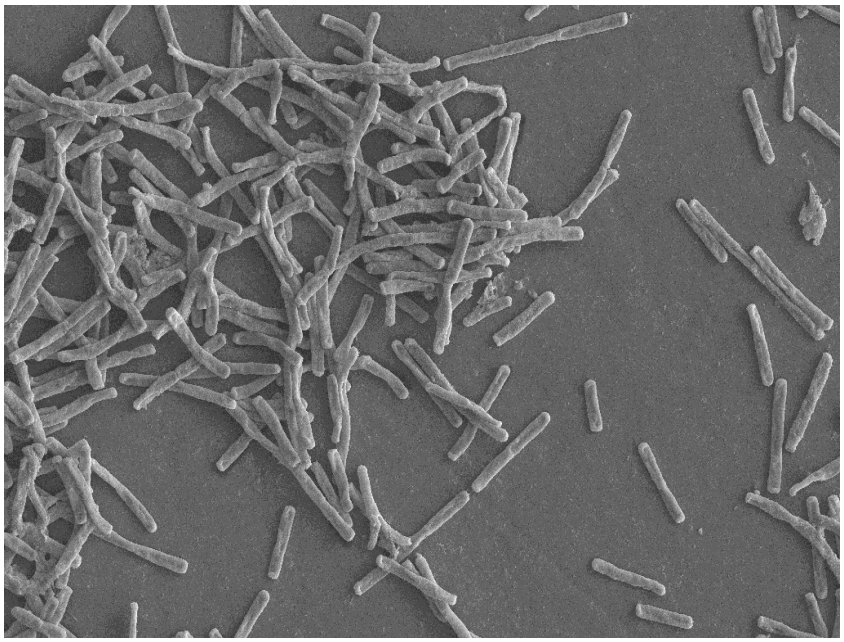


Figure 1.17. *Scanning electron image of C. difficile.*

It was also the primary isolate from the faeces of patients undergoing treatment with the broad-spectrum antibiotic clindamycin (Voth and Ballard, 2005). Antibiotic treatment was later identified as one of the most influential factors for the increase in cases of *C. difficile* infection (CDI) (McFarland, 1998) as it disturbs the natural gut flora, which serves as a major barrier against *C. difficile* proliferation (Lyerly *et al*., 1988).

Transmission of the pathogen is by the faecal-oral route. Most ingested vegetative cells succumb to the acidity of the stomach whilst spores remain unaffected (fig 1.18; A).

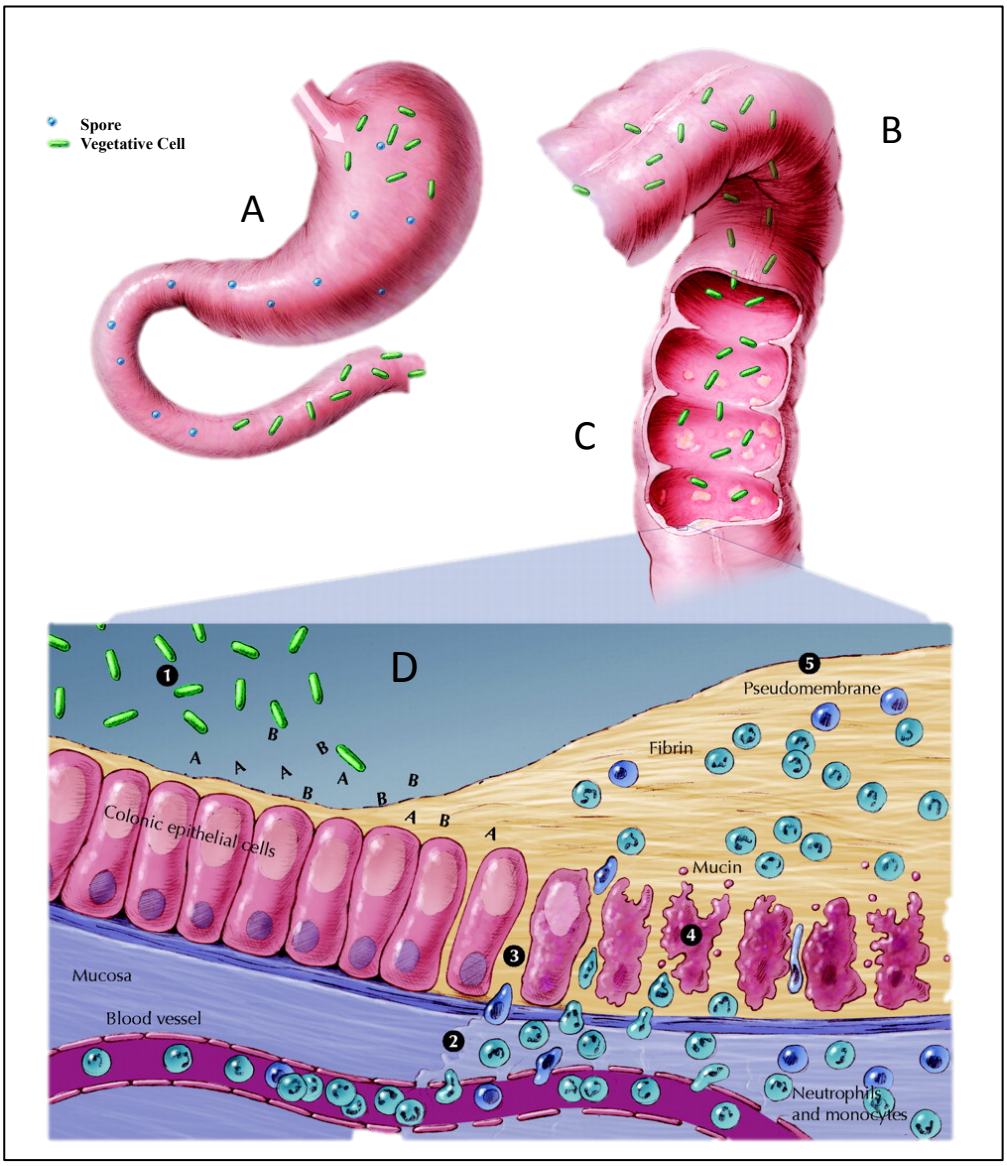


Figure 1.18. *Diagram of C. difficile transmission. Key: A – ingestion of C. difficile spores and vegetative cells; B – proliferation of C. difficile in the colon; C – adherence of C. difficile to the epithelial cells of the colon; D – pathogenesis of C. difficile in the colon; 1 – release of toxins A/B; 2 – neutrophil and monocyte recruitment; 3 – Junction formation between epithelial cells; 4 +5 – connective tissue degradation and pseudmomembrane formation due to local hydrolytic enzymes. Adapted from Poutanen and Simor, 2004).*

The ingested spores start to germinate in the small bowel on exposure to bile acids (fig 1.18; B). Vegetative cells start to multiply in the colon and then adhere to colonic epithelium aided by the gut mucosa (fig 1.18; C). The bacterial cells then release toxins (A and/or B), which cause epithelial apoptosis (fig 1.18; D, legend 4) and pseudomembrane formation (fig 1.18; D, legend 5) leading to the symptoms of CDI (Poutanen and Simor, 2004).

It is estimated that 3 – 15 % of the population are asymptomatic carriers of the pathogen (van Nood *et al*., 2009), however antibiotic use and immune-suppression can lead to proliferation of *C. difficile* in the colon resulting in CDI (Kelly *et al*., 1994). The main symptoms of mild to moderate CDI include diarrhoea and abdominal pains (Kelly *et al*., 1994). However, in more severe cases, diarrhoea is more profuse and may be accompanied by fever, abdominal distension (toxic megacolon) and colonic bleeding (Kelly *et al*., 1994). In 1 to 3 % of cases fulminant colitis can develop, which can cause perforation of the colon and death (Kelly and LaMont, 2008).

C. difficile is particularly prevalent in hospital and care settings as it can form spores, which persist in the environment for many years (Fekety *et al.*, 1981). The spores are very resistant to cleaning and hospital disinfectants and can be passed between patients through contact with hospital staff (Fekety *et al.*, 1981).

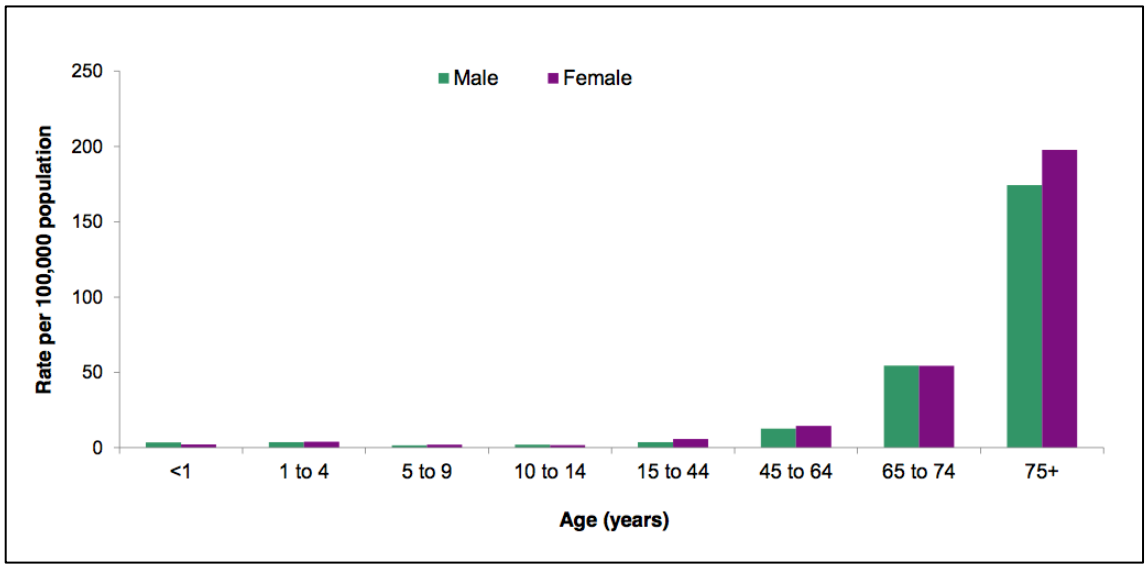


Figure 1.19. *Reported cases of C. difficile in England, Wales and N. Ireland in 2012 by age group (HPAa, 2012).*

A factor that makes *C. difficile* a particularly difficult infection to treat and thus a heavy burden in the health care environment is the high recurrence rate in patients after treatment (~20 %; Kelly *et al*., 2008). The recurrence risk rises dramatically after first (40 %) and second recurrence (60 %) (Kelly *et al*., 2008). It is thought that this increase might be explained by a reduced immune response, which is often seen in the elderly and immuno-compromised patients (Kyne *et al*., 2000). This sub-group of patients are also the most susceptible to the effects of CDI and are more likely to present with severe cases of the disease (Mylonakis *et al*., 2001). In figure 1.19 it can be observed that the majority of patients presenting with CDI in England, Wales and N. Ireland in 2012 were 75 years and above.

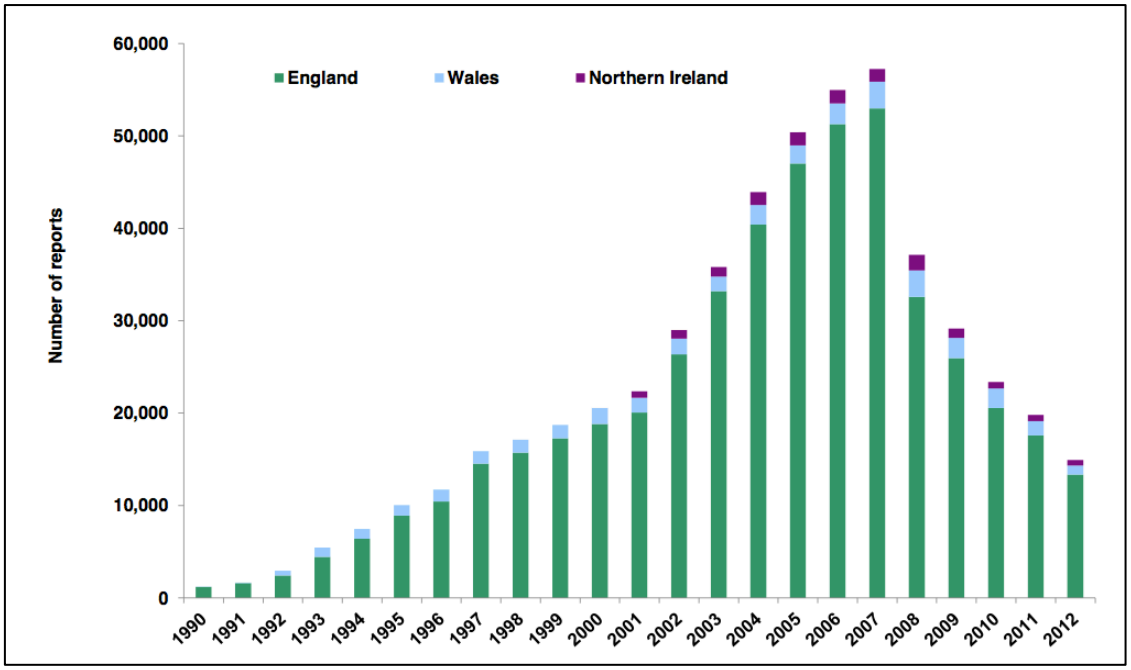


Figure 1.20. *Reported cases of C. difficile in England, Wales and N. Ireland from 1990 to 2012 (HPAa, 2012).*

The prevalence of *C. difficile* has increased dramatically since the emergence of the epidemic 027 ribotype in 2003 (O'Conner *et al*., 2009). This ribotype was the subject of many outbreaks of CDI in hospitals worldwide and was found to produce much higher quantities of toxin compared to other strains leading to increased mortality (Warney *el*

al., 2005). This coincided with the phase of increased of CDI's in the UK. Since reporting started in 1990, cases *C. difficile* in the UK reached an all time high in 2007 (fig. 1.20). Although cases have been steadily falling, *C. difficile* continues to be a significant problem in hospitals in the UK. In 2012, there were 14,910 reports of CDI, comprising of 13,352 cases from England, 969 cases from Wales and 589 cases from N. Ireland (HPAa, 2012).

1.7.1 *C. difficile* Toxins

C. difficile produces two major enterotoxins, TcdA (toxin A) and TcdB (toxin B), which are responsible for exerting the clinical symptoms of *C. difficile* infection (Voth and Ballard, 2005). The toxins are glucosyltransferases, which inactivate GTP-binding proteins in cells such as Rho, Rac, and Cdc42 (Voth and Ballard, 2005). Toxins A and B show a high degree of both sequence and structural homology (Kelly and LaMont, 2008).

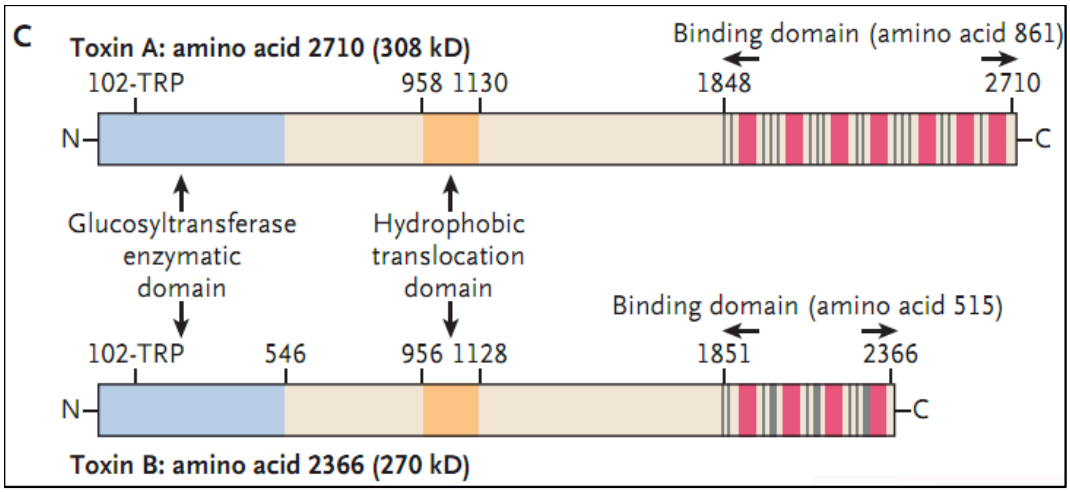


Figure 1.21. *An illustration of the different sites on the C. difficile enterotoxins. (taken from Kelly and LaMont, 2008).*

Each of the toxins have a C-terminal receptor-binding domain and a central hydrophobic domain, believed to mediate the insertion of the toxin into the membrane

of the endosome, which allows the N-terminal glucosyltransferase enzymatic domain to enter the cytosol (Kelly and LaMont, 2008; fig 1.21 and 1.22).

A third toxin, a binary toxin (CDT), is also produced in some strains of *C. difficile*. This binary toxin is an actin-specific ADP-ribosyltransferase that has a high homology to the Clostridium *perfringens* toxin (Popoff et al., 1988). It is not considered as important as toxin A or B in the pathogenicity of *C. difficile*, as 2% of toxin A and B negative strains, which are thought not to cause CDI, produce CDT.

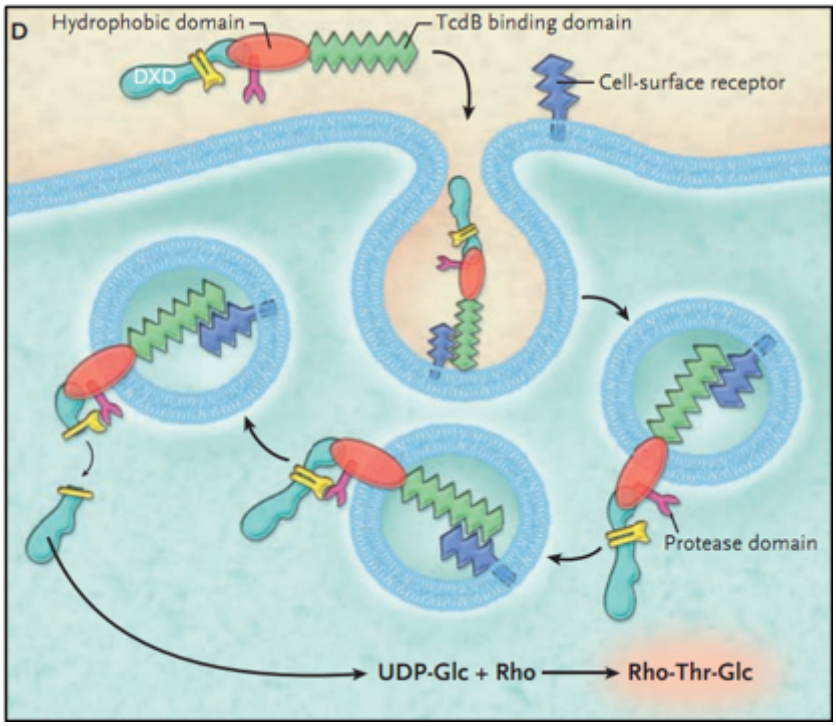


Figure 1.22. *The uptake of C. difficile toxins into the cell. (taken from Kelly and LaMont, 2008).*

Identification of the toxins in a faecal specimen is key to diagnosing CDI due to the high frequency of asymptomatic carriers of *C. difficile* (Johnson *et al*., 1992) and the occurrence of non-toxigenic *C. difficile* (Djuretic *et al*., 1999). As a result, the current gold standard for the clinical diagnosis of CDI is identification of cytotoxin B alongside an isolated culture of *C. difficile* on selective medium (Mylonakis *et al*., 2001).

1.7.2 Treatment of *C. difficile* Infection (CDI)

1.7.2.1 Antibiotics

Metronidazole and vancomycin are the current antibiotics of choice for the treatment of *C. difficile* infections (Martin *et al*., 2013) and have similarly high success rates (93 – 98 %; Olson *et al*., 1994). Vancomycin is a glycopeptide that inhibits cell wall synthesis by binding to peptidoglycan precursors (Barna *et al*., 1984). The FDA approved it for use against CDI in 1986 (FDA, 1986). Administered at the recommended dose of 125 mg four times a day (Martin *et al*., 2013), the concentration of vancomycin in the colon exceeds the MIC concentration for *C. difficile* by 1000 fold due to the limited metabolism and absorption of the drug when orally administered (Tedesco *et al*., 1978). Unlike vancomycin, metronidazole has not been approved by the FDA for CDI but is regularly used for the condition as studies have shown it to be as equally effective (Gerding, 1997). Metronidazole has now overtaken vancomycin as the drug of choice due to the high cost of vancomycin and the emergence of vancomycin resistant enterococci (Dever *et al*., 1995). The British National Formulary (BNF) recommends a dose of 800 mg initially followed by 400mg every 8 hours for the treatment of CDI (Martin *et al*., 2013).

Nevertheless, vancomycin is preferred treatment in pregnant and lactating women and in patients who are on medication that interacts with metronidazole (i.e. warfarin) (Gerding *et al*., 2008). As mentioned, both antibiotics are very effective in treating CDI but relapse of CDI occurs in 15 – 20 % of cases. There has also been an emergence of vancomycin and metronidazole (Pelaez *et al*., 2002) resistant isolates of *C. difficile*, although it is yet to influence the clinical outcome.

More recently, the FDA approved fidaxomicin, a macrocyclic antibiotic for the treatment of CDI (Crook *et al*., 2011). A clinical trial showed that fidaxomicin was non-inferior to vancomycin for the treatment of CDI. Furthermore, patients receiving fidaxomicin had lower rates of recurrent infection (15.4% vs. 25.3%).

1.7.2.2 Probiotics

The term probiotics was first coined in 1974 as it was used to describe ‘organisms and substances which contribute to intestinal balance’ (Fuller, 1989). This definition has

now been revised to 'a live microbial feed supplement, which beneficially affects the host animal by improving its microbial balance' (Fuller, 1991). After administration, they are thought to enhance and replenish the natural gut flora and protect against diseases in the gut (Fuller, 1989). The use of probiotics is supported by the fact that CDI is predominately a consequence of disruption to the gut micro flora caused by antibiotic treatment (Bartlett *et al*., 1978). Probiotics come in a range of different products and are also differentiated by the microorganisms they contain and the dosage. There have been many clinical trials looking at the effects of probiotics in CDI, but the results are conflicting. A few studies suggest that probiotics may be beneficial in primary prevention, secondary prevention and adjuvant therapy of CDI. A large (n=246) double blind randomised controlled trial comparing a probiotic containing *S. boulardii* and placebo in conjunction with vancomycin, was conducted in patients with CDI (McFarland *et al*., 1994). After treatment for four weeks, it was found that treatment with *S. boulardii* greatly reduced relapse of CDI. However, further analysis of the data revealed that this finding was limited to patients treated for recurrent CDI (McFarland *et al*., 1994). A further three trials followed the publishing of this result (Surawicz *et al*., 2000; Wullt *et al*., 2003; Lawrence *et al*., 2005), but only one found a significant benefit of probiotics over placebo in combination with high dose vancomycin in patients with CDI (Surawicz *et al*., 2000). This study failed to demonstrate any benefit when probiotics were administered with metronidazole (1 g/day) or low dose vancomycin (500 mg/day) (Surawicz *et al*., 2000). It should also be noted that all three of these studies were statistically underpowered.

The safety of probiotics has also been brought into question as a study reported that administration of probiotics to patients with severe inflammatory gut conditions might actually increase mortality (Besselink *et al*., 2008). It is clear that further research is needed in this area to confirm the efficacy and safety of probiotic use in the prophylaxis and treatment of CDI.

1.7.2.3 Faecal Infusion

Faecal infusion may be seen as the 'ultimate' probiotic therapy for CDI, in the respect that it involves supplementing the patient's gut flora with the faeces of a healthy donor via a colonoscopy, enema or nasogastric tube (Aas *et al*., 2003). This method was first

described in 1958 as a potential treatment for severe antibiotic associated diarrhoea (Eiseman *et al*., 1958). The reported success rate of this method is approximately 91 % (van Nood *et al*., 2009) and recently, a case series has reported a 100% success rate (n=7) in treating CDI using a low volume faecal transplantation (Silverman *et al*., 2010). Due to a lack of randomised controlled trials and the potential risk of secondary infection, faecal transplants are usually only offered as a treatment after two or more relapses and is still considered as a last line treatment for CDI (van Nood *et al*., 2009).

1.7.3 Tea as a Possible Treatment for *C. difficile* Infection

Tea is known to have antibacterial properties and anecdotal evidence has suggested that *C. difficile* may be inhibited by catechins and green tea extract (Ahn *et al*., 1990, 1991; Lee *et al*., 2006). The central problem with using tea polyphenols in a clinical setting is their extremely poor oral bioavailability. Several studies have shown that the proportion of ingested polyphenols absorbed in the systemic circulation after consumption varies from 0.2 % to 0.9 % for tea catechins to 20 % for quercetin and isoflavones (Hollman *et al*., 1995; Wang *et al*., 1995). Although this may be a problem for treating systemic infections, *C. difficile* resides in the gut, an area where polyphenols have been reported to concentrate after ingestion (Lee *et al*., 1995). One study observed that after 1 h of ingesting radiolabeled EGCG, 72 % was found in the digestive track and the majority was located specifically in the colon (Lee *et al*., 1995).

Specific targeting of the *Clostridia* species in the gut provides another strong argument for using tea as a potential treatment for CDI. A study conducted by Ahn *et al*. (1990) reported that green tea extract selectively inhibited the *Clostrida species* in the gut without any detrimental effect on other commensal flora. In addition, green tea was found to promote the growth of the healthy gut bacteria *lactobacillus* suggesting that tea may be useful for maintaining a healthy gut flora and could possibly be used to prevent diseases of the gut (Axling *et al*., 2012).

Tea is also a long established food product that is regarded as safe for consumption and has not been associated with any serious adverse effects. Furthermore, a recent phase 2 clinical trial reported that high doses of EGCG (4000 mg daily) and a purified green tea extract (Polyphenon E®) was well tolerated by participants. (Shanafelt *et al*., 2010).

In summary, the body of evidence presented suggests that tea may have potential in the treatment or prevention of *C. difficile* infection. The combination of its selectivity against the *Clostridia* species in the gut, its long established safety profile in man and the possibility of selecting tea plants with exceptional polyphenol content makes it an exciting candidate as a potential nutraceutical for treating one of the most prevalent hospital infections.

1.8 Aim and Objectives of this Study

1.8.1 Thesis Aim

The aim of this thesis was to determine if tea infusions have inhibitory activity against clinical isolates of *C. difficile* and how the polyphenol content and physiochemical properties of tea infusions affect their antibacterial activity.

1.8.2 Thesis objectives

The objectives of this thesis were to:

- Determine the catechin content and the chemical properties of a comprehensive range of tea infusions.
- Establish how the catechin content of tea infusions is affected by source, country of origin and degree of fermentation.
- Develop assays to determine the antibacterial activity of tea infusions in line with current international standards for antimicrobial testing.
- Quantify the antibacterial activity of tea infusions against a representative collection of clinical *C. difficile* isolates.
- Compare the catechin content and chemical properties of tea infusions with their antibacterial activity to help determine the antimicrobial components and potential mechanism of action.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2. General Materials and Methods

2.1 Aim and Objectives

The aim of this chapter was to describe all the methods and materials used throughout this thesis. The objectives of this chapter were to:

- Detail the source and preparation methods of all materials used in this study.
- List the bacterial cultures used in this investigation.
- Describe the methods used for identification and determination of pure bacterial cultures.
- Describe the method used to prepare tea infusions.
- Explain the statistical methods used in this study.

2.2 Materials

2.2.1 Chemicals and Reagents

All chemicals and reagents were from Sigma Aldrich Ltd., UK unless otherwise stated in the text.

All solvents were from Thermo Fisher Scientific Ltd., UK, unless otherwise stated in the text.

2.2.2 Biological Culture Media

2.2.2.1 Agars and Broths

Tryptone Soya Agar (TSA), Tryptone Soya Broth (TSB), Brain Heart Infusion agar (BHI), Nutrient Broth (NB), Iso-Sensitest® agar and Mueller-Hinton agar (MH) were from Thermo Fisher Scientific Ltd., UK.

Brucella agar was from Sigma Aldrich Ltd., UK and Lysogeny agar (LB) was purchased from Thermo Fisher Scientific Ltd., UK.

All agars and broths were made according to the manufacturer's instructions and sterilised by autoclaving at 121 °C for 15 min.

2.2.2.2 Diluents

Deionised water (diH_2O) was obtained from an ELGA Purelab Option BP15 dispenser (ELGA labwater, UK). The inorganic content of the water at 25 ^oC was between 1 – 15 MΩ-cm with a total organic carbon content of <30 ppb.

Phosphate buffered saline (PBS) tablets were from Sigma Aldrich Ltd., UK. Once prepared in diH_2O they produced solution containing 0.01 M phosphate buffer at pH 7.4 (composed of 0.0027 M potassium chloride and 0.137 M sodium chloride). Solutions were sterilised by autoclaving at 121 ^oC for 15 min.

2.2.3 Bacterial Cultures

2.2.3.1 *Staphylococcus aureus*

A culture of methicillin sensitive *Staphylococcus aureus* (9518) from the National Collection of Industrial and Marine Bacteria (NCIMB, UK) was kindly provided by Dr Jean-Yves Maillard (School of Pharmacy and Pharmaceutical Sciences, Cardiff University). The culture was stored in 10% glycerol on Microbank^{TM} cyro-protective beads (Pro-Lab Diagnostics Ltd., UK) at -80 ^oC until further use.

2.2.3.2 *Clostridium difficile*

Freezer stocks of *C. difficile* strains (table 2.1a) obtained from the Anaerobic Reference Centre (University Hospital Wales, Cardiff, UK). All cultures were stored in 10% glycerol on Microbank^{TM} cyro-protective beads at -80 ^oC until further use.

***Table 2.1a**. Clinical isolates of C. difficile obtained from the Anaerobic Reference Library, UHW, Cardiff, UK*

Strain No.	PCR ribotype	Isolation year	Location Isolated
R13929	001	2000	Edinburgh
R14057	106	2000	Ealing, London
R14496	020	2000	Liverpool
R14933	002	2000	Liverpool
R14935	014	2000	Liverpool
R14936	070	2000	Paisley
R15213	072	2001	Manchester
R15627	078	2001	Preston
R15632	001	2001	Preston
R15691	023	2001	Dublin
R16631	005	2002	Dumbartonshire
R16632	137	2002	Dumbartonshire
R16633	138	2002	Dumbartonshire
R16762	027	2002	Birmingham
R17015	001	2002	Paisley
R17060	106	2004	Poole
R17083	027	2004	Ayelsbury
R17302	106	2002	Ashford
R17849	078	2003	Preston
R17857	106	2003	Sutton Coldfield
R18080	018	2003	Fife
R18083	001	2003	Sheffield
R18413	002	2003	Kent
R18603	106	2003	Wolverhampton
R18738	005	2003	Glasgow
R19157	001	2004	Preston
R19630	015	2004	Preston
R19885	002	2004	Poole
R20063	106	2004	Birmingham
R20700	045	2005	Whittington, London
R20702	001	2005	Whittington, London
R20703	106	2005	Whittington, London
R20831	001	2005	Birmingham
R20962	106	2005	Kingston
R20976	106	2005	Aberystwyth
R21028	014	2005	Stoke Mandeville
R22487	017	2006	Nottingham
R22753	001	2006	St Georges, London

Table 2.1b. *Clinical isolates of C. difficile obtained from the Anaerobic Reference Library, UHW, Cardiff, UK*

Strain No.	PCR ribotype	Isolation year	Location Isolated
R22814	106	2006	Cheltenham
R22887	174	2006	Truro
R22888	015	2006	St Georges, London
R22897	106	2006	Gloucester
R23121	027	2006	Cardiff
R23800	106	2007	Rhyl
R24565	027	2007	Swansea
R26452	020	2008	Cardiff
R26720	106	2008	Wrexham
R26797	027	2008	Bridgend
R28972	014	2010	Newport
R29035	002	2010	Rhyl
R29039	078	2010	Rhyl
R29078	027	2010	Withybush
R29096	106	2010	Swansea
R31056	027	2011	Cardiff
R31234	106	2011	Bangor
R31312	056	2011	Cardiff
R31315	013	2011	Cardiff
R31350	220	2011	Rhyl
R31382	118	2011	Wrexham
DS1407	015	2007	Southport
DS1447	081	2007	St Marys, London
DS1507	106	2007	Worthing
DS1758	023	2007	Maidstone
DS1859	001	2007	Sunderland
DS2090	124	2008	Manchester
DS2098	023	2008	Cumberland
DS2107	081	2008	Warrington
DS2117	014	2008	Liverpool
DS2205	001	2009	Plymouth
DS2285	106	2009	Leeds
DS2305	174	2009	Truro
DS2315	011	2009	Maidstone
DS2325	027	2009	Tameside
DS2335	027	2009	Stafford
DS2345	027	2009	Hillingdon, London

2.2.4 Tea Samples

Tetley® (C Donnelly, Head of Analytical Services, Global Compliance, Tata Global Beverages) provided samples of dried tea leaves from individual plantations shown in

table 2.2. Commercial teas were obtained from markets in the UK, Japan and India as shown in table 2.3.

Table 2.2. *A list of single plantations teas used in this investigation.*

Tea Code	Batch No.	Cultivation Year	Type	Country of Origin
GP-1	20111753	2006	Green	Kenya
GP-2	20114725	2007	Green	Kenya
GP-3	20116921	2008	Green	Vietnam
GP-4	20111996	2006	Green	Indonesia
GP-5	20114392	2007	Green	Kenya
GP-6	20111891	2006	Green	Sri Lanka
GP-7	20114677	2007	Green	China
GP-8	20113252	2008	Green	Indonesia
GP-9	20109727	2005	Green	Indonesia
GP-10	20114172	2007	Green	China
GP-11	20111754	2006	Green	China
GP-12	20111755	2006	Green	China
GP-13	20114709	2007	Green	India
GP-14	20112242	2006	Green	China
GP-15	20113252	2007	Green	Indonesia
BP-1	20114171	2007	Black	Papa New Guinea
BP-2	20161612	2008	Black	India
BP-3	20112177	2006	Black	India
BP-4	20116985	2008	Black	Tanzania
BP-5	20111193	2006	Black	Rwanda
BP-6	20112729	2007	Black	Malawi
BP-7	20116165	2008	Black	India
BP-8	20112733	2007	Black	Zimbabwe
BP-9	20117048	2008	Black	India
BP-10	20116934	2008	Black	Argentina
BP-11	20114294	2007	Black	India
BP-12	20111180	2006	Black	Kenya
BP-13	20113667	2007	Black	India
BP-14	20117049	2008	Black	Sri Lanka
BP-15	20116555	2008	Black	Indonesia
BP-16	20111663	2006	Black	Vietnam
BP-17	20111440	2006	Black	China
BP-18	20111454	2006	Black	China

Table 2.3. *A list of commercially sourced teas used in this investigation ('-' = information unknown).*

Tea Code	Batch No.	Type	Country of Origin	Brand
GC-1	L225498	Green	-	Tesco Green Tea
GC-2	L213898	Green	Vietnam	Tesco Finest Green Tea
GC-3	A15553996	Green	-	Twinning's Green Tea
GC-4	442235	Green	China	Asda Green Tea
GC-5	L2164	Green	-	Pukka Green Tea
GC-6	G7073	Green	-	Clipper Green Tea
GC-7	XJT 500	Green	China	Sea Dike Jasmine Tea
GC-8	-	Green	Japan	Yamakaen Matcha Powder
GC-9	-	Green	Japan	Hojo Japanese Sencha
GC-10	-	Green	China	Dragon Well Tea
GC-11	-	Green	Japan	Catagreen Matcha Tea
GC-12	442200	Green	-	Asda Jasmine Tea
GC-13	XGT 200	Green	China	Sea Dike Green Tea
GC-14	13216362	Green	China	Whittard Sencha
GC-15	-	Green	Japan	Mt. Fuji Region Sencha
GC-16	-	Green	Japan	Kyoto Region Sencha
GC-17	502186	Green	China	Whittard Jasmine Tea
GC-18	23403310118	Green	China	Camel Gunpowder
GC-19	14165651	Green	China	Whittard White Tea
GC-20	-	Green	China	Chinese Jasmine Pearl Tea
GC-21	-	Green	Taiwan	Pouchong Oolong Tea
GC-22	-	Green	Taiwan	Alishan Oolong Tea
GC-23	-	Green	Taiwan	Lishan Oolong Tea
BC-1	2114NBO	Black	-	Taylors Yorkshire Tea
BC-2	-	Black	Kenya	Morgan's Welsh Brew
BC-3	A15525830	Black	-	Twinning's Afternoon Tea
BC-4	-	Black	China	Chinese Red Oolong Tea
BC-5	A15510750	Black	-	Twinning's Darjeeling
BC-6	L2261	Black	-	Tetley Best of Both
BC-7	A15510751	Black	-	Twinning's English Breakfast
BC-8	442214	Black	-	Asda Loose Leaf Black Tea
BC-9	-	Black	India	San-Cha Blue Mountain
BC-10	502322	Black	*	Whittard English Breakfast
BC-11	-	Black	India	San-Cha Rainforest Black Tea
BC-12	-	Black	India	San-Cha Darjeeling Tea
BC-13	-	Black	China	Tieguanyin Tea
BC-14	AT220	Black	China	Bai Maohuo Tea
BC-15	-	Black	China	Whittard Puh Er Tea
BC-16	21853J132	Black	-	Pg Tips Strong Black Tea

2.3 Methods

2.3.1 Tea Infusion Preparation

Tea infusions were prepared by grinding 2.5 g of dried tea leaf into a course powder (>1 mm) using a pestle and mortar. The tea powder was then added to 200 mL of boiling diH_2O (100 °C) in a 250 mL glass beaker (Fisher Scientific Ltd., UK) and infused for 5 min. After the infusion period, the solution was filtered through Whatman® grade 1 paper using vacuum filtration to remove any solids. The supernatant was used immediately for any subsequent experiments. Although green teas can be brewed at temperature ranging from 70 – 100 °C, this extraction temperature was chosen to ensure a fair extraction profile between all teas and thus make all the comparisons between teas possible, as its been reported that catechin extraction in water is temperature dependant (Labbé *et al*., 2006).

2.3.2 Streak Plate Method for Determining Culture Purity

The streak plate method was used to confirm the purity of bacterial stocks and as a prerequisite for the Gram stain analysis. The objective of the technique was to spread a concentrated bacterial colony across an agar plate in a series of steps in order to obtain isolated colony forming units (CFU's) on the last streak. Between each step, a fresh inoculation loop (10 µL) was used to streak the colonies. After the procedure has been completed, the plate was incubated at 37 °C for 24 h and the colonies were inspected (fig 2.1).

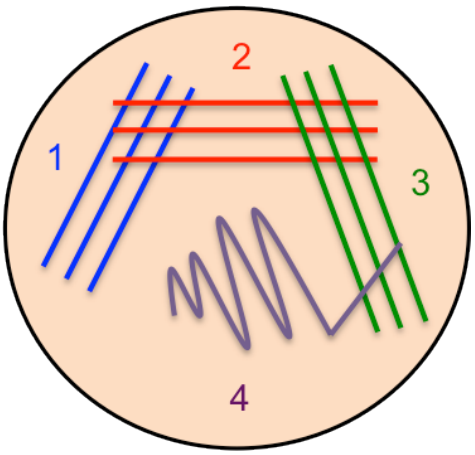


Figure 2.1. *The streak plate method showing 4 steps of streaking to isolate single colonies.*

2.3.3 Gram Staining of Bacterial Cells

An individual CFU was selected from a streaked culture (section 2.3.2) on an agar plate using a sterile inoculation loop (10 μL). The loop was spread on a microscope slide with sterile diH_20 to create a thin film. The slide was left to air dry and the bacteria were heat fixed to the slide by passing it rapidly through a Bunsen flame. Crystal violet reagent (Pro-Lab Diagnostics Ltd., UK) was used to stain the bacteria for 1 min, followed by application of Gram's iodine (Pro-Lab Diagnostics, UK) for 1 min. The slide was then rinsed with 70 %(v/v) ethanol for 15 sec and then finally, the red counterstain safranin (Pro-Lab Diagnostics, UK) was applied for 1 min. In between each staining step the slide was rinsed with distilled water. Slides were viewed under a microscope under oil immersion at a total magnification of 1000× (Olympus BX-50, Olympus, Japan).

2.4 Statistical Analysis

The results presented in this study are the mean of three results, unless otherwise stated. All statistics were performed using IBM® SPSS® Statistics version 20.

2.4.1 Independent Samples t-test

To determine the statistical difference in the means between two groups, the parametric independent students t-test was used if the following assumptions were met (Cohen, 1988):

1) The observations of the data were independent from each other.
2) The results follow normal distribution and pass normality tests.
3) The data sets to be compared show homogeneity of variance.

To assess normality, a histogram plot of the data was inspected to determine if a normal bell shaped curve was observed with minimal kurtosis and skewedness. The Q-Q plots and de-trended Q-Q plots were also reviewed in conjunction with the results of the Kolmogorov-Smirnov and Shapiro-Wilk tests for normality.

Levine's test of the equality of variances was conducted to determine if the data sets showed homogeneity of variance.

If any of the assumptions were violated, an appropriate transformation of the data will be attempted (Log_{10}, square root, etc.) to normalise the data or the equivalent non-parametric test will be used (e.g. Mann-Whitney U).

To determine the effect size between the two groups, or the magnitude of difference, the eta-squared value (η^2) was determined by the following equation:

$$\eta^2 = \frac{t^2}{t^2 + (n1 + n2 - 2)}$$

Where t = t value from t-test, $n1$ = sample size of group 1 and $n2$ = sample size of group 2. To interpret the η^2 value, the guidelines set out by Cohen (1988) shown in table 2.4 were implemented.

Table 2.4. *Interpretation of effect size of η^2 between two statistically different groups (Cohen, 1988)*

Effect size	**η^2**
Small	0.10 - 0.29
Moderate	0.30 - 0.49
Large	=> 0.50

2.4.2 Mann-Whitney U Test

If the assumptions were not met to perform the independent samples t-test, then the non-parametric Mann-Whitney U test was used to compare the difference between the medians of two groups.

If a significant difference was found (p =<0.05) then the effect size (r) will be calculated using the following equation:

$$r = \frac{z}{\sqrt{n}}$$

Where r = effect size, z = z value generated from test and n = total sample size. Effect size was interpreted using Cohen's (1988) criteria as shown in table 2.5.

Table 2.5 *Interpretation of effect size of r between two statistically different groups.*

Effect size	***r***
Small	0.10 - 0.29
Moderate	0.30 - 0.49
Large	=> 0.50

2.4.3 One-way Analysis of Variance (ANOVA)

The parametric one-way ANOVA test was used to determine if there was significant difference in the means between two or more groups of data if the assumptions described in section 2.4.1 were met.

If a significant difference between groups was found, Tukey's honestly significant difference test was used to determine which groups were significantly different from each other.

2.4.4 Kruskal Wallis Test

The Kruskal Wallis test is the non-parametric equivalent to the one-way ANOVA and was used if the assumptions described in section 2.4.1 were not met. It determined if the medians of each group were significantly different.

If a significant difference was found, the Bonferroni-Dunn's multiple comparisons test was used to determine which groups are significantly different.

2.4.5 Pearson's Correlation

Correlation analysis was used to determine the strength and direction of a linear relationship between two variables. Pearson's correlation, or parametric correlation, was used if the following assumptions were met (Cohen, 1988):

1) The relationship between two or more data sets is linear.
2) Observations in the data sets were independent.
3) Each data set in the analysis followed normal distribution.
4) The data sets showed homoscedasticity.

The strength of the relationship between variables was determined by the guidance set out by Cohen (1988) in table 2.5.

The coefficient of determination (r^2) was calculated by squaring the Pearson product-moment correlation coefficient (r) obtained between two variables.

2.4.6 Spearman's Correlation

If the data sets to be analysed did not follow normal distribution but do meet the other assumptions described in section 2.4.5, then Spearman's correlation was used to determine the strength and direction of the linear relationship between variables. Results were expressed as Spearman's rho.

CHAPTER 3

CHEMICAL ANALYSIS OF TEA INFUSIONS

3. Chemical Analysis of Tea Infusions

3.1 Chapter Introduction

Dried tea leaves contain a complex mixture of polar and non-polar compounds that can be extracted into solution using a variety of solvents. When investigating the polar contents of tea, mid to high polarity solvents such as acetone (Sasaki *et al.*, 2004), methanol (Anesini *et al.*, 2008), ethanol (Nishimura *et al.*, 2007) and water (Lin *et al.*, 2003) are normally used. However, very few researchers choose to use an extraction method, which is representative of the daily tea drinking habits of the general population, primarily because they aim to extract high yields of flavonoids and other polyphenols. In this thesis, the primary focus is to investigate the presence of polyphenols, particularly catechins, in aqueous extracts, which are comparable to everyday tea infusions. This can be achieved by using two different types of investigation; quick screening colourimetric assays and thorough quantitative analysis. The advantage of using quick screening assays is that they can give an estimation of the chemical components in tea infusions relativity quickly and help to differentiate unique teas from a large sample size.

3.1.1 Colourimetric Assays

The most widely used quick screening assays that have been reported in multiple papers are microtitre plate assays for determining total polyphenol content (TPC) (Lin *et al*., 2003b; Yao *et al*., 2005; Ku *et al*., 2010) and total flavonoid content (TFC) (Chang *et al*., 2002; Cheruiyot *et al*., 2007; Kim *et al*., 2010). They are high throughput screens that estimate the total concentration of phenolic compounds and more specifically, flavonoids.

3.1.1.1 Total Polyphenol Content (TPC) Assay

The TPC assay is a rapid method for estimating the total concentration of polyphenols in a solution based on a standard phenol derivative, most commonly gallic acid. The reagent employed is a mixture of tungsten and molybdenum oxides, which is reduced by polyphenols and phenolic acids in the tea solution to produce an intense blue colour (Singleton and Rossi, 1965). The intensity of the colour produced can be measured using a spectrophotometer at 750 nm and compared against a standard to estimate the

TPC. In this investigation, gallic acid was used as a standard as it is present in both green and black tea.

3.1.1.2 Total Flavonoid Content (TFC) Assay

Like the TPC assay, the TFC assay is another fast colourimetric method of estimating the concentration of compounds in solution, in this case flavonoids. In contrast to the TPC assay, this experiment specifically identifies the flavonoid polyphenols, which are claimed to be the most biologically active components in tea (Hamilton-Miller, 1995). In this assay, a combination of aluminium chloride, sodium nitrate and sodium hydroxide produces an intense red/orange colour when in contact with the flavonoids. This is due to acid stable complexes formed between aluminium chloride and the C-4 ketone group in flavonols or the C-3 or C-5 hydroxyl group in flavanols or catechins. Complexes are also formed with the ortho and dihydroxyl groups on the A or B ring of flavonoids (Mabry *et al*., 1970). Catechin is used as a standard as it is common to both black and green teas and contains both C-3 and C-5 hydroxyl groups (Ch. 1, fig 1.5).

3.1.1.3 Ferric Reducing Antioxidant Power (FRAP) Assay

Another microtitre assay routinely used is the ferric reducing antioxidant power (FRAP) assay. Antioxidants are described as substances that help prevent oxidation of other molecules, such as oxidative damage to cells and DNA (Sies, 1991). Since the antioxidant power of tea components is associated with the many health benefits of tea (Borse *et al*., 2007), it is a useful assay to quickly determine if a tea is likely to possess above average biological activity and merit further investigation. Again, this method is routinely reported in the literature and is often coincided with TPC and TFC measurements (Chang *et al*., 2002; Lin *et al*., 2003b; Yao *et al*., 2005; Ku *et al*., 2010).

The FRAP assay is one of a few assays that can be used to estimate the total antioxidant power of a solution. The standard of choice for this assay is the vitamin E derivative Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). In contact with the buffered FRAP solution, compounds that have antioxidant properties, turn the solution from straw yellow to a light blue colour due to the reduction of Fe(III)-2,4,6-Tri(2-pyridyl)-s- triazine complex to Fe(II) (Benzie and Strain, 1996). This can be measured

using a spectrophotometer at 595 nm and compared to the intensity of standard solutions of Trolox®.

3.1.1.4 Hydrogen peroxide production

Although tea polyphenols can act as antioxidants, they have been shown to act as pro-oxidants by generating H_2O_2 (Nakayama *et al*., 2002) in neutral (pH 7) and in weak alkaline solutions (pH 7.5-8.5), in the presence of heavy metals and oxygen (Dai *et al*., 2010). This can be measured using the ferrous oxidation-xylenol orange (FOX) assay (Jiang *et al*., 1990). As a result, H_2O_2 has been implicated in contributing to the antimicrobial activity of tea (Arkawa *et al*., 2004). The proposed mechanism of polyphenol autoxidation is shown in figure 3.1.

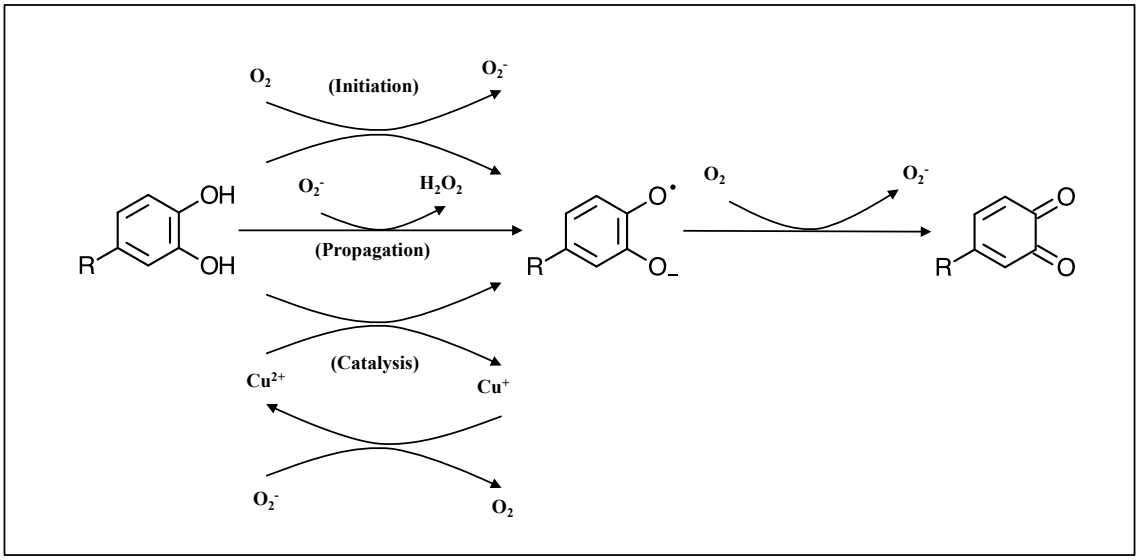


Figure 3.1. *Proposed mechanism of the autoxidation of flavonoids. Adapted from Akagawa et al. (2003).*

In contrast to the FRAP assay, Fe(II) is oxidised to Fe(III) by hydroperoxides in acidic conditions in the FOX assays. When using the aqueous assay (FOX-1), hydroperoxides produce a peroxide radical by reacting with sorbitol initiating oxidation of Fe(II). However, in the lipid compatible assay (FOX-2), hydroperoxides react directly with Fe(II) (Jiang *et al*., 1990). The subsequent Fe(III)-xylenol orange complex in both assays produces an intense purple colour that can be measured by spectrophotometry.

3.1.2 Quantitative Analysis - High Performance Liquid Chromatography (HPLC)

Ultimately, to characterise and determine the concentration of specific compounds in tea, techniques such as high performance liquid chromatography (HPLC) need to be employed. Quantitative analysis of the polyphenols in tea samples has traditionally been performed using HPLC analysis. It has proven to be a very effective technique and due to the vast number of different compounds in crude tea samples, many different methods have been published, each one tailored to identify one or multiple components**.** However, due to the complex stereochemistry of the catechin polyphenols, the methods used in the current literature are very diverse, ranging from isocratic two-phase solvent systems to complex gradient elution systems with acidic modifiers (Lee *et al*., 1995; Yao *et al*., 2005; Kim *et al*., 2010). The most sophisticated liquid separation method to date was conducted by Nováková *et al*., (2010), using ultra high performance liquid chromatography. In this study, the simultaneous separation of twenty-nine common phenolic compounds in tea was achieved.

3.2 Chapter Aim and Objectives

The main aim of this chapter was to determine if selected tea samples differ significantly in their polyphenol content and anti/pro-oxidant properties. The experimental objectives were to:

1. Determine the polyphenol content of aqueous tea extracts.

2. Assess the pro and anti-oxidant properties of aqueous tea extracts.

3. Compare the polyphenol content and anti/pro-oxidant properties of green, black, single plantation and commercial tea infusions.

4. Quantify the levels of EGCG, EC, EGC, ECG, C, GCG and caffeine found in selected tea infusions and determine if there are significant differences between different types of tea.

3.3 Methods

3.3.1 Colourimetric Assays

For each colourimetric assay, aqueous tea extracts of single plantation and commercially sourced teas (Ch. 2, table 2.2 and 2.3) were prepared using the method described in chapter 2 (section 2.3.1). Each measurement for individual teas was performed in triplicate (technical repeat; average value recorded) and the assays were repeated three times (experimental repeat; n = 3).

3.3.1.1 Determining the Total Polyphenol Content (TPC) of Aqueous Tea Infusions

A microtitre plate method for measuring TPC was adapted from Ku *et al*. (2010), as follows. Each infusion was diluted 10-fold in diH_2O and then 10 µL was pipetted into separate wells of a 96 well microtitre plate followed by 100 µL of 0.2 M Folin-Ciocalteu reagent. The solutions were mixed and left to develop for 5 min. After this time, 80 µL of saturated sodium carbonate solution was added to each well and the solutions were mixed on a shaking platform (Stuart Gyro-rocker, Bibby Scientific Ltd., UK) for 1 h at room temperature. The absorbance of each solution was then measured using a microtitre plate spectrophotometer (Anthos htII Plate-reader, Richmond Scientific Ltd.) at a wavelength of 750 nm. Using linear regression the TPC of each tea was calculated by a linear equation generated by constructing a standard curve with gallic acid. The standard solution concentrations of gallic acid ranged from 15 to 500 µg/mL in diH_2O. The base line absorbance was set using a negative control (diH_2O) solution. Results were expressed in milligrams of gallic acid equivalent (GAE) per gram of tea. The difference in TPC between types of tea (green and black), source (single plantation and commercially sourced) and origin was conducted using the appropriate statistical test as described in chapter 2 (section 2.4).

3.3.1.2 Determining the Total Flavonoid Content (TFC) of Aqueous Tea Infusions

TFC was determined for each tea infusion and standard using the method described by Yoo *et al*. (2008). Of each tea infusion, 20 µL was pipetted into separate wells of a 96 well microtitre plate followed by 40 µL of diH_2O and 6 µL of 5 % (w/v) sodium nitrate solution. The solution was mixed on a shaking platform and after 5 min, 12 µL of 10 % (w/v) $AlCl_3$ was added. After 6 min, 40 µL of 1 M NaOH was added followed by 42 µL

of diH_2O. The absorbance of each solution was measured in using a microtitre plate spectrophotometer at a wavelength of 515 nm. The TFC of each tea was calculated by the linear equation generated by constructing a standard curve with (+)-catechin. The standard solution concentrations of (+)-catechin ranged from 15 to 500 µg/mL in diH_2O. The base line absorbance was set using a negative control solution (diH_2O). Results were expressed in milligrams of catechin equivalent (CE) per gram of tea. The difference in TFC between types of tea (green and black), source (single plantation and commercially sourced) and origin was conducted using the appropriate statistical test as described in chapter 2 (section 2.4).

3.3.1.3 Determining the Ferric Reducing/Antioxidant Power (FRAP) of Aqueous Tea Infusions

3.3.1.3.1 Preparation of FRAP Reagent

The FRAP reagent was prepared by adding 2.5 mL of a 10 mmol/L 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) solution to 40 mmol/L hydrochloric acid (HCl). 2.5 mL of 20 mmol/L $FeCl_3 \bullet 6H_2O$ and 25 mL of 0.3 mol/L acetate buffer at pH 3.6 (Ku *et al*., 2010). The reagent was freshly prepared on the day and warmed to 37 °C in an incubator.

3.3.1.3.2 Determining the Antioxidant Power of Aqueous Tea Infusions

The antioxidant capacity of each tea infusion and standard was estimated according to the procedure described by Benzie and Strain, (1996), with alterations explained by Ku *et al*. (2010). Each tea infusion was diluted 10-fold in diH_2O and 10 µL was pipetted into separate wells on a 96 well microtitre plate followed by 300 µL of FRAP reagent. The solutions were mixed on a shaking platform and left to develop for 6 min. The absorbance of the solutions was then measured at 595 nm using a microtitre plate spectrophotometer. The antioxidant power of each tea was calculated by the equation linear equation generated by constructing a standard curve with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®). The standard concentrations of Trolox® ranged from 0.2 mM to 2 mM in diH_2O. The base line absorbance was set using a negative control solution (diH_2O). Results were expressed in millimols of Trolox® equivalent concentration (TE) per gram of tea. The difference in antioxidant

power between types of tea (green and black), source (single plantation and commercially sourced) and origin was conducted using the appropriate statistical test as described in chapter 2 (section 2.4).

3.3.1.4 Determining the H_2O_2 production of Aqueous Tea Infusions using the FOX assay

3.3.1.4.1 Preparation of working reagent

The working reagent (WR) was prepared from 1 volume of reagent A (2.5 mM ammonium ferrous (II) sulphate in 1 mL 2.5 M H_2SO_4) and 100 volumes of reagent B (100 mM sorbitol, 125 µM xylenol orange in 50 mL diH_2O). The WR was prepared on the day of use.

3.3.1.4.2 Determining the H_2O_2 production of Aqueous Tea Infusions

The H_2O_2 production of tea infusions was determined using the method developed by Jiang *et al*. (1990), as described by the Pierce™ quantitative peroxide assay kit instructions. Each tea infusion was diluted 10-fold in PBS to buffer the solution at pH 7.4. Of this solution, 20 µL was pipetted into separate wells on a 96 well microtitre plate followed by 200 µL of WR. The solutions were mixed on a shaking platform and left to develop for 20 min. The absorbance of the solutions was then measured using a microtitre plate spectrophotometer at 600 nm. The H_2O_2 production of each tea was calculated by the linear equation generated by constructing a standard curve with H_2O_2 standards. The standard concentrations of H_2O_2 ranged from 1 µM to 1 mM in diH_2O. The base line absorbance was set using a negative control solution (diH_2O). Results were expressed in micromoles of H_2O_2 produced per gram of tea. The difference in H_2O_2 production between green and black tea was conducted using the appropriate statistical test as described in chapter 2 (2.4).

3.3.2 HPLC Analysis of Aqueous Tea Extracts

3.3.2.1 Preparation of tea extracts and standards

Tea infusions were prepared fresh on the day as described in chapter 2 (section 2.2.1) and were diluted 10-fold in diH_2O. Standards of gallic acid (GA), gallocatechin gallate

(GCG), EC, EGCG, EGC, ECG, and caffeine (concentration ranges between 1.5 µg/mL and 0.15 µg/mL) were dissolved in 100% HPLC grade methanol.

3.3.2.2 HPLC Analysis

A Waters 2695 liquid chromatograph system comprising of a vacuum degasser, quaternary pump, auto- sampler, thermostatted column compartment and photodiode array detector was used. The column used was a C18 reversed phase Kingsorb 5 mm (150 x 4.6 mm) (Phenomenex Ltd., UK) with a Kingsorb 5 mm C18 (30 x 4.6 mm) guard column. Mobile phases consisted of 0.1% ortho-phosphoric acid in ultra-purified water (v/v) (mobile phase A) and 0.1 %(v/v) ortho-phosphoric acid in methanol (mobile phase B). The gradient elution was performed as shown in table 3.1.

Table 3.1 *Composition of the mobile phase in the gradient elution system between 0 and 30 min. Mobile phase A consists of 0.1% ortho-phosphoric acid in ultra-purified water and mobile phase B 0.1 %(v/v) ortho-phosphoric acid in methanol (eluent B). All gradients were linear.*

	Mobile phase (%)	
Time (min)	**A**	**B**
0 - 5	80	20
5 - 7	76	24
7 - 10	76	24
10 - 20	60	40
20 - 25	50	50
25 - 25.5	80	20
25.5 - 30	80	20

Post-run time was 5 min. Elution was performed at a solvent flow rate of 1 mL/min. Detection was accomplished with a diode array detector and chromatograms were recorded at 210 and 280 nm. The column was maintained at 30 °C. The sample injection volume was 15 µL. Peaks were identified by comparing their retention times

and UV spectra in the 200 to 400 nm range with authentic standards. A calibration curve for each standard was constructed and the corresponding peaks in the tea extracts were determined using linear regression.

3.4 Results and Discussion

3.4.1 The Polyphenol and Flavonoid Content of Aqueous Green and Black Tea Infusions

3.4.1.1 The TPC of Aqueous Tea Infusions

The TPC of aqueous infusions was determined by constructing a concentration versus optical density curve using gallic acid as a control. As shown in figure 3.2, the relationship between concentration and absorbance was linear and therefore the TPC of each tea infusion could be estimated using the derived equation.

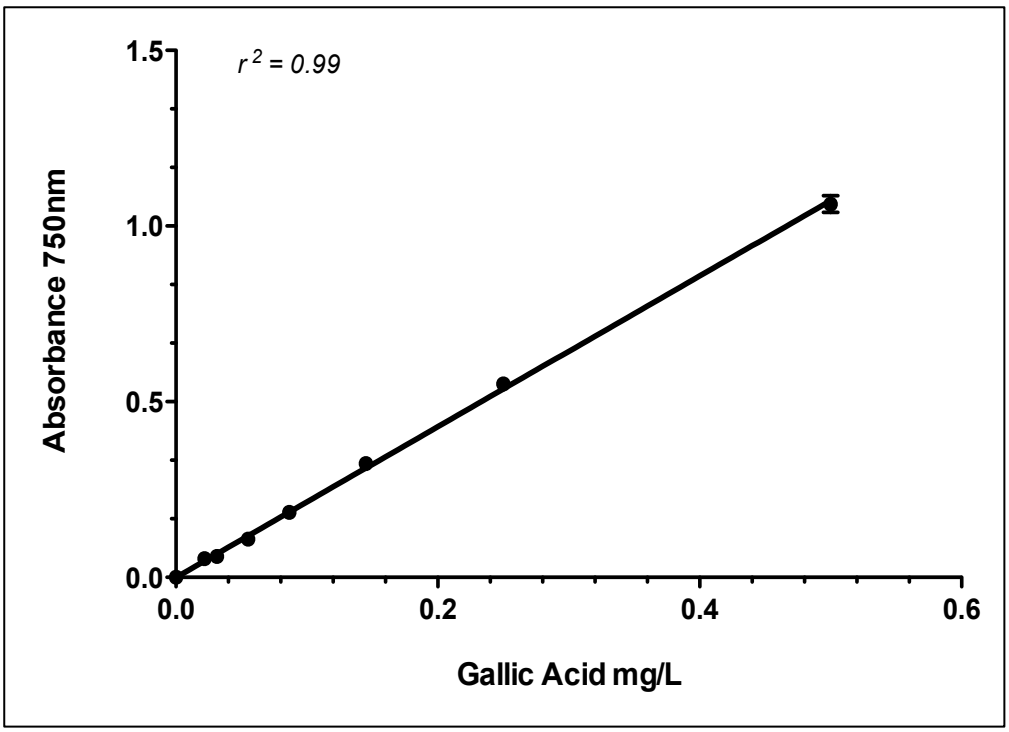


Figure 3.2. *Calibration curve of absorbance at 750 nm versus gallic acid concentration (error bars = SE; n = 3).*

It was found that the mean TPC of the green teas examined in this study was 77.5 ± 4.7 mg/g (± = *SE; n = 38*) whilst the mean for the black tea was lower at 54.4 ± 4.3 mg/g (± = *SE; n = 34*). The range of TPC within green teas (23.9 – 144.9 mg/g; fig 3.3) and black teas (6.0 – 100.7 mg/g; fig 3.4) was considerable.

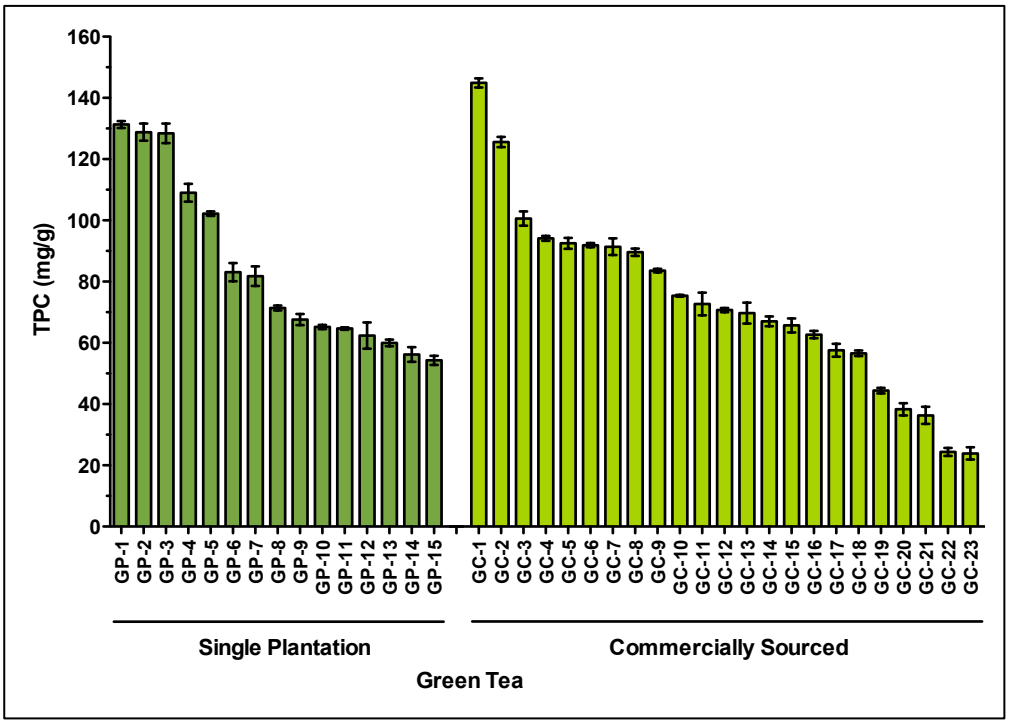


Figure 3.3 *The TPC (GAE) of 38 green tea samples (error bars = SE; n = 3).*

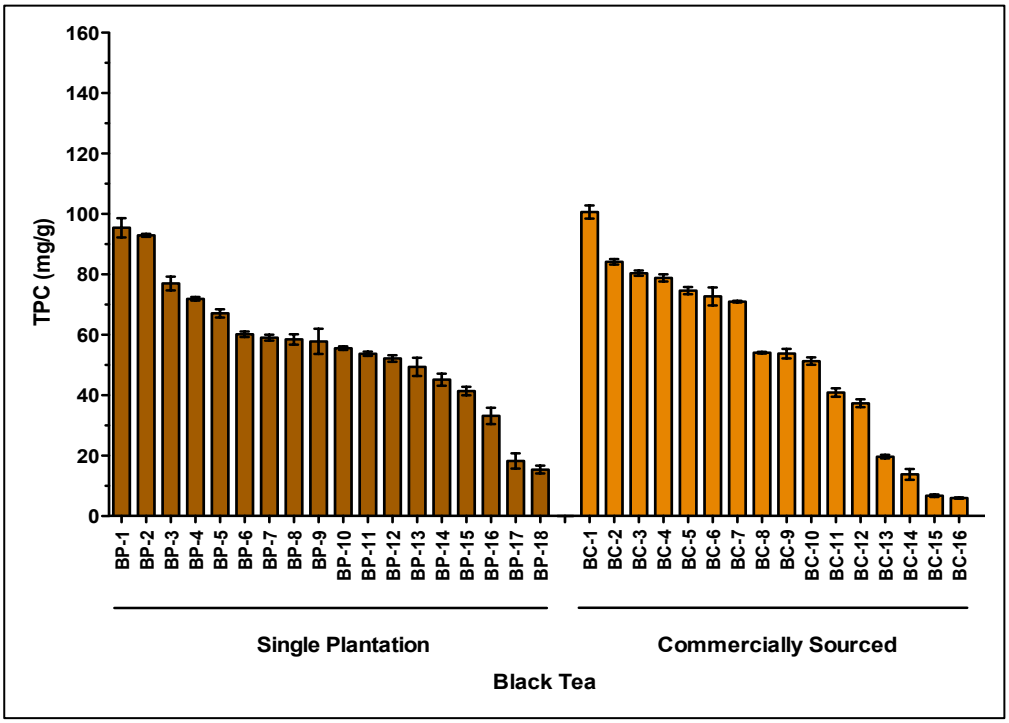


Figure 3.4 *The TPC (GAE) of 34 black tea samples (error bars = SE; n = 3).*

It was found that both data sets followed normal distribution, therefore it was appropriate to compare the TPC values of green and black teas using the student's t-test.

A significant difference between the TPC of green and black teas was found ($t = 3.57$; $p < 0.001$, two-tailed; $n = 72$; fig. 3.5). The magnitude of the difference in means (mean difference = 20.6 mg/g, 95% *CI*: 10.2 and 36.0 mg/g) was large ($\eta^2 = 0.16$).

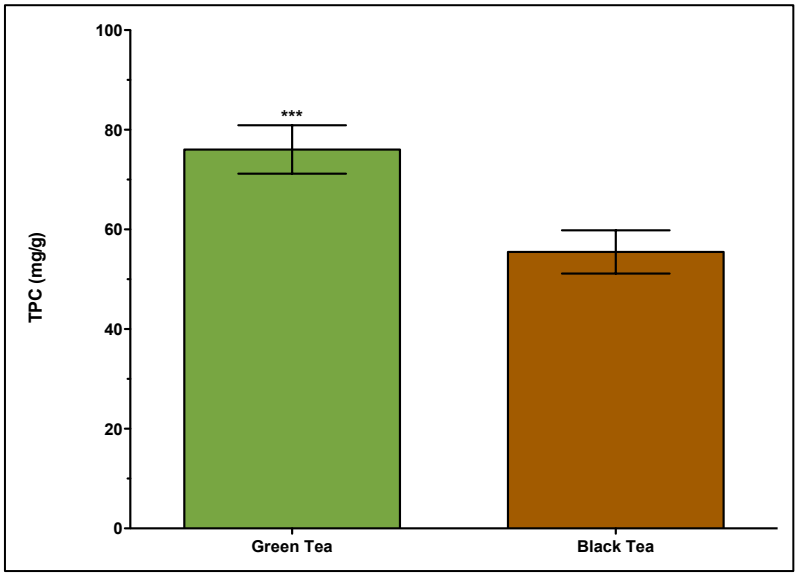


Figure 3.5 *Comparison of TPC of green (n=38) and black (n=34) teas by an independent students t-test (***=p<0.01).*

As previously mentioned, very few studies have looked at the properties of aqueous tea infusions based on typical tea drinking practices. Thus, it is not surprising to find in the current literature studies that agree (Manzocco *et al*., 1998; Anesini *et al*., 2008) and disagree (Khokhar *et al*., 2002; Hoff and Singleton, 1977) with the results of this investigation. Upon review of the studies, the most likely factors leading to the disagreement in results is the different extraction methods used and the relatively small numbers of samples included in these earlier studies (n = up to 12).

There may be multiple reasons why black tea infusions have been shown to contain less phenolic compounds than green teas. The most likely explanation is due to the nature of black tea polyphenols. An example of this would be the theaflavin polyphenols in black tea, which have three accessible phenol groups. However, theaflavin is the sum of two monomeric catechins, which have two accessible phenol groups each. The same would

hold true for the black tea thearubigins, which are much larger in size. A study by Hagerman *et al.* (1998), found that condensed tannins had slightly lower reducing ability than monomeric polyphenols over pH 3 – 8. Another possibility is that the extraction profiles of black tea polyphenols may be different to that of green tea polyphenols in water. Furthermore, it is also worth considering the limitations of the assay such as the influence of non-phenolic reducing agents and the difference in reductive power of unconjugated and conjugated polyphenols. Therefore, it would be unsound to suggest on the basis of this assay alone that there is a significant difference between the polyphenol content of green and black teas. Nevertheless, it can be concluded that green teas have more reducing power than black teas.

Evaluation of the TPC data for commercially sourced and single plantation teas found that both data sets did not follow normal distribution. Therefore, a non-parametric Mann-Whitney U test was used to determine if there was a significant difference between the groups. The outcome of the test revealed no significant difference (U = 626; z = -0.198; p = 0.84; r = 0.02; fig. 3.6) in the TPC of single plantation teas (Md = 62.44 mg/g, n = 33) and commercially sourced teas (Md = 69.8 mg/g, n = 39).

When comparing the TPC of green and black teas by origin, a non-parametric Kruskal Wallis test was used as the samples sizes were uneven and the data did not follow normal distribution.

A significant difference ($p < 0.05$) in TPC was found between green teas from Africa and China, however no difference was found between any of the other green teas (fig 3.7). This may be due to the vastly different climates in both countries or a difference in variety of tea bush. In general, teas originating from China and Japan are of the *Camellia* var. *sinensis* variety whilst those from India are usually *Camellia* var. *assamica* (Takeda *et al*., 2004; Yao *et al*., 2005; Chan et al., 2007). If this is true of the samples in this study, then the results are consistent with what was found in a previous study on green teas by Takeda *et al*. (2004), which found that green teas from China and Japan had significantly lower polyphenol content than green teas from other regions. Sabhapondit *et al*. (2012) also observed a reduced polyphenol content in green teas from China. Although, in this study there was no significant difference between Japanese teas and the other groups, it can be seen that the mean TPC is lower than that

of African and Indonesian teas. Arguably, with a larger sample size this may prove to be significant.

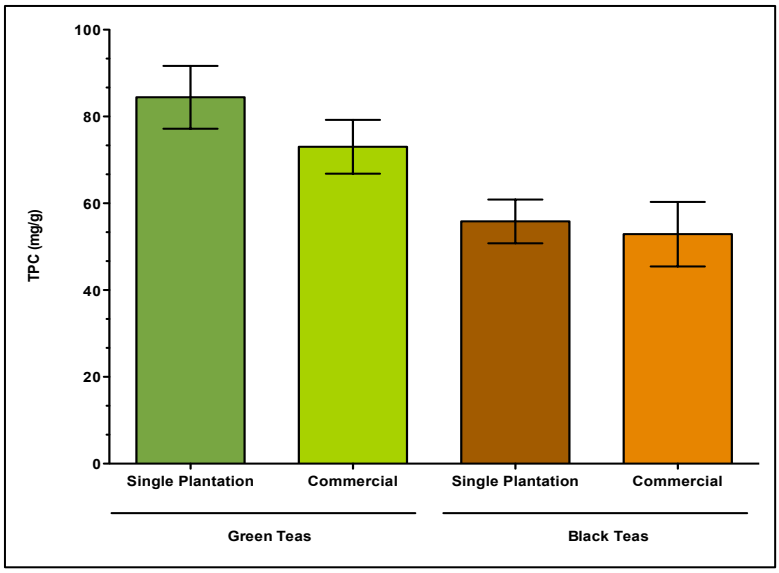


Figure 3.6. *Mean TPC of single plantation green (n=15), commercially sourced green (n=23), single plantation black (n=18), commercially sourced black (n=16) teas (error bars = SE).*

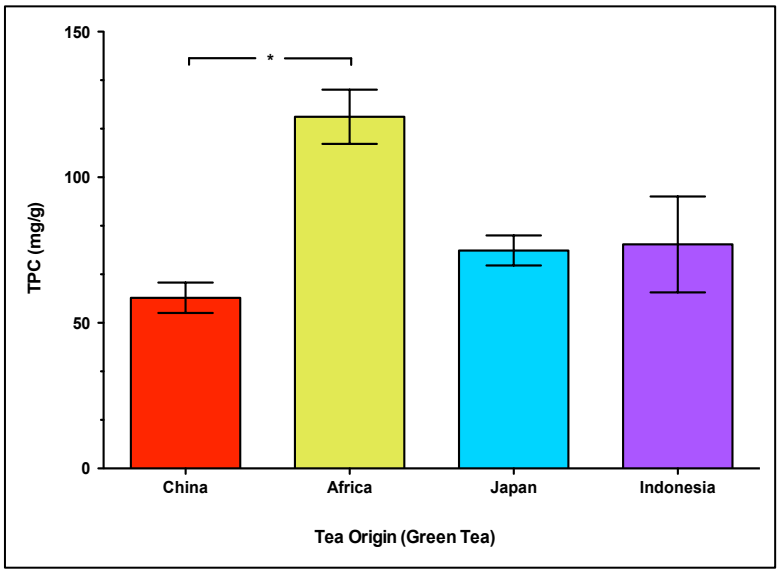


Figure 3.7 *Comparison of TPC of green teas from China (n=15), Africa (n=3), Japan (n=5) and Indonesia (n=4) using the Kruskal-Wallis test, (error bars = SE; *=p<0.05)*

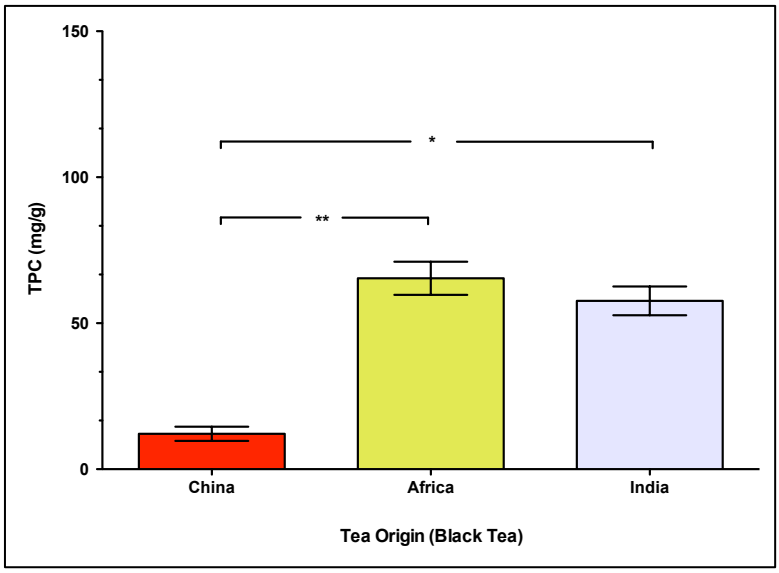


Figure 3.8. *Comparison of TPC of black teas from China (n=5), Africa (n=5) and India (n=10) using the Kruskal-Wallis test (error bars = SE; *=p<0.05; **=p<0.01).*

A similar trend was found with black teas (fig 3.8). Both African and Indian black teas had significantly higher TPC than teas from China. This is comparable to a study by Astill *et al*., (2001), which showed a similar result. Again this may be due to climate or tea bush variety and appears to be consistent been green and black teas originating from the country (Takeda *et al*., 2004; Yao *et al*., 2005; Chan *et al*., 2007).

3.4.1.2 The TFC of Aqueous Tea Infusions

The TFC of aqueous tea infusions was determined using a calibration curve constructed with catechin (C) as a standard. It was found that the relationship was linear and the therefore the TFC was estimated for each tea using the derived equation (fig 3.9).

The average TFC of the green teas included in this study was 16.3 mg/g ± 0.9 mg/g (± *SE; n = 38*) whilst the average for the black teas was lower at 12.9 mg/g ± 0.8 mg/g (± *SE; n = 34*). In contrast to the observations in TPC, the range of TFC within the green (4.6 – 28.0 mg/g; fig 3.10) and black teas (4.0 – 20.8 mg/g; fig 3.11) was similar. It was expected that black teas would have considerably lower TFC, as during the production of black tea the flavonoids conjugate to produce theaflavins and thearubigins, which reduce the concentration of monomeric flavonoids. Therefore, it may be possible that there is non-specific binding of the black tea pigments to the assay reagent.

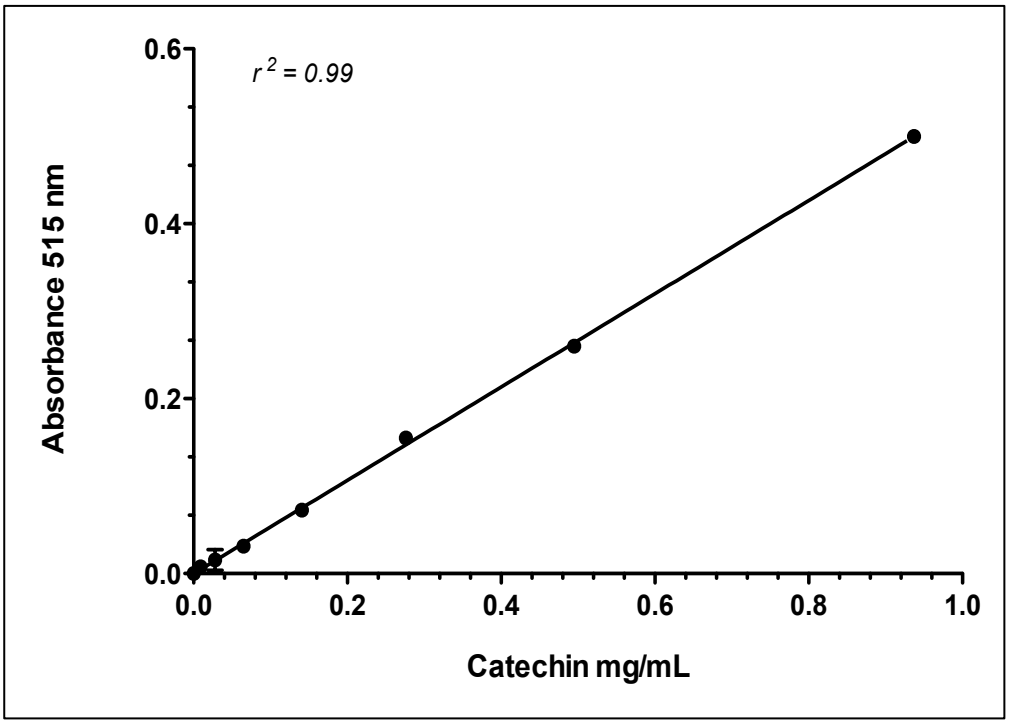


Figure 3.9. *Calibration curve of absorbance (515 nm) versus catechin concentration (n = 3).*

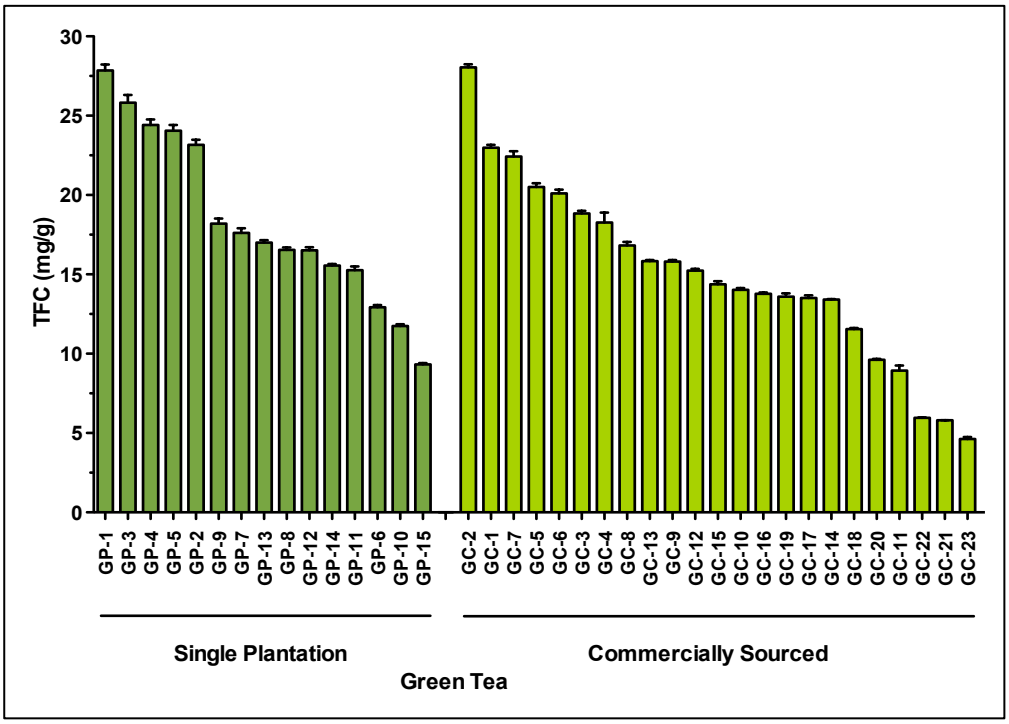


Figure 3.10. *The TFC (CE) of 38 green tea samples (error bars = SE; n = 3).*

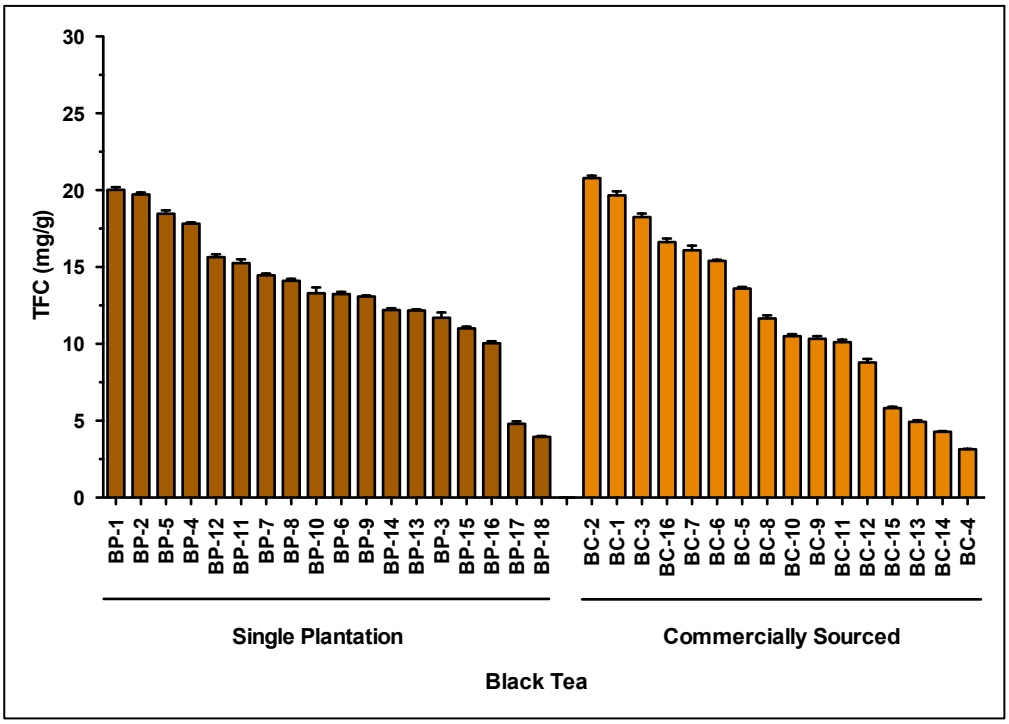


Figure 3.11. *The TFC (CE) of 34 black tea samples (error bars = SE, n = 3).*

Since the data followed normal distribution, an independent-samples t-test was conducted to compare the TFC of green and black teas (fig 3.12). There was a significant difference between the TFC of green tea (*mean* = 16.3 mg/g) and black tea (*mean* = 12.9 mg/g); t = 2.74; p <0.01, two-tailed; n = 72). The magnitude of the differences in means (mean difference = 3.5, 95% *CI*: 0.9 and 6.0 mg/g) was moderate (η^2= 0.10). Therefore, it can be seen that the difference of TPC between green and black tea is greater than their difference in TFC.

An independent-samples t-test was conducted to compare the TFC of single plantation and commercially sourced teas as both data sets showed normal distribution. No significant difference was observed between the TFC of single plantation (*mean* = 15.67 mg/g) and commercially sourced (*mean* = 13.86 mg/g); t = 1.38, p = 0.17, two-tailed; n = 72) teas (fig. 3.13). The magnitude of the differences in means (mean difference = 1.82 mg/g, 95% *CI*: 0.80 and 4.44 mg/g) was small (η^2= 0.02). These results were consistent with those seen for TPC, showing that the total phenolic and flavonoid

content between single plantation and commercially sourced teas is not significantly different (p>0.05).

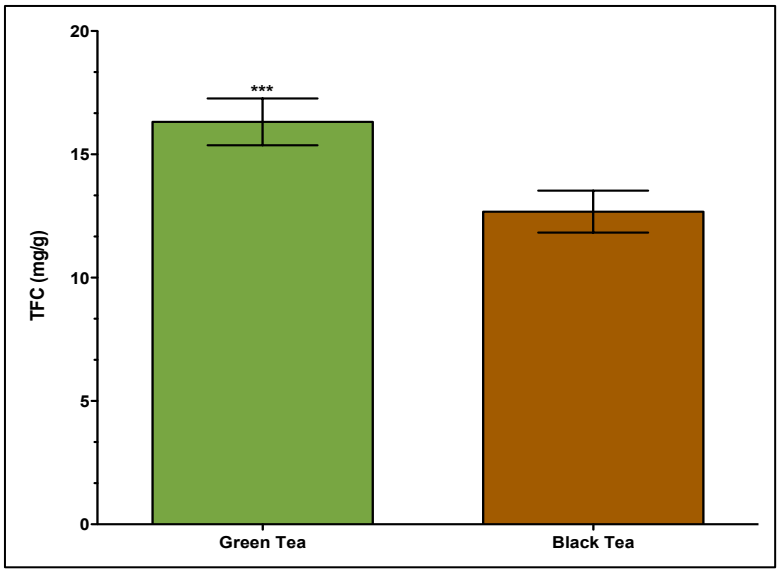


Figure 3.12. *Comparison of TFC (CE) of green (n=38) and black (n=34) teas by independent student's t-test (error bars = SE; ***=p<0.001).*

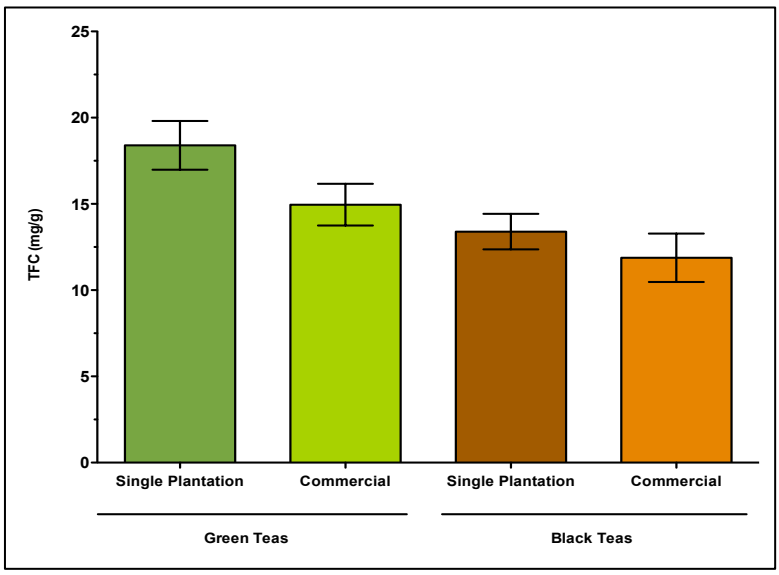


Figure 3.13. *Mean TFC (CE) of single plantation green (n=15), commercially sourced green (n=23), single plantation black (n=18) and commercially sourced black (n=16) teas (error bars = SE).*

Similar to what was found with TPC, the TFC of green and black teas when compared by country of origin did not follow normal distribution and contained unequal sample sizes, thus a non-parametric Kruskal-Wallis test was used to compare the groups. The test found a significant difference ($p<0.05$) in TFC was seen between green teas from Africa and China, however no difference was seen between any of the other green teas (fig. 3.14). Although this is a unique result to the assay, it is not surprising that this is similar to what was found when comparing TPC, as flavonoids would account for a large proportion of the total polyphenols in tea infusions (Balentine *et al.*, 1997).

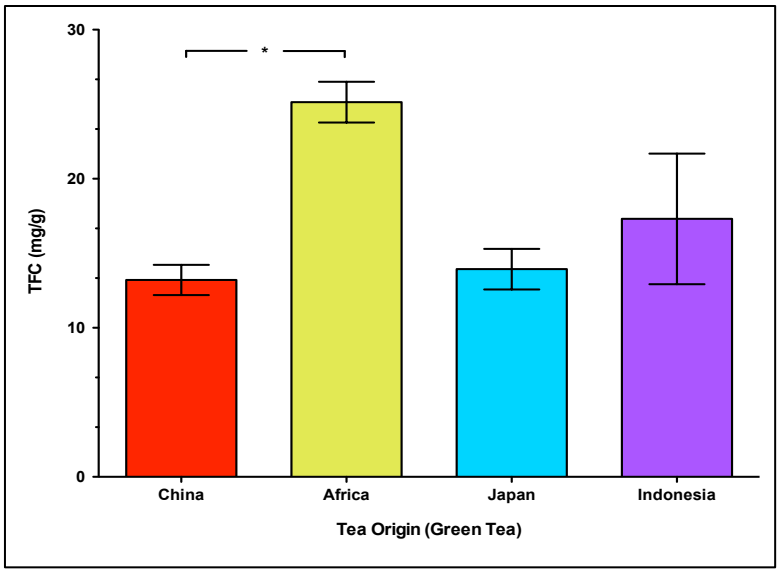


Figure 3.14. *Comparison of TFC (CE) of green teas from China (n=15), Africa (n=3), Japan (n=5) and Indonesia (n=4) using the Kruskal-Wallis test (error bars = SE; *=p<0.05).*

When comparing the black teas by origin, it was found that Chinese black teas had lower levels of flavonoids than African ($p<0.01$) and Indian ($p<0.05$) sourced teas (fig. 3.15). This is likely to be explained by the reasons mentioned previously for TPC such as variety of tea bush used and environmental conditions (Takeda *et al.*, 2004; Yao *et al.*, 2005; Chan *et al.*, 2007). However, since the results of the assay may have been skewed by the presence of black tea polyphenols due to non-specific binding, the results may be better explained by HPLC analysis.

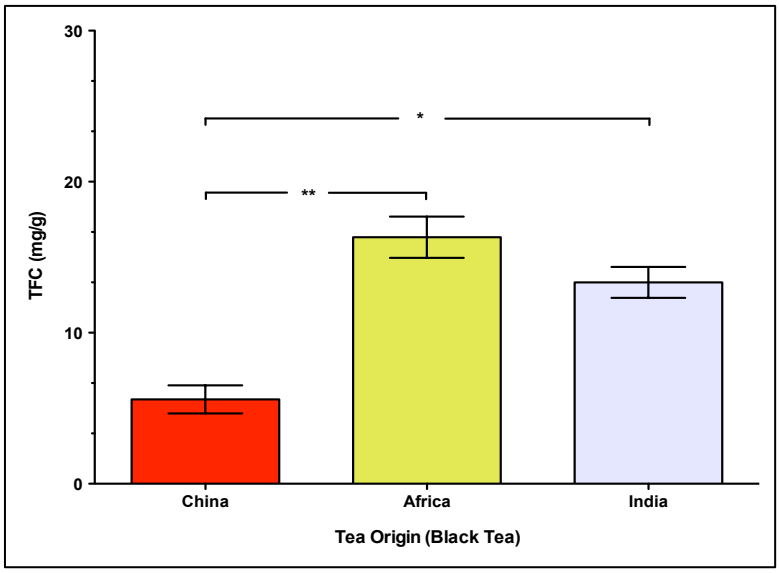


Figure 3.15. *Comparison of TFC (CE) of black teas from China (n=5), Africa (n=5) and India (n=10) using the Kruskal-Wallis test (error bars = SE; *=p<0.05; **=p<0.01).*

3.4.2 The Antioxidant and Pro-oxidant activity of Tea Infusions

3.4.2.1 The Antioxidant Power (FRAP) of Tea Infusions

The antioxidant power of aqueous infusion was determined using a concentration curve constructed with Trolox®. As shown in figure 3.16, the relationship between concentration and absorbance was linear and therefore the antioxidant power of each tea was estimated using the derived equation.

The mean antioxidant power of the green teas was 2.7 ± 0.1 mM/g (± = *SE; n = 38*). In comparison, the average antioxidant power for the black teas was approximately half that of green teas at 1.5 ± 0.1 mM/g (± = *SE; n = 34*). Again, there was a considerable difference in the range of antioxidant power within the green (0.9 - 5 mM/g; Fig 3.17) and black teas (0.4 – 2.5 mM/g; Fig 3.18).

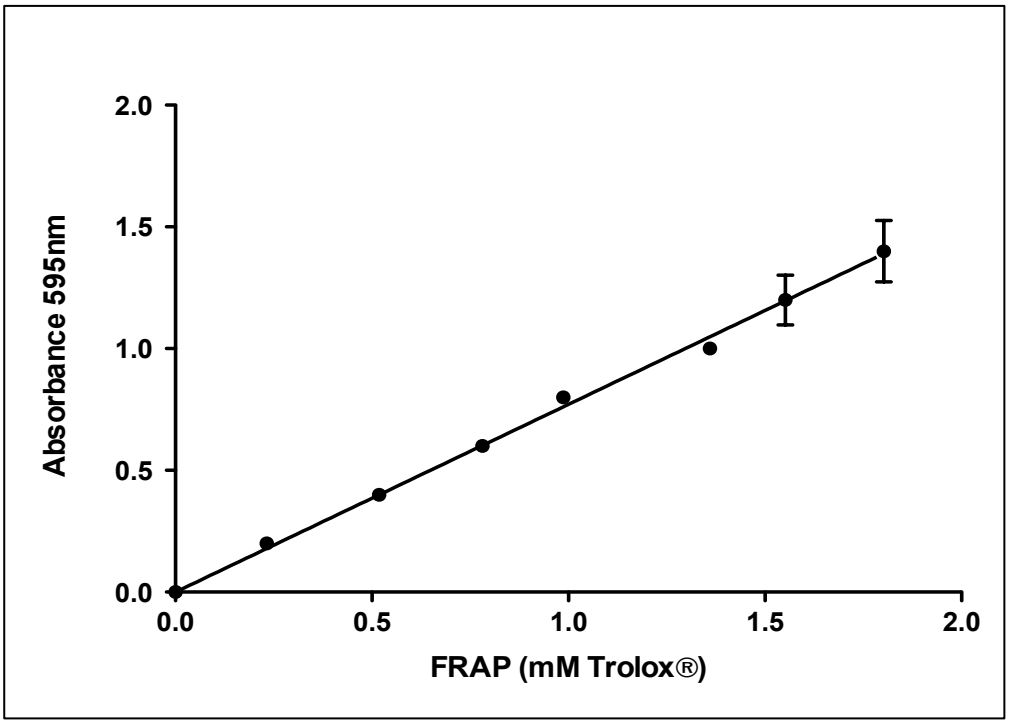


Figure 3.16. *Calibration curve of absorbance (595 nm) versus antioxidant power (error bars = SE; n = 3).*

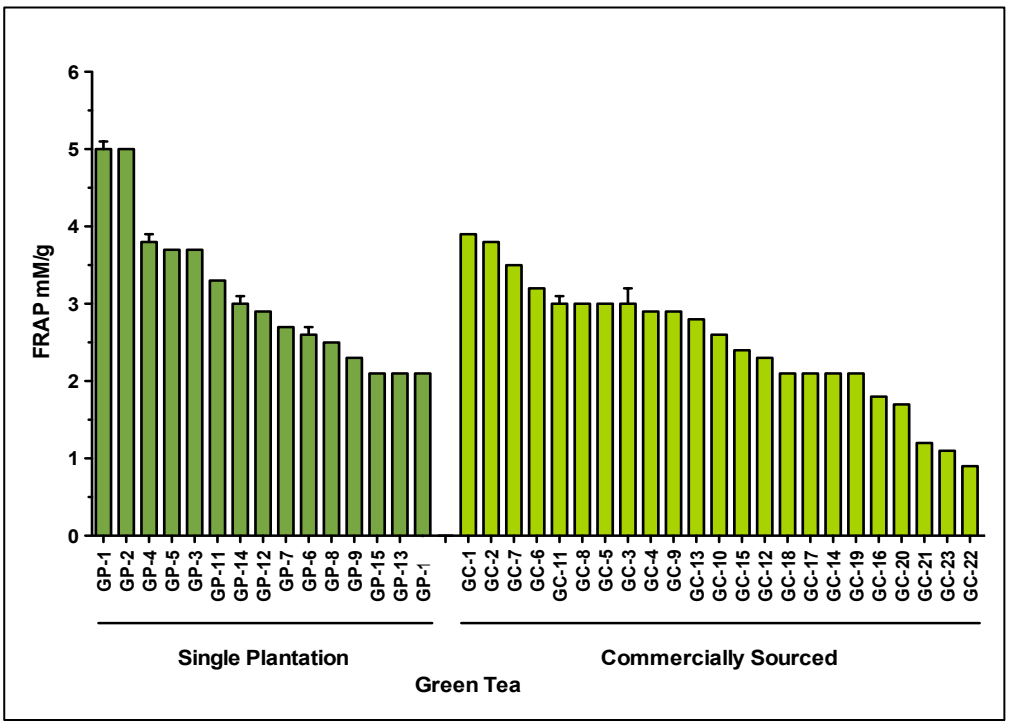


Figure 3.17. *The antioxidant power (TE) of 38 green tea samples (error bars = SE; n = 3).*

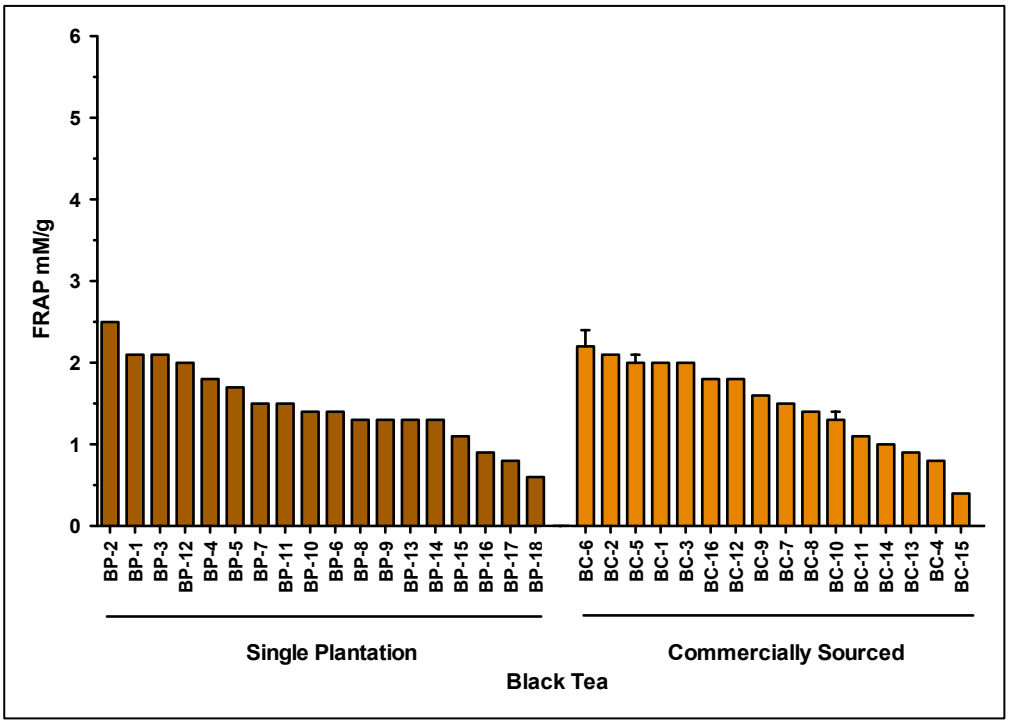


Figure 3.18. *The antioxidant power (TE) of 34 black tea samples (error bars = SE; n = 3).*

To compare the difference in antioxidant power, the student's t-test was conducted. A significant difference was found between the antioxidant capacity of green (*mean* = 2.7 mM/g) and black tea (*mean*= 1.5 mM/g; t = 7.09; p <0.001, two-tailed; n = 72; Fig. 3.19). Furthermore, the magnitude of the differences in means (mean difference = 1.3 mM/g, 95% *CI*: 0.9 and 1.6 mM/g) was very large (η^2= 0.42).

The catechins in green tea are known to have considerable antioxidant activity, which can explain the high values observed in the green tea samples tested. However, the condensation products of the catechins in black teas, theaflavins and thearubigins, are also reported to have antioxidant activity (Paquay *et al*., 2000). Therefore, the results of the assay suggest that the black tea infusions have less antioxidant power than green tea infusions. This may the result of different extraction kinetics or that the black teas samples may have had lower catechin content than the green tea samples post-harvesting resulting in lower concentrations of condensed polyphenols.

These results are also consistent with the TPC of the teas, which was expected, as it is another measure of reductive power (Benzie and Szeto., 1999). Other studies such as Quan *et al*. (2007), and Almajano *et al*. (2008), have found similar trends, however there was no statistical significance applied to their findings.

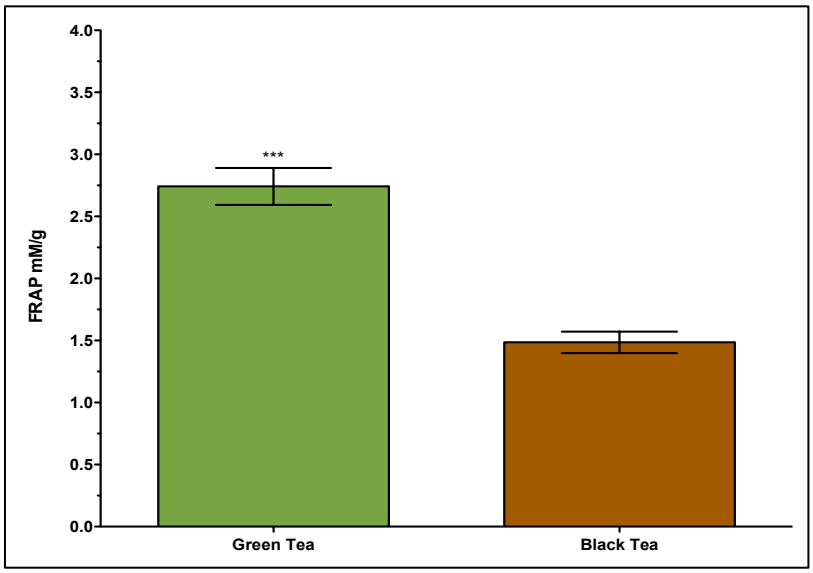


Figure 3.19. *Comparison of FRAP (TE) of green (n=38) and black (n=34) teas (error bars = SE; ***=p<0.001).*

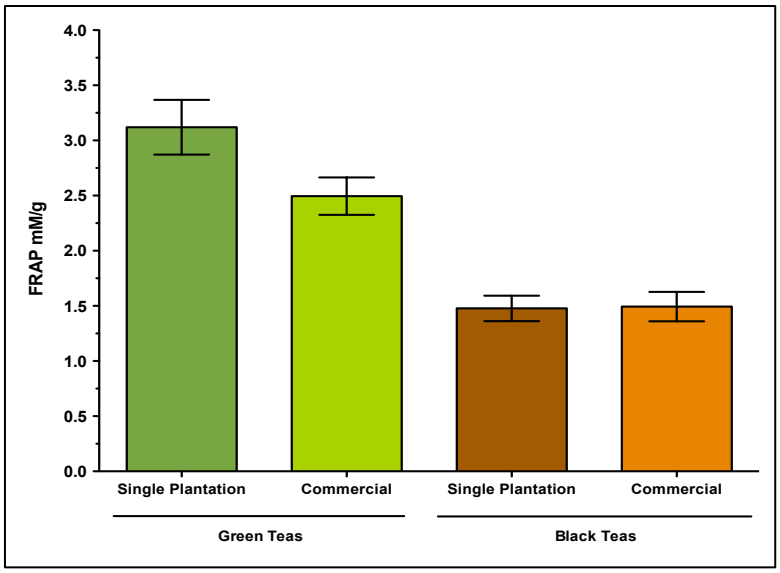


Figure 3.20. *Mean antioxidant power of single plantation green (n=15), commercially sourced green (n=23), single plantation black (n=18) and commercial sourced black (n=16) teas (error bars = SE).*

Since both data sets followed normal distribution, an independent-samples student t-test was conducted to compare the antioxidant power of single plantation and commercially sourced teas. The results of the analysis showed that there was no significant difference found between the single plantation (*mean* = 2.2 mM/g) and commercially sourced (*mean* = 2.1 mM/g); t = 0.60; p = 0.55, two-tailed; n = 72) teas (fig. 3.20). This is consistent with the results observed for TPC and TFC.

As a result of the uneven sample sizes and non-normal distribution of the groups, the Kruskal-Wallis test was used to compare the antioxidant power of teas based on origin. Consistent with TPC and TFC, a significant difference ($p < 0.05$) in antioxidant power was seen between green teas from Africa and China. There was no statistical difference between any of the other green teas (fig. 3.12). Furthermore, African ($p<0.01$) and Indian ($p<0.01$) black teas had significantly higher antioxidant power than Chinese black teas (fig. 3.13). Again this was similar to the conclusions of the TPC and TFC assay.

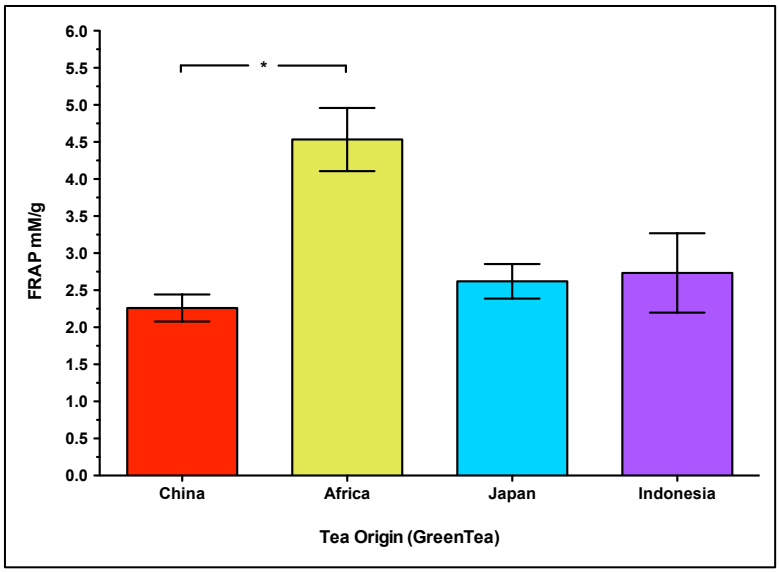


Figure 3.21. *Comparison of the antioxidant power (TE) of green teas from China (n=15), Africa (n=3), Japan (n=5) and Indonesia (n=4) using the Kruskal-Wallis test (error bars = SE; *=p<0.05).*

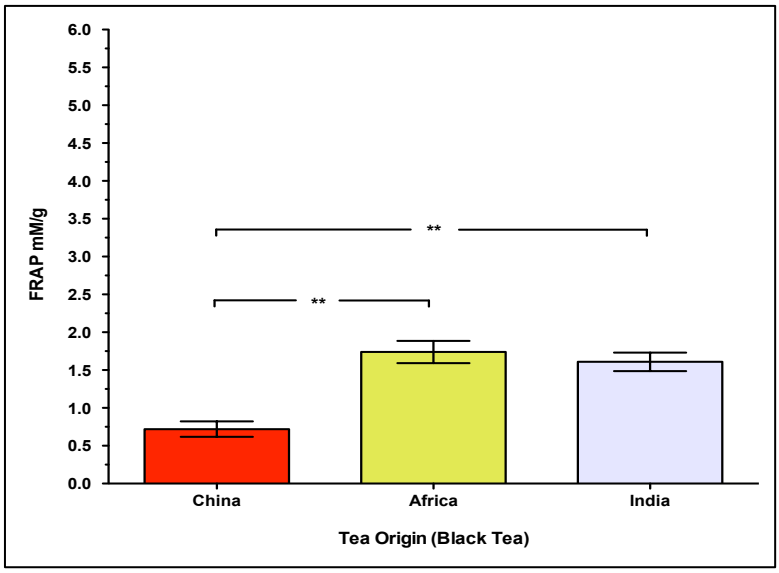


Figure 3.22. *Comparison of the antioxidant power of black teas from China (n=5), Africa (n=5) and India (n=10) using the Kruskal-Wallis test (error bars = SE; **=p<0.01).*

3.4.2.2 The Hydrogen Peroxide Production of Tea Infusions

The hydrogen peroxide (H_2O_2) production of aqueous infusions was determined using a concentration curve constructed with H_2O_2 as a standard (fig. 3.23) The relationship between concentration and absorbance was found to be linear, therefore the H_2O_2 production of each tea infusion was estimated using the derived equation. For this assay a representative sample of green (n =12) and black teas (n =12) were chosen based on their TPC, TFC and antioxidant power.

After 48 hours, the tea that produced the most H_2O_2 was GP-1 (1196 ± 53 µM/g; ± = SE) whilst GP-21 produced the least (393 ± 45 µM/g). The range in H_2O_2 production of the green and black teas was 393 – 1196 µM/g and 518 – 933 µM/g, respectively.

The initial production of H_2O_2 (after the 5 min brew, t = 0 h) by the green teas was 53 ± 2.5 µM/g (n = 12; ± = SE) whilst for black teas it was 47.2 ± 2.2 µM/g (n = 12; ± = SE).

Between the initial brew and 12 hours of incubation there was a rapid increase in H_2O_2 production by both green and black teas (more than 10 times). The maximum production of H_2O_2 was achieved after 48 hours for green and black teas, 928.3 ± 74.5 and 725.6 ± 38.5 µM/g, respectively (n = 24; ± = SE).

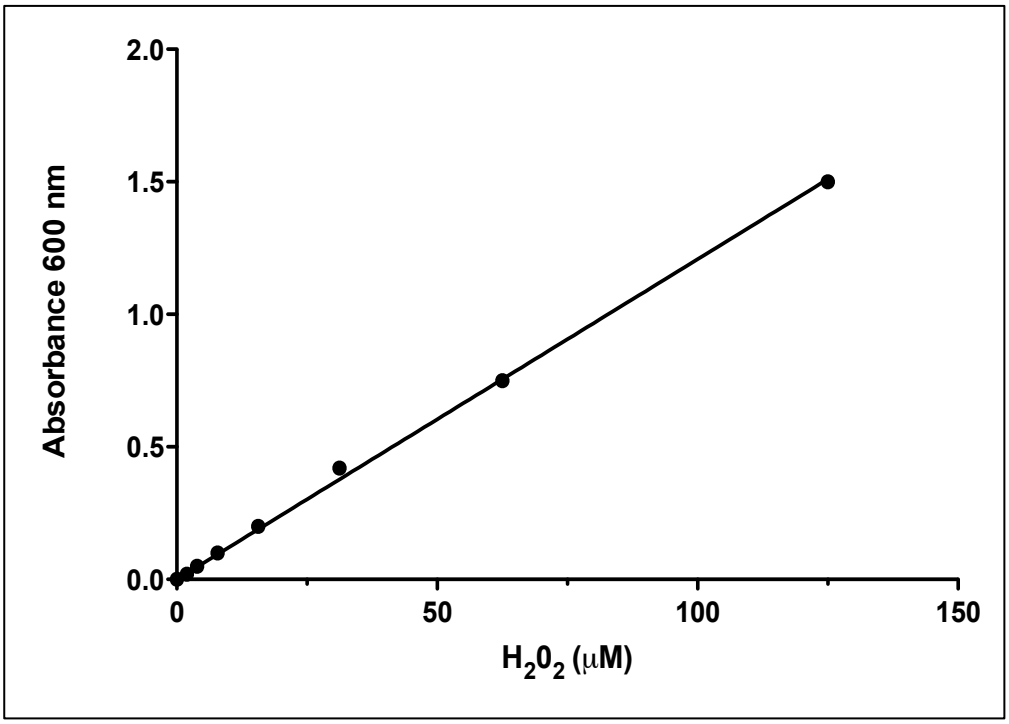


Figure 3.23. *Calibration curve of absorbance (600 nm) versus the concentration of H_2O_2 (μM) using the FOX assay (n = 3).*

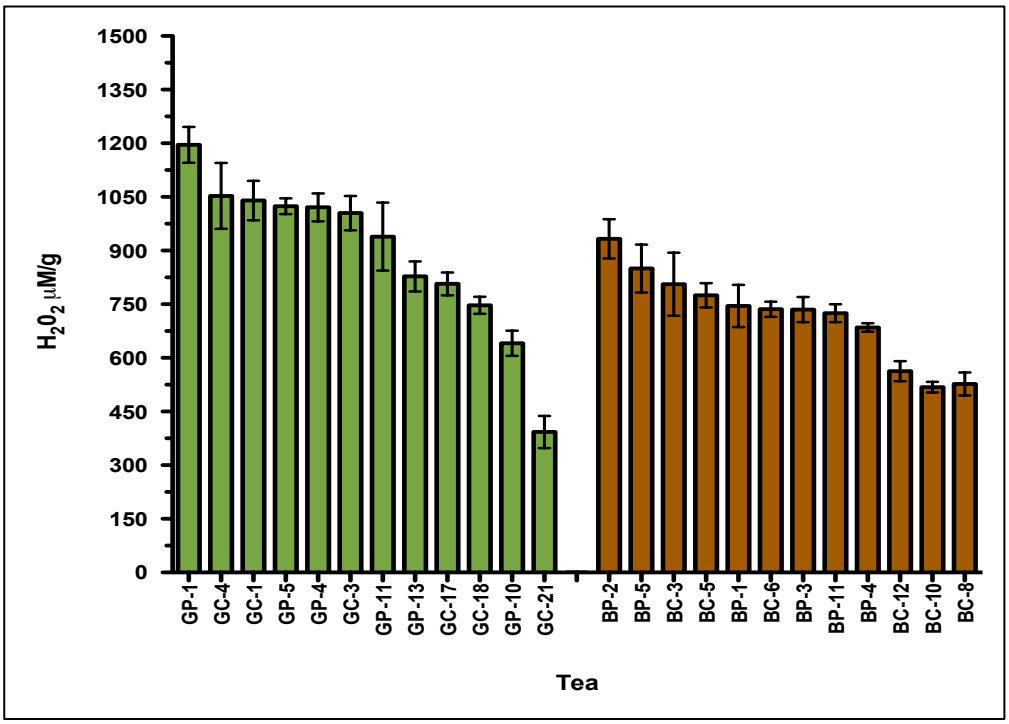


Figure 3.24. *The H_2O_2 production of green and black tea samples at pH 7.4 after incubation at 37 °C for 48 h (error bars = SE; n=3).*

The data points at each time point for both green and black teas were normally distributed, therefore a t-test was used to determine any significant differences. No significant differences were found in H_2O_2 production between 0 and 12 hours (p>0.05), although significant differences were found at 24 ((t = 2.38; p>0.05, two-tailed; mean difference = 174.6 µM/g; 95% *CI*: 22.4 and 326.9 µM/g; η^2= 0.20; n = 24; Fig 3.25) and 48 hours (t = 2.42; p>0.05, two-tailed; mean difference = 202.7 µM/g, 95% *CI*: 28.9 and 376.6 µM/g; η^2= 0.20; n = 24; fig 3.25).

The difference in H_2O_2 production between green and black teas is most likely due to higher catechin content in green teas (Nakayama *et al.,* 2002; Akagawa *et al*., 2003). Catechins have been shown to produce significant amounts of H_2O_2 between pH 7 and pH 9 (Nakayama *et al.,* 2002), whilst black tea polyphenols are thought to have little or no pro-oxidant activity (Hagerman *et al.,* 1998).

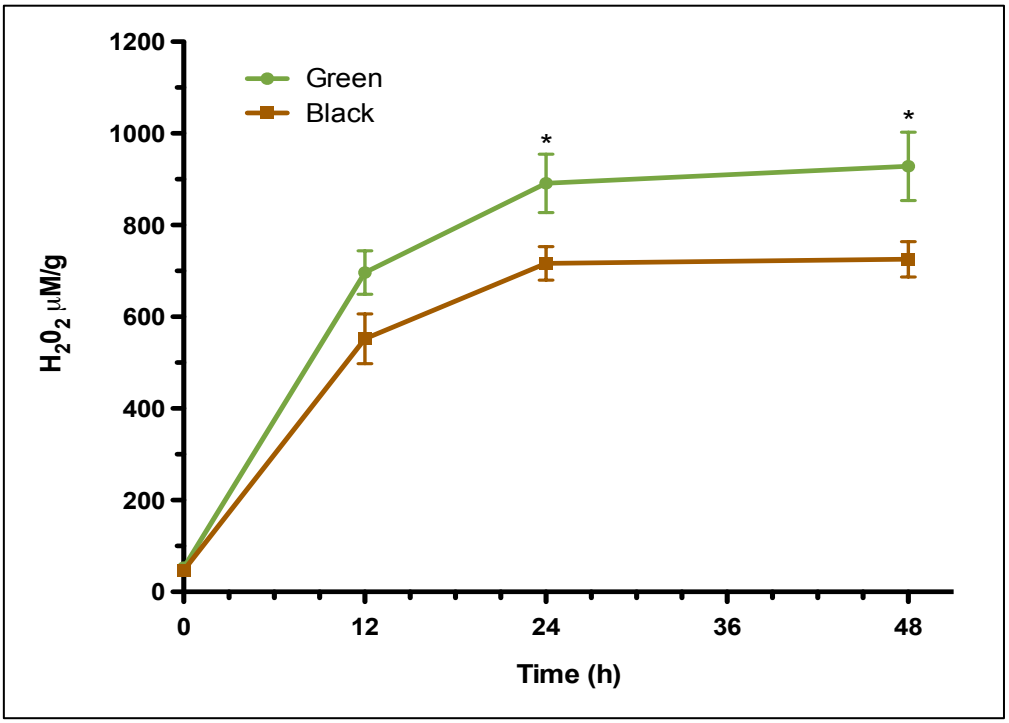


Figure 3.25. *The H_2O_2 production of green (n=12) and black tea samples (n=12) over 48 hours at pH 7.4 after incubation at 37 °C (error bars = SE; *=p <0.05).*

Although limited by a small sample size (n=1), a study by Akagawa *et al*. (2003) reported similar results with green tea producing more H_2O_2 than black teas with peak production occurring at 48 hours (Akagawa *et al.,* 2003).

In summary, green and black teas produce considerable amounts of H_2O_2 at physiological pH. Therefore in the context of this thesis, its potential involvement in the antimicrobial mechanism of tea must be considered.

3.4.3 Correlation of TPC, TFC and Antioxidant Power and H_2O_2 Production

The assays to measure TPC, TFC, hydrogen peroxide production and antioxidant power all measure specific parameters of tea infusions, but it is obvious from the results that there are clear trends and agreement between the observations. To determine the extent of the relationship between these assays, a correlation analysis was performed.

After reviewing the data, it was found that all the assumptions were met to perform Pearson's correlation.

Table 3.2 *Pearson correlation matrix of the TFC, TPC, H_2O_2 production and FRAP of green and black teas (**=p<0.01). Values are expressed as Pearson's product-moment correlation (coefficient of determination 'r^2').*

	TFC	**TPC**	**FRAP**	**H_2O_2**
TFC	-	0.84 (0.70)**	0.82 (0.67)**	0.78 (0.61)**
TPC		-	0.84 (0.70)**	0.72 (0.52)**
FRAP			-	0.86 (0.74)**
H_2O_2				-

From the analysis it can be seen that there are strong positive correlations between all the assays, the strongest being between H_2O_2 and FRAP (0.86; table 3.2). This supports the dual physiochemical properties of tea polyphenols (Nakayama *et al.,* 2002; Akagawa *et al.,* 2003; Arakawa *et al.,* 2004), as the correlation suggests that teas with

high antioxidant power can also produce higher concentrations of H_2O_2 depending on the pH of the solution.

The analysis also shows that total measured polyphenols (TPC) and flavonoids (TFC) in tea infusions explain most of the variance of H_2O_2 production, 52% (r^2= 0.52) and 61% (r^2= 0.61), respectively (table 3.2). That is to say, the difference in the amount of H_2O_2 a tea can produce is explained in part by the abundance or lack of polyphenols and flavonoids.

Furthermore, the flavonoids (TFC) present in tea infusions explain the majority of the variance in polyphenol content (TPC) supporting that they are the major phenolic compounds present in tea (83%, r^2 = 0.82; table 3.2). A study by Ku *et al*. (2010) showed a similar finding between TPC, TFC and antioxidant activity in black pu-erh teas.

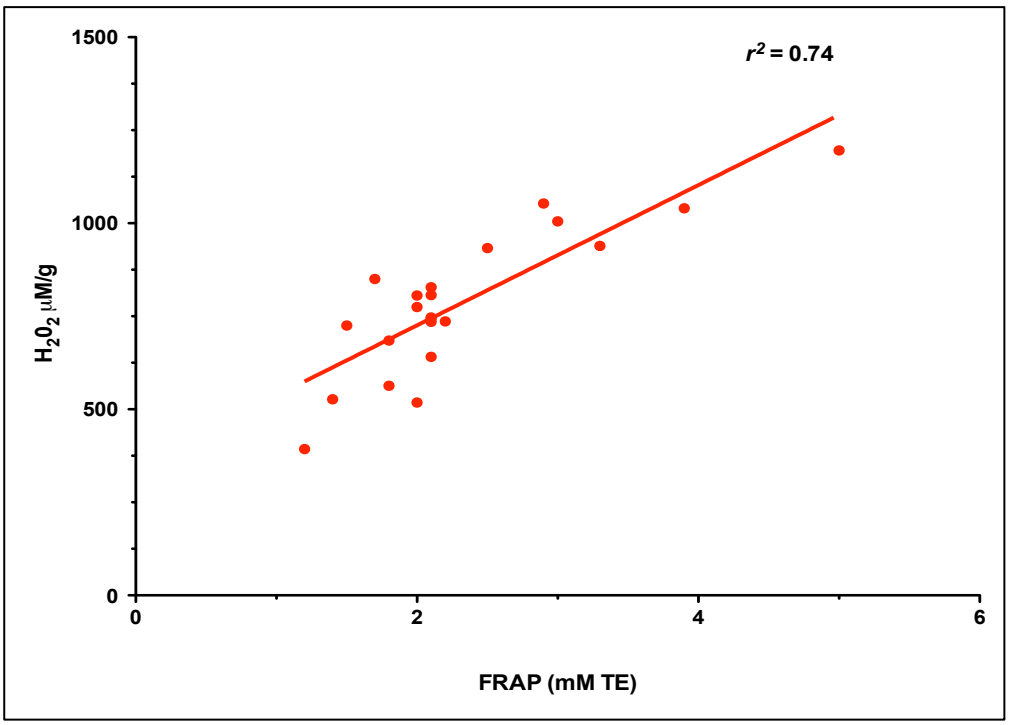


Figure 3.26. *Pearson correlation of the H_2O_2 production and antioxidant power (FRAP) of green and black teas (n =24).*

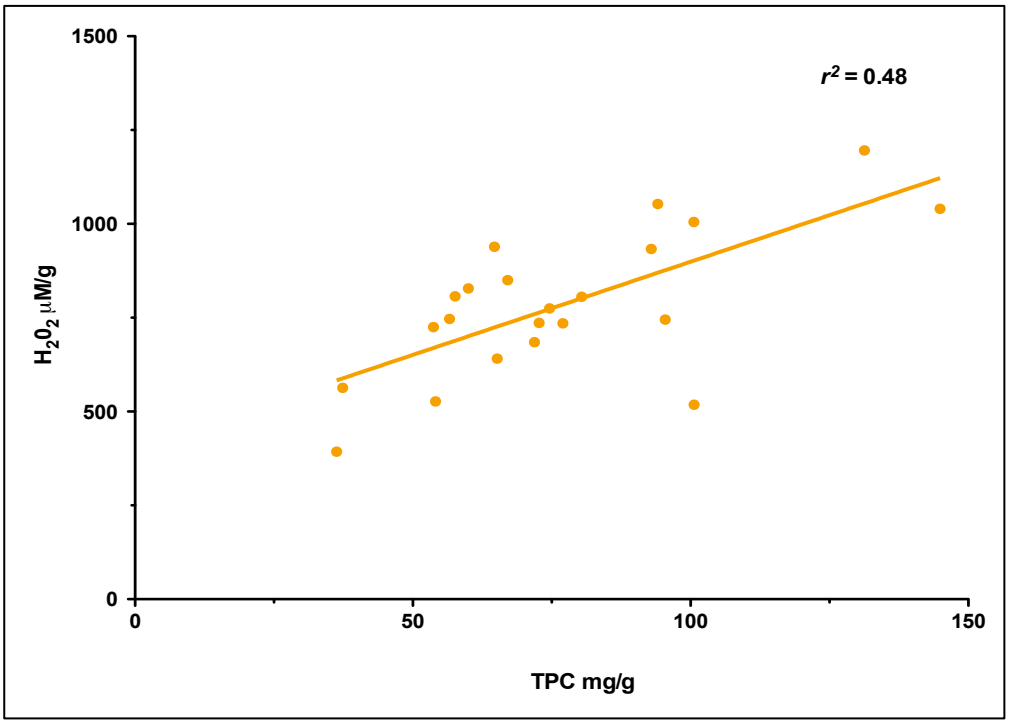


Figure 3.27. *Pearson correlation of the H_2O_2 production and TPC of green and black teas (n =24).*

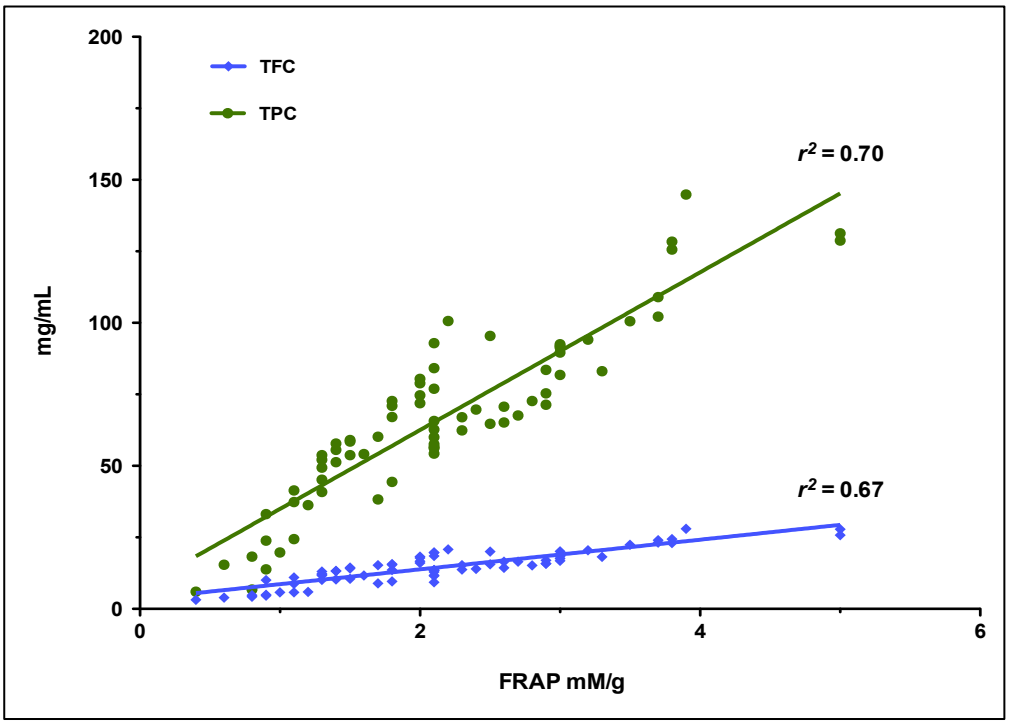


Figure 3.28. *Pearson correlation of the TFC, TPC and antioxidant power (FRAP)of green and black teas (n =72).*

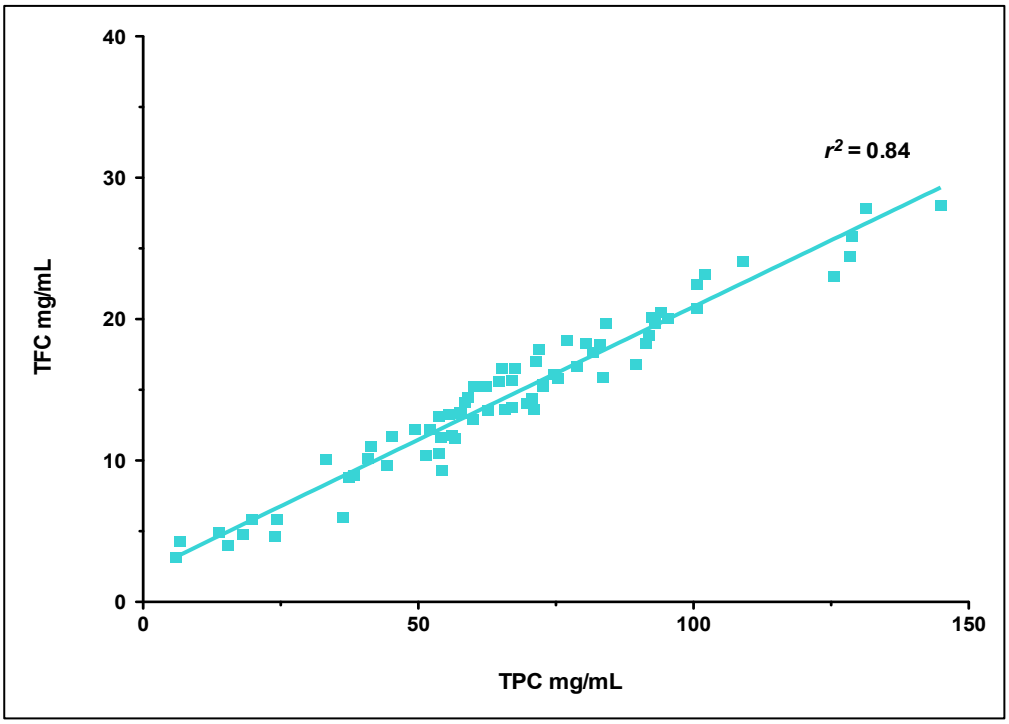


Figure 3.29. *Pearson correlation of the TFC and TPC of green and black teas (n =72).*

In summary, these assays have shown the diversity of the phenolic content and antioxidant/pro-oxidant properties of tea infusions. Good correlations between these assays help to give confidence in the selection teas with which to test the hypothesis that tea infusions with higher phenolic content and antioxidant/pro-oxidant power will have greater antimicrobial activity.

3.4.4 HPLC Analysis of Aqueous Tea Infusions

In this investigation the HPLC method developed by Wang *et al*. (2003), was chosen, as it allowed simultaneous identification of six different catechins, gallic acid and caffeine (fig 3.30). Initially, the PDA detector was set between 200 – 400 nm and after analysing a mixture of authentic standards; it was found that the optimal detection wavelength for this study was 210 nm. From the profiles of each standard compound (fig. 3.31) it can be seen that they also absorb strongly at 280 nm. A potential advantage of using higher wavelengths for detection is the reduction in the signal to noise ratio arising from impurities in the mobile phase solvents. However, in this study, it was found that at 280 nm the limit of detection was reduced considerably.

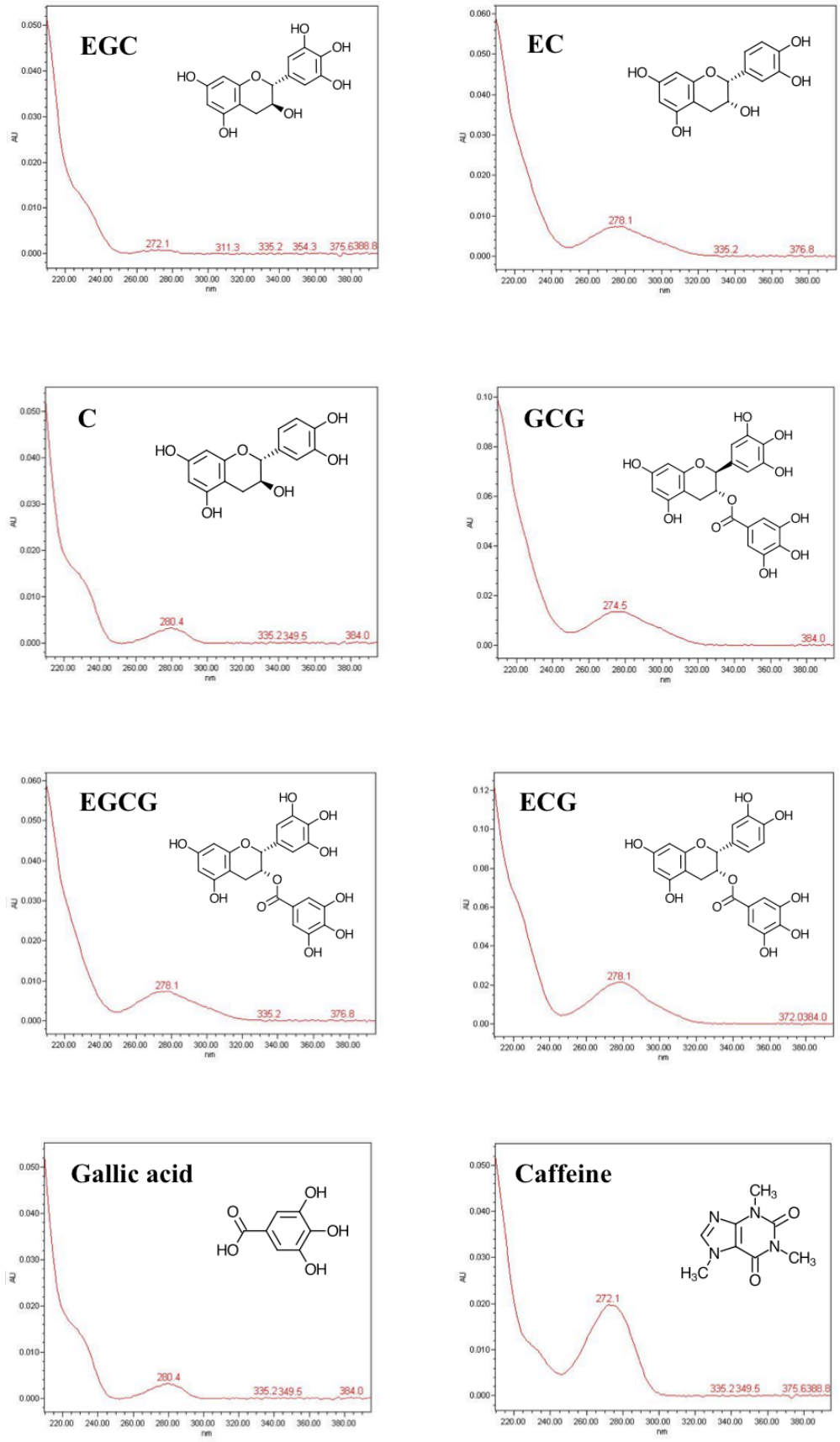


Figure 3.30. *The UV spectra of catechins, gallic acid and caffeine between 200 – 400 nm.*

The concentration curve for each standard was linear between 0.15 – 1.5 µg/mL (fig 3.31). Therefore, the concentrations of standards in the tea infusions were determined by their peak area using linear regression.

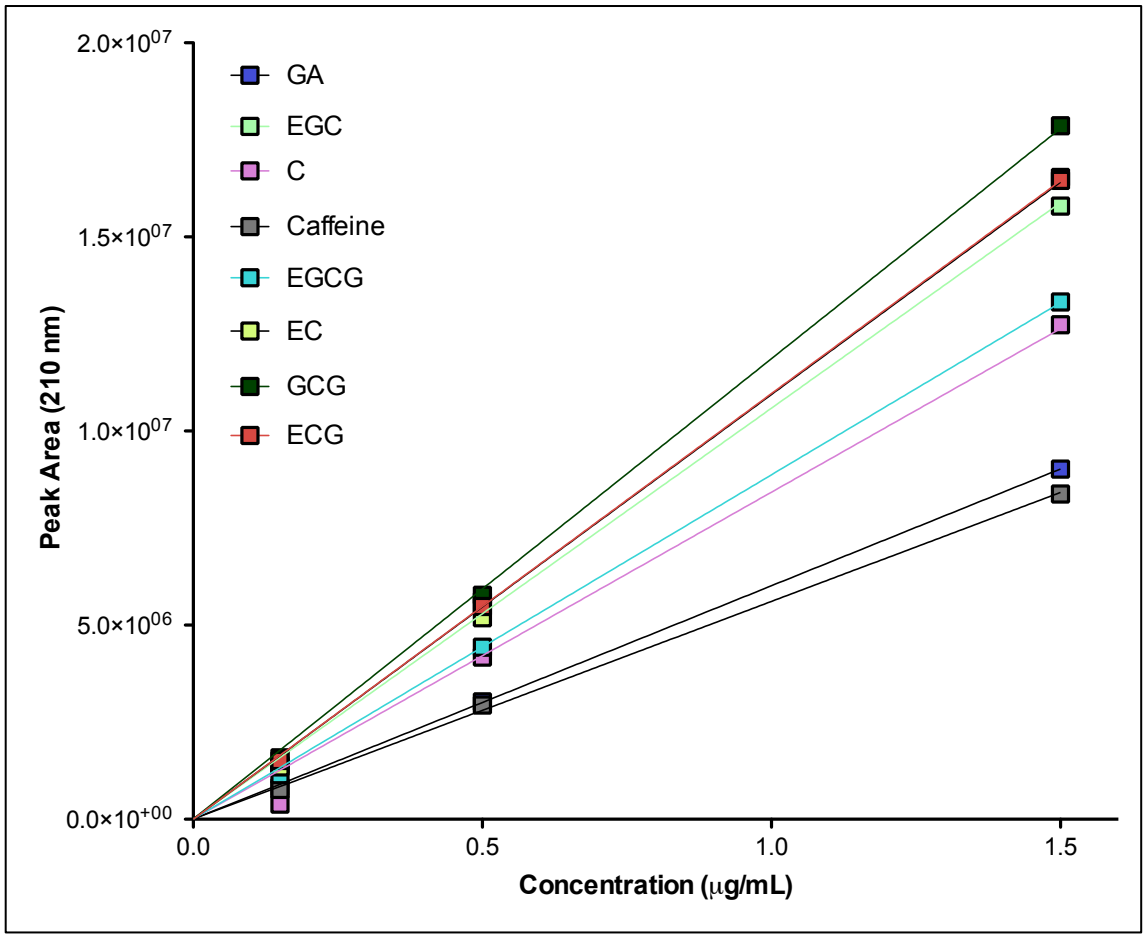


Figure 3.31. *Calibration curve of the peak area of absorbance (210 nm) for each standard versus their concentration (n = 3; r^2 = 0.99 for all standards).*

Apart from gallic acid, the resolution of the standard peaks in the tea samples was comparable to the study by Wang *et al*., (2003) (fig 3.31 and 3.32). In that study, slight co-elution of gallic acid with other peaks occurs, although it was more pronounced in this study. This is likely due to differences in sample preparation and efficiency of the HPLC apparatus.

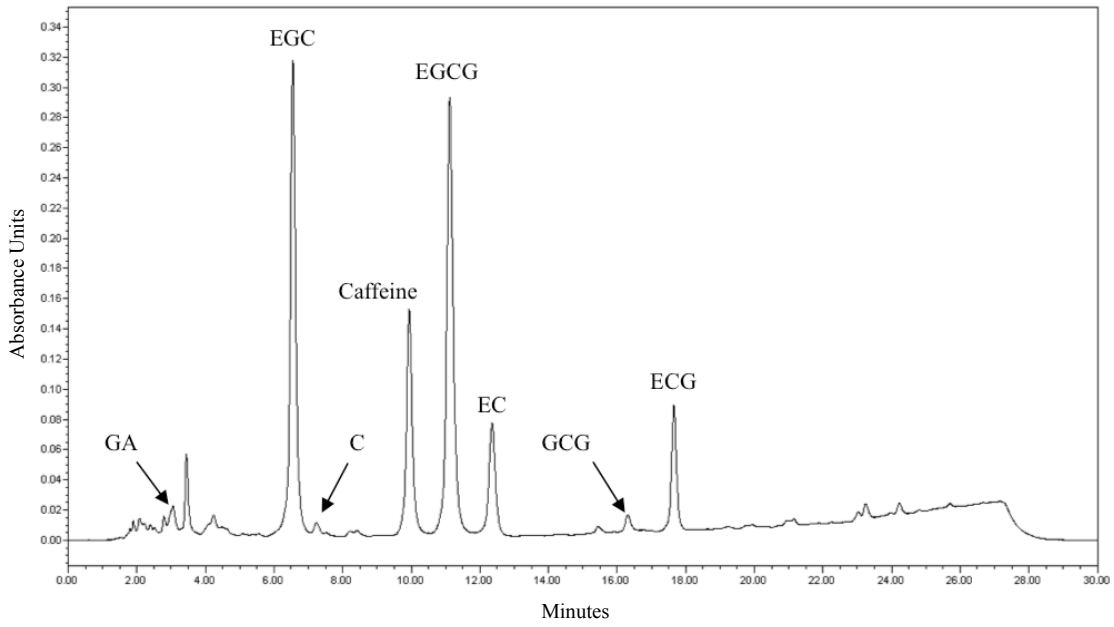


Figure 3.32. *HPLC profile of green tea GP-2 at 210 nm.*

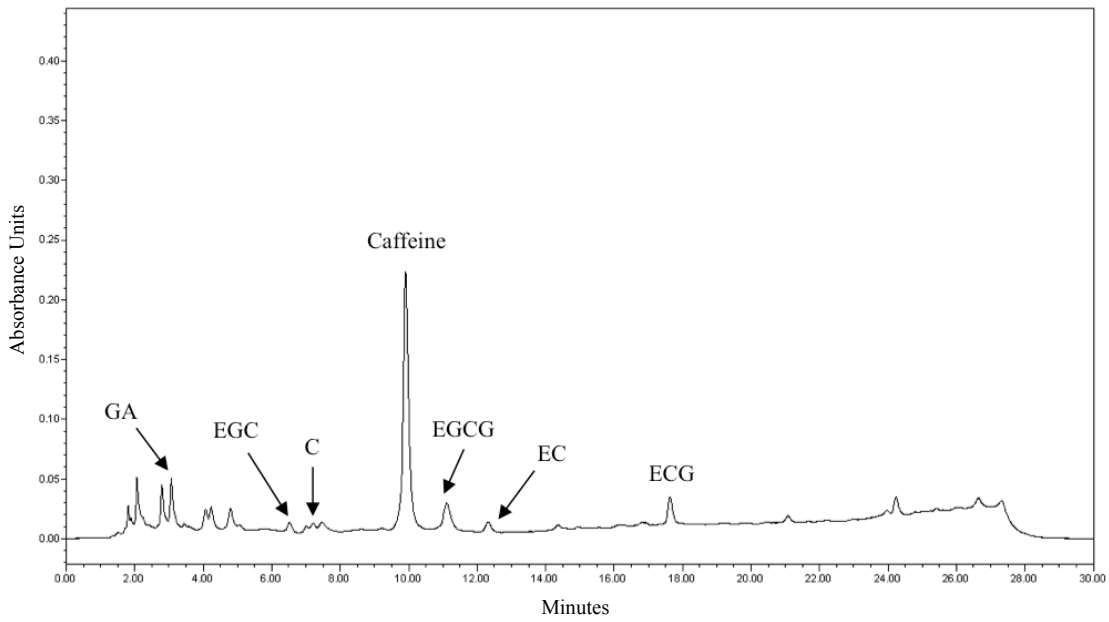


Figure 3.33. *HPLC profile of black tea BP-5 at 210 nm.*

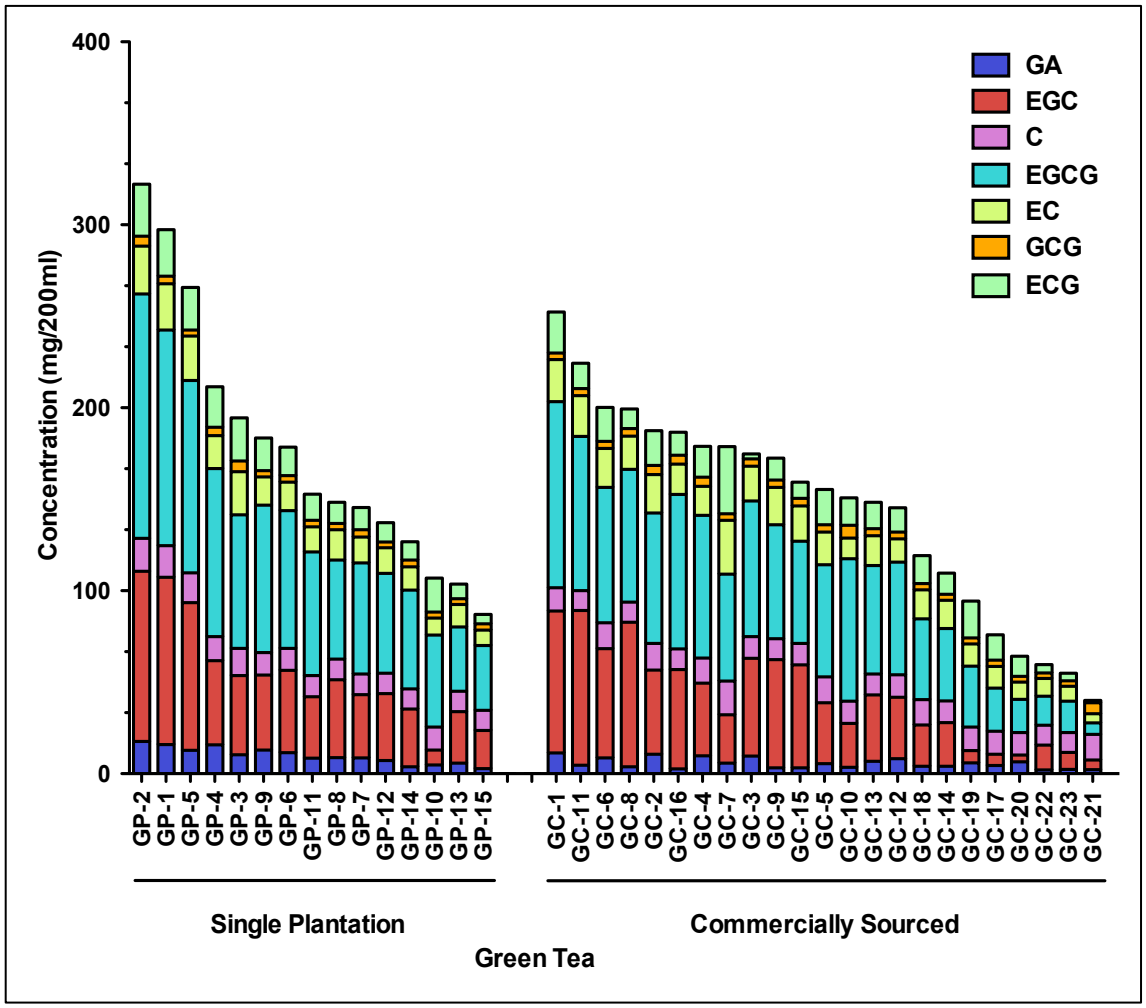


Figure 3.34. *Concentration of catechins and gallic acid in 38 green tea samples (n = 3).*

It is clear that the two most abundant catechins in green tea are EGCG and EGC; they are also the catechins that showed the most variation between the different samples (fig. 3.34). In contrast, the levels of GA, C and GCG were fairly consistent between all the green teas regardless of total catechin content (full results matrix in appendix I). The relative levels of each catechin and order of abundance, EGCG>EGC>ECG>EC>C>GCG, was comparable to that reported in other studies (Wang *et al*., 2003; Yao *et al*., 2005; Saito *et al*., 2006).

Individually, tea infusion GP-2 contained the highest catechin content with more than twice the average amount of EGCG (132.77 ± 0.86 mg/200mL) and EGC (92.12 ± 0.84 mg/200mL) of all the samples tested. Teas GP-2 and GP-5 also had higher than average

catechin content. Similar to GP-2, they are also single plantation teas originating from Kenya. This suggests that either the environmental conditions, processing or the variety of tea bush used in this region may be optimal for catechin production. However, to prove this hypothesis, more samples from this area would need to be investigated.

The teas with the lowest catechin content were found to be GC-21, GC-22 and GC-23. These teas are all green oolong teas, which have all undergone partial fermentation. While the fermentation of these specific samples is minimal, it is the most likely reason why these teas have considerably lower than average catechin contents. On the other hand, it cannot be ruled out that these teas had lower levels of polyphenols post-processing.

Table 3.3. *Comparison of the mean catechin, gallic acid and caffeine content (mg/200mL) of green (n=38) and black teas (n=36) by the Mann-Whitney U test (***=p<0.001; ns = not significant).*

	Green Tea	Black Tea	Significance
GA	7.27 ± 0.67	9.54 ± 0.62	ns
EGC	40.28 ± 4.02	2.11 ± 0.50	***
C	12.78 ± 0.32	10.72 ± 0.33	ns
EGCG	62.21 ± 4.57	14.18 ± 3.45	***
EC	16.35 ± 0.91	6.51 ± 0.58	***
GCG	3.98 ± 0.15	1.11 ± 0.26	ns
ECG	14.87 ± 1.19	5.86 ± 0.89	***
Caffeine	55.58 ± 3.00	61.75 ± 2.64	ns
Total Catechins	158.26 ± 10.30	50.01 ± 5.53	***

On average, black teas only had a third of the catechin content of green teas (table 3.3). This difference was shown to be significant (p<0.001) after performing a Mann-Whitney U test (U = 56; z = -6.66; r = 0.78). This was expected, as catechins are the building blocks of black tea polyphenols (Roberts, 1958). However, like green teas,

EGCG was found to account for most of the variation between black teas. Again, C and GA remained fairly consistent between black teas and were not found in significantly different concentrations to the green teas (table 3.3). This appears to support the current theory that C is not involved in black tea pigment formation, as thus far, black tea pigments containing catechin subunits have not been found (Tanaka *et al*., 2009a). The caffeine content between green and black teas was also not significantly different (table 3.3).

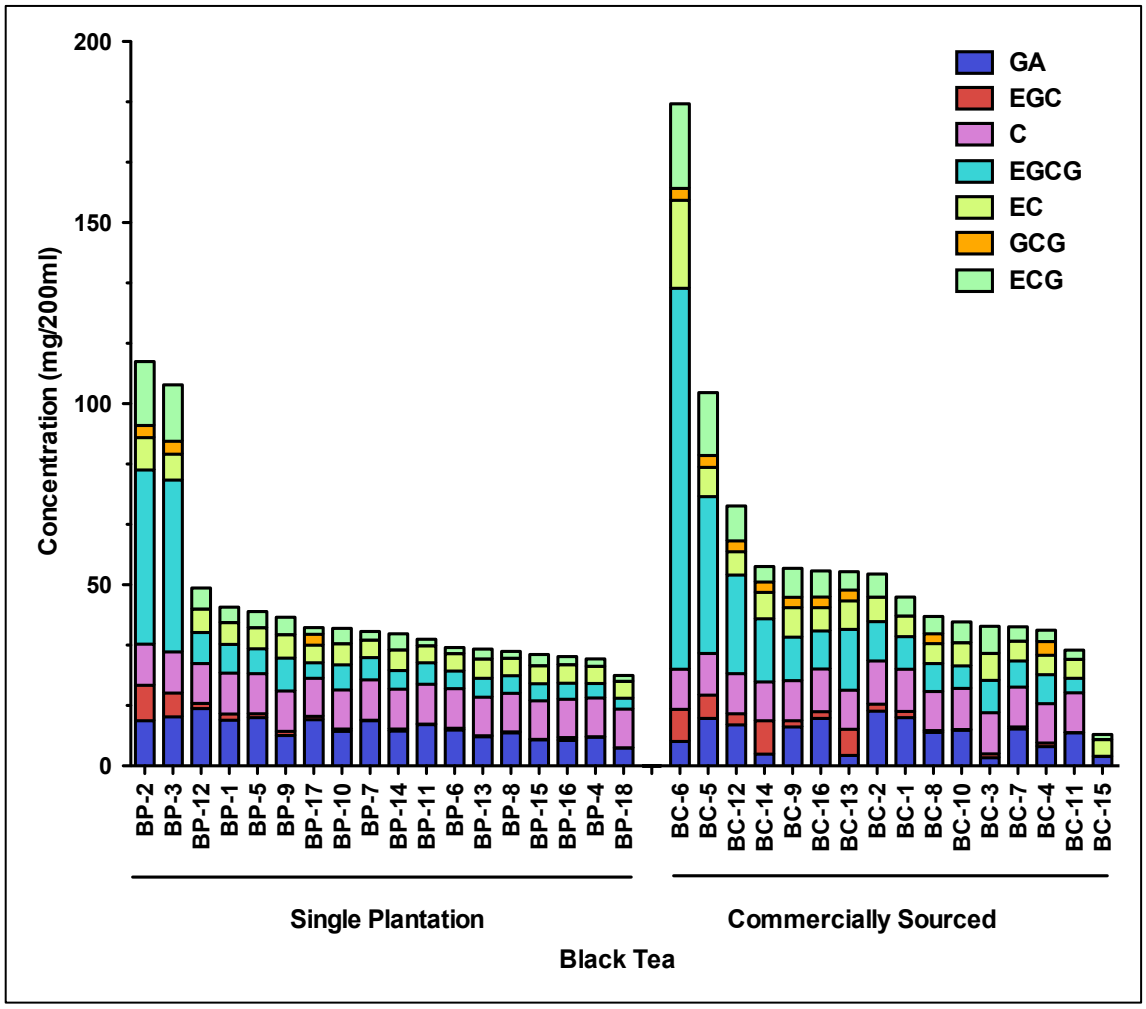


Figure 3.35. *Concentration of catechins and gallic acid in 34 black tea samples (n = 3).*

Collectively, the catechin profiles of black teas are relativity similar apart from a few examples. In particular, tea BC-6 was shown to contain more than three times the total catechin content of other black teas (182.8 mg/200mL). This is because BC-6 is a

commercial tea marketed for health purposes and is composed of green and black tea at a ratio of 1:2. In comparison with the other black tea samples and with reference to the average green tea catechin content, it is obvious that a large proportion of the catechins in this tea blend are accounted for by the addition of the green tea leaves. However, this tea did not stand out in the TPC and TFC assays highlighting the limitations of these assays. In addition to BC-6, black teas BP-2, BP-3, BC-5 and BC-12 also showed a substantially higher than average catechin content (fig 3.35). These four teas are of the Darjeeling variety, which means that they have been grown and processed in the Darjeeling district of West Bengal, India. As previously discussed, many factors may be involved in determining the catechin content of tea leaves; however of the tested samples, it is evident that Darjeeling teas have disproportionately higher catechin content than other black teas. Although no specific reference to the observation was made, studies by Astill *et al*. (2001), and Fernández *et al*. (2002), also show that Darjeeling teas have higher catechin content that other types of black tea. Again these samples did not stand out in the TPC and TFC assays. However, they did appear to have higher than average antioxidant power compared to the black tea samples in this study.

Another noticeably different black tea was BC-15. With only small concentrations of EC, ECG and GA, it had the lowest total catechin content (8.67 mg/200mL) of all the black tea samples. This can be explained by the processing of this tea; it is a Pu-erh tea variety that undergoes microbial fermentation after enzymatic fermentation is exhausted (Xie *et al*., 2009). This takes place over many years and ultimately produces teas that have little or no catechin content as seen with tea BC-15.

Although a significant difference was found between green and black teas ($p<0.001$), a Kruskal–Wallis one-way ANOVA showed that there was no difference between commercially sourced and single plantation teas (fig. 3.36), which was consistent with the findings of the colourimetric assays.

Grouping the green teas by country of origin and performing a Kruskal–Wallis one-way ANOVA showed that African teas contained significantly more catechins than those from China ($p<0.01$). However, there was no difference observed between any of the other groups (fig. 3.37).

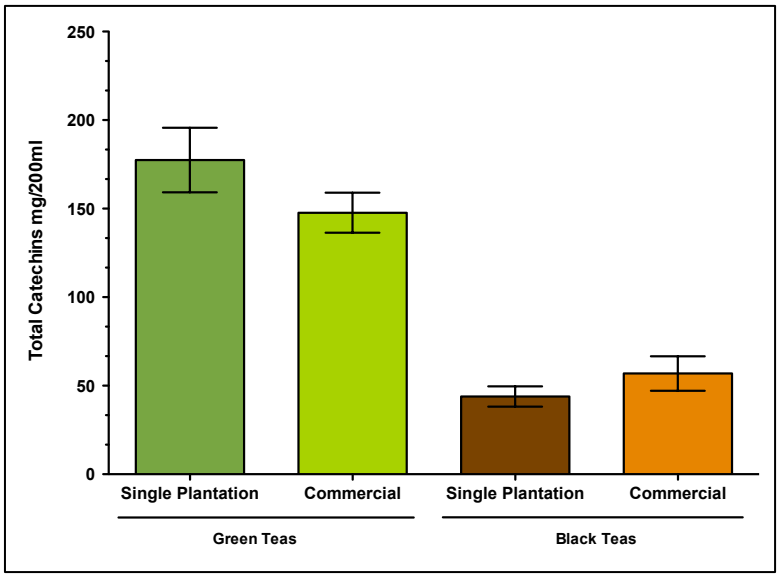


Figure 3.36. *Mean catechin content of single plantation green (n=15), commercially sourced green (n=23), single plantation black (n=18) and commercially sourced black (n=16) teas (error bars = SE).*

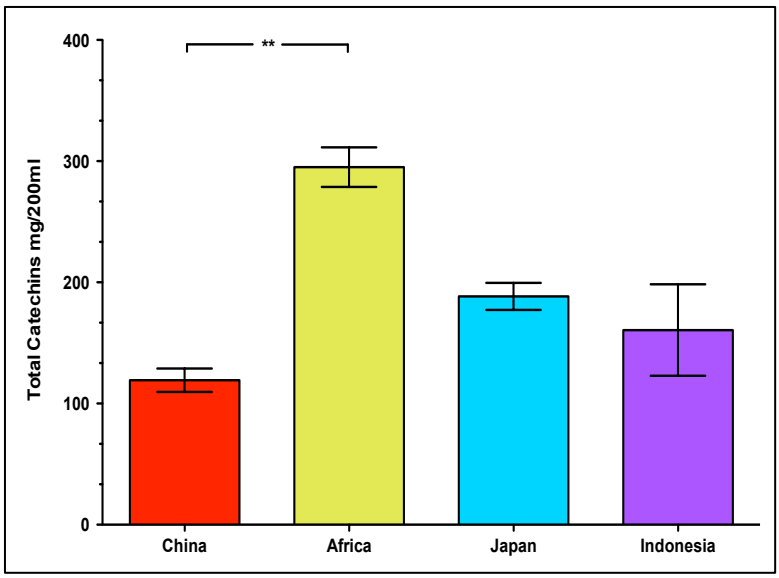


Figure 3.37. *Comparison of the total catechin content of green teas from China (n=15), Africa (n=3), Japan (n=5) and Indonesia (n=4) using the Kruskal-Wallis test (error bars = SE; **=p<0.01).*

African green teas had the highest concentration of gallic acid and all six catechins apart from GCG (fig. 3.38). The concentration of EGCG in African teas was particularly high (>120mg/mL); a characteristic which is seen of the *assamica* variety of plant (Yang *et al*., 2007). The opposite was true for Chinese green teas, which specifically showed a marked reduction in concentrations of EGC and EGCG (fig. 3.38). Japanese teas had higher levels of EGC in comparison to Indonesian teas (66.6 ± 6.3 vs. 35.9 ± 7.7 mg/200mL; ± = SE), however they had both had similar levels of other catechins. The findings of this analysis reinforce the conclusions of the colourimetric assays.

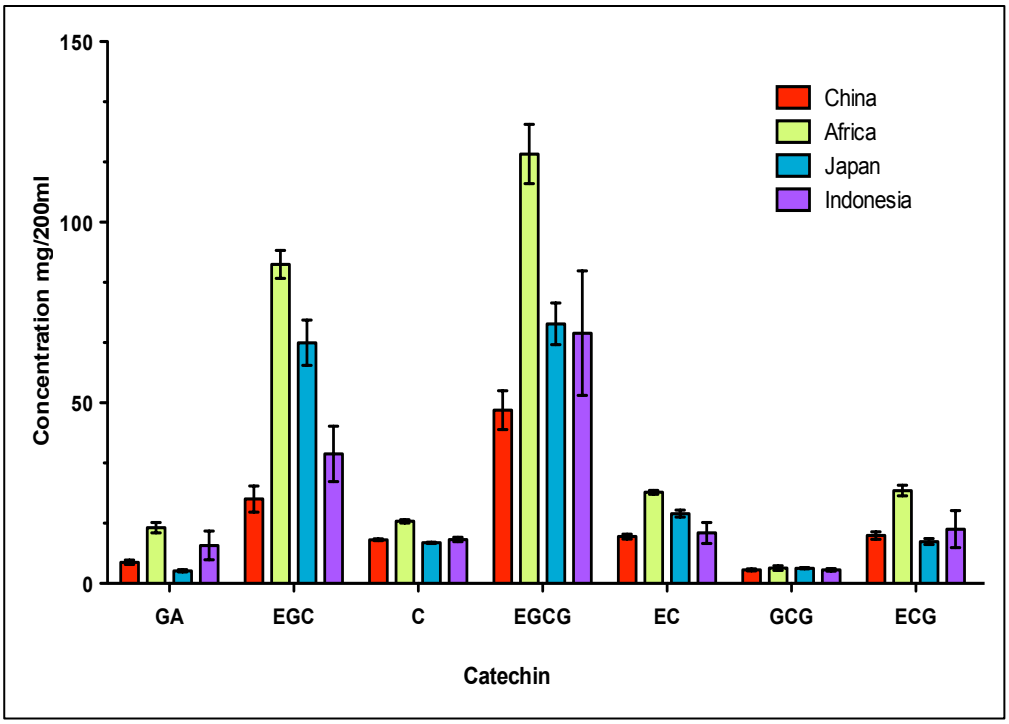


Figure 3.38. *Comparison of the individual catechin content of green teas from China (n=15), Africa (n=3), Japan (n=5) and Indonesia (n=4; error bars = SE).*

In contrast to the previous findings, a Kruskal–Wallis one-way ANOVA revealed no significant difference (p>0.05) in the catechin content of black teas based on country of origin (fig 3.39). It was previously found that Chinese black teas had significantly lower TPC, TFC and antioxidant power (section 3.4.1, 3.4.2 and 3.4.3). Therefore, this result

would imply that other flavonoids or black tea pigments rather than catechins in black teas are responsible for these differences.

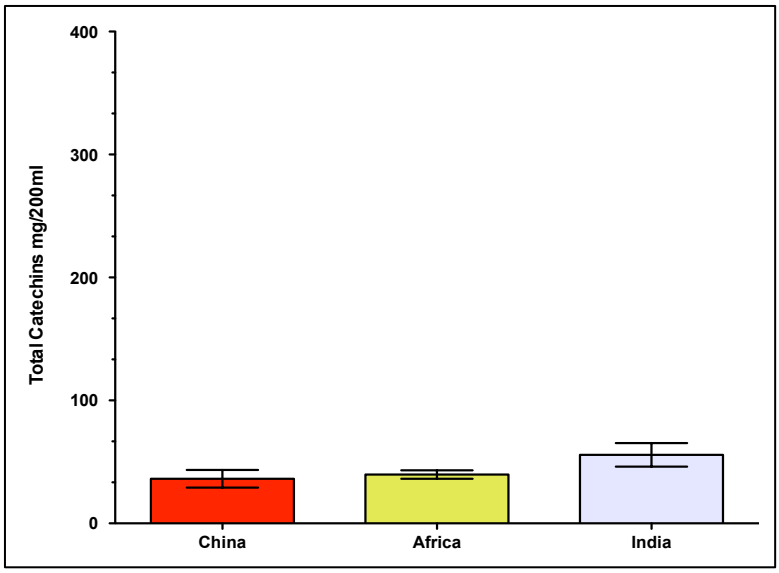


Figure 3.39. *Comparison of the total catechin content of black teas from China (n=5), Africa (n=5) and India (n=10) using the Kruskal-Wallis test (error bars = SE).*

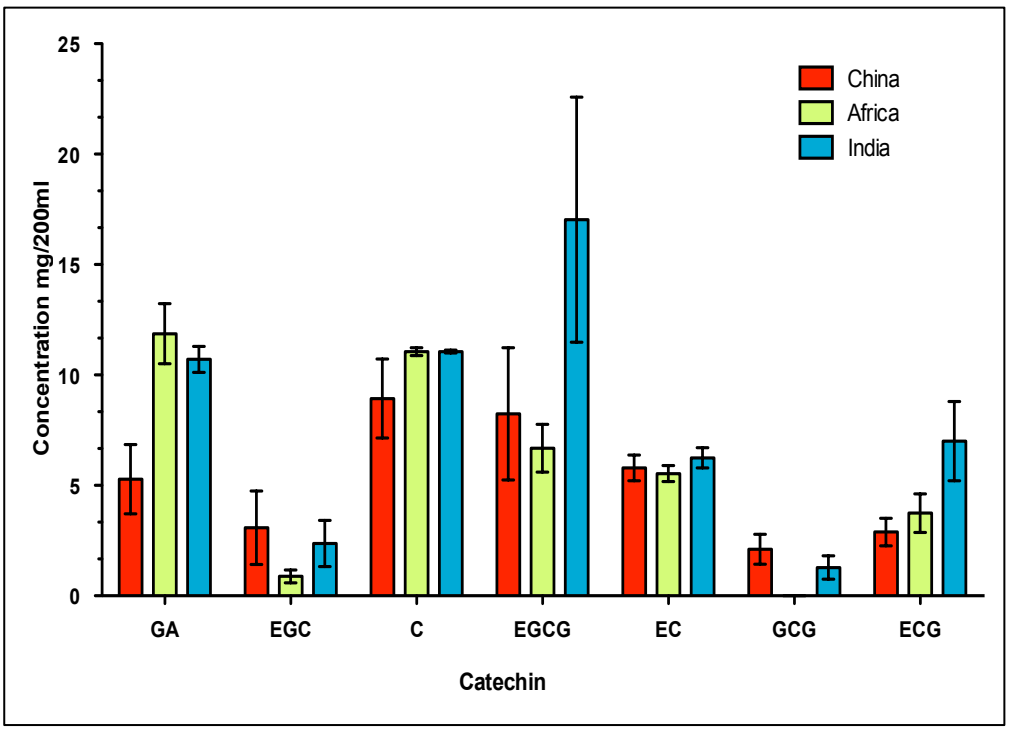


Figure 3.40. *Comparison of the individual catechin content of black teas from China (n=5), Africa (n=5) and India (n=10; error bars = SE).*

Reviewing the breakdown of the individual levels of catechins in each country, it was found that Indian teas have higher levels of EGCG and ECG. This variation is likely due to the influence of the four Darjeeling samples, which were previously shown to be catechin rich. In addition, African black teas contained no GCG and relatively low levels of EGC. This might be attributed to the method and extent of tea leaf processing at these cultivars.

3.4.5 Correlation of Colourimetric assays with total catechin levels measured by HPLC

The results of the HPLC analysis appear to complement the findings of the colourimetric assays. A correlation analysis was performed to determine the relationship between these assays. Pearson's bivariate correlation was used to determine the relationship between the assays for green teas, as all the data set met the assumptions detailed in chapter 2 (section 2.3.5). Conversely, the catechin content of black teas did not follow normal distribution. Therefore, the data was transformed using a log transformation, which was subsequently found to follow normal distribution.

Table 3.4. *Correlation of total catechins measured by HPLC in teas with their TFC, TPC, FRAP (n=72) and H_2O_2 production (n=24). Data represented as Pearsons r (r^r).*

Total Catechins	TPC	TFC	FRAP	H_2O_2
Green	0.86 (0.73)**	0.76 (0.58)**	0.89 (0.79)**	0.86 (0.74)**
Black[1]	0.41 (0.17)*	0.28 (0.08)	0.71 (0.50)**	0.27 (0.07)

[1] *Data transformed using a log transformation; **=p<0.01, *=p<0.05.*

For green teas, strong correlations were observed between total catechins (as measured by HPLC) and all four colourimetric assays (r >0.70; table 3.4). The strongest correlations were found between total catechins, antioxidant power, H_2O_2 production and TPC in green teas, which had a shared variance of 79 %, 74 % and 73 %, respectively. These strong correlations support the hypothesis that catechins are the

main anti/pro-oxidants and account for the majority of the polyphenols present in green tea infusions. This would be expected as catechins are the predominant polyphenols in green tea and make up 30 – 40% of the extracted solids in tea infusions, whilst other flavonoids only account for 5 – 10% (Graham, 1992).

In comparison, the total catechin content of black teas was weakly correlated to TFC (r=0.28; table 3.4) and H_2O_2 production (r=0.27; table 3.4). This would infer that H_2O_2 production in black teas is due to other components, possibly thearubigins and theaflavins. In addition, the weak correlation between TFC and catechins would suggest that other compounds are the predominant flavonoids in black tea. The flavonols quercetin and kaempferol are present in black teas at similar levels to that of green teas, as they are not affected by the fermentation process to produce black tea (Wang and Helliwell, 2001). Alternatively, it may be due to non-specific binding of black tea polyphenols to the assay reagent or differential extraction kinetics.

A moderate correlation (r=0.41; table 3.4) was found between the TPC and catechin content of black teas. This can be explained by the lower levels of catechins found in black teas and therefore the black tea polyphenols would account for the majority of the TPC.

Black teas were previously shown to have significantly less antioxidant power than green teas (fig. 3.19). Thus, the strong correlation between the catechin content of black teas and antioxidant power (r=0.71; fig 3.41) would imply that the black tea polyphenols are less powerful antioxidants.

3.4.6 Comparison of the Colourimetric assays with the HPLC analysis

The TPC assay would appear to be an accurate estimation of the total catechin content of green and black infusions. In table 3.5 it can been seen the catechin content of green teas measured by HPLC and the estimated TPC are in agreement, as catechins make up the majority of polyphenols in green tea (Graham, 1992).

The catechin content of black tea infusions is reported to be approximately 18 % dependant on the type of black tea (Graham, 1992). In this study it was found on

average that catechins made up approximately 38 % of the total phenolic content of black tea infusions. This slight over estimation may be due to the limitations of TPC assay or differences in extraction kinetics.

On the contrary, the TFC assay severely underestimated the catechin content in both green and black teas (table 3.5). Based on these results and the correlation analysis, it would appear that the TFC assay could be used to differentiate teas by catechin content. However, the value obtained from this assay may not reflect the true concentration in the tea infusion.

Table 3.5. *The mean TPC, TFC and total measured catechins (HPLC) of black and green teas (values expressed as mg/200ml).*

Tea	TPC	TFC	Catechins
Green	193.75	40.75	158.26
Black	136.25	32.35	50.01

3.5 Conclusions

The analysis of a substantial collection of single plantation and commercially sourced teas (n=72) has allowed the ability to perform a comprehensive statistical analysis of the following factors, TPC, TFC, FRAP and H_2O_2 production. This has shown that green teas on average have higher antioxidant power, produce more H_2O_2 at physiological pH (pH 7.4) and contain more catechins than black teas. It was also found that there is considerable variation in these properties within examples of the same type of tea.

From this data it would appear that the country of origin might play a key role in defining the chemical makeup of individual tea infusions. The factors responsible are likely to include the variety of the tea plant and the environmental conditions under which the plant is grown. To confirm the validity of these findings more tea samples from each country would need to be investigated and each sample would need to be supported by details of the tea variety, environmental growth conditions and processing method.

On an experimental note, this investigation has found that the colourimetric assays for measuring TPC, TFC, H_2O_2 production and antioxidant power are robust methods with which to determine relative differences in the properties of tea samples, as long as the limitations of their use are taken into consideration. The high throughput of these techniques also enables a rapid preliminary determination to be made of unique or representative examples prior to quantitative analysis using a method such as HPLC. Furthermore, this chapter has demonstrated the dual antioxidant/pro-oxidant activity of tea and has emphasised the need to account for the pH of the testing environment when determining the potential health benefits or antimicrobial properties of a tea infusion.

Ultimately, this chapter has quantified the attributes thought to contribute to the antimicrobial actives of tea. Thus, these findings will prove fundamental in interpreting the biological results of the following chapters.

CHAPTER 4

EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF TEA INFUSIONS

4. Evaluation of the Antibacterial Activity of Tea Infusions

4.1 Chapter Introduction

Tea infusions are known to contain compounds with antimicrobial properties, particularly against Gram-positive organisms (Ahn *et al.,* 1990; Ikigai *et al*., 1993; Taguri *et al.,* 2004). However, contrary to the abundance of research into specific tea compounds, particularly catechins, relatively few studies have compared and screened the antimicrobial activity of whole tea infusions. As a consequence there are currently no standard methods for testing the antibacterial activity of tea infusions or an understanding of how the catechins contribute to the antibacterial activity of tea.

4.1.1 *Staphylococcus aureus* as a Model Organism for Method Development

Before determining the sensitivity of *C. difficile* to tea infusions and individual polyphenols, an assay for accurately quantifying the antimicrobial activity needed to be developed. Unfortunately, *C. difficile* is a particularly difficult organism to culture (Hall and O'Tool, 1935) and for this reason, *Staphylococcus aureus*, a bacterium that is relatively simple to handle and manipulate, was employed to develop the various assays to be used in future studies.

S. aureus is a Gram-positive cocci that measures between 0.7 and 1.0 μm in size (Cowan and Shaw, 1954). They are non-motile bacteria that form grape like structures in culture and unlike *C. difficile*, do not form spores (Shaw *et al*., 1951). Described as facultative anaerobes, they can grow in both aerobic and anaerobic conditions on a variety of general culture media preparations (Murray *et al*., 2007). Clinically, they are a common cause of community-acquired sepsis and are one of the most prevalent nosocomial infections in the UK (Murray *et al*., 2007). In 2012 there were 8000 cases of *S. aureus* reported in England alone (HPAa. 2012).

Of particular interest to this study is the fact that the bacterium and its antibiotic resistant variants, *Methicillin Resistant Staphylococcus aureus* (MRSA), have been previously used by researchers to assess the antimicrobial effect of tea extracts and polyphenols (Chou *et al*., 1999; Hamilton-Miller and Shah. 1999; Hu *et al.,* 2002; Stapleton *et al*., 2004). This has helped to provide an understanding of the sensitivity of

S. aureus to tea compounds and undoubtedly has prompted further investigation into the sensitivity of other Gram-positive bacteria to tea.

In addition to the direct antimicrobial action, pre-treatment with tea polyphenols have been reported to reduce the oxacillin resistance of MRSA by interfering with penicillin binding protein 2a (Bernal *et al.,* 2009). In this study ECG was found to be the most effective tea polyphenol and had profound effects on the cell surface causing cell wall thickening and aggregation of cells (Bernal *et al.,* 2009). At a concentration of 12 mg/mL the MIC of oxacillin was reduced from 256 μg/mL to 4 and 16 μg/mL with ECG and EGCG respectively. However, the non-galloyl catechin EC had no effect on the MIC of oxacillin against MRSA (Stapleton, 2007), suggesting that the galloyl moiety is important for this function. It was also demonstrated in this study that ECG treated MRSA prevented biofilm formation; a key function that is required for the cells to adhere to surfaces.

In summary, *S. aureus* is an ideal model organism as like *C. difficile*, it is a Gram-positive bacteria, it is relatively easy to culture and has been shown to be sensitive to the antimicrobial properties of tea.

4.1.2 Methods for Determining the Antibacterial Activity of Tea

Tea infusions are known to contain antimicrobial compounds (Ahn *et al.,* 1990; Ikigai *et al*., 1993). However, dependence on qualitative susceptibility tests, such as the agar diffusion assay and a lack of robust standard methods to quantify the degree of antimicrobial activity, has made it difficult to compare the results of different studies and determine the antimicrobial profile of individual tea infusions (Ahn *et al*., 1991; Yi *et al*., 2010; Tariq and Reyaz 2012). To the best of our knowledge, quantitative antimicrobial testing has only been performed on dried tea extracts, obtained using extensive extraction techniques (> 30 min) with solvents such as ethyl acetate, ethanol or methanol (Ahn *et al*., 1991; Yam *et al*., 1997; Neyestani *et al*., 2007; Tahir and Moeen, 2011). To date, no standard assay has been developed to determine the minimum inhibitory concentration of aqueous tea infusions.

A further point that warrants consideration when designing antimicrobial assays is to determine the possibility of negative interactions between the active compounds and the constituents of the assay. For example, the biological growth medium used for testing tea infusions or polyphenols may contain elements that inhibit activity.

The addition of milk to tea is thought to have a detrimental effect on its health benefits due to the presence of casein proteins in milk, particularly *β*-casein (Lorenz *et al*., 2007), binding to the major tea catechins (EC, EGC, ECG and EGCG) by a combination of hydrophobic and hydrophilic interactions (Bourassa *et al*., 2013). The relevance of these observations to microbial culture media is that many preparations contain casein proteins to aid microbial growth. Hence, it is plausible that these compounds, particularly in solid based media, may affect the antimicrobial activity of polyphenols and tea infusions. Furthermore, other components such as heavy metals in the media may also interact with polyphenols, as they may instigate Fenton reactions resulting in polyphenol oxidation (Nakagawa *et al*., 2002). Therefore, it is essential that these factors are considered when attempting to design an assay which accurately and reproducibly determines the antibacterial activity of a tea infusion.

4.1.3 Ascertaining the Antimicrobial Compounds in Tea Infusions

4.1.3.1 Combining HPLC Analysis with Antimicrobial Activity

The chemical complexity of the tea infusion inherently makes it difficult to establish the contribution of individual compounds to the overall antimicrobial activity. To date the sole factors have not been determined. Further compounding this problem is the observation that tea polyphenols can act synergistically, potentiating the activity of other polyphenols (Sasaki *et al*., 2004).

At present, it is thought that the catechin polyphenols are the primary contributors to tea's antimicrobial activity, as they represent by weight, the major components in aqueous tea infusions and individually have been shown to possess antimicrobial activity (Ikigai *et al*., 1993; Chou *et al*., 1999; Sakanaka *et al*., 2000). However, other compounds in tea, such as the flavonols quercetin (Hamburger and Cordell, 1987), kaempferol (Koo *et al*., 2000) and myricetin (Bylka *et al*., 2004) have also been shown

to possess antimicrobial activity. Although present in smaller quantities than the catechins, these compounds represent 2 – 3 % of the aqueous tea infusion (Balentine *et al*., 1997).

Combining HPLC analysis, standardised antimicrobial assays and statistical analysis such as correlation and multiple regression, may help to determine the most important factors in tea and their contribution to the overall activity of the infusion. A similar approach was attempted by Sasaki *et al*., (2004). This study compared the HPLC profiles of fractionated oolong tea extracts in relation to their antimicrobial activity (Sasaki *et al*., 2004). The study reported that synergism was present between the main catechins, but due to the design of study and limited number of catechin combinations, it was not able to rule out if other components in the tea infusion may have contributed to the activity (Sasaki *et al*., 2004).

4.1.3.2 High Performance Counter Current Chromatography (HPCCC)

In addition to HPLC, non-destructive fractionation techniques such as high performance counter current chromatography (HPCCC) may also prove useful in determining active components of tea. Described as an orthogonal approach to HPLC, it has become an increasingly popular technique in natural product drug discovery in the last 10 years (Sutherland and Fisher, 2009), largely due to its ability to isolate large quantities of pure compounds from crude extracts. The application of this technique to tea extracts is relatively new, however a notable example of its use demonstrated the ability to separate gram quantities of pure EGCG and ECG (Baumann *et al*., 2001). The principals are the same as HPLC in that the crude mixture is carried in the mobile phase (MP) and then is separated in a stationary phase (SP) due to the different affinities of the individual compounds to the stationary phase (Sutherland and Fisher, 2009). Thus resulting in compounds eluting from the system at different times, and if a preparatory method is used, the individual peaks, detected by UV, can be collected as they elute. In HPLC, the SP is solid, usually a column of packed C-18 silica.

In HPCCC the SP is liquid and separation of individual components does not arise due to physical interaction, but on basis of the different partition coefficients (*Kd*) of the individual compounds in the mixture to the SP and the MP (Martin and Synge, 1941).

This is advantageous, as there is no loss of compounds due to physical adsorption onto the column (Sutherland *et al*., 1987). To aid the separation of the compounds in CCC and increase the contact of the compounds to each phase, the column (which is filled with SP liquid) is spun at high speed in a planetary motion (Sutherland *et al*., 1987). This results in hundreds of individual mixing and separation waves throughout the column, which can be controlled by the flow rate of the solvent, the temperature of the oven and the speed of the column (Sutherland *et al*., 1987). The MP then flows through the column in an 'Archimedes Screw' like fashion through the SP and is collected at time points defined by the user (Sutherland *et al*., 1987). Ultimately, using this technique, crude mixtures can be separated for further analysis or desired peaks can be extracted in large volumes. An overview of this system is shown figure 4.1.

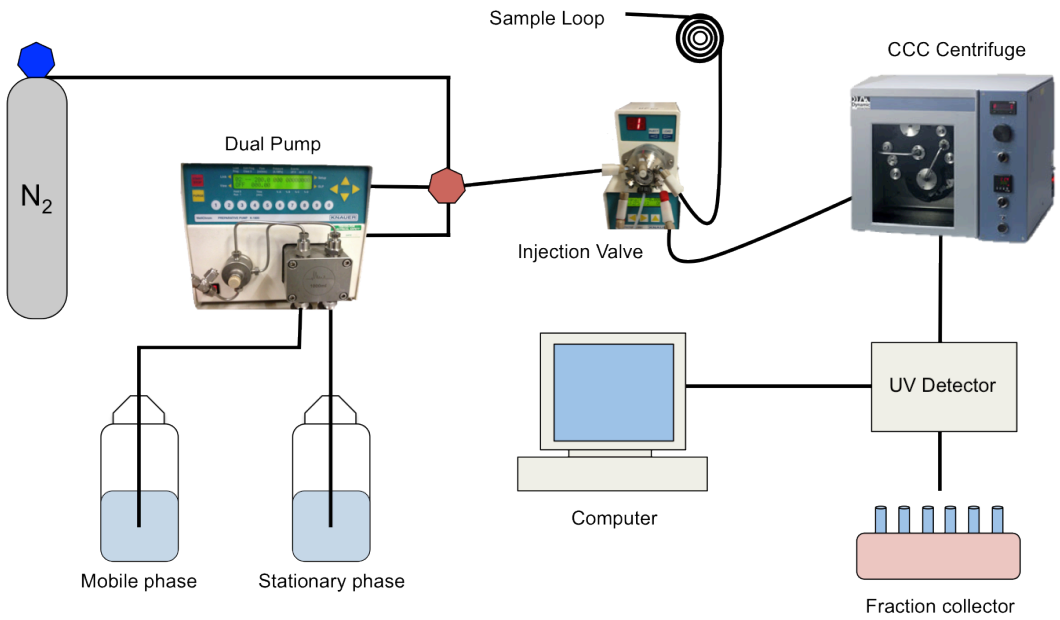


Figure 4.1. *A schematic diagram of HPCCC equipment and set up.*

Although HPCCC is a powerful tool for separating crude mixtures (Sutherland *et al*., 2007), selecting a solvent system to use in the apparatus is the limiting step and the major drawback to progression of the technology (Ignatova *et al*., 2011). The solvent

selection is crucial for successful isolation of compounds, as their interaction with the two phases is key to their separation (Sutherland *et al*., 1987). If a compound or groups of compounds only have affinity for one phase, they will not mix during the run and will therefore co-elute with other compounds (Martin and Synge. 1941). Therefore, to determine the ideal solvent system, the crude mixture must be tested in a wide variety of solvent systems in the HPCCC, often with limited success (Ignatova *et al*., 2011). Not only is this a labour intensive process, it is impractical if a limited amount of starting material is available.

In context, using this method to sub-fractionate tea extracts into its component parts in combination with HPLC and antimicrobial activity assays may be a powerful method to discover new compounds or attribute activity to known components. However, to achieve the potential of this technique, a method must be developed to ensure desired fractionation of compounds and selection of an appropriate solvent system.

4.1.4 The Involvement of H_2O_2 in the Antimicrobial Mechanisms of Tea Infusions and Catechins

The antimicrobial mode of action of catechins or tea infusions is not well understood, yet the body of evidence suggests that the likely targets are the outer structures of the bacterial cell such as the lipid membrane or cell wall, at least for Gram-positive bacteria (Stapleton *et al*., 2004; fig 4.2). It has been proposed that binding of catechins to peptidoglycan via hydrogen bonds, results in the degradation of peptidoglycan crosslinking (Yoda *et al*., 2004). It has also been observed that polyphenols with higher affinity for lipid membranes, such as catechin gallates, have more pronounced antimicrobial activity and therefore, exert their action by perturbing the membrane (Hashimoto *et al*., 1999). More recently, catechins have been shown to interact with bacterial DNA gyrase (Gradišar *et al*., 2007) suggesting that more than one mechanism of action may exist.

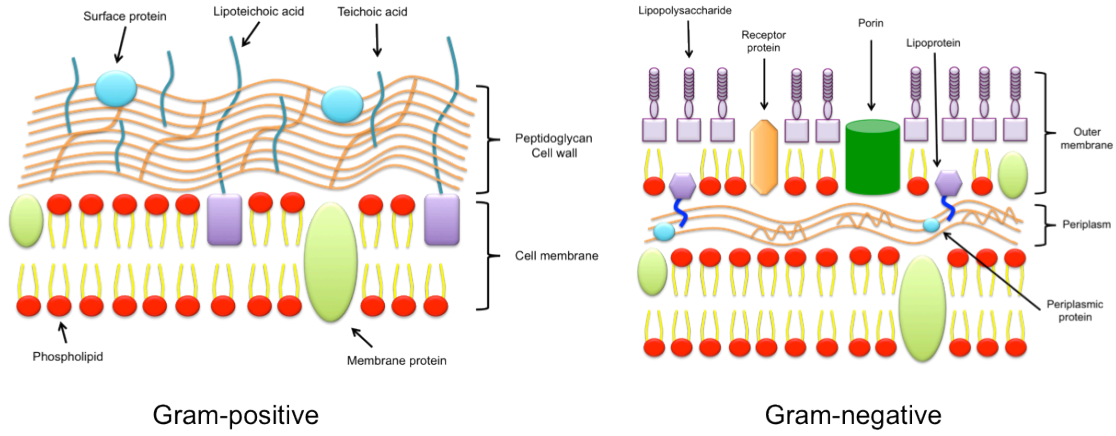


Figure 4.2. *The structure of the cell envelope of Gram-positive and Gram-negative bacteria. Adapted from Denyer et al. (2004).*

In addition to the direct interaction of polyphenols with bacterial components, it has been proposed that antimicrobial activity could be a consequence of hydrogen peroxide production by tea polyphenols and it is this, which is key to their antimicrobial action (Arakawa *et al*., 2004). A study by Arakawa *et al*. (2004), found that the most biologically active polyphenols generated the most H_2O_2 by the mechanism proposed in chapter 3 (section 3.1.1.4). They also reported that the addition of catalase, an enzyme, which degrades H_2O_2, eliminated the antibacterial activity of EGCG in a dose-dependent manner (Arakawa *et al*., 2004). This was supported by a previous study, which showed that H_2O_2 production was related to the presence of pyrogallol and galloyl moieties (Akagawa *et al*., 2003) of catechins, as it is known that these types of catechins have more potent antimicrobial activity (Sasaki *et al*., 2004). Furthermore, EGC, which is one of the most potent antimicrobial catechins, produces more H_2O_2 than other catechins (except EGCG) and yet has the lowest affinity for lipid bilayers (Nakayama *et al*., 2002). The galloyl and pyrogallol groups of the catechin EGCG is shown in figure 4.3.

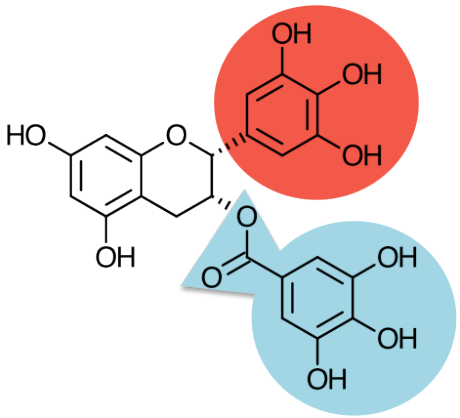


Figure 4.3. *The structure of EGCG. The galloyl moiety is highlighted in blue whilst the pyrogallol moiety is highlighted in red. Adapted from Bradfield and Bate-Smith, (1950).*

Contrary to these findings, a recent study has suggested that H_2O_2 is involved in the antimicrobial activity of catechins against Gram-negative bacteria but not Gram-positive bacteria (Cui *et al*., 2012). The study observed, using Atomic Force Microscopy (AFM) that *S. aureus* treated with catechins caused cell aggregation and distortion of the outer cell structure, a phenomenon that was also observed with MRSA by Taylor *et al*. (2009) using Scanning Emission Microscopy (SEM). However, when the bacteria were treated with H_2O_2, these changes were not found (Cui *et al*., 2012). Although, in the same study, it was found that H_2O_2 was most likely to be implicated in the antimicrobial mechanism of catechins against Gram-negative bacteria, as AFM images showed similar types of damage to the outer membrane of *E. coli* treated with H_2O_2 (Cui *et al*., 2012).

It is clear that there is uncertainty surrounding the involvement of H_2O_2 in the antimicrobial action of catechins. However, in the context of tea infusions, a complex mixture in which synergy is implicated (Sasaki *et al*., 2004), the role of H_2O_2 in their antibacterial action is unknown.

To help determine the antimicrobial mechanism of tea infusions, this study will attempt to investigate how H_2O_2 production contributes to activity and if structural derivatives of the pyrogallol or galloyl moiety in catechins can further improve the understanding of these underlying mechanisms.

4.2 Aim and Objectives

The aim of this chapter was to determine the antimicrobial activity of a comprehensive range of tea infusions and attempt to underpin their antimicrobial components.

The objectives of this chapter were to:

1) Develop a reproducible and accurate method for quantitatively determining the antimicrobial activity of tea infusions.

2) Quantify the antimicrobial activity of tea infusions and determine the difference in activity between green, black, commercial and single plantation teas.

3) Determine the relationship between the chemical properties, composition and the antimicrobial activity of different tea infusions.

4) Evaluate the relationship between chemical structure, hydrogen peroxide production and the antibacterial activity of catechin polyphenols.

5) Develop a novel method to fractionate tea using HPCCC to help the identification of the antimicrobial components in tea infusions.

4.3 Methods

4.3.1 Culture Preparation and Enumeration

4.3.1.1 Preparation of Fresh Bacterial Culture Slopes

A 10 mL volume of molten sterile tryptone soya agar (TSA) was added to a sterile 20 mL McCartney bottle and allowed to set at a gradient. The agar slope was inoculated with a loop of *S. aureus* (NCIMB 9581) obtained from a freshly thawed freezer culture and then incubated at 37 °C (Memmert Ltd., UK) for 24 h. The resulting culture was then checked for purity using the streak plate method (Ch. 2; section 2.3.2) followed by Gram staining isolated colonies (Ch. 2; section 2.3.3). Cultures were then stored in a fridge (2 ^{o}C - 8 ^{o}C) (Scandinova, Comet Ltd., UK) until further use. Fresh culture slopes prepared from frozen stocks every two weeks.

4.3.1.2 Determining the Concentration of Re-suspended Bacterial Cultures

Centrifuge tubes (Thermo Fisher Scientific Ltd., UK) containing 30 mL of tryptone soya broth (TSB) were inoculated from a bacterial slope culture using a 10 µL inoculation loop (Microspec, Ld., UK). The inoculated broths were incubated at 37 °C for 24 h in a shaking incubator set at 100 revolutions per minute (rpm) (Thermo Fisher Scientific Ltd., UK). After 24 h the broths were centrifuged at 2600 x*g* for 15 min (Avanti J-20 XP, Beckman Coulter Inc. UK). The supernatant was removed and the pellet was re-suspended in sterile PBS to produce a stock solution with an optical density of 0.8 as determined by a spectrophotometer (Ultrospec 3100 Pro, Amersham Biosciences Ltd., USA) set a wavelength of 600 nm (OD_{600}). The neat bacterial culture stock was diluted with PBS to produce a range of OD_{600} between 0.1 – 0.8. Simultaneously, a viable count of the colony forming units (CFU)/mL for each dilution was performed using the drop counting method by Miles *et al.,* (1938). The method by Miles *et al.,* (1938) was performed as follows. A 10 µL aliquot of each culture dilution was pipetted onto a sector of a circular TSA plate in triplicate and incubated for 24 h. Following incubation, colonies were counted on each sector that contained between 3 - 30 countable CFU's per spot and the average of the three spots was calculated.

The log CFU/mL was then calculated by the following equation:

$$Log\ CFU/mL = \left(\frac{mean\ CFU}{dilution\ factor}\right)$$

The results were then recorded into an Excel® spread sheet and an OD_{600} versus CFU/mL graph was constructed (fig 4.4). This produced the linear equation to predict the CFU/mL in a sample based on the optical density of the solution at 600 nm. This method was repeated five times.

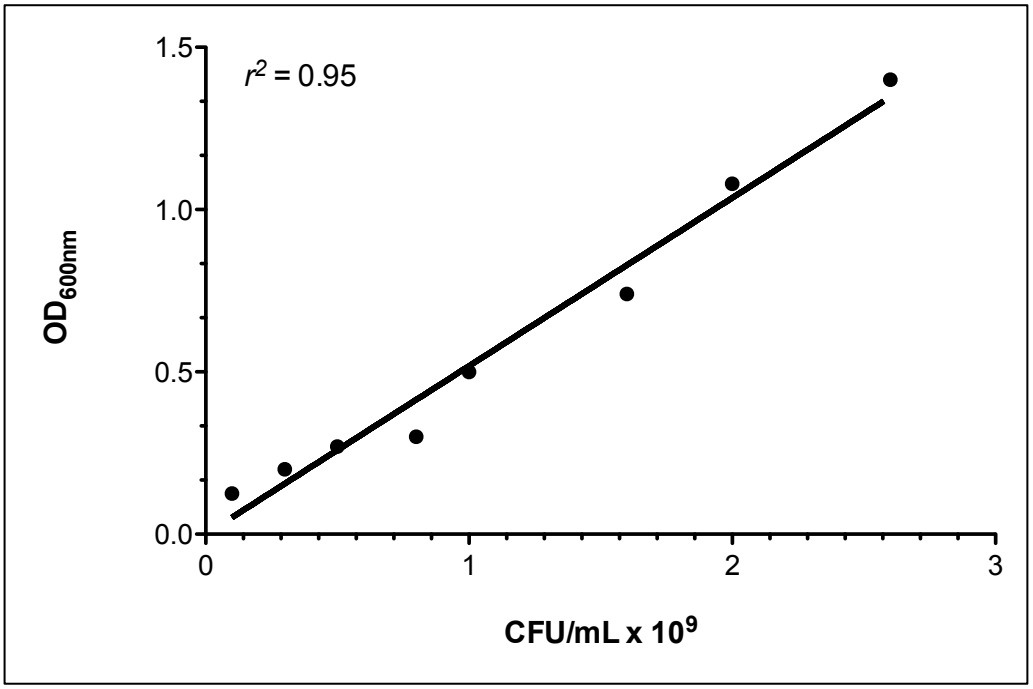


Figure 4.4. *An OD_{600} versus CFU/mL x 10^9 for S. aureus NCIMB 9518.*

4.3.1.3 Preparation of a Working Culture

Bacteria were cultured as described in section 4.3.1.2 and diluted in PBS to an OD_{600} indicative of a concentration 1 x 10^9 CFU/mL. This working culture was then further diluted in PBS using serial dilution to achieve the CFU/mL required for subsequent assays.

4.3.2 Developing a Method for Determining the MIC of Tea Infusions

All experiments in this section were repeated in triplicate unless otherwise stated. The MIC of ampicillin was tested simultaneously in each experiment between the concentration ranges of 0.015 – 0.48 %(w/v) in sterile diH_2O as a positive control. Tea infusions were prepared using the method previously described in chapter 2 (section 2.3.1).

4.3.2.1 Determining the MIC of Tea Infusions and Catechins Using the Broth Dilution Method

To each well of a 96 well microtitre plate, 20 µL of 10 times concentrated LB broth was added. A 1 x 10^8 CFU/mL bacterial suspension of *S. aureus NCIMB 9518* was prepared as described in section 4.3.1. The suspension was serially diluted and 10 µL of each dilution (1 x 10^3 to 1 x 10^8 CFU/mL) was added to each row of a 96 well plate. Next, 170 µL of EGCG at solutions between 0.18 – 2.4 mg/mL (diluted in sterile diH_2O) were added to each row in duplicate. The same procedure was performed for each tea infusion. Of each tea infusion, 170 µL of samples containing 100, 50 and 25 %(v/v) dilutions in sterile diH_20 were added. The plates were then incubated for 18 h at 37 °C on a shaking platform at 80 rpm. After 24 h wells were checked for growth by comparing the OD_{600} of each well to the negative and positive control wells. Negative control wells contained 10 µL of sterile PBS while positive control wells contained 10 µL of each bacterial inocula in 10 times concentrated LB broth.

4.3.2.2 Determining MIC of Tea Infusions by a Modified Agar Dilution Method

Neat tea infusions were serially diluted with sterile diH_2O to produce a concentration range between 3.13 and 100 %(v/v). To the wells of a 6 well cell culture plate (Corning, UK), 2 mL of each tea dilution was added with 2 mL of autoclaved and cooled (60 ^oC) double concentrated LB agar and left to set, which produced agar wells containing 1.6 to 50 %(v/v) of tea infusion agar (fig. 4.5). A standard inoculum of *S. aureus* was prepared as described in section 4.3.1 containing 10^4 CFU/10 µL. Three 10 µL inocula of *S. aureus* were pipetted onto separate areas of each tea agar well and the plate was incubated for 18 h at 37 ^oC. After incubation, wells were checked for the growth of colonies. The minimum concentration of tea incorporated agar that prevented growth of

bacteria after 18 h was recorded as the MIC of that tea infusion. The negative control was 2 mL double strength LB agar diluted with 2 mL of sterile diH_2O. Tea infusion plates and control plates were produced in triplicate. This method was adapted from the BSAC agar dilution MIC protocol (BSAC., 2012).

The appropriate statistical analysis as described in chapter 2 (section 2.4) was used to determine if there was a significant difference in the MIC of green, black, commercial and single plantation tea infusions.

The experiment was also repeated at the end of the study period to validate the stability of the stored tea leaf material.

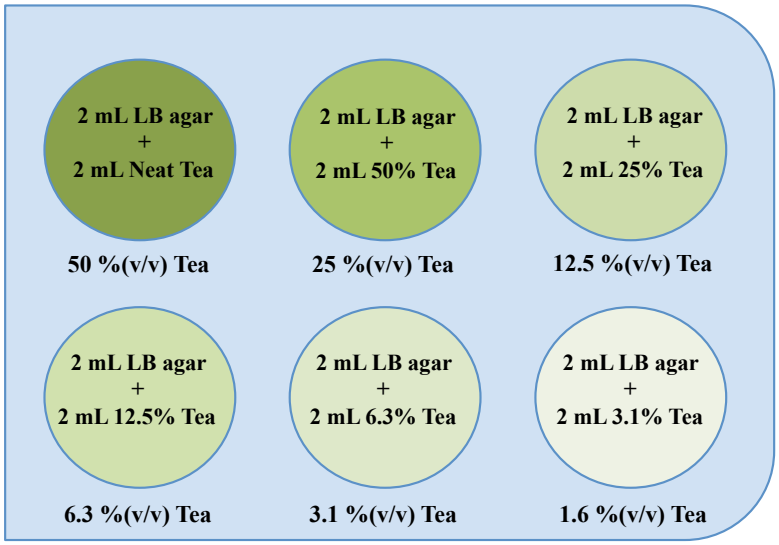


Figure 4.5. *Tea incorporated agar placed into 6 well culture plates at different concentrations.*

4.3.2.3 Determining MIC of Tea Components by a Modified Agar Dilution Method

Stock solutions of each catechin or tea components were prepared to achieve a concentration of 2 mg/mL in sterile diH_2O. Compounds with a solubility limit lower than 2 mg/mL were made up to the highest achievable concentration. The stock solutions were serially diluted in sterile diH_2O to produce a concentration range between 31.25 µg/mL – 1 mg/mL. Dilutions were made up to a volume of 2 mL. The agar dilution plates were produced and the MIC was determined as described in section 4.3.1.2. The negative control was 2 mL double strength LB agar diluted with sterile

diH_2O. Compounds with an MIC of 31.25 μg/mL were retested at a dilution range between 0. 97 – 31.25 μg/mL.

4.3.2.4 The Effect of Casein Hydrolysate on the MIC of Tea Infusions and Catechins

Agar dilution plates of tea infusions and catechins were produced as described in 4.3.2.2 with LB agar and with Muller-Hinton agar. To the LB agar plates, 0, 1, 1.75, 2 or 3 %(w/v) casein hydrolysate was added. The MIC was then determined as described in section 4.3.2.2. The negative control was 2 mL double strength LB agar diluted with sterile diH_2O at each casein concentration.

4.3.2.5 The Effect of Different Agars on the MIC of Tea Infusions and Catechins

Agar dilution plates of tea infusions were produced with LB, MH, Iso-sensitest®, Brucella, BHI and TSA agars and the MIC of tea infusions was determined using the method described in section 4.3.2.2. The negative control was 2 mL of each agar (double concentrated) diluted with sterile diH_2O.

4.3.3 Evaluation of the Relationship Between the Properties of Catechins and Tea Infusions with Antimicrobial activity

4.3.3.1 Determining the MIC of Gallic Acid Derivatives

The MIC's of gallic acid derivatives: 3,4,5-trimethoxybenzyl alcohol, shikimic acid, 3,4,5-trimethoxybenzoic acid, 3,4,5-trihydroxybenzamide, pyrogallol, gallacetophenone, 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid were determined using the method previously described in section 4.3.2.2. All compounds were dissolved in sterile diH_2O.

4.3.3.2 Determining the Relationship Between Hydrogen Peroxide Production from Tea Infusions and Antimicrobial Activity

The relationship between H_2O_2 production and antimicrobial activity was determined using the appropriate correlation analysis as described in chapter 2 (section 2.4).

4.3.3.3 The Effect of the Addition of Catalase to the MIC of Tea Infusions and Catechins

Tea infusion or catechin agar plates were prepared as described in section 4.3.2.2 with the following modifications. To each tea or catechin dilution, 100 µL of catalase solution (1000 Units/mL in sterile diH_2O) or diH_2O (negative control) was added. The MIC was then determined as described in 4.3.2.2.

4.3.3.4 Determining the Relationship Between Total Polyphenol Content, Antioxidant Power, Total Flavonoid Content, Hydrogen Peroxide Production, Total Catechins and the MIC of Tea Infusions

The relationship between TPC, FRAP, TFC, H_2O_2, total catechins and the MIC of tea infusions was determined using the appropriate correlation analysis as described in chapter 2 (section 2.4).

4.3.3.5 Determining the MIC of a Synthetic Copy of Tea Infusion GP-2

A synthetic version of tea GP-2 was made using the same quantities of GA (17.3 mg/mL), EGCG (132.8 mg/mL), ECG (28.6 mg/mL), EGC (92.1 mg/mL), EC (26.1 mg/mL), C (19.3 mg/mL) and GCG (5.5 mg/mL) in diH_2O found in GP-2 based on the HPLC analysis (appendix 1). The MIC of the synthetic tea was determined as described in section 4.3.2.2.

4.3.3.6 Determining the Relationship Between The Concentration of Individual Catechins and the MIC of Tea Infusions

The relationship between the concentration of individual catechins and the MIC of tea infusions was determined using the appropriate correlation analysis as described in chapter 2 (section 2.4). The relationship was also determined between galloyl and pyrogallol containing catechins and MIC.

4.3.4 Bioactivity Guided Solvent Selection for HPCCC

4.3.4.1 Preparation of Green Tea Extract

A methanol extract of green tea GP-2 was prepared as follows. To 500 mL of methanol, 25 g of tea was added and left to stir at room temperature for 24 h using a magnetic stirrer to create a vortex. The supernatant was then collected by vacuum filtration using Whatman® grade 1 filtration paper. The solvent was then removed using a Büchi Rotavapor R-124 (Büchi Ltd., UK) at room temperature and the remaining solid was stored between 2 – 8 °C.

4.3.4.2 Solvent Selection

Two-phase solvent systems were made using an automated Multiprobe II Plus liquid handling system (PerkinElmer Ltd., UK). Each system was made up to a total volume of 5 mL in test tubes (table 4.1). Next, the tea extract was prepared. To 5 mL of analytical grade methanol, 300 mg of a dried methanol extract of green tea GP-2 was dissolved. To each solvent system, 50 µL of the green tea solution was added and the test tube was manually shaken to aid distribution of the compounds between the two solvent phases. The solvent systems were then left to settle at room temperature for 10 min. After settlement, a 500 µL sample was taken from the upper phase and lower phase of each system and transferred to labelled HPLC vials in duplicate. The solvent in each vial was then evaporated using a solvent concentrator (Eppendorf® Concentrator 5301, UK). After evaporation, the vials were then stored between 2 – 8 °C until needed for HPLC analysis or biological activity screening.

4.3.4.3 HPLC Analysis of Solvent System Partitions

Samples of the lower and upper phase of each solvent system were analysed by HPLC using the method described in Chapter 3 (section 3.3.2.2). Standards of EGCG, EC, ECG, EGC, GA and caffeine were used to identify reference peaks in the chromatogram. The area under each peak was recorded for compounds in the lower and upper phase of each solvent system.

4.3.4.4 Bioactivity Screen of Solvent Systems Against *S. aureus*

4.3.4.4.1 Preparation of Bacterial Susceptibility Plates

Plates were prepared by adding 30 mL of sterile molten LB agar to sterile square petri dishes (100 mm x 100 mm; Sterilin Ltd., UK), which were left to set at room temperature. The agar plate was inoculated with 500 µL of *S. aureus* (1×10^5 CFU/mL) and evenly spread across the surface of the agar using a cotton swab under aseptic conditions. A cork borer with a diameter of 1 cm was sterilised with ethanol and a Bunsen flame. It was then used to bore 12 individual wells into the agar plate.

4.3.4.4.2 Bacterial Susceptibility Assay

The upper and lower phase samples of each solvent system were suspended in 100 µL of 2 %(v/v) dimethyl sulfoxide (DMSO) in diH_2O. Of each sample, 50 µL was pipetted into a well of a bacterial susceptibility plate in triplicate and labelled. A volume of 50 µL (2 %(v/v)) dimethyl sulfoxide was also pipetted into triplicate wells as a negative control. The plates were then incubated at 37^{o}C for 24 h. Following incubation plates were inspected for growth and zones of inhibition were determined using the following equation: *Zone of Inhibition = Total Diameter of Inhibition - Diameter of Well*

4.3.4.5 Preliminary HPCCC method

Based on the bioactivity assay, solvent system 5 was selected for a preliminary HPCCC run. The solvent system of heptane, methanol and water was made up to 1 L using the ratios shown in table 4.1 and mixed in a separating funnel. After vigorous shaking, solvents were left to settle until both phases become transparent and, therefore, equilibrated. Then the two phases were separated shortly before HPCCC separation. The upper aqueous phase (more polar) was used as the stationary phase and the lower organic phase (less polar) as the mobile phase, which corresponds to normal phase mode.

Table 4.1. *Composition of the solvents (mL) used in each solvent system to determine the ideal mobile and stationary phase for HPCCC analysis*

Solvent System	Heptane	Ethyl acetate	Methanol	Butanol	Water	Acetonitirile	Trifluroacetate	Acetic acid	Acetone	Tert butyl methyl ether	Dichloromethane	Ethanol	Propanol
1				2.50	2.50								
2		1.00		1.50	2.50								
3		2.00		0.50	2.50								
4		2.50			2.50								
5	0.36	2.14	21.40		0.36								
6	0.63	1.87	0.63		1.87								
7	1.00	1.50	1.00		1.50								
8	1.36	1.14	1.36		1.14								
9	1.79	0.71	1.79		0.71								
10	2.09	0.41	2.09		0.41								
11	2.38	0.12	2.38		0.12								
12	2.50		1.00			1.50							
13				2.48	2.48			0.04					
14				2.49	2.49			0.02					
15	0.13	2.37			2.48			0.02					
16	2.00		1.40		0.60						1.00		
17				0.59		0.88	2.37			1.16			
18	0.83	0.83			0.83				2.51				
19			2.00		1.33						1.67		
20	2.50					2.00					0.50		
21	1.88				1.56							1.56	
22	1.28	1.28			0.77							1.67	
23		2.25			2.25							0.50	
24		1.25		2.00	1.50							0.25	
25				2.22	2.22							0.66	
26	2.50				1.89								0.61
27	2.86		1.14		0.29								0.71
28						1.43	2.15			1.42			
29	0.63		1.24		0.63						2.50		
30	0.30		1.76		0.88						2.06		
31	1.75			1.75	2.50								
32													
33													

Total volume of solvent system made up to 5 mL.

To 3 mL of stationary phase (upper phase), 300 mg of methanolic tea extract GP-2 was dissolved and added to the 3.66 mL sample loop. In each HPCCC preparative separation run, the column was filled with the stationary (upper) phase in the head to tail mode into a Midi HPCCC system (Dynamic Extractions Ltd., UK). Then the mobile (lower) phase was pumped into the coil at a flow rate of 2 mL/min with a centrifuge rotational speed of 1600 rpm at 30 °C to equilibrate. When hydrodynamic equilibrium was established, the sample solution was injected into the coil and the flow rate was increased to 4 mL/min. The eluent was continuously monitored by connecting the tail outlet of the coiled column to an Algilent PDA detector set at wavelengths 250, 275, 310 nm. Fractions were collected every minute from the eluting solvent (4 mL per fraction). After the separation was completed, pressurised nitrogen gas was used to force out the stationary phase from the column. Stationary phase fractions were collected every minute (4 mL per fraction).

4.3.4.6 Bioactivity Screen of HPCCC fractions against *S. aureus*

Fractions of the eluting mobile phase were collected at time points corresponding to peaks on the UV spectra for antimicrobial testing. Of each fraction, 500 µL was pipetted into a vial and the solvent was evaporated using a solvent concentrator. The dried samples were then re-suspended in 100 µL of 2 %(v/v) DMSO. The antimicrobial activity was then determined using the method described in section 4.3.4.4.

4.3.5 Determining the Antibacterial Components of Green Tea using HPCCC

4.3.5.1 HPCCC Method (Normal Phase)

The HPCCC method described in 4.3.4.5 was repeated for the same methanol extract of green tea.

4.3.5.2 HPCCC Method (Reverse Phase)

The HPCCC method described in 4.3.4.5 was repeated for the same methanol extract of green tea, however, the mobile phase in the first run (lower phase) was used as the stationary phase and vice versa. This means that the eluting fractions in the first run

(section 4.3.5.1) now remained in the column whilst the compounds previously retained in the column were fractionated.

4.3.5.3 Bioactivity Screen of Normal and Reverse Phase Fractions

Fractions from the normal phase run (every minute from 20 – 64 min) and reverse phase run (every minute from 18 – 64 min) were selected for antimicrobial testing. Of each fraction, 500 µL was pipetted into a vial and the solvent was evaporated in a solvent concentrator. The dried samples were then re-suspended in 100 µL of 2 %(v/v) DMSO. The antimicrobial activity was then tested using the method described in section 4.3.4.4.

4.3.5.4 HPLC Analysis of HPCCC Fractions

The same HPLC method in Chapter 3 (section 3.3.2.2) was used to analyse the fractions collected in the normal and reverse phase HPCCC experiments. The chromatograph for every fraction was reviewed and the area of each peak was calculated. Fractions were collected every min, therefore, when looking at the HPLC chromatograms, from fraction 18 (time = 18 min) to fraction 64 (time = 64 min) the elution of each peak could be observed over time. This data was used to produce a fractogram. A fractogram quantifies the elution of compounds or a compound over time.

4.4 Results and Discussion

4.4.1 Development of a Quantitative Antimicrobial Assay for Tea Infusions

4.4.1.1 Evaluation of the Broth Dilution Method

Testing antimicrobial compounds using an MIC broth dilution assay is an alternative method to using agar dilution. The advantage of performing a broth dilution test is that it can be done on a 96 well microtitre plate and many concentrations of a potential antibacterial agent can be tested simultaneously. The compounds are added to the wells along with broth and a known inoculum of bacteria. The optical density (OD) of each well is read in a spectrometer at 600 nm before and after the plate is incubated overnight. The OD readings of each well pre-incubation are compared to the readings post-incubation. If the concentration of the antibacterial compound is less than the amount needed to inhibit the growth of the bacteria, an increase in OD would be seen post-incubation. Bacterial inhibition was determined as no change in OD and no visible bacterial growth.

Initially, using the broth dilution method, different concentrations of EGCG were tested against *S. aureus* and it was found following incubation that optical density in all wells had increased considerably (> 1 absorbance unit), even at the maximum concentration of EGCG tested (2.4 mg/mL). This was unexpected and closer examination of the wells revealed that there was a significant amount of precipitate in the solutions. At first, it was thought that the large volume of broth compared to the volume of EGCG solution might have caused the EGCG to precipitate out of the solution. Therefore, the experiment was repeated using a more concentrated (10 ×), but lower volume of broth (20 µL vs. 200 µL). By reducing the volume of broth, the final solutions in the wells were clear and no precipitate was evident. However, after incubation, the OD of each well had increased again, suggesting that EGCG had no inhibitory effect. However, media control wells (no EGCG or bacteria) had remained clear , and although the OD of the positive growth control wells (bacteria but no EGCG) had increased, the colour of the well had not changed, indicating that the wells containing EGCG were changing colour over time regardless of bacterial growth (fig 4.6).

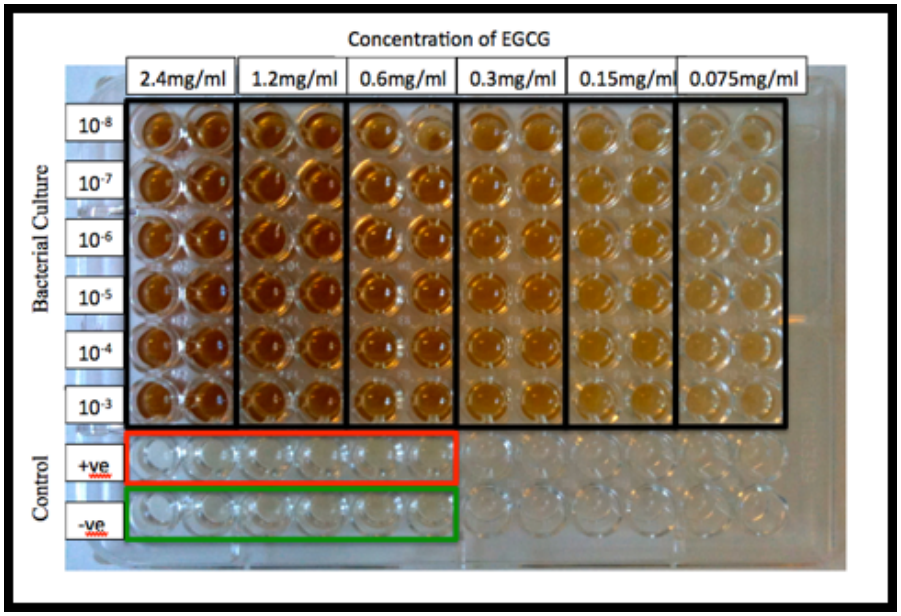


Figure 4.6. *A 96 well plate containing different concentrations of EGCG inoculated with S. aureus after 18 h incubation at 37 ℃.*

A similar effect was observed when testing both green and black tea samples. A possible explanation of this observation was the oxidation of polyphenols over time.

Since a change in optical density could not be used as a reliable indication of bacterial growth, an agar dilution based method was adopted instead.

4.4.1.2 Evaluation and Modification of the Agar Dilution Method

The agar dilution method is commonly used to determine the activity of antimicrobial agents. This method involves incorporating the antimicrobial agent in different concentrations into the agar, allowing it to set, then inoculating the surface of the agar with a known quantity of bacteria. With antibiotics or any other pure compounds, they can simply be added into cooled agar to create a stock solution of a desired concentration, which is then diluted with further agar to achieve different concentrations in the testing range (BSAC, 2012). The starting stock solution can be as high as dictated by the solubility of the compound. Therefore small, concentrated volumes of the antibiotic can be added to the agar without having any detrimental effect to its consistency. With aqueous tea infusions however, this is not achievable. When using a

standard aqueous tea infusion, the concentration of the infusion is determined by the initial brewing, therefore it cannot be further concentrated to achieve a higher stock solution at a lower volume. This is a problem, as when making a dilution series using the agar dilution method, only small amounts of the tea infusion could be added to the agar without disturbing its consistency. Consequently, the stock solution is so dilute that any antibacterial activity is likely to be quenched. To overcome this problem, double strength agar was used and the tea infusions were diluted with diH_2O to achieve a doubling dilution series. As a result, once 2 mL of tea dilution was added to 2 mL of double strength agar, the resulting agar mix retained the correct consistency and set after cooling.

This method proved to work well with good reproducibility when testing tea infusions and tea polyphenols (section 4.4.2). Therefore, throughout this investigation this modified agar dilution method was used to determine the MIC of any tea samples, polyphenols or related compounds.

4.4.1.3 The Interaction of Casein Hydrolysate with Tea Infusions and Polyphenols

It is important to determine which microbial media are suitable for assessing the antimicrobial activity of tea infusions. Many broths and solid media preparations for antibacterial testing and culturing contain casein or components of casein, such as casein hydrolysate, casein peptones and other digests of casein proteins. In agar, these casein components are usually found at concentrations between 1 and 1.75 %(w/v). As previously discussed, casein may interact with tea components and if these components are responsible for the antibacterial activity of tea, then any testing performed on media containing casein could potentially affect the results of the test.

To determine if the presence of casein hydrolysate in media affected the antibacterial activity of tea, different concentrations of the protein were added to LB agar and its effect on the antibacterial activity of selected green and black teas was determined (table 4.2). The following teas GP-1, GP-2, GP-3, BP-1, BP-2 and BP-3 were selected for this experiment as their catechin content was representative of the complete sample set (Ch. 3, section 3.4.6). LB agar was chosen as the base agar since it supports the

growth of *S. aureus,* is casein hydrolysate free and contains only the basic ingredients to support microbial growth.

Table 4.2. *The effect of the addition of different concentrations of casein hydrolysate %(w/v) to LB agar on the antimicrobial activity of tea infusions and ampicillin against S. aureus.*

	Added casein hydrolysate %(w/v)					
Tea	**0.00**	**0.50**	**1.00**	**1.75**	**2.00**	**3.00**
GP-1	6.3	6.3	6.3	6.3	12.5	50
GP-2	3.1	3.1	3.1	3.1	6.3	12.5
GP-3	12.50	12.50	12.50	12.50	50	>50
BP-1	50	50	50	>50	>50	>50
BP-2	25	25	25	>50	>50	>50
BP-3	25	25	25	>50	>50	>50
Control						
Ampicillin	0.03	0.03	0.03	0.03	0.03	0.03

Values represent the MIC of tea infusions (% v/v) and ampicillin (μg/mL) (n=3).

It was found that the MIC of green teas increased when the amount of casein hydrolysate present in the agar was 2 %(w/v) or greater. For black teas, which were less active than green teas, the antibacterial activity was inhibited after more than 1.75 %(w/v) of casein hydrolysate was added to the agar. Contrary to this, the antimicrobial activity of ampicillin was unaffected by the addition of casein. Therefore, this study highlights that casein hydrolysate, a common additive to microbial media, specifically interacts with antimicrobial compounds in tea infusions and should be considered when selecting an appropriate medium to test tea or related compounds.

To determine if this effect was due to the binding of casein to a specific polyphenol, 3 %(w/v) casein hydrolysate was added to LB agar containing either EGCG or GA (table 4.3). The assay revealed that the MIC of EGCG increased by 2-fold compared to the un-supplemented media whilst the antibacterial activity of gallic acid was completely eliminated. This would suggest that there is a stronger interaction between GA and the casein proteins than EGCG, or that its structure plays a key role in the

interaction. It may also suggest that the gallic acid group or the galloyl moiety of catechins is important for antimicrobial activity but not essential.

Table 4.3. *The effect of adding different concentrations of casein hydrolysate %(w/v) to LB agar on the antimicrobial activity of EGCG, GA and ampicillin against S. aureus. Values represent the MIC of tea compounds (% v/v) and ampicillin (μg/mL) (n=3).*

	Added casein hydrolysate %(w/v)	
Compound	**0**	**3**
GA	62.5	>1000
EGCG	125	250
Control		
Ampicillin	0.03	0.03

Given the effect of casein on the antibacterial activity of tea, it was important to determine if casein containing microbiological growth media had similar inhibitory effects. Therefore, the inhibitory activity of different green and black tea samples against *S. aureus* was determined using a variety of common aerobic and anaerobic media (table 4.4), as shown in table 4.5.

Table 4.4. *The casein components and concentrations (% w/v) in common commercially available bacterial growth media.*

Agar	Casein Component	Concentration %(w/v)
LB	Tryptone	1.00
TSA	Tryptone	1.50
Brucella	Peptone	1.00
BHI	Proteose petones	1.00
Mueller-Hinton	Hydrolysate	1.75
Iso-Sensitest™	Hydrolysate	1.10

LB = Lysogeny broth agar; TSA = Trytone soya agar; BHI = Brain heart infusion agar.

***Table 4.5.**. The effect of different media on the MIC of tea infusions (% v/v) and ampicillin (μg/mL) against S. aureus (n=3).*

	Agar					
Tea	**Mueller-Hinton**	**TSB**	**Brucella**	**BHI**	**LB**	**Iso-Sensitest™**
GP-1	6.3	6.3	6.3	6.3	6.3	25
GP-2	3.1	3.1	3.1	3.1	3.1	12.5
GP-3	12.5	12.5	12.5	12.5	12.5	50
BP-1	50	50	50	50	50	>50
BP-2	25	25	25	25	25	>50
BP-3	25	25	25	25	25	>50
Control						
Ampicillin	0.03	0.03	0.03	0.03	0.03	0.03

Muller-Hinton is the agar of choice for the susceptibility testing of antibiotics in the United States of America. This recommendation comes from the Clinical and Laboratory Standards Institute (CLSI), as they concluded that this agar is compatible with most antibiotics and supports the growth of a large number of microorganisms (CLSI., 2007). It contains approximately 1.75 %(w/v) of casein hydrolysate, therefore, based on the previous results, it was expected that an MIC value similar to LB agar supplemented with 1.75 %(w/v) casein hydrolysate would be attained for green and black teas. Although, this was true for green tea, it was not the observation for black tea, since the MIC values of black tea incorporated into this media did not change compared to the control of un-supplemented LB agar. This may be due to other components in the media that prevented the binding of casein to the antimicrobial compounds, as Muller-Hinton is a more complex mixture than LB agar. Similarly, in other media containing casein such as TSA, Brucella and BHI, the MIC of both black and green teas did not change in comparison to LB agar, suggesting that they could be potentially used for MIC testing.

Although, the MIC values of the teas were reproducible in most media, using Iso-Sensitest™, the most complex media, had a detrimental effect. In this media the MIC of green tea increased 4-fold whilst the activity of black tea was completely abolished. This is most likely due to the presence of trace metals in the media, which may cause

oxidation of polyphenols through Fenton reactions (Dai and Mumper, 2010). There was no change in the MIC of the antibiotic control, suggesting that this effect is limited to tea compounds. Iso-Sensitest™ is currently the media of choice for antimicrobial susceptibility tests in the UK, as recommend by the British Society for Antimicrobial Chemotherapy (BSAC, 2012). However, based on this study, it is unsuitable for testing the inhibitory concentration of tea infusions and related compounds.

It is evident that the antimicrobial factors in tea infusions can be affected by components in different biological media, however, the extent of this effect may be influenced by other unrelated components. In light of this, LB agar was used for all subsequent MIC testing of activity against *S. aureus*, as it does not contain any components known to interfere with tea or polyphenols. Furthermore, based on the MIC of ampicillin in each agar, it would appear that using LB agar, does not give the bacteria either an advantage or disadvantage when testing antimicrobial compounds. This is important, as it could be potentially argued that using a less rich media such as LB would put the bacteria at a disadvantage and therefore yield MIC values for compounds that are overestimated.

4.4.2 The Antimicrobial Activity of Tea Infusions

4.4.2.1 The MIC of Tea Infusions

A wide range of antibacterial activity was observed between the different tea infusions (table 4.6). Green teas had an mean MIC of 13.7 %(v/v) whilst the mean MIC of black teas was much higher at 40.8 %(v/v) (geometric means). The variation of MIC between the green tea samples was larger than the black tea samples, as the MIC ranged from 3.13 – 50 %(v/v) for green teas and 12.5 – >50 %(v/v) for black teas. The most active tea was a Kenyan green tea GP-2, which was also found to have the highest catechin content (322.2 mg/200mL; ± = SE). The other Kenyan green teas GP-1 and GP-5 also had very low MIC values (6.25 %(v/v)) and had above average catechin content (297.3 mg/200mL and 265.8 mg/200mL, respectively; ± = SE).

Table 4.6 *The MIC %(v/v) of green and black tea infusions against S. aureus on LB agar (n=3).*

Green tea		Black tea	
Code	**MIC %(v/v)**	**Code**	**MIC %(v/v)**
GP-1	6.3	BP-1	50
GP-2	3.1	BP-2	25
GP-3	12.5	BP-3	25
GP-4	12.5	BP-4	50
GP-5	6.3	BP-5	25
GP-6	12.5	BP-6	50
GP-7	12.5	BP-7	25
GP-8	12.5	BP-8	50
GP-9	12.5	BP-9	25
GP-10	25	BP-10	50
GP-11	25	BP-11	25
GP-12	12.5	BP-12	25
GP-13	12.5	BP-13	50
GP-14	12.5	BP-14	50
GP-15	12.5	BP-15	50
GC-1	6.3	BP-16	>50
GC-2	12.5	BP-17	>50
GC-3	12.5	BP-18	>50
GC-4	12.5	BC-1	25
GC-5	12.5	BC-2	25
GC-6	12.5	BC-3	25
GC-7	12.5	BC-4	>50
GC-8	6.3	BC-5	50
GC-9	12.5	BC-6	25
GC-10	12.5	BC-7	25
GC-11	12.5	BC-8	50
GC-12	12.5	BC-9	50
GC-13	12.5	BC-10	50
GC-14	25	BC-11	50
GC-15	12.5	BC-12	12.5
GC-16	12.5	BC-13	50
GC-17	25	BC-14	50
GC-18	12.5	BC-15	>50
GC-19	25	BC-16	25
GC-20	50		
GC-21	25		
GC-22	50		
GC-23	25		

Conversely, oolong teas GC-21, GP-22 and GC-23 were the least active green tea samples with MIC values of 50, 25 and 50 %(v/v), respectively. This is most likely due to the reduced catechin content, which on average was 58.2 ± 2.8 mg/200mL (± = SE), as the result of partial fermentation.

In contrast, the majority of black teas showed no activity at the highest concentration achievable (50 %(v/v)) or had an MIC of 25 or 50 %(v/v). Only one black tea had an MIC lower than 25 %(v/v), which was the Darjeeling tea BC-12 (12.5 %(v/v)). Surprisingly, this Darjeeling tea was not the most catechin rich black tea, which would suggest factors other than catechin content in this tea are responsible for its antimicrobial activity.

Furthermore, after repeated study during the investigation period (2010 – 2013), MIC's for all tea infusions remained consistent suggesting that no degradation of active components of the stored tea leaf material had taken place.

4.4.2.2 Comparison of the MIC of Tea Infusions by Type, Source and Origin

To compare the difference between the antimicrobial activity of green and black teas using statistical analysis, the MIC values were transformed using a log transformation. This is because the MIC values were obtained by a doubling dilution series and therefore will inherently follow a logarithmic scale, which is incompatible with the statistical tests.

Following a log transformation and after assessing the normality of the data sets, it was found that the data did not follow normal distribution, therefore the teas were compared using a Mann-Whitney U test. Teas that had MIC values of >50 %(v/v) were excluded from the analysis.

The test revealed that there was a significant difference in the MIC values between green and black teas (U= 107.5; z = -5.899; p <0.001; r = 0.73; fig 4.7). The results in the preceding chapter support the theory that this may be due to a significantly higher catechin content in green teas (section 3.4.4).

In support of these results, several other studies have shown that extracts of green teas contained more potent antibacterial activity than black teas, however the samples sizes in these studies were considerably lower than the numbers examined in this work (Neyestani *et al*., 2007, n= 2; Almajano *et al*., 2008, n = 10).

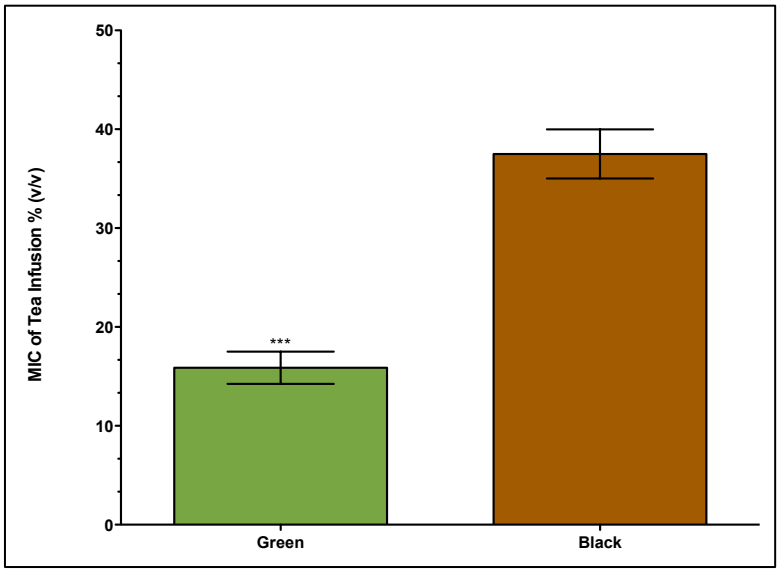


Figure 4.7. *Comparison of the antimicrobial activity of green (n=38) and black tea (n=29) infusions against S. aureus by the Mann-Whitney U test (***=p<0.001; error bars = 95 %CI).*

Comparison of the MIC values obtained with commercial and single plantation teas was conducted using a Kruskal-Wallis test, as the samples sizes were uneven and the data did not follow normal distribution. The analysis concluded that there was no significant difference in MIC between commercial and single plantation green teas (p>0.05; fig 4.8). There was also no significant difference in MIC between commercial and single plantation black teas (p>0.05; fig 4.8). These results were supported by the analysis of the catechin content of the two groups in chapter 3 (section 3.4.4).

To determine if there was any significant difference between the teas based on country of origin, the Kruskal-Wallis test was used due to the unequal sample sizes.

Green teas from China showed significantly less antimicrobial activity than green teas from other regions (p <0.01; fig 4.9). This is most likely due to their lower catechin content (Ch. 3; section 3.4.4). However, no significant differences in MIC were found

between black teas based on origin (p>0.05; fig 4.10). Though, it is important to note that 4 of the 6 Chinese black tea samples had no activity at all, which were not accounted for in this analysis. These teas also had lower TPC and catechin content than black teas from Africa and India (Ch. 3; section 3.4.4), suggesting that these compounds play the most important role in antimicrobial activity.

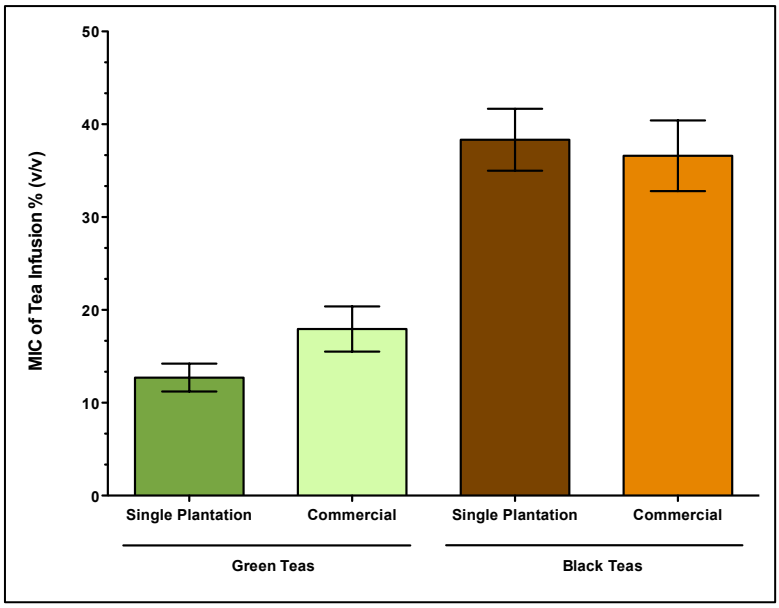


Figure 4.8. *The antimicrobial activity of commercially sourced green (n=23), single plantation green (n=15), commercially sourced black (n=12) and single plantation black (n=15) tea infusions against S. aureus using the Kruskal-Wallis test (error bars = 95 %CI).*

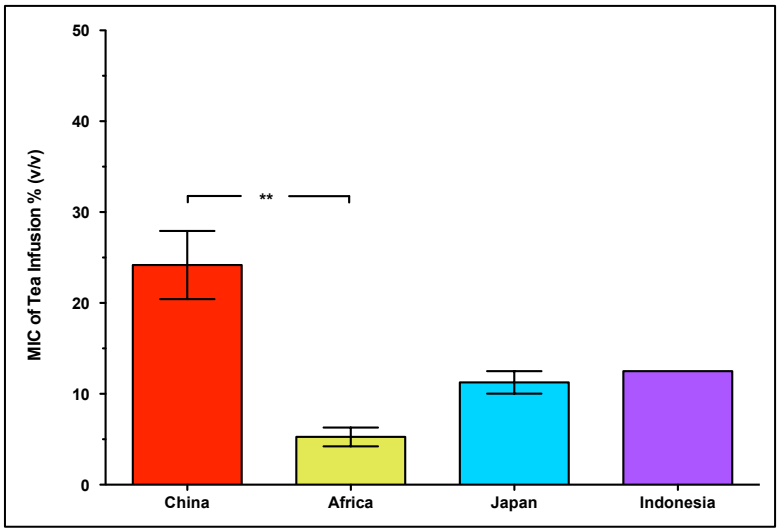


Figure 4.9. *Comparison of the antibacterial activity of green teas from China (n=15), Africa (n=3), Japan (n=5) and Indonesia (n=4) against S. aureus using the Kruskal-Wallis test (**=p<0.01; error bars = 95% CI).*

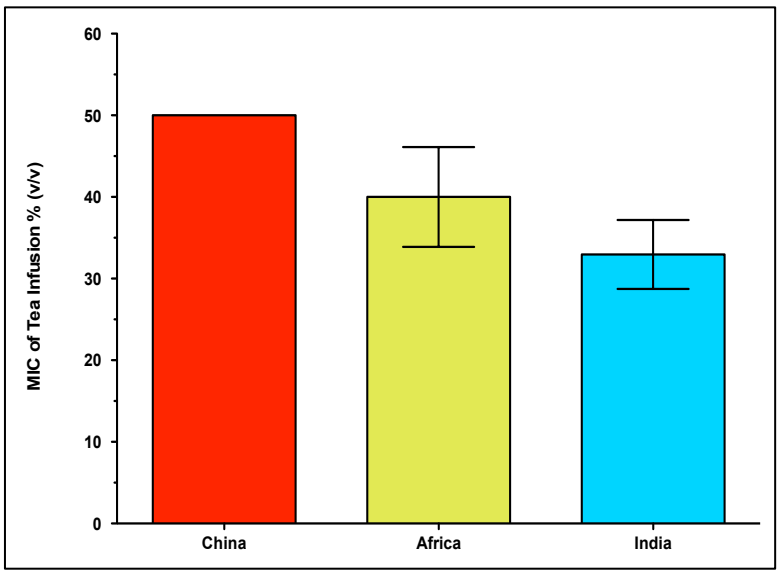


Figure 4.10. *Comparison of the antibacterial activity of black teas from China (n=2), Africa (n=5) and India (n=10) against S. aureus using the Kruskal-Wallis test (**=p<0.01; error bars = 95 %CI).*

It is evident that green teas possess significantly more potent antimicrobial activity than black teas, most likely due to their higher catechin content (Ch. 3; section 3.4.4). This would explain why green teas from Africa have particularly high antimicrobial activity,

as they were shown to have the highest catechin content of all the green tea samples (Ch. 3; section 3.4.4). It would also explain why there was no regional difference in antimicrobial activity seen in black teas, as the HPLC analysis revealed that their catechin profiles were not significantly different (Ch. 3; section 3.4.4). Therefore, since catechin levels appear to be good indicators of antimicrobial activity in tea infusions, it is important to determine their individual activity and to rule out other contributing factors.

4.4.3 The Antimicrobial Activity of Catechins and Tea Components

Tea infusions are a complex mixture of polyphenols, methylxanthines, amino acids and other molecules. As demonstrated, tea infusions have quite varied antibacterial activity, hence, it is important to understand which individual components may be responsible for this activity. The MIC values of the major catechins and other non-catechin components of tea infusions can be seen in table 4.7. Of the selected compounds, only the catechin polyphenols showed antibacterial activity. The most active components by weight were GA and the pyrogallol containing catechins (EGCG and EGC), followed by the EGCG epimer GCG and the catechin gallate ECG. Based on molar mass, the activity of the catechins and gallic acid follows the order EGCG>GA>EGC>GCG>ECG>EC=C. This is consistent with the findings of other studies (Hara, 2001; Stapleton *et al*., 2004). Furthermore, the MIC values obtained are also similar, supporting the transferability of the modified agar MIC assay (Stapleton *et al*., 2004; Hara., 2001).

Table 4.7. *The antibacterial activity of catechins and other tea components against S. aureus (n=3).*

Compounds	MIC (µg/mL)
Catechins	
Gallic acid (GA)	63
Epigallocatechin gallate (EGCG)	125
Epigallocatechin (EGC)	125
Epicatechin gallate (ECG)	250
Gallocatechin gallate (GCG)	250
Epicatechin (EC)	2000
Catechin (C)	2000
Methylxanthines	
Theobromine	>1000
Theophylline	>1000
Caffeine	>2000
Other components	
Coumarin	>500
Rutin	>500
L-Theanine	>2000
Caffeic acid	>2000

Although the methylxanthines are utilised for their potent respiratory and stimulant effects, they are generally not considered to be antibacterial agents. However, there is some evidence to suggest that at high doses (> 1 g/100 mL), they can inhibit the growth of Gram-negative bacteria such as *E. coli* (Ramanavièienë *et al*., 2003). As shown in table 4.7, none of the methylxanthines had antimicrobial activity below 2 mg/mL, which is a considerably higher concentration than would be found in tea infusions (e.g. average caffeine content = ~ 0.3 mg/mL; Ch. 3, 3.4.4).

4.4.4 The Relationship of Catechin Structure to Antimicrobial Activity and H_2O_2 Production

4.4.4.1 The Antibacterial Activity of Gallic Acid Derivatives

From the MIC study, it would appear that the structure of the catechins is key to their inhibitory activity. It has been previously suggested that the pyrogallol and to a lesser extent, the galloyl group is responsible for the antimicrobial activity of the catechins (Ikigai *et al*., 1993). To try and understand more about this relationship, the antibacterial activity of various gallic acid derivatives (fig 4.11) was determined (table 4.8).

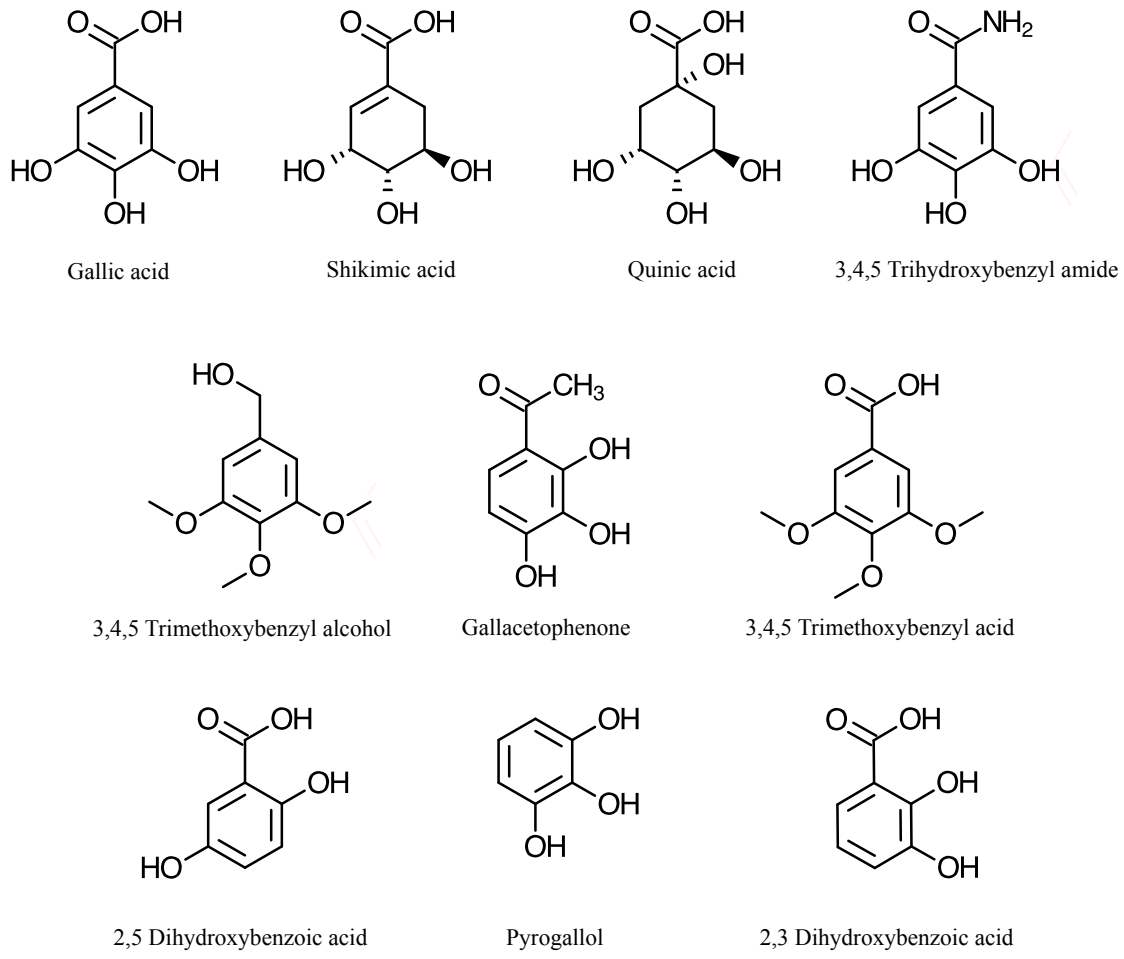


Figure 4.11. *The structure of gallic acid and similar compounds.*

Table 4.8. *The antibacterial activity (MIC) of gallic acid and similar compounds against S. aureus (n=3).*

Compound	MIC (µg/mL)
Pyrogallol	31
Gallic acid	63
3,4,5 Trihydroxybenzyl amide	63
Galloacetophenone	125
Quinic acid	>2000
Shikimic acid	>2000
2,3-Dihydroxybenzoic acid	>2000
2,5-Dihydroxybenzoic acid	>2000
3,4,5 Trimethoxybenzoic acid	> 2000
3,4,5 Trimethoxybenzyl alcohol	> 2000

Of all the tested compounds, pyrogallol was the most active followed by gallic acid, 3,4,5 trihydroxybenzyl amide and gallacetophenone. It is clear that the conjugated system of overlapping electrons in the benzene is important for activity, as quinic acid (a tea phenolic acid) and shikimic acid did not show any activity. Due to the bonds of these two compounds within the carbon ring, they lack the planar structure required for sharing pi electrons in order to create a conjugated system and will prevent the withdrawal of electrons from the hydroxyl groups and subsequent benzoquinone formation.

The tri-hydroxyl group is also an important feature for activity as when the tri-hydroxyl group is replaced with methoxy groups, or one hydroxyl group is missing, the activity is completely abolished. Furthermore, adding electron-withdrawing groups to the benzene such as –COOH (e.g. gallic acid), -$CONH_2$ (e.g. 3,4,5 trihydroxybenzyl amide) and -$COCH_3$ (gallacetophenone) reduces activity. This would suggest, as previously thought by Nakayama *et al*., (2002), that reducing the electron density in the benzene ring reduces the electron-donating ability of the compound resulting in attenuation of the

autoxidation cycle. In the case of gallacetophenone, the reduction in activity in comparison to other tri-methoxy compounds is likely due to the proximity of the ortho-position hydroxyl group in relation to the ketone group.

These results support the MIC of the tea catechins as C and EC, which only have 2 hydroxyl groups on the B-ring (Ch. 1, fig 1.5), have no antimicrobial activity, whilst EGCG and EGC, which have tri-hydroxyl groups are the most active. Furthermore, the catechin ECG, which only has a galloyl group, has less activity than EGC that only has the pyrogallol group. In addition, EGCG, which contains both groups, is the most active catechin.

This structure activity investigation agrees with a study showing that compounds such as pyrogallol with more reactive hydroxyl groups are more readily converted to quinones forming H_2O_2 as a byproduct in the autoxidation cycle (Akagawa *et al*., 2003). This would therefore support the theory that H_2O_2 is involved in the antimicrobial action of polyphenols (Akagawa *et al*., 2003). Furthermore, they showed that the pyrogallol moiety produced more H_2O_2 than the dihydroxyl pyrocatechol group and that the order of H_2O_2 production of the catechins was EGCG>ECG>GA>EGC>EC>C (Akagawa *et al*., 2003). This is similar to the order of activity of catechins by molar mass in this thesis and in other studies (Stapleton *et al*., 2004; Hara, 2001).

4.4.4.2 The Effect of Catalase on the Antimicrobial Activity of Tea and Catechins

To confirm the role of hydrogen peroxide in antibacterial activity the enzyme catalase which specifically converts H_2O_2 to O_2 and H_2O was added to green and black tea infusions. As shown in table 4.9, the addition of catalase completely abolished the antibacterial activity of the tea infusions. The addition of catalase also inhibited the antibacterial activity of EGCG and GA (table 4.9). A previous study by Arakawa *et al*. (2004) demonstrated the same effect with the catechin EGCG.

This provides evidence that the production of H_2O_2 is involved in the antibacterial mechanism of tea infusions. Therefore, based on these results, it would be rational to hypothesise that teas that produce more H_2O_2 would have greater antibacterial activity. To test the validity of this hypothesis, the relationship between antibacterial activity and

H_2O_2 production of tea infusions was compared using Pearson correlation (fig 4.12). It was found that there was a strong inverse correlation between MIC and H_2O_2 production (*r=- 0.66; p=<0.01*). In other words, tea infusions that produce more H_2O_2 have more potent antimicrobial activity. Although, correlation alone does not prove causation, the latter experiments with catalase support the interaction of these two variables.

Table 4.9. *The effect of catalase on the antibacterial activity of tea infusions, GA, EGCG and ampicillin against S. aureus (n=3). The MIC values for tea infusions are expressed in %(v/v) while the values for EGCG, GA and ampicillin are expressed in µg/mL.*

	Catalase (Units/mL)	
Tea	**0**	**1000**
GP-1	6.25	> 50
GP-2	3.13	> 50
GP-3	12.50	> 50
BP-1	50	> 50
BP-2	25	> 50
BP-3	25	> 50
EGCG	63	> 1000
GA	125	> 1000
Control		
Ampicillin	0.03	0.03

A correlation analysis was also performed between antimicrobial activity and H_2O_2 production for green and black teas separately. Due to the normality of the data sets, Person correlation was performed for green teas whilst Spearman correlation was performed for black teas. A strong inverse correlation was found between antimicrobial activity (Log MIC) and H_2O_2 production for green teas (r = -0.733; p<0.01) whilst there was poor correlation in black teas (r = -0.171; p = 0.59). This would suggest that H_2O_2 production is more important to the inhibitory activity of green teas than black teas. However, this would contradict the observation that the addition of catalase removes the inhibitory activity of both green and black teas. In light of this, it is possible the small

sample size (n=14) and poor distribution (highly skewed) of antibacterial activity of black tea may be responsible for this ambiguity.

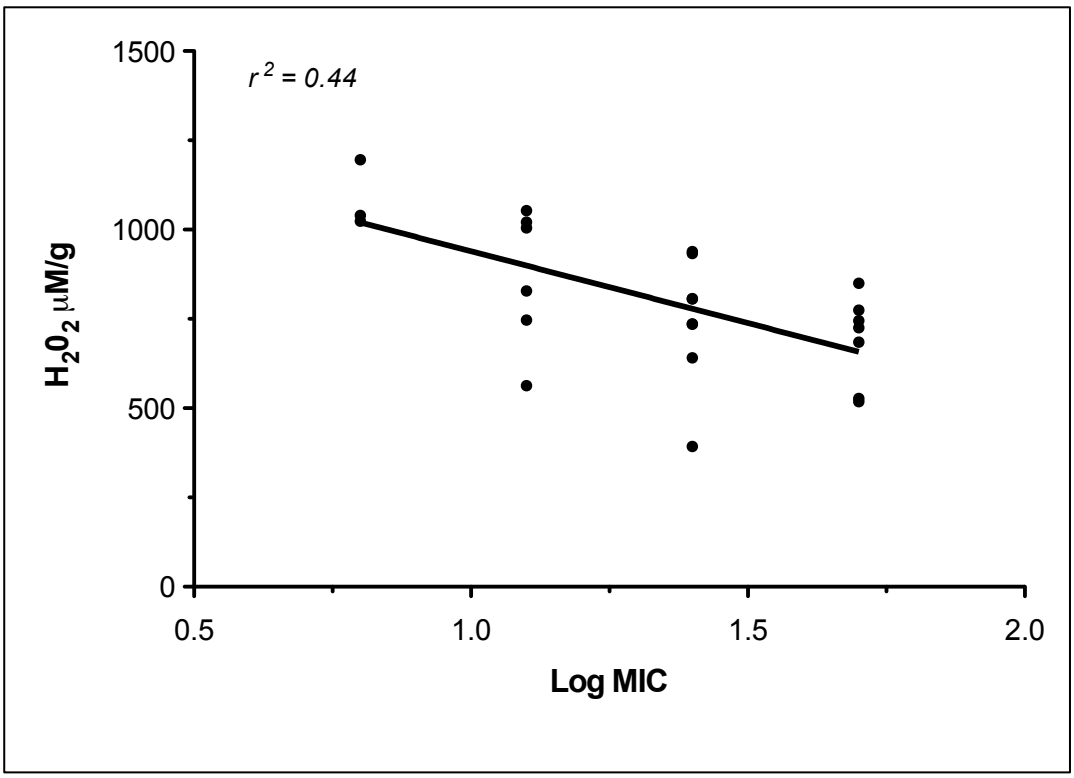


Figure 4.12. *Pearson correlation of antimicrobial activity (Log MIC) and H_2O_2 production (µM at 24 h) of green and black tea infusions ($p<0.01$; $r^2=0.44$; n=24).*

It was found in chapter 3 (section 3.4.2.2) that green tea infusions generated higher concentrations H_2O_2 than black teas, however this was only significant after 24 h. Furthermore, the rate of production between 0 – 12 h was similar for both types of tea. Since the inhibitory effect of teas is likely to occur before 12 h, it would suggest that amount of H_2O_2 produced does not fully explain the difference in activity between green and black teas.

It has been shown that catechins may insert themselves into the membranes of bacteria and can protrude into the cell cytoplasm (Sirk *et al*., 2008). Therefore, it is a possibility that local H_2O_2 production at these sites give rise to the antibacterial activity of tea. This would explain why black teas, which produce similar amounts of H_2O_2 over 12 h, are

less potent, as black tea polyphenols have lower affinities for bacterial membranes compared to catechins due to their high molecular weight (Sirk *et al*., 2011).

Overall, these studies would suggest while H_2O_2 production is essential for antibacterial activity of tea, the manner by which it contributes to this effect is yet to be determined. Therefore, understanding the contribution of catechins in the tea infusion to the overall antimicrobial activity of the infusion may help to provide an insight into the basis of the antimicrobial mechanism of tea infusions.

4.4.5 The Antimicrobial Components in Tea Infusions

4.4.5.1 Correlation of Log MIC with Total Catechins, TPC, TFC and Antioxidant Power

When comparing the antimicrobial activity to the chemical properties of tea infusions, it is clear from this study that trends exist and that polyphenols, most likely catechins, may account for the majority of antimicrobial activity seen. To test this hypothesis, a correlation analysis between the antimicrobial activity of green and black teas and the chemical properties of the teas (TPC, TFC, antioxidant power and total catechins) were performed to identify any significant relationships. Since black and green teas differ significantly in their chemical composition and in antimicrobial potency, separate correlation analyses of each groups was performed. Pearson correlation was used for green teas as the data met all the assumptions described in chapter 2 (2.3.5). Spearman's correlation was used for analysing black teas, as the data was not normally distributed. Teas with MIC values >50 %(v/v) were excluded from the analysis as their antimicrobial activity was outside the tested range (i.e. did not represent a valid integer).

Table 4.10. *Correlation of Log MIC, TPC, TFC, FRAP and total catechin content of green (n=38) and black (n=34) teas (**=p<0.01; *=p<0.05).* [1] *Pearson correlation;* [2] *Spearman correlation.*

Log MIC	TPC	TFC	FRAP	Total Catechins
Green[1]	-0.747**	-0.661**	-0.761**	-0.849**
Black[2]	-0.377*	-0.439*	-0.544**	-0.408*

The analysis showed a strong inverse correlation between Log MIC and TPC, TFC, FRAP and total catechins (table 4.10). Therefore, an increase in antimicrobial activity was seen when tea infusions have higher polyphenol content, flavonoid content, antioxidant power and more catechin polyphenols. In green teas the correlation between catechins and Log MIC would appear stronger than in black teas (-0.849 vs. -0.408). This would support the suggestion that other compounds in black tea, apart from the green tea catechins, such as theaflavins, contribute to antimicrobial activity (Betts *et al*., 2011). This correlation also supports the hypothesis that catechins are the predominant antimicrobial compounds in green tea, as the difference in concentration of catechins between the tea samples explains 72 % of the variance in MIC ($r^2=0.72$), which is very high. Furthermore, antioxidant power appears to be a good predictor of antibacterial activity for both green and black teas (table 4.10). This is in agreement with the observed H_2O_2 production and antibacterial activity of green teas, but not with black teas (section 4.4.4.2).

Overall, it would appear that there are stronger correlations with TFC, antioxidant power and TPC with regards to the inhibitory activity of green teas. However, to determine if these differences are significant, more black teas with a wider range inhibitory activity would need to be tested. The black teas in this study have a poor distribution of antimicrobial activity in comparison to green teas, which currently limits the interpretation of these correlations.

4.4.5.2 Synthetic Tea as a Model to Determine the Contribution of Catechins to Antimicrobial Activity in Green Tea Infusions.

To understand the contribution of catechins on the antimicrobial activity of green and black teas, the average concentration of each catechin present at the minimum inhibitory concentration of the tea infusions was determined (table 4.11).

Comparing these values to the inhibitory concentrations of the catechins in isolation (table 4.7), it is clear that they are present at sub-MIC concentrations in tea infusions. That is to say, when the tea infusions are diluted to the lowest possible active concentration, the levels of catechins are considerably below their individual antimicrobial concentration. This would suggest that the antimicrobial activity of tea

infusions is the sum of additive or synergistic interactions between catechins rather than the action of a single antimicrobial compound. However, although unlikely, it cannot be ruled out that the activity may be due to completely different compounds not accounted for in this study or that the catechins are interacting with other non-catechin compounds.

Table 4.11. *The mean concentration of catechins present in green (n=38) and black (n=34) tea infusions (µg/mL± SE) at their minimum inhibitory concentrations.*

	Mean Concentration of Catechin in Tea at the MIC of the Infusion	
Compound	**Green**	**Black**
GA	4.9 ± 0.5	20.9 ± 1.9
EGC	23.7 ± 1.6	3.3 ± 0.9
C	10.8 ± 1.3	24.2 ± 2.4
EGCG	39.5 ± 2.1	21.1 ± 3.7
EC	11.5 ± 0.7	13.4 ± 1.1
GCG	3.1 ± 0.3	2.4 ± 0.8
ECG	10.4 ± 1.0	10.3 ± 1.4

To strengthen the hypothesis of this thesis, that the catechins are the predominant antimicrobial factors in tea infusions, the catechin content of the most active green tea GP-2 was replicated using pure standards in water according to the results from the HPLC analysis (appendix I).

As predicted, a synthetic version of GP-2 consisting of EGCG, EC, C, EGC, ECG, GCG and GA at the same concentrations found in the original tea demonstrated the same level of antibacterial activity with an MIC of 3.13 %(v/v). This provided compelling evidence to suggest that these compounds are the sole factors responsible for the antimicrobial activity of green tea infusions. It also demonstrated that in this green tea, and most probably other green tea infusions, that antimicrobial activity is the sum of synergistic or additive interactions between these particular catechins.

Although synergy has been shown previously to exist with combinations of individual catechins (Sasaki *et al*., 2004), this is the first time to our knowledge that it has been shown to be responsible for the antimicrobial activity of green tea infusions.

4.4.5.3 Determining the Individual Contribution of Catechins to the Antimicrobial Activity of Tea Infusions

Multiple regression is a powerful statistical analysis that can be used to help determine the contribution of individual factors to a particular outcome (Tabachnick and Fidell, 2007). Since it has been demonstrated that the 6 catechins and gallic acid are likely to be the sole antimicrobial factors, it would seem an appropriate technique to determine the individual contribution of each catechin in the tea infusion to the antimicrobial activity.

Prior to building a multiple regression model, correlation analysis of each factor must be undertaken to determine if any factors, in this case catechins, show co-correlation. This is an essential first step, as strongly co-correlating factors cannot be used to build a robust multiple regression model (Tabachnick and Fidell, 2007).

The results of the correlation show a significant inverse correlation with the concentration of all the catechins and Log MIC, apart from C (table 4.12). In other words, an increase in catechin concentration, apart from C, in the green tea infusion would indicate a tea with more potent antimicrobial activity. Overall, this was expected, as the majority of catechins have individual activity. However, the strong inverse correlation of Log MIC with EC (-0.749; table 4.12) was unusual, as like C, it has negligible antimicrobial activity when tested alone (table 4.7). Therefore, this correlation may suggest that EC plays a synergistic role in the antibacterial activity of the tea infusion. Although, without further analysis such as multiple regression, this observation may be the result of other confounding factors. In support of this hypothesis is a study by Kajiya *et al*. (2002) observed that the presence of EC increased the incorporation of EGCG and ECG into lipid bilayers, suggesting that it potentiated the antimicrobial activity of these polyphenols. Furthermore, EC has also been reported to increase apoptosis in cancer cell lines in combination with EGCG, yet it had no activity alone (Suganuma *et al*., 1999)

Table 4.12. *Spearman correlation of catechin concentration and the antimicrobial activity (Log MIC) of green teas (n=38; **=p<0.01; *=p<0.05). Highlighted values in red show co-correlation (i.e =>0.7).*

	GA	EGC	C	EGCG	EC	GCG	ECG	Log MIC
GA		0.483**	0.535**	0.658**	0.537**	0.153	0.688**	-0.502**
EGC			0.122	0.835**	0.846**	0.492**	0.350*	-0.816**
C				0.347*	0.429**	0.371*	0.729**	-0.253
EGCG					0.735**	0.558**	0.624**	-0.764**
EC						0.457**	0.598**	-0.749**
GCG							0.244	-0.460**
ECG								-0.442**

The correlation analysis revealed that there are several co-correlating factors in the matrix. Since some of the factors in the model are so closely correlated, a technique such as multiple regression would not be able to adequately differentiate between the factors, meaning that this approach it is not suitable for determining how much contribution each catechin in the infusion makes to the antimicrobial activity. Generally, to overcome co-correlation, similar factors would be combined to form new factors and an analysis such as principle components would be implemented. However, in relation to this study, it would not make sense to combine factors, as it would not help answer the initial question of the study.

Although multiple regression cannot be used to determine the contribution of individual catechins in the tea infusion to the antimicrobial activity, correlation can be used to determine the relationship between antimicrobial activity and the ratio of pyrogallol or galloyl catechins in the green tea infusions.

Using spearman correlation, it was found that a high ratio of pyrogallol containing catechins (EGCG, GCG and EGC) in relation to the other catechins in green tea infusions is strongly correlated with more potent antimicrobial activity (*rho*= -0.739, *p*<0.01; n=38). Conversely, a high ratio of galloyl catechins (EGCG, GCG and ECG) did not show any correlation with antimicrobial activity (*rho*= 0.056, *p*=>0.05; n=38). This would suggest, as hypothesised in the MIC study, that the pyrogallol containing catechins are more active and are also more important for antimicrobial activity in tea infusions.

In summary, the body of evidence in this chapter supports the hypothesis that the catechins are likely to be the sole contributors to antimicrobial activity in green tea infusions. However, to help reinforce this hypothesis, HPCCC was used to confirm the identity of the active components and to rule out the presence of other contributory factors.

4.4.6 High Performance Counter Current Chromatography (HPCCC)

4.4.6.1 Bioactivity Guided Solvent Selection for HPCCC

High performance counter current chromatography is a powerful technique to fractionate samples into their component parts or to remove specific compounds from a crude mixture (Sutherland and Fisher, 2009). In this study, HPCCC was used to fractionate an extract of green tea, GP-2, to help determine which compounds in the extract are responsible for antimicrobial activity.

Before HPCCC was used to fractionate the green tea sample, a suitable solvent system had to be selected (Ignatova *et al*., 2011). To overcome the trial and error nature of this selection process (Ignatova *et al*., 2011), a biological based assay was designed to select the ideal solvent system to separate the tea extract in the HPCCC. For an ideal run, the active components need to have affinity for both solvent systems. Therefore, by adding the tea extract directly to a 2 phase solvent system and then testing the upper and lower phase for antibacterial activity the presence of active components in both phases could be determined. Complementary to this process, HPLC was used to determine the distribution ratios of each identified peak in the crude mixture within the two phases. In table 4.13 (a-c) the distribution ratios of each identified peak in each solvent system are shown with the presence (green) or absence (red) of antimicrobial activity in each phase, as determined by bacterial susceptibility well assays.

Table 4.13a. *The solvent systems screened in this study to determine a system suitable for analysing tea by HPCCC. Green columns indicate antibacterial activity against S. aureus in that layer whilst red indicates no activity. For each compound, the area under the peak is listed for each phase. UP = upper phase, LP = lower phase*

Solvent System	Compound	UP Area	LP Area
1	GA	133870	1021037
	C	561812	102447
	EGCG	17735688	882929
	EC	1335551	94330
	EGC	7363257	149795
	ECG	803650	18842

Solvent System	Compound	UP Area	LP Area
2	GA	1368912	0
	C	838820	0
	EGCG	27926596	0
	EC	1874608	0
	EGC	10023040	0
	ECG	1106006	0

Solvent System	Compound	UP Area	LP Area
3	GA	156031	0
	C	1454340	0
	EGCG	30362850	4616
	EC	2780192	0
	EGC	12127568	0
	ECG	2210482	0

Solvent System	Compound	UP Area	LP Area
4	GA	367339	1299758
	C	529104	520009
	EGCG	22101244	2617357
	EC	1374456	402933
	EGC	8479784	698779
	ECG	911100	143562

Solvent System	Compound	UP Area	LP Area
5	GA	7816	1976086
	C	396069	1137754
	EGCG	8367722	19696724
	EC	1038894	1962487
	EGC	7218673	4237197
	ECG	1197372	1215634

Solvent System	Compound	UP Area	LP Area
6	GA	0	62155
	C	131861	7014
	EGCG	2900601	486853
	EC	414892	31083
	EGC	3677483	122433
	ECG	424104	10959

Solvent System	Compound	UP Area	LP Area
7	GA	0	1597255
	C	0	781865
	EGCG	64355	22863735
	EC	18573	1788958
	EGC	90537	8844139
	ECG	0	927723

Solvent System	Compound	UP Area	LP Area
8	GA	0	1611846
	C	0	690741
	EGCG	4323	2809380
	EC	0	1864744
	EGC	10672	9101989
	ECG	0	938120

Solvent System	Compound	UP Area	LP Area
9	GA	245	1513561
	C	0	779997
	EGCG	2326	26461533
	EC	0	1727457
	EGC	7361	8487831
	ECG	0	900348

Solvent System	Compound	UP Area	LP Area
10	GA	140437	1023936
	C	0	720431
	EGCG	16660	10720439
	EC	0	1114102
	EGC	0	4518224
	ECG	0	692616

Solvent System	Compound	UP Area	LP Area
11	GA	0	1777916
	C	0	772005
	EGCG	228298	29305814
	EC	190138	1949107
	EGC	0	9666633
	ECG	0	984808

Solvent System	Compound	UP Area	LP Area
12	GA	0	745509
	C	0	864072
	EGCG	0	29802598
	EC	0	2014758
	EGC	0	10164781
	ECG	0	113087

Table 4.13b. *The solvent systems screened to determine a suitable system for analysing tea by HPCCC. Green columns indicate antibacterial activity against S. aureus in that layer whilst red indicates no activity. For each compound, the area under the peak is listed for each phase. UP = upper phase, LP = lower phase*

Solvent System	Compound	UP Area	LP Area
13	GA	875033	1013237
	C	7373	218836
	EGCG	23297021	3318367
	EC	2054803	377214
	EGC	9044396	393433
	ECG	1749557	0

Solvent System	Compound	UP Area	LP Area
14	GA	806902	1117325
	C	626225	211995
	EGCG	24698113	3386928
	EC	1541355	387782
	EGC	8591867	694094
	ECG	940023	100541

Solvent System	Compound	UP Area	LP Area
15	GA	150262	1267082
	C	721441	725929
	EGCG	21932022	3002528
	EC	1799060	466831
	EGC	11117954	626105
	ECG	1374771	153386

Solvent System	Compound	UP Area	LP Area
16	GA	0	2261402
	C	6142	1119943
	EGCG	139939	36178413
	EC	0	286024
	EGC	201583	1497335
	ECG	0	164930

Solvent System	Compound	UP Area	LP Area
17	GA	0	2105593
	C	832961	165511
	EGCG	29125128	3650913
	EC	1956055	336284
	EGC	11080604	328643
	ECG	148719	0

Solvent System	Compound	UP Area	LP Area
18	GA	652854	1277878
	C	779892	345018
	EGCG	21444187	4497685
	EC	1972591	684490
	EGC	9101020	1340963
	ECG	1027242	428973

Solvent System	Compound	UP Area	LP Area
19	GA	91767	1760831
	C	600640	497481
	EGCG	19605851	5568998
	EC	1685176	487673
	EGC	8315085	708170
	ECG	896584	48431

Solvent System	Compound	UP Area	LP Area
20	GA	0	2657067
	C	44617	2031481
	EGCG	891585	36074276
	EC	187056	3640187
	EGC	722950	14013310
	ECG	0	2935416

Solvent System	Compound	UP Area	LP Area
21	GA	89828	1894
	C	286874	0
	EGCG	3947097	12329
	EC	302274	0
	EGC	3177741	3844
	ECG	305518	0

Solvent System	Compound	UP Area	LP Area
22	GA	1234131	0
	C	630834	0
	EGCG	21183356	395509
	EC	1395205	71710
	EGC	6945703	921305
	ECG	730986	0

Solvent System	Compound	UP Area	LP Area
23	GA	89701	0
	C	0	516843
	EGCG	472563	16431304
	EC	0	1564968
	EGC	57989	867928
	ECG	23207	61715

Solvent System	Compound	UP Area	LP Area
24	GA	20327	1753092
	C	15962	1382659
	EGCG	494408	27759514
	EC	41521	2682508
	EGC	208528	10225709
	ECG	14125	2127152

Table 4.13c. *The solvent systems screened to determine a suitable system for analysing tea by HPCCC. Green columns indicate antibacterial activity against S. aureus in that layer whilst red indicates no activity. For each compound, the area under the peak is listed for each phase. UP = upper phase, LP = lower phase*

Solvent System	Compound	UP Area	LP Area
25	GA	0	1585775
	C	0	669206
	EGCG	0	22770803
	EC	0	17201768
	EGC	13532	807649
	ECG	0	480939

Solvent System	Compound	UP Area	LP Area
29	GA	2633	1785982
	C	1580	1430265
	EGCG	72762	27402295
	EC	8457	2696401
	EGC	61798	10224620
	ECG	2063	2116928

Solvent System	Compound	UP Area	LP Area
32	GA	1624441	53876
	C	723242	54992
	EGCG	27299440	1154290
	EC	1821964	89982
	EGC	1074519	385669
	ECG	116172	34779

Solvent System	Compound	UP Area	LP Area
26	GA	0	1874905
	C	731868	495648
	EGCG	28854975	7973374
	EC	2194463	989191
	EGC	11194705	1712913
	ECG	2228686	571100

Solvent System	Compound	UP Area	LP Area
30	GA	151760	1625985
	C	613076	691481
	EGCG	8784557	8641425
	EC	866785	1191957
	EGC	572942	2482956
	ECG	807459	863412

Solvent System	Compound	UP Area	LP Area
33	GA	0	2168795
	C	50447	1396995
	EGCG	1411685	25622289
	EC	101997	2625899
	EGC	1078178	6616052
	ECG	86797	1867617

Solvent System	Compound	UP Area	LP Area
28	GA	461986	2435934
	C	924861	599773
	EGCG	21131088	7713279
	EC	1924749	868582
	EGC	8296180	1783582
	ECG	1551271	425884

Solvent System	Compound	UP Area	LP Area
31	GA	3428544	0
	C	2108363	0
	EGCG	46240019	2055
	EC	5283651	0
	EGC	18985135	1347612
	ECG	3985889	0

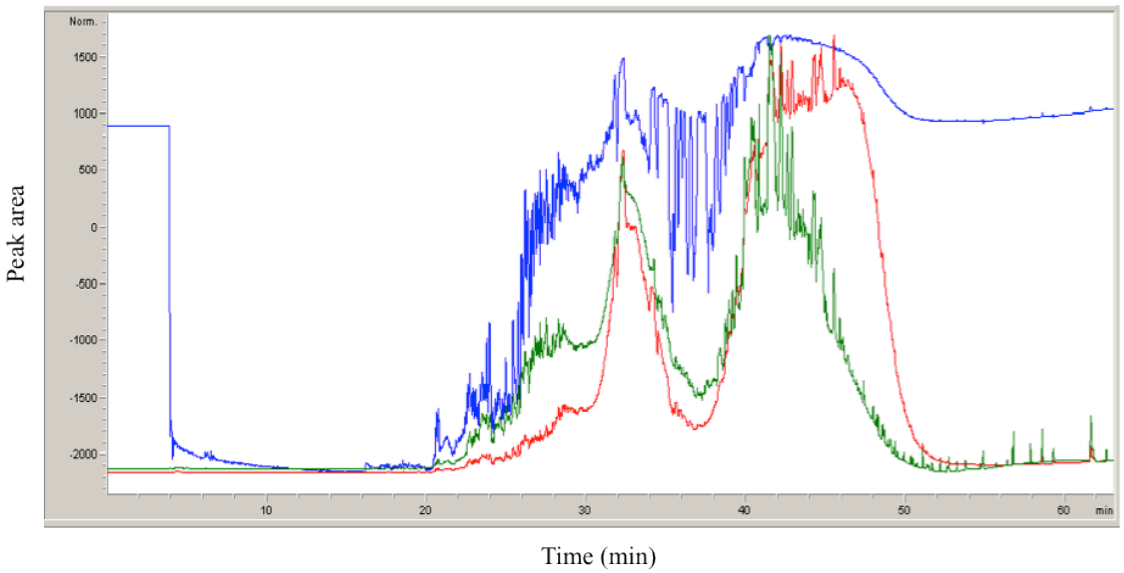


Figure 4.13. *HPCCC chromatogram of a methanol extract of green tea GP-2 at wavelengths 210 (blue), 280 (red) and 310 (green) nm.*

There were several solvent systems that demonstrated antibacterial activity in both phases. However, solvent system 5 consisting of heptane (0.36 mL), ethyl acetate (2.14 mL), methanol (2.14 mL) and water (0.36 mL) was selected for the first HPCCC run as it had good distribution of all the compounds between the two phases and consisted of four solvents. The advantage of the latter point is that there is more scope for solvent adjustment when seeking to further optimise the system (Ignatova *et al*., 2011).

Table 4.14. *The mean zone of inhibition (mm) against S. aureus for mobile phase fractions (min) collected from the HPCCC run of green tea extract GP-2 using the bacterial susceptibility well assay (n=3). Activity after 18 h incubation at 37* ***°C***

Mobile Phase Fractions (min)	Zone of Inhibition (mm)
24	0 ± 0
28	0 ± 0
34	8 ± 0.1
42	18 ± 0.3
48	17 ± 0.1

As predicted, solvent system 5 appeared to give good separation of the green tea extract as two distinct peaks were observed on the HPCCC chromatogram (fig. 4.13). To determine if these peaks contained compounds that had antibacterial activity, samples collected at time points 24, 28, 34, 42 and 48 min were tested for activity using the bacterial susceptibility well assay. Of these time points, only the fractions that fell within the area of the two peaks had activity (table 4.14). The stationary phase was extruded from the column over 30 min, which produced several coloured fractions and fractions with precipitate. Of these fractions, time points 8 min, 15 min and 22 min produced the most intense colours and precipitate. These fractions were tested for antimicrobial activity and as shown in table 4.15, fractions at time points 8 and 15 min had activity. Demonstrating that compounds left in the stationary phase after separation had antimicrobial activity.

Table 4.15. *The mean zone of inhibition (mm) against S. aureus for stationary phase fractions (min) collected from the HPCCC run of green tea extract GP-2 using the bacterial susceptibility well assay (n=3). Activity after 18 h incubation at 37* ***°C***

Stationary Phase Fractions (min)	Zone of Inhibition (mm)
8	9 ± 0.2
15	10 ± 0.1
22	0 ± 0

4.4.6.2 Determining the Antibacterial Compounds of Green Tea using HPCCC

The preliminary HPCCC run revealed that solvent system 5 was an appropriate solvent for separating compounds in the green tea fraction. It also showed that antibacterial activity resided in the fractions at time points coinciding with the two major peaks and also in the residual stationary phase. Therefore, the experiment was repeated and each time point from 20 – 64 min was screened for antibacterial activity (fig. 4.14a). In figure 4.14b it was found that activity was exclusively within the two major peaks as seen in the preliminary run.

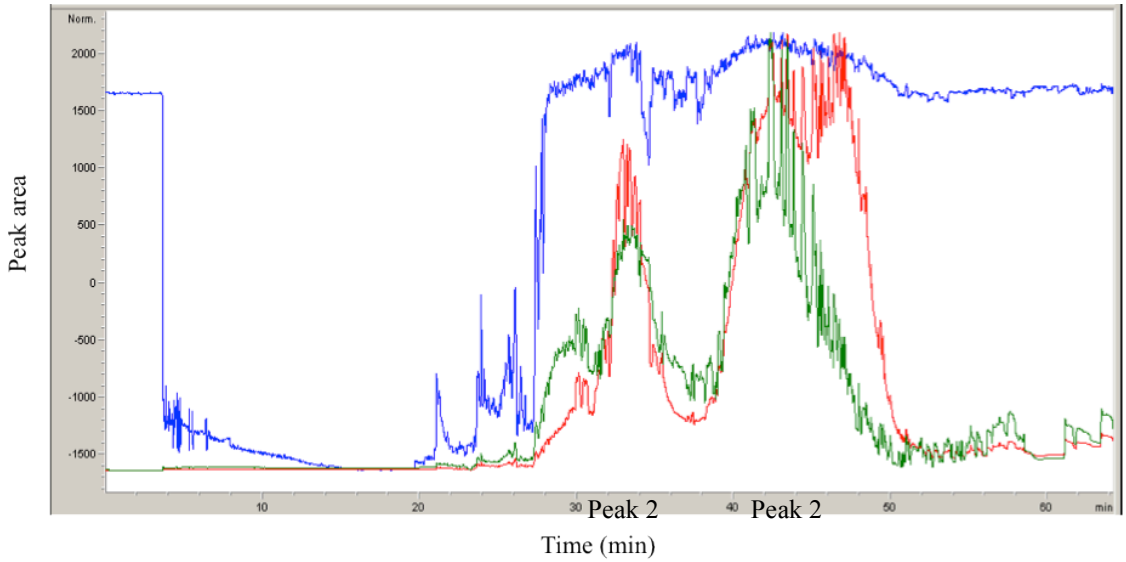


Figure 4.14a. *Normal phase HPCCC chromatogram of a methanol extract of green tea GP-2 at wavelengths 210 (blue), 280 (red) and 310 (green) nm.*

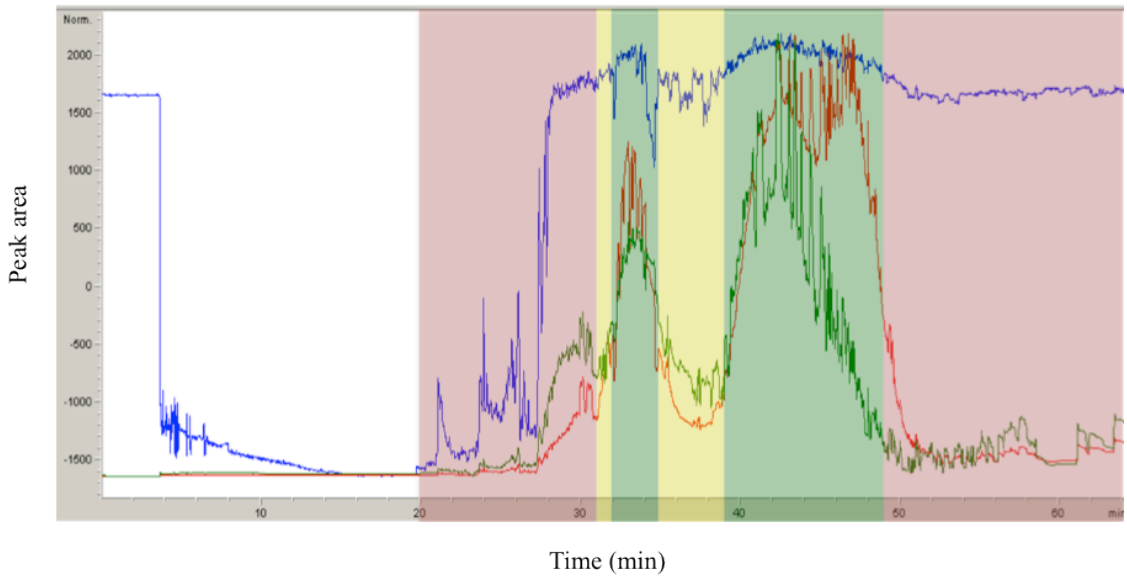


Figure 4.14b. *Normal phase HPCCC chromatogram of a methanol extract of green tea GP-2 with an indicator of antibacterial activity against S. aureus overlaid. At each time point, the mean zone of inhibition against S. aureus: 0 - 2 mm = red, 3 - 4 mm = yellow and >4 mm = green (n=3).*

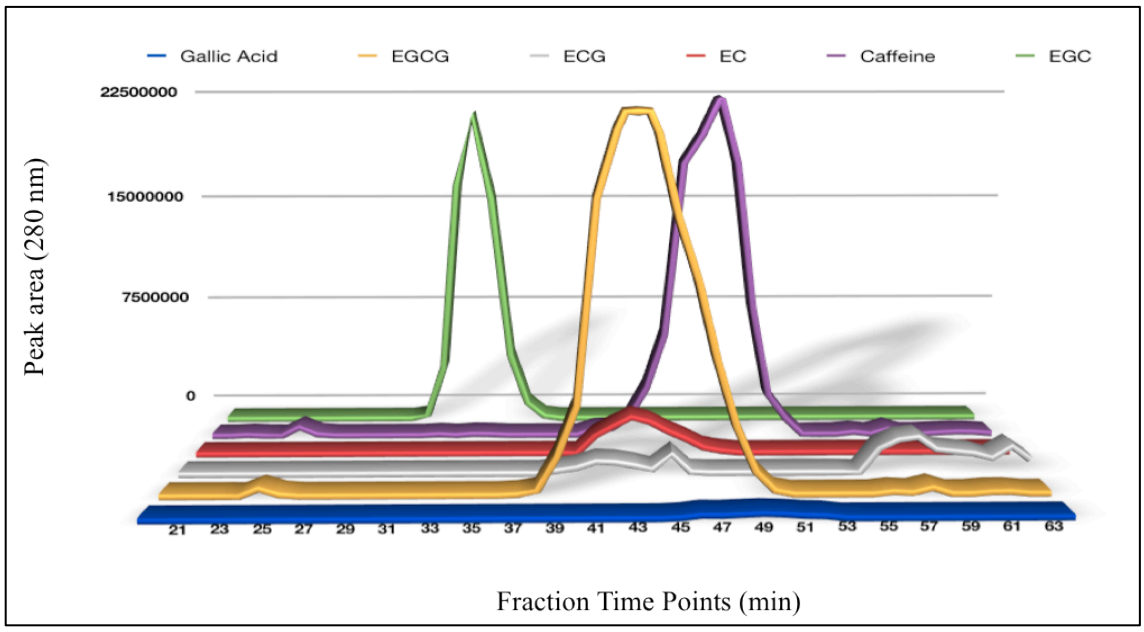


Figure 4.15a. *A fractogram showing of the peak area of each compound in GP-2 identified by HPLC at each time point on the normal phase HPCCC run.*

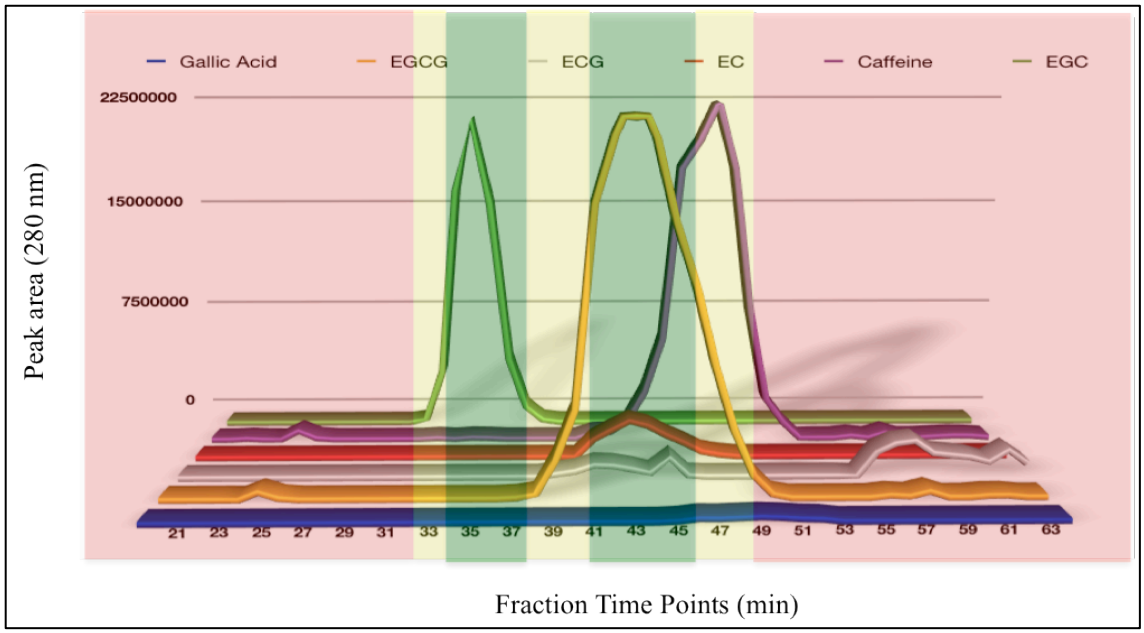


Figure. 4.15b. *A fractogram showing of the peak area of each compound in GP-2 identified by HPLC at each time point on the normal phase HPCCC run with an indicator of antibacterial activity overlaid. At each time point, the mean zone of inhibition against S. aureus: 0 - 2 mm = red, 3 - 4 mm = yellow and >4 mm = green (n=3).*

To identify which compounds were responsible for the antibacterial activity, HPLC was performed on each fraction from time points 20 - 64 min. This information was then plotted on a fractogram (fig. 4.15a) and the fractions were tested for antimicrobial activity (fig. 4.15b). It was found that peak 1 in the HPCCC spectra (32 - 34 min) was EGC, whilst peak 2 contained EGCG and caffeine. Caffeine has been shown not to have any antimicrobial activity against *S. aureus* (table 4.7), therefore the antibacterial activity in these fractions must be attributed to EGC and EGCG.

In the preliminary HPCCC run, it was found that there was residual antibacterial activity left in the stationary phase. Therefore, the green tea sample GP-2 was run again, but this time the mobile phase was used as the stationary phase and vice versa (reverse phase). This would result in the fractions collected in the first HPCCC run being retained in the column whilst the compounds left in the stationary phase were fractionated.

Three major peaks were observed when the green tea sample was run in reverse phase HPCCC (fig. 4.16a). An antimicrobial assay of the time points showed that the activity was due to peaks 2 and 3 in the chromatogram (fig. 4.16b). When cross-referenced with the fractogram of the fractions (fig. 4.17a) collected from this run, the activity in peak 2 was attributed to EGCG, C and EC whilst the activity in peak three was due to ECG (fig. 4.17b). In light of the results from the MIC study, it is likely that the activity of peak 2 is due to EGCG rather than of C or EC.

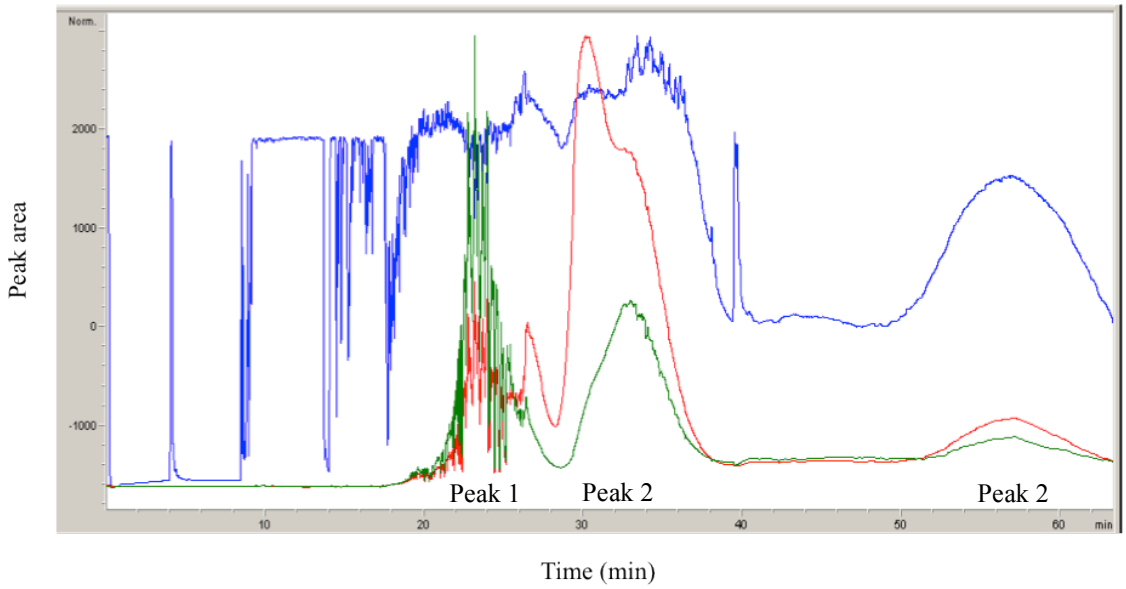


Figure 4.16a. *Reverse phase HPCCC chromatogram of a methanol extract of green tea GP-2 at wavelengths 210 (blue), 280 (red) and 310 (green) nm.*

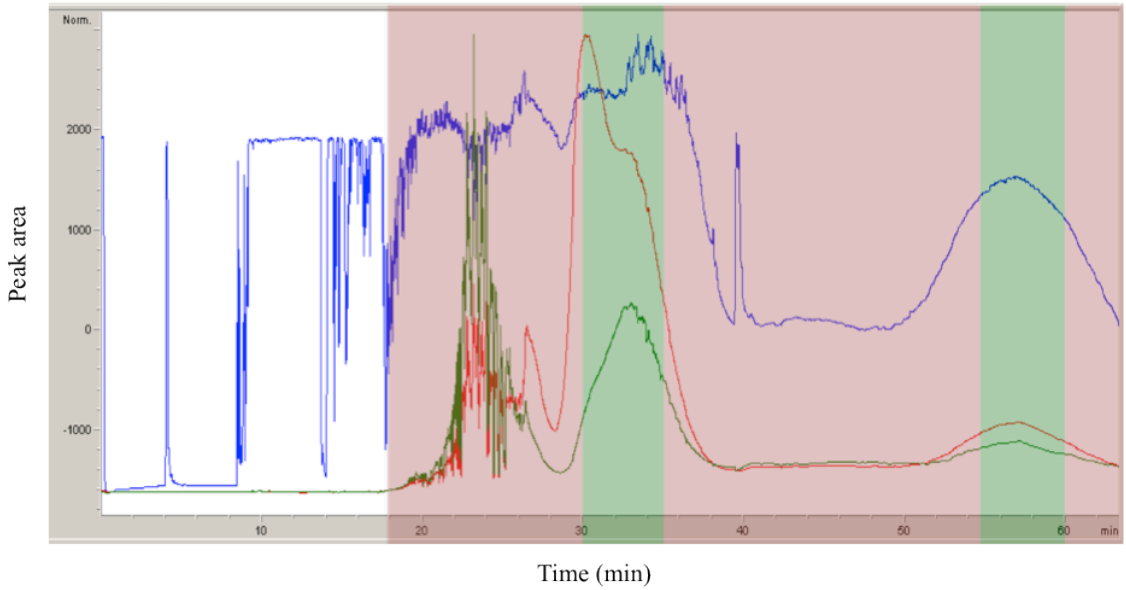


Figure 4.16b. *A reverse phase HPCCC chromatogram of a methanol extract of green tea GP-2 with an indicator of antibacterial activity against S. aureus overlaid. At each time point, the mean area of inhibition against S. aureus: 0 - 2 mm = red, 3 - 4 mm = yellow and >4 mm = green (n=3).*

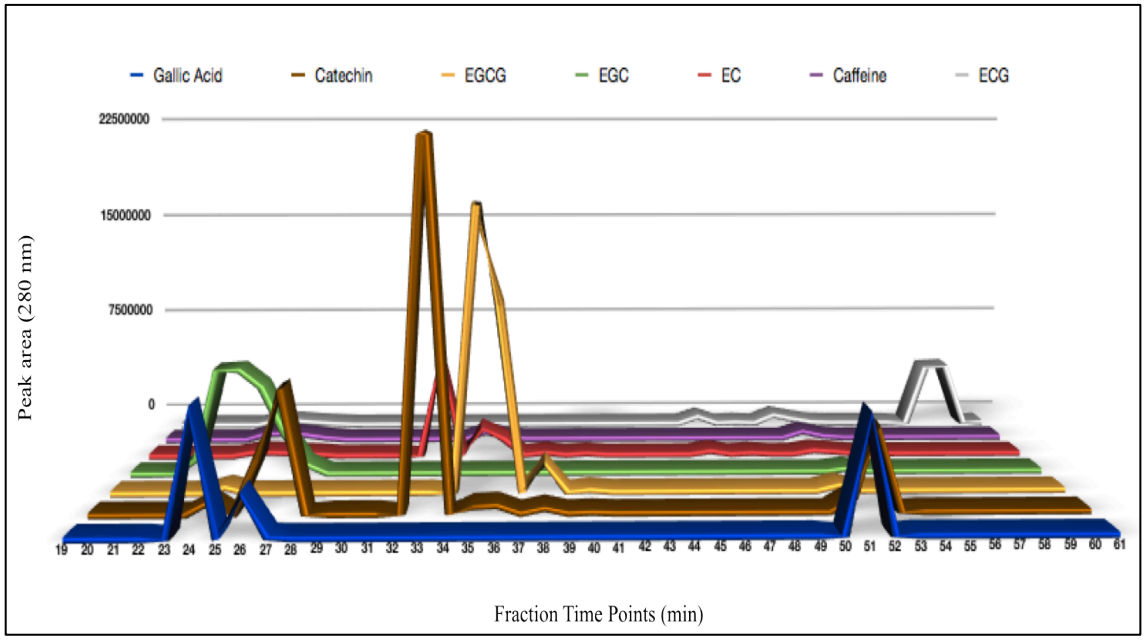


***Figure 4.17a**. A fractogram showing the peak area of each compound in GP-2 identified by HPLC at each time point on the reverse phase HPCCC run.*

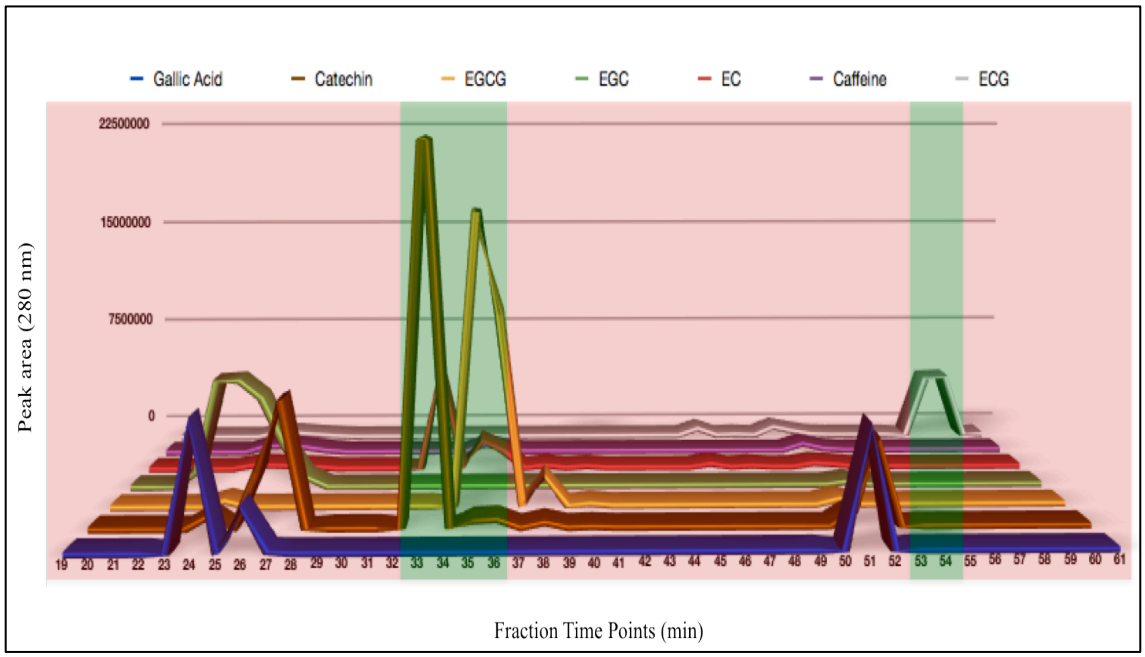


***Figure 4.17b.** A fractogram showing of the peak area of each compound in GP-2 identified by HPLC at each time point on the reverse phase HPCCC run with an indicator of antibacterial activity overlain. At each time point, the mean area of inhibition against S. aureus: 0 - 2 mm = red, 3 - 4 mm = yellow and >4 mm = green (n=3).*

In both the normal phase and reverse phase runs, there were no other major peaks in the HPLC spectra in the fractions with antimicrobial activity. This would suggest that in this tea, there were no other major antimicrobial compounds. Furthermore, since the extract is more concentrated than a typical tea infusion, it also helps to rule out the presence of antimicrobial compounds present in only trace amounts in tea infusions. Overall this supports the results from the MIC study of the individual catechins and the hypothesis that the catechins are the key antimicrobial compounds in tea.

4.5 Conclusions

In this chapter, a method for testing tea infusions has been successfully developed and enabled the screening of a large sample of single plantation and commercial teas for antimicrobial activity. In comparison to other studies, the MIC values for catechins determined in this study are similar to published MIC's, thus supporting the validity of this agar based method (Stapleton *et al*., 2004; Hara, 2001).

For the first time, using this modified agar method, the antimicrobial activity of standard tea infusions has been quantified to a standard similar to that used for antibiotics and other antibacterial agents, and as a result, has allowed for robust screening and selection of teas with exceptional antimicrobial activity.

A large variation in the antimicrobial activity of teas was observed even if they were of the same type (i.e. green or black) suggesting that the growth environment or variety of tea plant may play a key role in its antimicrobial activity. A large part, if not all, of this variation in green teas can be attributed to the levels of catechin polyphenols. In black teas, this trend was not so clear, and although residual catechins appear to still play an important role, other factors are contributing to the antimicrobial activity. However, as can be seen from generally low activity of black teas, these other compounds are either not as active as the green tea catechins or are present in very small amounts in black tea infusions.

The ability of the synthetic tea to recreate the exact antimicrobial activity of the tea on which it was based, strengthened the hypothesis that the six catechins and gallic acid identified in this study are the sole factors contributing to the antimicrobial activity of green teas. This was further supported by the HPCCC analysis, which helped to rule out the presence of other antimicrobial compounds. Furthermore, for the first time, the synthetic tea also provided a clear demonstration of the synergistic and/or additive effects of the antimicrobial action of tea infusions. Unfortunately, statistical analysis and review of the data was inadequate to determine the nature of these synergies, but did provide some insight into the importance of the galloyl containing catechins to activity.

Comparing the antimicrobial activity of individual tea infusions, catechins, gallic acid and galloyl like compounds has shown that chemical structure is very important for antimicrobial activity. Overall, it would appear that catechins with galloyl or pyrogallol groups, which favour benzoquinone formation and subsequent hydrogen peroxide production, are the most potent antimicrobials. Furthermore, it was observed that teas that produced more H_2O_2 had more potent inhibitory activity. Clearly, hydrogen peroxide production plays an important role in antimicrobial activity of teas. However, whether it has a direct antimicrobial effect or is a component required for further reactions is still unknown.

CHAPTER 5

ANTIMICROBIAL ACTIVITY OF TEA INFUSIONS AGAINST *CLOSTRIDIUM DIFFICILE*

5. Antimicrobial Activity of Tea Infusions Against *Clostridium difficile*

5.1 Chapter Introduction

The antibacterial effects of tea against *C. difficile* are relatively unknown, however some anecdotal evidence has shown that the *Clostridia* species are susceptible to the inhibitory effects of green tea (Ahn *et al*., 1990) and catechins (Ahn *et al*., 1991; Lee *et al*., 2006). It was also been observed that tea catechins had relatively poor inhibitory activity against other bacteria that form the commensal gut flora (Ahn *et al*., 1991; Lee *et al*., 2006) and may help to promote the growth of the *lactobacillus* species (Axling *et al*., 2012). This would suggest that teas inhibitory effects in the gut may have a degree of selectivity, promoting growth of the healthy intestinal microbiota whilst inhibiting the growth of potential pathogenic strains.

Based on this evidence it is important to establish if tea or its constituents could be employed as a potential treatment for *C. difficile* and it is essential that any such therapeutics is active against as wide a range of strains and ribotypes as possible. Evaluation of whether tea interacts with the antibiotics used to treat the infection is also warranted to access the possibility of combination treatment. Furthermore, with the availability of advanced microscopy technology such as scanning electron microscopy (SEM), visualising the effect of tea treatment against *C. difficile* may provide an insight into the antimicrobial mechanism of action.

5.1.1 Distribution and Relevance of *C. difficile* ribotypes

Ribotyping is a tool that can be used for differentiating between different strains of bacteria according to their DNA, or more specifically the 16S and 23S regions of their genome (Grimont and Grimont, 1986). The 16S and 23S regions of the *C. difficile* genome are extremely heterogeneous and thus has proved to be the best method for determining Polymerase Chain Reaction (PCR) ribotypes (Aktories and Wilkins, 2000). To date a large number of *C. difficile* ribotypes have been identified (>116; Aktories and Wilkins, 2000) and the most commonly occurring ribotypes in England and Northern Ireland are 027, 002, 106, 015 and 001 (HPAb, 2012; table 5.1). In Europe the most common Ribotypes are 027 and 001 (HPAb, 2012). In 2008, the majority of *C. difficile* cases were from ribotypes 027, 106 and 001. However, over the last three years

their incidence has become less prevalent and the distribution of ribotypes has become more uniform (table 5.1).

The 027 ribotype has been linked with several global *C. difficile* epidemics (Carlson *et al*., 2013) and is also the most prevalent *C. difficile* pathogen worldwide (O'Connor *et al.,* 2008). Clinically, it has been shown to have a higher rate of spore production and produce higher levels of toxin, which is thought to be responsible for more severe cases of CDI (Carlson *et al*., 2013). Furthermore, clinical isolates of ribotype 027, 106 and 001 have been shown to more resistant to the first line antibiotics vancomycin and metronidazole (HPAb, 2012; Moura *et al*., 2013), highlighting the need to screen the activity of novel antimicrobials against multiple ribotypes.

Table 5.1*. The percentage of C. difficile ribotypes accounting for CDI's reported from hospitals in England and Northern Ireland between 2008 and 2011 (HPAb., 2012).*

	Reporting year			
Ribotype	**2008**	**2009**	**2010**	**2011**
027	55.3	36.1	22.1	12.4
106	13.0	12.7	7.3	7.3
001	8.7	7.3	7.4	6.8
002	2.7	5.7	6.0	7.5
014	2.8	5.4	2.6	1.2
015	2.4	5.3	6.6	7.7
078	1.8	3.5	5.7	6.2
005	1.4	2.9	4.3	5.4
023	1.0	2.7	3.0	2.5
026	0.2	2.1	0.8	0.8

5.1.2 Combinational Effects of Tea and Antibiotics

Combinational synergy or additive interactions of antimicrobial compounds are much sought after due to their potential to improve clinical outcomes (Lambert *et al.,*2003). Synergy is present when the combined activity of the antimicrobials exceeds the sum of their individual activities, whereas additive interactions are defined as a linear combination of the two individual effects (Lambert *et al.,* 2003). Bacteriostatic and bactericidal synergistic interactions have been shown to take place between compounds with different mechanisms of action. A good example of bactericidal synergy is the

facilitation of streptomycin penetration into bacterial cell wall by the presence of penicillin (Lopardo *et al.,* 2005). In addition, combinations of the preservatives chlorocresol and 2-phenylethanol produce bacteriostatic synergy by acting in a complementary manner against the cell membrane, ultimately resulting in disruption of proton gradient-supported transport processes (Denyer *et al.,* 1986).

There have been multiple reports of synergistic effects of tea or catechins with antibiotics against bacteria *in vitro* (Zhao *et al*., 2001; Hu *et al*., 2002; Stapleton *et al*., 2004). One such study observed that EGCG interacted synergistically with β-lactam antibiotics against *S. aureus* and its resistant type MRSA (Zhao *et al*., 2001). It was hypothesised that this synergy was due to both agents attacking the cell wall and was supported by observations of synergy between EGCG and another cell wall inhibitor DL-cycloserine (Zhao *et al*., 2001). Further supporting this theory was observation of indifferent interactions of EGCG and ofloxacin (nucleic acid synthesis inhibitor) and additive interactions with minocycline (protein synthesis inhibitor) (Zhao *et al*., 2001).

Another notable study observed largely additive or indifferent interactions between EGCG and protein or nucleic acid inhibitors such as tetracycline, chloramphenicol and erythromycin against *S. aureus* (Hu *et al*., 2002). However they also found that combinations of EGCG and glycopeptide antibiotics such as vancomycin and polymyxin B showed antagonistic tendencies (Hu *et al*., 2002). This was thought to be due to physical binding of the polyphenol to the glycopeptide antibiotics (Hu *et al*., 2002).

Synergistic interactions have also been reported with green tea extracts and antibiotics against Gram-negative bacteria (Tiwari *et al*., 2004). These included the protein synthesis inhibitors chloramphenicol and gentamycin, and also the DNA gyrase inhibitor nalidixic acid (Tiwari *et al*., 2004). However, the mechanism of their interaction is currently unknown.

Clearly there is a body of evidence to suggest that tea or its catechins can interact with antibiotics both synergistically and antagonistically against bacterial pathogens, which

emphasises the importance of determining the interactions of tea with the antibiotics used in the treatment of CDI.

5.1.3 Microscopic Evaluation of the Antibacterial Effects of Tea

The effect of tea infusions against bacteria at the microscopic level has yet to be determined. Nevertheless, a limited number of studies have shown that individual tea catechins can cause gross changes to the outer cell structures of both Gram-positive and Gram-negative bacteria (Taylor *et al*., 2009; Cui *et al*., 2012). These effects are evident when *S. aureus* is treated with ECG as seen in figure 5.1. It is thought that these surface changes are the result of cell membrane perturbation and thickening of the cell wall due to the retention of autolysins (Taylor *et al*., 2009).

Using Atomic Force Microscopy (AFM), similar irregularities have been observed on the outer structures of *E. coli* and *S. aureus* following treatment with EGCG (Cui *et al*., 2012; fig 5.1). This would suggest that tea, which is a combination of catechin polyphenols, may produce similar result against *C. difficile* and therefore warrants investigation.

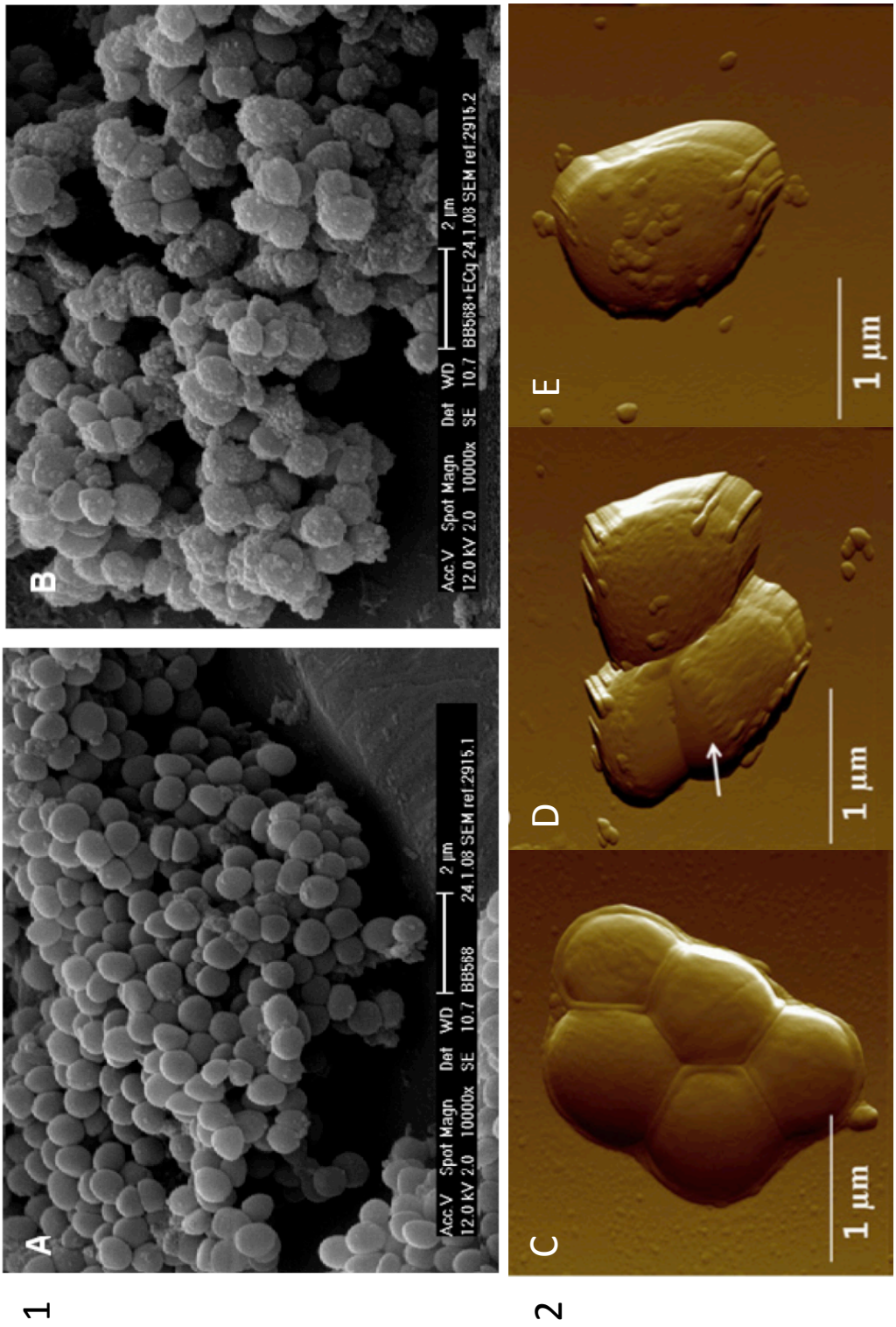


Figure 5.1. *1. The effects of ECG against S. aureus viewed by SEM; (A) Untreated cells, (B) Cells treated with ECG 12 mg/L (taken from Taylor et al., 2009). 2. The effects of EGCG against S. aureus viewed by AFM; (C) Untreated cells, (D) Cells treated with EGCG 12.5 mg/L for 1 h, (E) Cells treated with EGCG 12.5 mg/L for 2 h (taken from Cui et al., 2012).*

5.2 Aim and Objectives

The aim of this chapter was to determine the antimicrobial effects of tea infusions against *C. difficile*. The objectives of this chapter were to:

1. Determine the antimicrobial activity of tea and catechins against *C. difficile.*

2. Determine if the antimicrobial activity of tea infusions against *C. difficile* is affected by strain, ribotype or year of isolation.

3. Determine the interactions (antagonism, synergy or indifference) between tea infusions and the antibiotics vancomycin and metronidazole against *C. difficile.*

4. Investigate the effects of tea and EGCG on the structure of *C. difficile* cells using scanning electron microscopy.

5.3 Methods

5.3.1 Determination of the MIC of Tea Infusions and Catechins against *C. difficile* using the Clinical and Laboratory Standards Institute Method for Testing Anaerobic Bacteria

5.3.1.1 Preparation of Agar plates

Agar plates containing tea or catechin were prepared as described in chapter 4 (section 4.3.2.2) with the following modifications. To accommodate multiple isolates of *C. difficile,* square petri dishes (100mm x 100mm) were used for each tea dilution rather than a well of a 6 well culture plate. Double strength Brucella agar supplemented with 5 %(v/v) laked sheep's blood, 0.0001 %(w/v) vitamin K_1 and 0.0005 %(w/v) hemin was used instead of double strength LB agar as recommend by the CLSI (2003).

Vitamin K_1 was added to the agar as described by CLSI, (2003). Briefly, a 0.098 %(w/v) solution vitamin K_1 was prepared by diluting 200 µL of neat vitamin K_1 (density 0.984 g/mL) into 19.8 mL 95% ethanol and then diluting this solution 1 in 10 with sterile diH_2O. Of this solution, 500 µL was added to 500 mL of double concentrated Brucella agar (CLSI, 2003). The final concentration of vitamin K_1 in the agar was 984 ng/mL.

Hemin was prepared as described by CLSI, (2003). To 2 mL of 1M NaOH, 100 mg of hemin was added. Once the hemin dissolved in solution, the mixture was made up to 20 mL with sterile diH_2O. Of this solution, 500 µL was added to 500 mL of double concentrated Brucella agar. The final concentration of hemin in the agar was 5 µg/mL.

Agar plates were produced by adding 15 mL of tea/polyphenol dilution with 15 mL of cooled (60 °C) supplemented double strength Brucella agar. Serial dilutions of tea were prepared as described in chapter 4 (4.3.2.2). An agar plate composed of 15 mL sterile diH_2O and 15 mL of cooled (60 °C) supplemented double strength Brucella agar was used a positive growth control.

5.3.1.2 Preparation of Cultures from Freezer Stocks and Standard Inoculum Preparation

Frozen stocks of *C. difficile* strains (table 5.2) obtained from the Anaerobic Reference Centre (University Hospital Wales, Cardiff, UK) were thawed, streaked for purity (Ch.2; section 2.2.3 and 2.2.5) on pre-prepared fastidious anaerobe agar plates and incubated at 37 °C for 18 h in an anaerobic cabinet (A95 Workstation, Don Whitley Ltd., UK). The gas mixture of the cabinet was composed of 10 % CO_2, 10 % H_2 and 80 % N_2 as recommend by the CLSI, (2003). Post incubation, plates were inspected for purity and visible CFUs were added to 3 mL of sterile Brucella broth using a 10 µL inoculation loop to give the broth turbidity equal to a McFarland standard of 1 (~10^8 CFU/mL).

5.3.1.3 Quality Control of McFarland Standard

To ensure that suspensions of cultures with turbidity equal to a McFarland standard of 1 approximately contained 1 x 10^8 CFU/mL, viable counts were performed on strains *DS2315* (PCR ribotype 001), *DS1507* (PCR ribotype 106) and *DS2335* (PCR ribotype 027) using the Miles and Misra method (Miles *et al*., 1938) as described in chapter 4 (section 4.3.1.2) with the following modifications. Briefly, Brucella agar was used to perform viable counts in place of TSA and all cultures were incubated anaerobically at 37 °C for 48 h. These strains were chosen as they covered the major ribotypes in the samples set (Ch. 2; table 2.1).

5.3.1.4 Measuring MIC values

Of the standardised cultures (~10^8 CFU/mL) and negative control solution (sterile Brucella broth), 100 µL was added to a 96 well grid (fig. 5.2) suitable for use in a Denley® multipoint inoculator (Denley Ltd, UK). Each tea infusion agar plate and control plate (50% double strength agar and 50% sterile dH_2O) were inoculated with the isolates of *C. difficile* and the control solutions using a Denley® Multipoint Inoculator. The pins of the inoculator delivered 1 µL of each solution to the surface of the agar plate (approximately 10^5 bacteria per spot). Control plates were inoculated at the start and finish of each tea plate series to check growth and potential contamination during the inoculation process. After the plates were inoculated, they were allowed to dry

briefly for up to 10 min and placed in an anaerobic cabinet at 37 °C. After 48 h, plates were checked to determine the MIC value of each tea sample using the MIC endpoint criteria described by CLSI (2003). The data was evaluated by various statistical analysis described in (Ch.2; 2.4).

1	2	3	4	5	6	7	8	9	10
11	12	13	14	15	16	17	18	19	20
21	22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39	40
41	42	43	44	45	46	47	48	49	50
51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70
71	72	73	74	75		BF	SA		C

Figure 5.2. *A diagram of the 96 well Denley® multipoint inoculator grid. Numbers 1 – 75 represent cultures of C. difficile while 'C' represents the negative growth control (sterile Brucella agar), 'BF' represents B. fragilis NCTC 9343 and 'SA' represents S. aureus NCTC 6571.*

5.3.2 Determination of the MIC of Tea Infusions against *C. difficile* using a modified version the CLSI Method for Testing Anaerobic Bacteria.

This method was performed exactly as described in section 5.3.1 except the double strength Brucella agar was not supplemented with laked sheep's blood, vitamin K_1 or hemin.

5.3.3 Comparison of the CLSI method with the modified CLSI Method

The MIC of the antibiotics vancomycin (Flynn Pharma Ltd., UK) and metronidazole were determined for *C. difficile* strains *DS2315* (PCR ribotype 001), *DS1507* (PCR ribotype 106) and *DS2335* (PCR ribotype 027) using the method described in 5.3.1 and

5.3.2. Antibiotics were tested between the range of 0.03 – 8 µg/mL using serial dilutions with sterile diH_2O.

5.3.4 Determination of the effect of Tea Infusions in Combination with Antibiotics against *C. difficile*

The MIC of tea infusions and the antibiotics vancomycin and metronidazole were determined using the method described in 5.3.2.

Tea infusions and antibiotics were combined using the checkerboard method described by Moody (1992). The concentration ranges and combinations are described in tables 5.2 and 5.3. Solutions of metronidazole and vancomycin were diluted in sterile diH_2O and added to tea dilutions to obtain the desired concentration in each respective agar plate.

Bacteroides fragilis NCTC 9343 was used as a quality control strain for metronidazole sensitivity, whilst *S. aureus* NCTC 6571 was used for vancomycin as recommended by the CLSI (2003).

Table 5.2*. Combinations of different concentrations of vancomycin (µg/mL) and green tea infusion GP-2 (%v/v) tested for antimicrobial activity against C. difficile according to the checkerboard method (Moody, 1992).*

		GP-2 %(v/v)					
	Concentration	**1.6**	**3.1**	**6.3**	**12.5**	**25**	**50**
Vancomycin (µg/mL)	**31**	1A	2A	3A	4A	5A	6A
	63	1B	2B	3B	4B	5B	6B
	125	1C	2C	3C	4C	5C	6C
	250	1D	2D	3D	4D	5D	6D
	500	1E	2E	3E	4E	5E	6E
	1000	1F	2F	3F	4F	5F	6F
	2000	1G	2G	3G	4G	5G	6G
	4000	1H	2H	3H	4H	5H	6H
	8000	1I	2I	3I	4I	5I	7I

To determine the interaction of each combination, the Fractional Inhibitory Index (FIC) was calculated for each compound using the following equations (Moody, 1992):

$$FIC\ of\ antibiotic = \frac{MIC\ of\ antibiotic\ in\ combination\ with\ tea}{MIC\ of\ antibiotic\ alone}$$

$$FIC\ of\ tea = \frac{MIC\ of\ tea\ in\ combination\ with\ antibiotic}{MIC\ of\ agent\ tea\ alone}$$

Table 5.3. *Combinations of different concentrations of metronidazole (µg/mL) and green tea infusion GP-2 (%v/v) tested for antimicrobial activity against C. difficile according to the checkerboard method (Moody, 1992).*

		GP-2 %(v/v)					
	Concentration	**1.6**	**3.1**	**6.3**	**12.5**	**25**	**50**
Metronidazole (µg/mL)	**31**	1AA	2AA	3AA	4AA	5AA	6AA
	63	1BB	2BB	3BB	4BB	5BB	6BB
	125	1CC	2CC	3CC	4CC	5CC	6CC
	250	1DD	2DD	3DD	4DD	5DD	6DD
	500	1EE	2EE	3EE	4EE	5EE	6EE
	1000	1FF	2FF	3FF	4FF	5FF	6FF
	2000	1GG	2GG	3GG	4GG	5GG	6GG
	4000	1HH	2HH	3HH	4HH	5HH	6HH
	8000	1II	2II	3II	4II	5II	7II

The summation of FIC index (ΣFIC) was determined for each combination using the following equation (Moody, 1992):

$$\Sigma FIC\ of\ combination = FIC\ of\ tea + FIC\ of\ antibiotic$$

The ΣFIC was interpreted for each combination using the criteria in table 5.4. The geometric mean of the ΣFIC and range (ΣFIC_{min} - ΣFIC_{max}) for each combination was reported. The experiment was repeated three times.

Table 5.4. *The interpretation ΣFIC values for determining synergy, antagonism and indifference (Moody, 1992).*

Interpretation	**ΣFIC**
Synergism	$x < 0.5$
Indifference	$0.5 < x \leq 4$
Antagonism	$x \geq 4$

5.3.5 Scanning Electron Microscopy of *C. difficile* treated with Tea (GP-2) and EGCG

5.3.5.1 Treatment of *C. difficile* with Tea GP-2 and EGCG

A freezer culture of *C. difficile DS2335* was prepared as described in section 5.3.1.2. To 1 mL of neat tea GP-2, EGCG (0.5 mg/mL) or sterile diH_2O, CFU's of *C. difficile DS2335* were added to give a turbidity equalling that of a 1 McFarland standard (~10^8 CFU/mL). The samples were then incubated in triplicate anaerobically at 37 °C for 1, 5 and 10 h as shown in table 5.5. This was repeated three times to obtain three separate samples of each time point and treatment.

***Table 5.5**. Samples prepared for scanning electron microscopy.*

Sample No.	Treatment	Incubation Time (h)
1	Water (control)	1
2	"	5
3	"	10
4	EGCG (0.5 mg/mL)	5
5	"	10
6	Tea GP-2 (neat)	1
7	"	5
8	"	10

5.3.5.2 Sample Preparation and Imaging

Bacterial cultures suspended in treatment or control were pelleted by centrifuging at 2600 xg for 10 min and the supernatant was removed. The cultures were fixed at room temperature for 1 h by suspending in 500 µL of 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The samples were then centrifuged at 2600 xg for 10 min and the supernatant was removed. To each sample, 500 µL of a solution containing a ratio of 2:2:1 of 2 % osmium tetroxide: 0.1 M sodium cacodylate (pH 7.4): 25 % glutaraldehyde was added and incubated for 1 h at room temperature.

After the fixing stage, samples were centrifuged again and the supernatant was removed. The samples were re-suspended in 50 % ethanol in diH_2O and centrifuged at 5000 xg. After removing the supernatant this process as repeated with 70, 80 and 95 % ethanol followed by three washes with absolute ethanol. The samples were stored in 1 mL of absolute ethanol at room temperature before dehydration under vacuum on carbon grids, sputter coating with platinum and imagining on a scanning electron microscope.

For each sample, 10 different fields of view were examined at 25,000 x and 100,000 x magnification using a Zeiss SIGMA FEG-SEM scanning electron microscope.

5.4 Results and Discussion

5.4.1 Antibacterial activity of Tea against *C. difficile*

5.4.1.1 Comparison of Supplemented and Un-supplemented Brucella Agar

Initially the agar specified by the CLSI was used to determine the antimicrobial activity of tea infusions against *C. difficile.* Unfortunately it was found that Brucella agar supplemented with laked sheep's blood, hemin and vitamin K_1 inhibited the antibacterial activity of both green and black teas against *C. difficile*. In contrast, un-supplemented Brucella agar containing tea inhibited the growth of *C. difficile*. This would suggest that one of the agar supplements inhibited the antibacterial activity of tea. Based on previous findings (Ch. 4; 4.4.1.3) and other observations of trace metals (Dai *et al*., 2010), it is most likely due to either the hemin or laked sheep's blood, however further tests would be required to confirm this hypothesis.

Due to the incompatibility of the standard CLSI media with tea, the antibiotic susceptibility of *C. difficile* was determined on un-supplemented and supplemented Brucella agar (table 5.6). This was performed to determine if antimicrobial testing using un-supplemented agar affected the susceptibility of the bacteria to antimicrobials.

Table 5.6*. The effect of supplementing Brucella agar with vitamin K_1, laked sheep's blood and hemin on the antimicrobial activity of vancomycin and metronidazole (μg/mL) against C. difficile (n=3).* [1]*Results expressed as MIC range (geometric mean) of three repeats.*

	MIC Range (Mean[1])			
	Metronidazole		Vancomycin	
Strain	***Supplemented***	***Unsupplemented***	***Supplemented***	***Unsupplemented***
DS2335	0.13 - 0.25 (0.15)	0.13 (0.13)	1 (1)	0.5 - 1 (0.63)
DS1507	0.25 (0.25)	0.25 (0.25)	1 (1)	1 (1)
DS2315	0.13 (0.13)	0.13 - 0.25 (0.16)	0.5 - 1 (0.63)	1 (1)

From the observed results, it would appear that using either agar did not have an affect on antimicrobial susceptibility to these antibiotics. Therefore, throughout this chapter

the MIC of antibiotics and tea infusions against *C. difficile* isolates was determined using un-supplemented Brucella agar.

5.4.1.2 The MIC of Tea, EGCG and GA against 75 Clinical *C. difficile* Isolates

To determine the range of antimicrobial activity of tea infusions against *C. difficile*, a sample of the most prevalent ribotypes from 2000 – 2011 were selected for testing.

A range of antimicrobial activity was seen against *C. difficile* for green and black teas (table 5.7a-d). The trend in antibacterial activity was similar to that of *S. aureus NCTC 9518* although the mean MIC of the tea infusions was slightly lower against *C. difficile*, particularly for black teas (e.g. MIC of BP-5 = $9.5_{C.difficle}$ vs. $25.0_{S.aureus}$ %(v/v) (Ch. 4; table 4.6). However, to determine the significance of this observation, a broader collection of *S. aureus* strains would need to be tested.

The MIC of EGCG and GA against *C. difficile* was also similar compared to *S. aureus* (table 5.8). The mean MIC of EGCG was 53 µg/mL (95 %CI = 44 and 64 µg/mL) while for GA the mean MIC was 70 µg/mL (95 %CI = 58 and 85 µg/mL). The most active green tea was GP-2 with a mean MIC of 3.4 %(v/v) (95% CI = 3.1 and 3.8 %(v/v)) while the least active green tea was GP-8 with a mean MIC of 6.5 %(v/v) (95% CI = 5.7 and 7.5 %(v/v)). Within the black teas, BP-5 had the most potent antimicrobial activity with a mean MIC of 9.5 %(v/v) (95% Cl = 8.8 – 10.3 %(v/v)) while BP-17 was the least active black tea with a mean MIC of 25.3 %(v/v) (95% Cl = 22.3 – 28.8 %(v/v)).

To compare the difference between the antimicrobial activity within the green and black teas against *C. difficile* using statistical analysis, the MIC values were transformed using a log transformation. Following a log transformation and after assessing the normality of the data sets, it was found that the data did not follow normal distribution, therefore the teas were analysed using a Kruskal Wallis test. Groups were compared using the Bonferroni-Dunn's multiple comparisons test.

***Table 5.7a**. The antimicrobial activity (MIC %v/v) of green tea (n=5) infusions against clinical C. difficile isolates*

		GP-2		GP-5		GP-6		GP-7		GP-8	
Strain	**Ribotype**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**
R13929	001	1.56 - 3.13	2.21	3.13	3.13	6.25	6.25	3.13 - 6.25	4.42	6.25	6.25
R14057	106	6.25	6.25	6.25 - 12.5	8.84	12.5	12.50	12.5	12.50	6.25 - 12.5	8.84
R14496	020	3.13	3.13	3.13 - 6.25	4.96	1.56 - 12.5	4.96	3.13 - 6.25	4.96	6.25 - 12.5	7.87
R14933	002	3.13 - 6.25	3.94	3.13 - 6.25	4.96	6.25 - 12.5	7.87	6.25	6.25	6.25	6.25
R14935	014	1.56 - 3.13	2.48	3.13 - 6.25	4.96	6.25 - 12.5	7.87	6.25 - 12.5	7.87	3.13 - 6.25	4.96
R14936	070	1.56 - 6.25	3.12	3.13 - 6.25	4.96	3.13 - 12.5	6.25	3.13 - 12.5	7.88	3.13 - 12.5	7.88
R15213	072	3.13	3.13	3.13	3.13	6.25	6.25	6.25	6.25	6.25	6.25
R15627	078	12.5	12.50	8.33 - 25	14.43	12.5 - 50	25.00	12.5 - 50	25.00	12.5 - 50	19.84
R15632	001	3.13	3.13	3.13 - 6.25	4.42	6.25	6.25	3.13 - 6.25	4.42	6.25 - 12.5	8.84
R15691	023	1.56	1.56	1.56 - 3.13	2.21	3.13 - 6.25	4.42	12.5 - 50	17.68	1.56 - 12.5	4.42
R16631	005	1.56 - 6.25	3.12	1.56 - 3.13	2.21	3.13 - 6.25	3.94	3.13 - 6.25	4.96	1.56 - 6.25	3.12
R16632	137	3.13	3.13	1.56 - 3.13	2.48	3.13 - 6.25	4.96	1.56 - 6.25	3.94	1.56 - 3.13	2.48
R16633	138	3.13	3.13	1.56 - 6.25	3.12	1.56 - 6.25	3.94	3.13 - 6.25	4.96	1.56 - 6.25	3.12
R16762	027	1.56 - 3.13	2.21	3.13	3.13	3.13	3.13	1.56 - 6.25	3.12	1.56 - 6.25	3.12
R17015	001	1.56 - 3.13	2.48	1.56 - 6.25	3.12	1.56 - 12.5	4.96	1.56 - 12.5	4.96	6.25 - 25	9.92
R17060	106	1.56 - 3.13	2.48	1.56 - 6.25	3.12	6.25 - 12.5	7.87	1.56 - 6.25	3.12	1.56 - 6.25	3.94
R17083	027	1.56 - 3.13	2.21	6.25	6.25	3.13 - 12.5	6.25	3.13 - 12.5	6.25	6.25 - 12.5	8.84
R17302	106	3.13	3.13	1.56 - 3.13	2.21	1.56 - 3.13	2.21	1.56 - 3.13	2.21	1.56 - 6.25	3.12
R17849	078	6.25 - 12.5	9.92	12.5 - 25	17.33	6.25 - 50	19.84	6.25 - 50	15.75	12.5 - 50	19.84
R17857	106	1.56 - 6.25	3.12	3.13 - 6.25	3.94	3.13 - 12.5	6.25	3.13 - 12.5	4.97	3.13 - 12.5	6.25
R18050	001	1.56 - 3.13	2.48	3.13 - 6.25	4.96	6.25 - 12.5	9.92	3.13 - 6.25	4.96	6.25 - 12.5	7.87
R18080	018	1.56 - 3.13	2.48	3.13 - 6.25	3.94	1.56 - 12.5	4.96	3.13 - 12.5	6.25	1.56 - 12.5	4.96
R18413	002	1.56 - 3.13	2.21	3.13 - 6.25	4.42	6.25 - 12.5	8.84	6.25	6.25	6.25	6.25
R18603	106	6.25	6.25	6.25	6.25	25	25.00	12.5	12.50	12.5	12.50
R18738	005	3.13 - 6.25	4.42	3.13 - 6.25	4.42	25	12.50	6.25	8.84	6.25 - 12.5	8.84
R19157	001	1.56 - 3.13	2.21	3.13	3.13	3.13 - 6.25	4.42	3.13 - 6.25	4.42	3.13 - 6.25	4.42
R19630	015	1.56 - 6.25	3.94	3.13 - 6.25	3.94	6.25 - 25	9.92	6.25 - 12.5	9.92	6.25 - 25	9.92
R19885	002	1.56 - 6.25	3.94	3.13	3.13	6.25 - 25	12.50	6.25 - 12.5	9.92	3.13 - 25	7.88
R20063	106	1.56 - 6.25	3.12	3.13 - 6.25	6.25	12.5 - 25	12.50	6.25 - 12.5	7.87	6.25 - 12.5	7.87
R20700	045	3.13 - 12.5	7.88	12.5	12.50	12.5 - 50	31.50	6.25 - 12.5	9.92	6.25 - 12.5	9.92
R20702	001	3.13 - 6.25	3.94	1.56 - 6.25	3.94	1.56 - 6.25	3.94	3.13 - 12.5	6.25	6.25 - 12.5	7.87
R20703	106	1.56 - 3.13	2.48	1.56 - 3.13	1.97	1.56 - 3.13	1.97	1.56 - 3.13	1.97	1.56 - 6.25	3.12
R20831	001	1.56 - 6.25	3.12	3.13 - 6.25	3.13	1.56 - 6.25	3.12	3.13 - 6.25	4.42	3.13	3.13
R20962	106	1.56 - 3.13	2.48	3.13 - 6.25	3.94	1.56 - 6.25	3.12	1.56 -6.25	3.94	3.13 - 6.25	4.96
R20976	106	3.13 - 6.25	3.94	3.13 - 6.25	3.94	3.13 - 12.5	6.25	1.56 - 12.5	4.96	6.25 - 12.5	7.87
R21028	014	6.25	6.25	12.5	8.33	6.25	6.25	12.5	12.50	12.5	12.50
R22487	017	1.56 - 3.13	2.21	1.56 - 3.13	2.21	1.56 - 3.13	2.21	3.13	3.13	1.56 - 6.25	3.12
R22753	001	3.13	3.13	1.56 - 3.13	2.21	1.56	1.56	1.56	1.56	1.56	1.56

[a]Geometric mean of three repeats.

***Table 5.7b**. The antimicrobial activity (MIC %v/v) of green tea (n=5) infusions against clinical C. difficile isolates*

		GP-2		GP-5		GP-6		GP-7		GP-8	
Strain	**Ribotype**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**
R22814	106	3.13 - 6.25	4.42	3.13 - 6.25	4.42	3.13 - 12.5	6.25	6.25	6.25	6.25 - 12.5	8.84
R22887	174	3.13	3.13	3.13 - 6.25	4.42	6.25	6.25	6.25	6.25	12.5	12.50
R22888	015	3.13 - 12.5	7.88	6.25 - 25	15.75	12.5 - 50	25.00	6.25 - 12.5	8.84	12.5 - 25	17.68
R22897	106	1.56 - 3.13	2.48	3.13 - 6.25	3.94	1.56 - 6.25	3.94	3.13 - 6.25	4.96	1.56 - 12.5	4.96
R23121	027	1.56 - 3.13	2.48	3.13	3.13	1.56 - 6.25	3.94	3.13 - 6.25	4.96	1.56 - 6.25	3.94
R23800	106	3.13	3.13	1.56 - 6.25	3.12	3.13	3.13	1.56	1.56	1.56	1.56
R24565	027	1.56 - 3.13	2.21	3.13	3.13	1.56 - 3.13	2.21	1.56 - 6.25	3.12	3.13 - 6.25	4.42
R26452	020	3.13	3.13	3.13 - 6.25	4.96	6.25	6.25	6.25	6.25	6.25	6.25
R26720	106	1.56 - 3.13	2.48	1.56 - 3.13	2.48	1.56 - 3.13	2.48	1.56 - 6.25	3.12	1.56 - 6.25	3.12
R26797	027	1.56 - 3.13	2.48	1.56 - 3.13	2.48	3.13 - 6.25	3.94	6.25	6.25	1.56 - 3.13	2.48
R28972	014	1.56 - 3.13	2.48	3.13 - 6.25	3.94	3.13 - 6.25	4.96	3.13 - 6.25	4.96	1.56 - 6.25	3.13
R29035	002	3.13 - 6.25	4.96	3.13	3.13	6.25 - 12.5	7.87	6.25	6.25	3.13 - 12.5	7.87
R29039	078	1.56	1.56	1.56 - 3.13	2.21	1.56	1.56	3.13	3.13	3.13	3.13
R29078	027	1.56 - 3.13	1.97	3.13 - 6.25	4.96	6.25 - 12.5	7.87	6.25	6.25	6.25 - 12.5	7.87
R29096	106	1.56 - 3.13	2.48	3.13	3.13	3.13 - 6.25	3.94	1.56 - 6.25	3.94	3.13 - 6.25	4.96
R31056	027	1.56 - 3.13	2.21	3.13	2.21	1.56 - 12.5	2.21	1.56 - 3.13	2.21	3.13 - 6.25	4.42
R31234	106	1.56 - 6.25	3.12	1.56 - 6.25	3.12	1.56 - 12.5	4.42	1.56 - 12.5	4.42	3.13 - 12.5	6.25
R31312	056	1.56 - 3.13	2.48	1.56 - 3.13	1.97	1.56 - 6.25	3.12	1.56 - 12.5	3.12	3.13 - 12.5	6.25
R31315	013	1.56 - 6.25	3.12	3.13	3.13	3.13 - 6.25	3.94	1.56 - 6.25	3.94	3.13 - 12.5	6.25
R31350	220	3.13 - 6.25	4.42	1.56 - 3.13	2.21	3.13 - 6.25	4.42	3.13 - 6.25	4.42	6.25	6.25
R31382	118	3.13	3.13	1.56 - 3.13	2.21	3.13	3.13	1.56	1.56	1.56	1.56
DS1407	015	1.56 - 3.13	2.48	1.56 - 3.13	2.48	1.56 - 3.13	1.97	1.56 - 3.13	2.48	1.56 - 6.25	2.48
DS1447	081	1.56 - 6.25	3.12	1.56 - 6.25	3.12	1.56 - 25	6.25	1.56 - 12.5	4.96	6.25 - 12.5	6.25
DS1507	106	1.56 - 3.13	2.48	1.56 - 6.25	3.12	1.56 - 6.25	3.12	1.56 - 12.5	4.96	1.56 - 6.25	3.94
DS1758	023	3.13 - 6.25	4.42	3.13 - 6.25	4.96	6.25 - 25	12.50	3.13 - 12.5	6.25	6.25 - 12.5	8.84
DS1859	001	3.13 - 6.25	4.42	6.25	6.25	6.25 - 12.5	8.84	6.25 - 12.5	8.84	6.25	6.25
DS2090	124	3.13 - 6.25	4.42	1.56 - 6.25	3.12	1.56 - 3.13	2.21	1.56	1.56	1.56 - 3.13	2.21
DS2098	023	1.56 - 3.13	2.48	3.13 - 6.25	3.94	6.25	6.25	3.13 - 6.25	3.94	3.13 - 12.5	6.25
DS2107	081	1.56 - 3.13	2.48	1.56 - 3.13	1.97	1.56 - 6.25	3.12	1.56 - 6.25	3.94	3.13 - 6.25	4.96
DS2117	014	1.56 - 6.25	3.12	3.13 - 6.25	3.94	3.13 - 6.25	4.96	1.56 - 6.25	3.94	3.13 - 6.25	4.96
DS2205	001	3.13 - 6.25	3.94	3.13 - 6.25	4.96	6.25	6.25	3.13 - 6.25	5.46	6.25 - 12.5	7.87
DS2285	106	3.13	3.13	3.13 - 6.25	4.34	1.56 - 12.5	4.96	6.25	6.25	6.25 - 12.5	9.92
DS2305	174	3.13	3.13	3.13 - 6.25	4.42	6.25 - 12.5	8.84	6.25 - 12.5	8.84	6.25 - 12.5	8.84
DS2315	011	3.13 - 6.25	4.96	3.13 - 6.25	5.46	6.25 - 25	12.50	6.25 - 12.5	9.92	6.25 - 12.5	12.50
DS2315	027	3.13 - 6.25	3.94	3.13 - 6.25	3.94	3.13 - 12.5	7.88	6.25 - 25	9.92	6.25 - 25	12.50
DS2325	027	3.13	3.13	3.13	3.13	3.13	3.13	6.25	6.25	3.13 - 6.25	4.96
DS2335	027	3.13	3.13	3.13	3.13	3.13 - 6.25	4.96	3.13 - 12.5	4.96	6.25	6.25
DS2345	027	3.13 - 6.25	4.42	3.13 - 6.25	3.94	6.25	6.25	6.25	6.25	6.25 - 25	12.50

[a]*Geometric mean of three repeats.*

***Table 5.7c**. The antimicrobial activity (MIC %v/v) of black tea (n=5) infusions against clinical C. difficile isolates*

		BP-5		BP-8		BP-11		BP-17		BP-16	
Strain	**Ribotype**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**
R13929	001	6.25 - 12.5	8.84	12.5 - 50	25.00	6.25 -12.5	8.84	25 - 50	35.36	12.5 - 25	17.68
R14057	106	12.5	12.50	50	50.00	12.5	12.50	50	50.00	25	25.00
R14496	020	6.25 - 12.5	7.87	12.5 - 25	15.75	6.25 - 25	9.92	25 - 50	39.69	12.5 - 50	25.00
R14933	002	6.25 - 12.5	9.92	25	25.00	12.5	12.50	25 - 50	39.69	25 - 50	31.50
R14935	014	6.25 - 12.5	7.87	12.5 - 25	15.75	12.5	12.50	25 - 50	31.50	12.5 - 50	31.50
R14936	070	6.25 - 12.5	7.87	12.5 - 50	25.00	6.25 - 25	12.50	50	50.00	25 - 50	31.50
R15213	072	12.5	12.50	25	25.00	12.5	12.50	25	25.00	12.5	12.50
R15627	078	25 - 50	39.69	25 - 50	35.36	12.5 - 50	19.84	50	50.00	50	50.00
R15632	001	12.5	12.50	12.5 - 25	17.68	12.5	12.50	12.5 - 50	17.68	12.5 - 25	17.68
R15691	023	6.25	6.25	12.5 - 25	17.68	6.25 -12.5	8.84	12.5 - 50	25.00	25	25.00
R16631	005	6.25 - 12.5	7.87	12.5 - 25	15.75	6.25	6.25	12.5 - 50	25.00	12.5 - 50	15.75
R16632	137	6.25 - 12.5	7.87	6.25 - 12.5	9.92	6.25 -12.5	7.87	25 - 50	31.50	12.5 - 25	15.75
R16633	138	6.25 - 12.5	7.87	12.5 - 25	15.75	6.25 -12.5	9.92	12.5 - 50	25.00	12.5 - 25	19.84
R16762	027	6.25	6.25	12.5	12.50	6.25	6.37	12.5	12.50	12.5	12.50
R17015	001	6.25 - 12.5	7.87	12.5 - 25	15.75	6.25 -12.5	9.92	12.5 - 50	25.00	6.25 - 25	15.75
R17060	106	6.25 - 12.5	9.92	12.5 - 25	15.75	6.25 -12.5	9.92	25 - 50	31.50	6.25 - 50	19.84
R17083	027	6.25 - 12.5	8.84	12.5 - 25	17.68	6.25 -12.5	8.84	25 - 50	35.36	25	25.00
R17302	106	6.25 - 12.5	8.84	12.5	12.50	6.25	6.25	12.5 - 25	17.68	6.25 - 12.5	8.84
R17849	078	12.5 - 50	25.00	25 - 50	35.36	25 - 50	31.50	50	50.00	25	25.00
R17857	106	6.25 - 12.5	7.87	12.5 - 25	15.75	6.25 -12.5	7.87	50	50.00	25	25.00
R18050	001	6.25 - 12.5	9.92	12.5 - 25	15.75	12.5 - 25	15.75	25 - 50	39.69	25	25.00
R18080	018	12.5 - 25	15.75	12.5 - 25	15.75	6.25 -12.5	9.92	12.5 - 50	25.00	6.25 - 25	12.50
R18413	002	6.25 - 12.5	8.84	12.5	12.50	12.5	12.50	25	25.00	25	25.00
R18603	106	12.5	12.50	25	25.00	12.5	12.50	12.5	12.50	25	25.00
R18738	005	12.5 - 25	17.68	12.5 - 25	17.68	12.5	12.50	25 - 50	35.36	25	25.00
R19157	001	6.25 - 12.5	8.84	12.5 - 25	17.68	6.25 -12.5	8.84	12.5 - 50	25.00	6.25 - 25	12.50
R19630	015	6.25 - 12.5	12.50	12.5 - 50	25.00	6.25 - 25	12.50	25 - 50	35.36	12.5 - 50	31.50
R19885	002	6.25 - 25	12.50	12.5 - 50	25.00	6.25 - 25	12.50	50	50.00	12.5 - 50	25.00
R20063	106	6.25 - 12.5	12.50	12.5 - 25	15.75	6.25 -12.5	12.50	25 - 50	39.69	12.5 - 50	25.00
R20700	045	6.25 - 25	12.50	12.5 - 50	25.00	12.5 - 50	19.84	25	25.00	12.5 - 50	25.00
R20702	001	6.25 - 12.5	9.92	6.25 - 25	15.75	6.25 -12.5	9.92	25 - 50	31.50	12.5 - 25	15.75
R20703	106	6.25 - 12.5	7.87	12.5	12.50	6.25	6.25	6.25 - 25	12.50	6.25 - 12.5	7.87
R20831	001	6.25	6.25	12.5	12.50	6.25 -12.5	8.84	12.5 - 25	17.68	6.25	6.25
R20962	106	6.25 - 12.5	9.92	12.5 - 25	15.75	6.25 -12.5	9.92	12.5 - 50	25.00	12.5 - 25	19.84
R20976	106	6.25 - 12.5	9.92	12.5 - 25	19.84	6.25 -12.5	9.92	12.5 - 50	31.50	12.5 - 25	19.84
R21028	014	12.5	12.50	25	25.00	12.5	12.50	25	25.00	25	25.00
R22487	017	6.25	6.25	6.25 - 12.5	8.84	6.25	6.25	12.5 - 25	17.68	6.25 - 12.5	8.84
R22753	001	6.25	6.25	12.5	12.50	12.5	12.50	6.25	6.25	6.25	6.25

[a]*Geometric mean of three repeats.*

***Table 5.7d**. The antimicrobial activity (MIC %v/v) of black tea (n=5) infusions against clinical C. difficile isolates*

		BP-5		BP-8		BP-11		BP-17		BP-16	
Strain	**Ribotype**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Range**	**Mean[a]**
R22814	106	6.25 - 12.5	8.84	12.5 - 25	17.68	12.5	12.50	25 - 50	35.36	25	25.00
R22887	174	12.5	12.50	12.5	12.50	6.25 -12.5	8.84	25	25.00	12.5	12.50
R22888	015	12.5 - 50	25.00	25	25.00	12.5 - 50	25.00	50	50.00	25	25.00
R22897	106	6.25 - 12.5	7.87	6.25 - 12.5	9.92	6.25 -12.5	9.92	6.25 - 25	15.75	6.25 - 12.5	9.92
R23121	027	6.25	6.25	6.25 - 12.5	9.92	6.25 -12.5	9.92	12.5 - 25	19.84	6.25 - 12.5	9.92
R23800	106	6.25	6.25	12.5	12.50	6.25	6.25	6.25	6.25	6.25	6.25
R24565	027	6.25	6.25	6.25 - 25	12.50	6.25	6.25	12.5 - 25	17.68	6.25 - 12.5	8.84
R26452	020	6.25 - 12.5	9.92	25	25.00	6.25 -12.5	9.92	12.5 - 25	19.84	25 - 50	31.50
R26720	106	6.25	6.25	6.25 - 12.5	9.92	6.25 -12.5	7.87	6.25 - 25	12.50	6.25 - 25	12.50
R26797	027	6.25	6.25	12.5	12.50	6.25 -12.5	7.87	12.5 - 25	19.84	12.5	12.50
R28972	014	12.5	12.50	12.5 - 25	19.84	6.25 -12.5	7.87	12.5 - 50	25.00	12.5 - 25	15.75
R29035	002	12.5	12.50	12.5 - 25	19.84	6.25 -12.5	7.87	25 - 50	35.36	25 - 50	31.50
R29039	078	6.25	6.25	12.5	12.50	6.25	6.25	6.25	6.25	12.5	12.50
R29078	027	6.25 - 12.5	12.50	12.5 - 25	19.84	6.25 -12.5	9.92	25 - 50	39.69	12.5 - 25	19.84
R29096	106	6.25	6.25	6.25 - 12.5	7.87	6.25 -12.5	7.87	12.5	12.50	12.5 - 25	15.75
R31056	027	6.25	6.25	12.5	12.50	6.25	6.25	12.5 - 25	17.68	6.25	6.25
R31234	106	6.25 - 12.5	8.84	12.5	12.50	6.25 -12.5	8.84	6.25 - 25	12.50	6.25 - 25	12.50
R31312	056	6.25 - 12.5	9.92	12.5 - 25	15.75	6.25 -12.5	9.92	6.25 - 25	12.50	6.25 - 12.5	9.92
R31315	013	6.25 - 12.5	9.92	12.5	12.50	6.25 -12.5	9.92	12.5 - 50	25.00	6.25 - 25	12.50
R31350	220	12.5 - 25	17.68	12.5	12.50	6.25	6.25	12.5 - 50	25.00	12.5 - 25	17.68
R31382	118	6.25	6.25	12.5	12.50	6.25	6.25	6.25	6.25	6.25	6.25
DS1407	015	6.25	6.25	6.25 - 25	12.50	6.25 -12.5	7.87	12.5 - 25	15.75	6.25 - 25	12.50
DS1447	081	6.25 - 25	9.92	6.25 - 50	15.75	6.25 -12.5	7.87	25 - 50	25.00	12.5 - 50	25.00
DS1507	106	6.25 - 12.5	7.87	12.5 - 25	12.50	6.25	6.25	6.25	6.25	6.25 - 25	12.50
DS1758	023	6.25 - 12.5	8.84	25	25.00	12.5	12.50	12.5 - 50	25.00	25 - 50	35.36
DS1859	001	6.25 - 12.5	8.84	12.5 - 25	17.68	12.5	12.50	25 - 50	35.36	25	25.00
DS2090	124	6.25	6.25	6.25 - 25	8.84	6.25	6.25	12.5 - 25	17.68	6.25	6.25
DS2098	023	6.25 - 12.5	7.87	12.5	12.50	6.25 -12.5	9.92	12.5 - 50	31.50	12.5 - 50	25.00
DS2107	081	6.25 - 12.5	7.87	12.5	12.50	6.25 -12.5	9.92	6.25 - 25	15.75	6.25 - 25	9.92
DS2117	014	6.25	6.25	6.25 - 25	12.50	6.25 -12.5	7.87	12.5 - 25	19.84	6.25 - 12.5	9.92
DS2205	001	6.25 - 12.5	7.87	12.5 - 25	19.84	6.25 - 25	9.92	25 - 50	39.69	12.5 - 50	25.00
DS2285	106	6.25 - 12.5	9.92	12.5 - 25	19.84	12.5 - 25	15.75	25 - 50	39.69	12.5 - 25	19.84
DS2305	174	12.5	12.50	12.5 - 25	17.68	12.5	12.50	25 - 50	35.36	12.5 - 50	25.00
DS2315	011	12.5 - 25	15.75	25 - 50	31.50	12.5 - 25	15.75	50	50.00	12.5 - 50	25.00
DS2315	027	6.25 - 12.5	9.92	12.5 - 25	19.84	12.5	12.50	25 - 50	39.69	25	25.00
DS2325	027	6.25	6.25	6.25 - 12.5	9.92	6.25 -12.5	7.87	12.5 - 25	19.84	12.5	12.50
DS2335	027	6.25 - 12.5	9.92	12.5	12.50	6.25 -12.5	7.87	12.5 - 25	19.84	12.5 - 25	15.75
DS2345	027	6.25 - 25	12.50	12.5	12.50	6.25 -12.5	8.84	25 - 50	35.36	25	25.00

[a]*Geometric mean of three repeats.*

Table 5.8. *The antimicrobial activity (MIC µg/mL) of EGCG and GA against clinical C. difficile isolates*

		EGCG		GA				EGCG		GA	
Strain	**Ribotype**	**Range**	**Mean[a]**	**Range**	**Mean[a]**	**Strain**	**Ribotype**	**Range**	**Mean[a]**	**Range**	**Mean[a]**
R13929	001	64 - 125	99	64	63	**R22814**	106	125	125	64 -250	126
R14057	106	125	125	250 - 500	354	**R22887**	174	64	64	64	63
R14496	020	32 - 125	64	64 - 125	80	**R22888**	015	250	250	250	250
R14933	002	64 - 125	99	64 - 125	79	**R22897**	106	8 - 16	13	32 - 64	50
R14935	014	125	125	64 - 125	79	**R23121**	027	32 - 64	50	32 - 64	50
R14936	070	64 - 125	100	64 - 125	99	**R23800**	106	64	64	64	63
R15213	072	125	125	32	31	**R24565**	027	8 - 16	11	8 - 32	16
R15627	078	250	250	500	500	**R26452**	020	125	125	64 - 125	79
R15632	001	16	16	32 - 125	62	**R26720**	106	16 - 32	20	16 - 64	25
R15691	023	64	64	32 - 64	44	**R26797**	027	8 - 16	13	32 - 64	40
R16631	005	16	16	64 - 125	80	**R28972**	014	64 - 125	79	32 - 125	79
R16632	137	16 - 32	50	64	63	**R29035**	002	125	125	64 -250	125
R16633	138	32 - 64	50	125	125	**R29039**	078	64	64	64	63
R16762	027	16	16	64	63	**R29078**	027	64	64	64 - 125	79
R17015	001	16 - 32	20	32 - 64	50	**R29096**	106	32 - 64	39	16 - 64	40
R17060	106	125	125	64 - 125	79	**R31056**	027	16	16	16 - 64	32
R17083	027	64 - 125	89	64 - 125	89	**R31234**	106	16	16	125 - 250	177
R17302	106	64	64	64	63	**R31312**	056	32 - 125	49	32 - 125	63
R17849	078	125 - 250	198	125 - 250	198	**R31315**	013	32 - 64	39	32 - 125	49
R17857	106	8 - 16	13	8 - 32	13	**R31350**	220	16 - 32	45	64 - 125	89
R18050	001	125	125	64 -250	125	**R31382**	118	64	64	16	16
R18080	018	64	64	64 - 125	99	**DS1407**	015	16 - 32	20	16 - 64	25
R18413	002	64	64	64 - 125	89	**DS1447**	081	16 - 32	20	16 - 32	25
R18603	106	64	64	500.00	500	**DS1507**	106	16	16	8 - 16	13
R18738	005	64	64	500.00	177	**DS1758**	023	125	125	125 - 250	177
R19157	001	64 - 125	99	32 - 64	44	**DS1859**	001	125	125	64	63
R19630	015	64 - 125	99	64 - 125	79	**DS2090**	124	16	16	16 - 32	22
R19885	002	125	125	64 - 125	89	**DS2098**	023	64 - 125	99	64	63
R20063	106	64 - 125	99	125 - 250	157	**DS2107**	081	16	16	8 - 32	20
R20700	045	125 - 250	198	125 - 250	157	**DS2117**	014	8 - 16	13	64 - 125	80
R20702	001	64 - 125	100	64 - 125	79	**DS2205**	001	64 - 125	80	64 - 125	79
R20703	106	16 - 32	25	8	8	**DS2285**	106	64	64	64 - 125	79
R20831	001	16	16	16 - 64	32	**DS2305**	174	125	125	64 -250	125
R20962	106	32 - 64	50	64 - 125	109	**DS2315**	011	64 - 125	100	64 -250	125
R20976	106	125	125	125 - 250	157	**DS2315**	027	64	64	64 -250	125
R21028	014	125	125	250	250	**DS2325**	027	16 - 32	25	32 - 64	108
R22487	017	8 - 16	11	8 - 16	11	**DS2335**	027	32 - 125	49	64	63
R22753	001	64	64	64	63	**DS2345**	027	8 - 32	13	125 - 250	157

[a]*Geometric mean of three repeats.*

A significant difference in antimicrobial activity was found within the green teas as shown in figure 5.3.

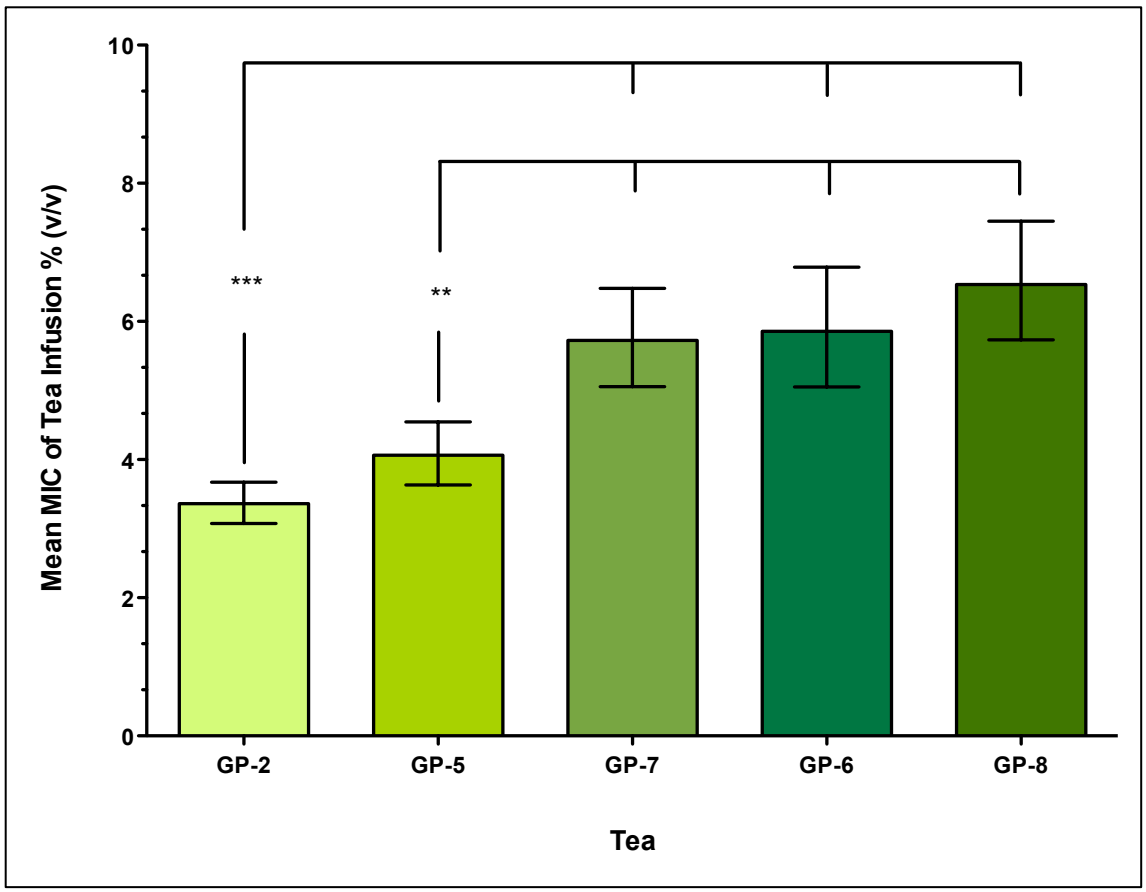


Figure 5.3. *Comparison of the mean (geometric) antimicrobial activity of green tea infusions against C. difficile (n=75). Groups were compared by the Kruskal-Wallis test (***=p<0.001; **= p<0.01; error bars = 95% CI).*

The antimicrobial activity of the Kenyan green teas GP-2 and GP-5 were significantly higher ($p<0.001$) than that of the other three green teas examined in this study, but were not significantly different from each other. These teas were also shown to be the most catechin rich (Ch.3; fig 3.34) and have the highest antimicrobial activity against *S. aureus* (Ch.4; table 4.6).

In comparison to the green teas, there was greater variation in the antimicrobial activities of the black teas (fig 5.4). Furthermore, it was observed that tea BP-17 had inhibitory activity across all strains of *C. difficile* in the study (table 5.7c-d). Previously,

it was shown to have no inhibitory activity against *S. aureus* (>50 %v/v) implying that *C. difficile* may be more susceptible to the antimicrobial effects of tea. However, more strains of *S. aureus* would need to be tested to confirm this theory.

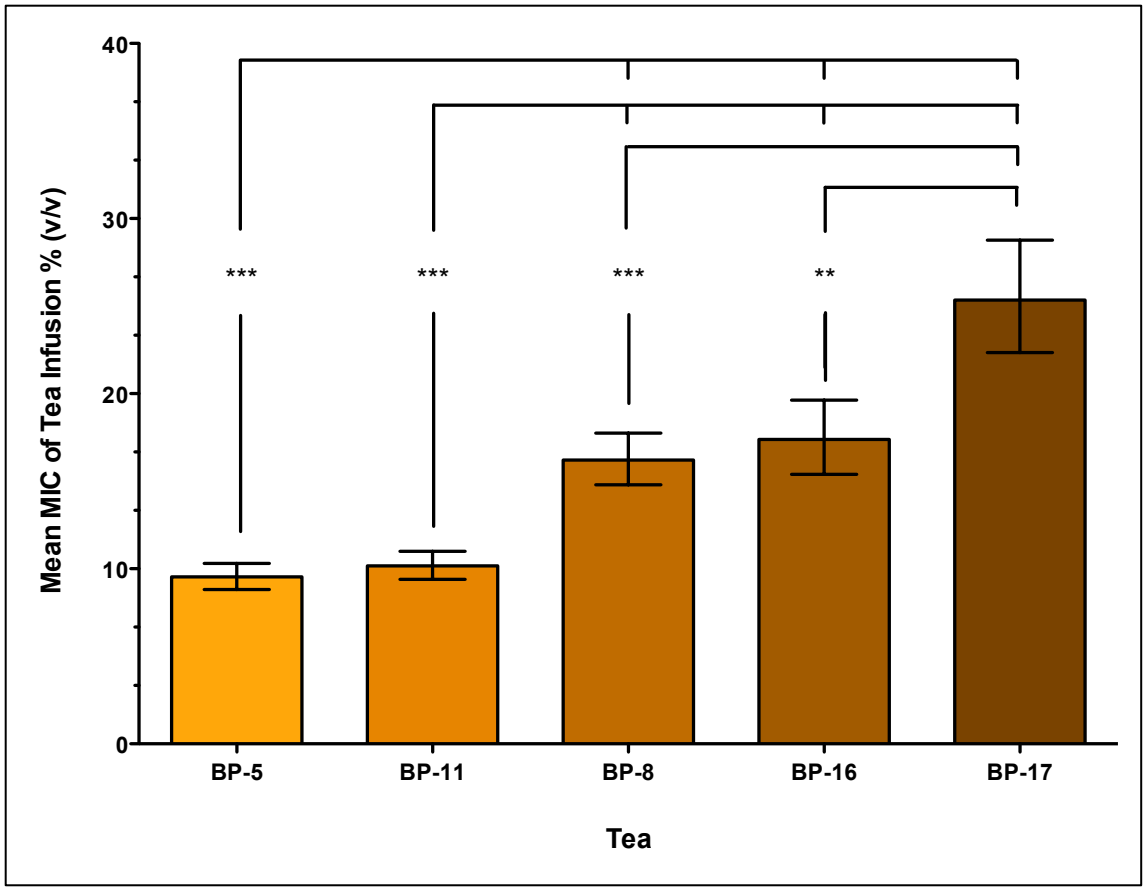


Figure 5.4. *Comparison of the mean (geometric) antimicrobial activity of black tea infusions against C. difficile (n=75). Groups were compared by the Kruskal-Wallis test (***=p<0.001, **= p<0.01; Error bars = 95% CI).*

In the preceding chapter (Ch.4; table 4.10) a strong correlation was found between the catechin content of tea and its antimicrobial activity against *S. aureus*. It would appear that this trend is also true for *C. difficile* (fig 5.5).

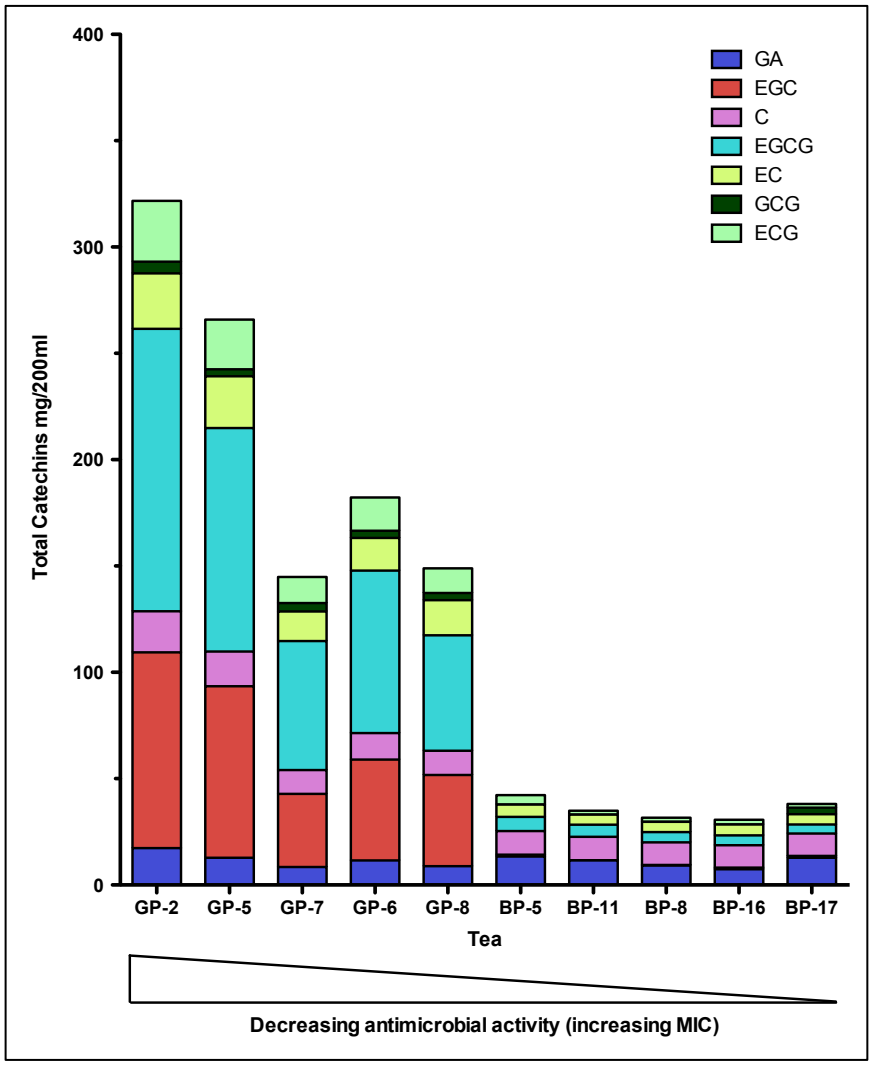


Figure 5.5*. Comparison of the catechin content of green (n=5) and black tea (n=5) ranked in order of descending antimicrobial activity against C. difficile (n=75).*

5.4.1.3 Comparison of the Antimicrobial Activity of Green and Black Tea Infusions against *C. difficile*

The mean MIC of green and black teas against all strains of *C. difficile* (n=75) was 5.9 and 17.4 %(v/v) respectively (fig 5.6). To determine if this difference was significant, the log MIC values were compared using a Mann-Whitney U test.

The test revealed that there was a significant difference in the MIC values between green and black teas (U= 12237; z = -19.35; p = <0.001; r = 0.71). This is similar to

what was found with *S. aureus* (Ch. 4; fig 4.7) and would support that green tea, which has higher catechin content than black tea, are more potent antimicrobials.

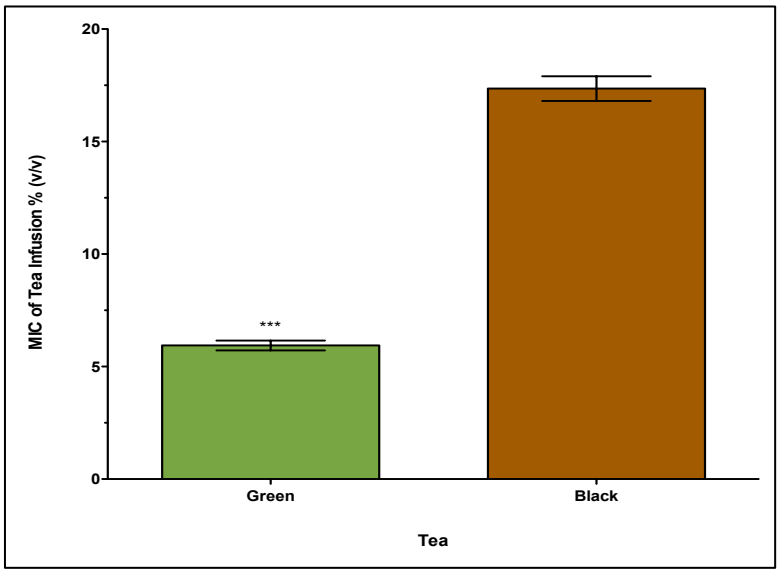


Figure 5.6. *Comparison of the mean (geometric) antimicrobial activity of green (n=5) and black tea (n=5) infusions against C. difficile (n=75). Groups were compared by the Mann-Whitney U test (***=p<0.001; error bars = 95% CI).*

5.4.1.4 Antimicrobial Activity of Tea against different *C. difficile* ribotypes

To determine if green and black teas were equally effective against the different ribotypes of *C. difficile*, the log MIC of tea against the three most common ribotypes, namely 027, 001 and 106, were compared using the Kruskal Wallis and the Bonferroni-Dunn's multiple comparisons test (fig 5.7).

It was found that there was no significant difference (p>0.05) in inhibitory activity of green and black teas between different ribotypes of *C. difficile.* As previously discussed, different ribotypes are related to different pathological attributes of the bacteria. However it would appear that this does not influence the susceptibility of the bacteria to the inhibitory activity of tea. This is advantageous, as it highlights the potential application of tea for treating patients presenting with both mild and severe cases of CDI.

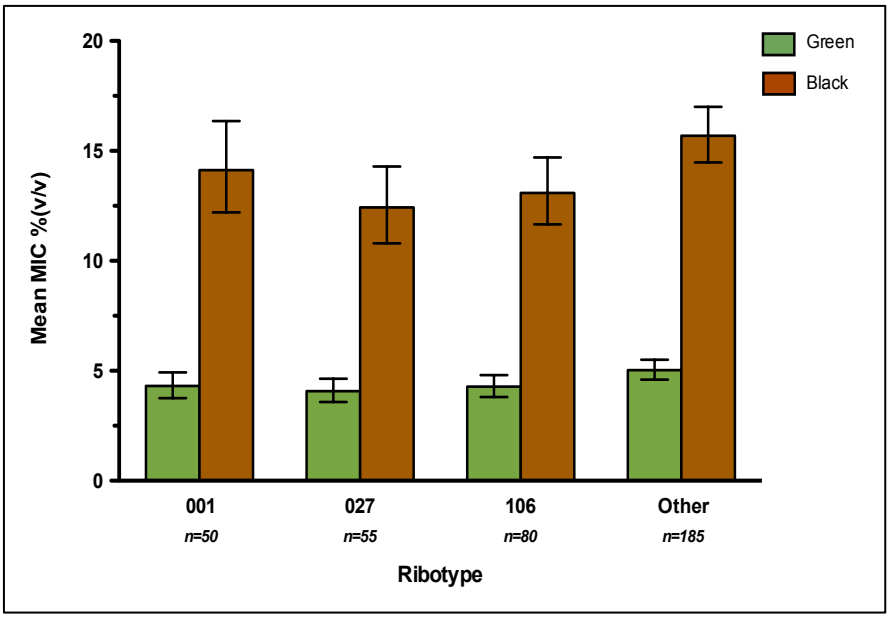


Figure 5.7. *Comparison of the mean (geometric) antimicrobial activity of green (n=5) and black tea (n=5) infusions against different C. difficile ribotypes. Ribotypes (n): 001 (10), 027 (11), 106 (16), other (37). Groups were compared by the Kruskal-Wallis test (error bars = 95% CI).*

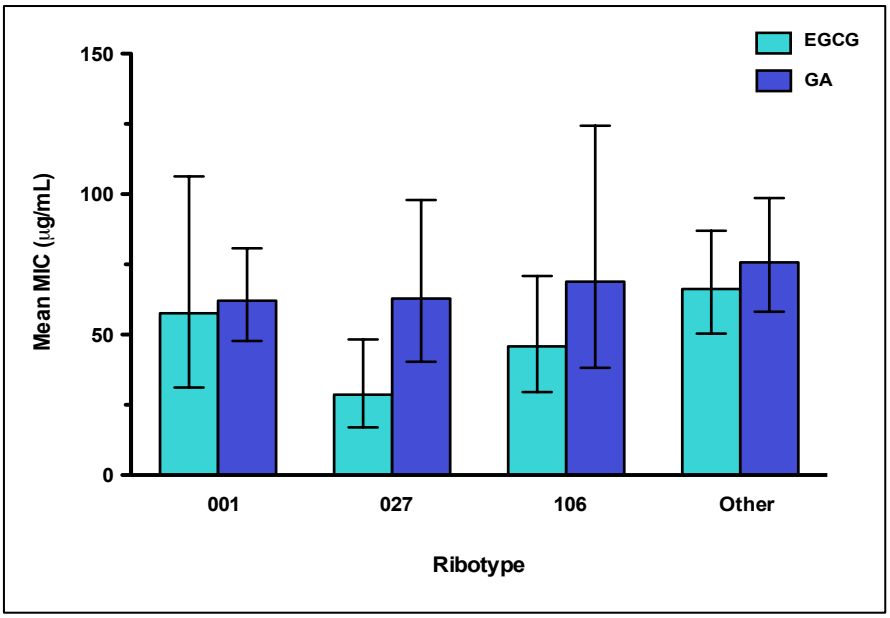


Figure 5.8. *Comparison of the mean (geometric) antimicrobial activity of EGCG and GA against different C. difficile ribotypes. Ribotypes (n): 001 (10), 027 (11), 106 (16), other (37). Groups were compared by the Kruskal-Wallis test (error bars = 95% CI).*

The Kruskal Wallis and the Bonferroni-Dunn's multiple comparisons test was also used to determine if different ribotypes has different susceptibility to EGCG and GA. Comparable to what was observed with tea infusions, no significant difference (p>0.05) was found (fig 5.8).

5.4.1.5 Susceptibility of *C. difficile* to Tea According to Year of Isolation

It is well known that bacteria can acquire resistance to antimicrobials over time due to a variety of different factors (Levy and Marshall, 2004). Therefore, it is useful to compare the susceptibility of bacteria to an antimicrobial over a period of time to determine if any strains have acquired any resistant mechanisms that counteract its efficacy. Although tea is not currently used as a treatment for CDI, exposure of *C. difficile* to different antibiotics over time may affect the sensitivity of the bacteria to tea indirectly, as seen with other antibiotics. (Webber and Piddock, 2003). Furthermore, since tea is one of the world's most popular drinks (Fernández-Cáceres *et al*., 2001) and at least 1 – 3 % of the population are asymptomatic carriers of *C. difficile* (Carlson *et al*., 2013), exposure of *C. difficile* to tea in the environment is very likely.

In figure 5.9, with the exception of 2002 and 2009, isolates of *C. difficile* appear to have become more sensitive to the effects of tea each year from 2000 to 2011. To determine if the inhibitory activity of tea was significantly different in 2000 compared to 2011 the Log MIC of green and black teas was compared using a t-test. A significant difference in the inhibitory activity of green tea against isolates from 2000 and 2011 was found (t = 5.18; p <0.001, two-tailed; n = 60; 95% *CI*: 0.15 – 0.33; fig 5.9.). The magnitude of the difference in means was large (η^2= 0.32).

Similarly, a significant difference in the inhibitory activity of black tea against isolates from 2000 and 2011 was found also (t = 4.35, p = <0.001, two-tailed; n = 60; 95% *CI*: 0.14 – 0.37; fig 5.9). Again, the magnitude of the difference in means was large (η^2= 0.35).

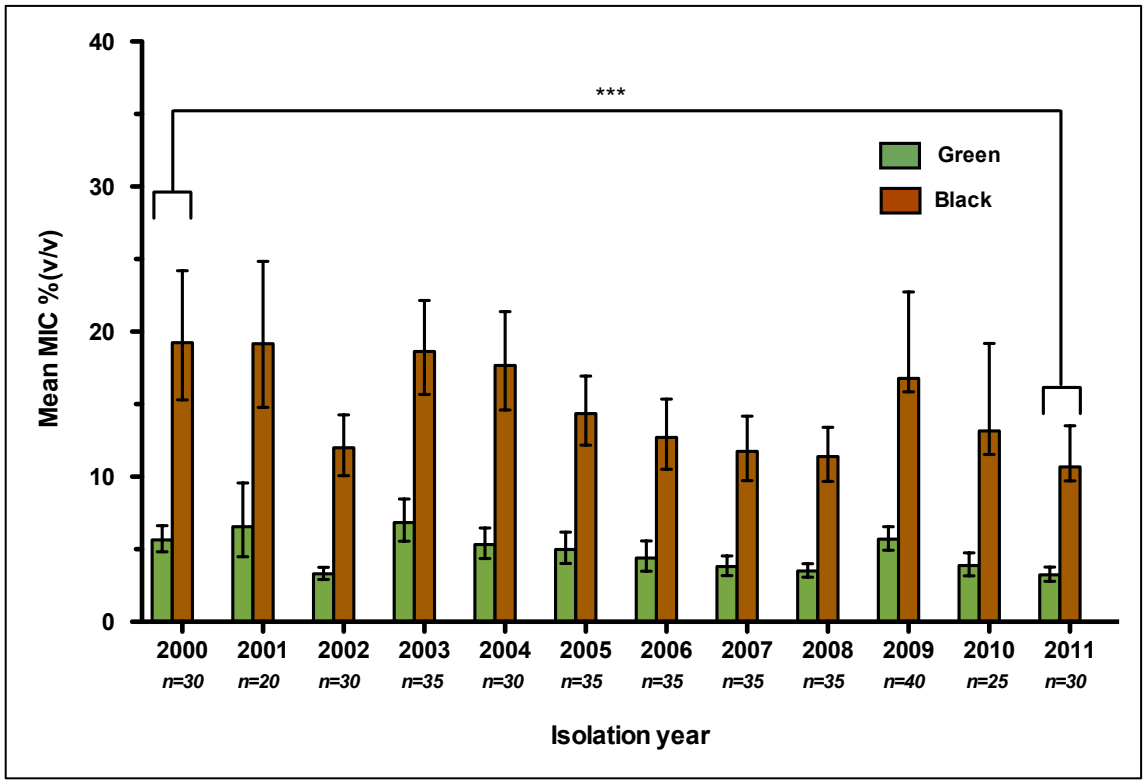


Figure 5.9. *Comparison of the mean (geometric) antimicrobial activity of green (n=5) and black tea (n=5) infusions against different C. difficile strains according to year of isolation. Strains per year (n): 2000 (6), 2001 (4), 2002 (6), 2003 (7), 2004 (6), 2005 (7), 2007 (7), 2008 (7), 2009 (8), 2010 (5), 2011 (6). The antimicrobial activity of tea against isolates from the year 2000 were compared to isolates arising in 2011 by the t-test (***=p<0.001; error bars = 95% CI).*

Even though a stable metronidazole resistant *C. difficile* has been isolated recently (Lynch *et al*., 2013), there is currently no evidence to suggest that globally, *C. difficile* is becoming more or less susceptible to the current antibiotics (Spigalia *et al*., 2011). Therefore, it is possible that other environmental factors may have given rise to the increased susceptibility of *C. difficile* to tea observed in this study, although further work on more isolates would be required to verify this observation.

5.4.1.6 Identification of Strains with Increased or Decreased Susceptibility to Tea

Collectively, the inhibitory concentration of tea infusions against *C. difficile* was comparable across different strains and ribotypes (table 5.7a-d). Furthermore, each tea within the tested dilution range inhibited all strains of *C. difficile*. However, it is

apparent that a few strains appeared to be more susceptible or more resistant to the inhibitory effects of tea. By reviewing the distribution of MIC values across strains for each tea, it was found that the extreme cases were present in the 15th and 85th percentile. The strains that had MIC's in these percentiles for black and green teas most often are shown in table 5.9.

Table 5.9. *The occurrence of C. difficile strains identified as being resistant (MICs in 85th percentile) or susceptible (MICs in the 15th percentile) to the inhibitory effects tea infusions and their sensitivity to EGCG, GA, vancomycin and metronidazole. '-' = data not available. [1] Mean (geometric) MIC across all tested isolates; EGCG (n= 75), GA (n =75), vancomycin (n = 44), metronidazole (n = 44).*

	% Occurrence (n) in teas		Antibiotic/Catechin MIC (µg/mL)			
Strain	**Green**	**Black**	**EGCG**	**GA**	**Vancomycin**	**Metronidazole**
Resistant **= 85th percentile of MIC values**						
R15627	100 (5)	100 (5)	250	500	1	0.5
R14057	100 (5)	100 (5)	125	354	-	-
R17849	100 (5)	100 (5)	198	198	0.5	0.5
R22888	80 (4)	100 (5)	250	250	1	0.12
R18603	100 (5)	80 (4)	64	500	-	-
R20700	100 (5)	80 (4)	198	157	1	0.12
R21028	80 (4)	80 (4)	125	250	1	0.12
R19885	40 (2)	100 (5)	125	89	1	0.12
R14933	0 (0)	80 (4)	99	79	-	-
Susceptible **= 15th percentile of MIC values**						
R22487	100 (5)	80 (4)	11	11	-	-
R29039	100 (5)	80 (4)	64	64	0.06	0.06
R20703	80 (4)	100 (5)	25	8	-	-
R31382	80 (4)	100 (5)	64	16	-	-
R23800	60 (3)	100 (5)	64	64	-	-
R16762	80 (4)	80 (4)	16	63	-	-
R22753	80 (4)	80 (4)	64	64	-	-
R31056	80 (4)	80 (4)	16	32	-	-
DS2090	60 (3)	80 (4)	16	22	1	0.12
R26720	60 (3)	60 (3)	20	25	1	0.12
		Mean[1]	53	70	1	0.12

As shown in table 5.9, strains *R14057*, *R15627* and *R17849* had MICs in the upper 85th percentile in every green and black tea tested in this study. Furthermore, the MIC's of EGCG and GA against these three strains are also considerably higher than the mean MIC for these compounds across all the strains. This trend is also seen for the other

strains as identified being less susceptible to the effects of tea (table 5.9). However, it did not appear that these stains are less susceptible to metronidazole or vancomycin, suggesting that that this affect is unique to tea (MIC range for metronidazole and vancomycin are 0.5 – 2 μg/mL and 0.06 – 2 μg/mL respectively; HPAb 2012).

No strains of *C. difficile* had MIC's in the lower percentile for every green and black tea. However, the distribution of susceptibility was even between green and black teas. Unlike with the resistant strains, the strains identified as susceptible to tea were not necessarily more susceptible to EGCG and GA, although strains *R22487* and *R20703* had noticeably lower than average MIC for EGCG and GA. Due to the lack of available data it is unclear if these strains are more or less susceptible to vancomycin and metronidazole.

5.4.2 The Interactions of Green Tea GP-2 with Vancomycin and Metronidazole Against *C. difficile*

Combinations of green tea GP-2, vancomycin and metronidazole were tested against *C. difficile* to determine the presence of any synergistic or antagonistic relationships. In table 5.10 it can be seen that collectively (mean ΣFIC), combinations of vancomycin and GP-2 are predominately indifferent (98 %). In 16 (37 %) strains, antagonism was observed in at least one combination of vancomycin and GP-2, whilst in 2 strains (5 %) synergy was observed.

In one strain of *C. difficile*, namely *R22888,* antagonism was the predominant interaction. Antagonism was seen with vancomycin at 0.25 and 0.06 μg/mL in combination with tea GP-2 at 12.5 %(v/v), and with vancomycin at 0.125 μg/mL and GP-2 at 6.1 %(v/v).

A previous study found that combinations of EGCG and vancomycin resulted in antagonism against MRSA (Hu *et al*., 2002). However, it would seem that with green tea, which is a mixture of compounds, antagonism is not the predominant interaction with vancomycin.

***Table 5.10**. The interaction of combinations of different concentrations of green tea GP-2 and the antibiotics vancomycin and metronidazole against clinical isolates of C. difficile (n=44).*

		Vancomycin + Tea GP-2		Metronidazole + Tea GP-2	
Strain	**Ribotype**	**ΣFIC Range (Mean[1])**	**Interpretation[2]**	**ΣFIC Range (Mean[1])**	**Interpretation[2]**
R13929	001	1.00 - 2.50 (1.72)	I	1.24 - 2.48 (1.71)	I
R14496	020	1.00 - 2.25 (1.50)	I	1.25 - 2.50 (1.77)	I
R14935	014	1.06 - 2.50 (1.69)	I	1.00 - 2.48 (1.60)	I
R14936	070	1.00 - 2.50 (1.86)	I	1.25 - 2.96 (1.84)	I
R15691	023	1.00 - 1.25 (1.10)	I	1.50 (1.50)	I
R16631	005	0.75 - 1.50 (1.08)	I	1.00 - 2.25 (1.50)	I
R16632	137	1.50 - 4.13 (2.52)	I	1.25 - 4.34 (2.04)	I
R16633	138	1.06 - 5.01 (2.03)	I	1.00 - 2.16 (1.48)	I
R17015	001	0.31 - 1.25 (0.81)	I	1.00 - 1.25 (1.12)	I
R17060	160	1.06 - 3.00 (1.76)	I	1.12 - 6.01 (2.55)	I
R17083	027	1.24 - 4.51 (2.30)	I	1.00 - 4.17 (2.34)	I
R17857	106	0.56 - 1.50 (0.89)	I	0.67 - 2.00 (1.08)	I
R18080	018	1.06 - 2.06 (1.47)	I	1.50 - 4.24 (1.99)	I
R18413	002	0.56 - 2.00 (0.95)	I	1.50 - 2.33 (1.85)	I
R19630	015	0.56 - 2.50 (1.08)	I	0.75 - 2.08 (1.30)	I
R19885	002	0.56 - 2.50 (1.08)	I	0.75 - 2.08 (1.17)	I
R20063	106	1.06 - 4.51 (2.16)	I	1.00 - 2.24 (1.00)	I
R20700	045	1.06 - 2.16 (1.50)	I	0.98 - 2.25 (1.35)	I
R20962	106	0.75 - 1.50 (1.09)	I	1.00 - 2.48 (1.28)	I
R20976	106	0.56 - 6.17 (1.92)	I	1.00 - 4.17 (1.92)	I
R21028	014	0.56 - 3.00 (1.54)	I	1.25 - 8.17 (2.68)	I
R22814	106	1.06 - 1.50 (1.25)	I	0.98 - 1.96 (1.22)	I
R22887	174	0.75 - 2.12 (1.24)	I	0.98 - 2.50 (1.56)	I
R22888	015	4.13 - 8.26 (12.78)	A	4.49 - 16.48 (8.60)	A
R23121	027	0.50 - 1.12 (0.70)	I	0.75 - 1.50 (1.06)	I
R26452	020	0.56 - 1.50 (1.03)	I	0.54 -1.48 (0.97)	I
R26720	106	0.56 - 2.50 (1.55)	I	0.50 - 4.96 (1.34)	I
R28972	014	1.06 - 4.50 (2.24)	I	1.00 - 2.24 (1.54)	I
R29035	002	1.06 - 4.50 (2.09)	I	0.98 - 2.24 (1.54)	I
R29039	078	1.06 - 5.01 (2.30)	I	4.51 (4.51)	A
R29078	027	0.56 - 1.50 (0.83)	I	0.74 - 1.50 (1.08)	I
R31234	106	0.74 - 2.20 (1.40)	I	0.74 - 2.50 (1.34)	I
R31315	013	1.06 - 2.12 (1.43)	I	1.25 - 1.50 (1.37)	I
R31350	220	1.00 - 2.25 (1.28)	I	0.75 (0.75)	I
DS1447	081	1.50 - 4.20 (2.70)	I	0.75 - 6.00 (2.23)	I
DS1507	106	2.07 - 4.50 (3.41)	I	0.75 - 4.96 (2.75)	I
DS1859	001	2.01 - 4.50 (3.55)	I	2.25 - 4.48 (3.37)	I
DS2090	124	2.05 - 8.05 (3.03)	I	2.07 - 4.21 (2.63)	I
DS2205	001	0.31 - 1.25 (0.76)	I	0.75 - 1.00 (0.87)	I
DS2285	106	0.75 - 4.12 (1.66)	I	0.75 - 6.00 (2.05)	I
DS2305	174	0.56 - 4.02 (1.35)	I	0.75 - 3.00 (1.65)	I
DS2315	011	0.55 - 4.50 (1.98)	I	0.74 - 4.24 (1.65)	I
DS2335	027	0.56 - 1.50 (1.06)	I	1.17 - 3.00 (1.17)	I
DS2345	027	1.06 - 1.50 (1.22)	I	1.12 - 3.00 (1.56)	I

[1]Geometric mean of three repeats. [2]Interpetation of FIC: S = synergy, I = indifference, A = antagonism.

Similarly, the predominant interaction between green tea GP-2 and metronidazole was indifferent (95%). In 15 (34 %) strains, antagonism was observed in at least one combination of metronidazole and GP-2, however unlike with vancomycin, no synergy was observed.

Again with strain *R22888*, the predominant interaction between GP-2 and metronidazole was antagonism. This was seen with metronidazole at 0.06 μg/mL in combination with GP-2 at 3.1 %(v/v), and with metronidazole 0.03 μg/mL at and GP-2 at 12.5 %(v/v). Furthermore, antagonism was also the predominant interaction in strain *R29039*. In this strain antagonism was observed with metronidazole at 0.03 μg/mL and GP-2 at 6.1 %(v/v).

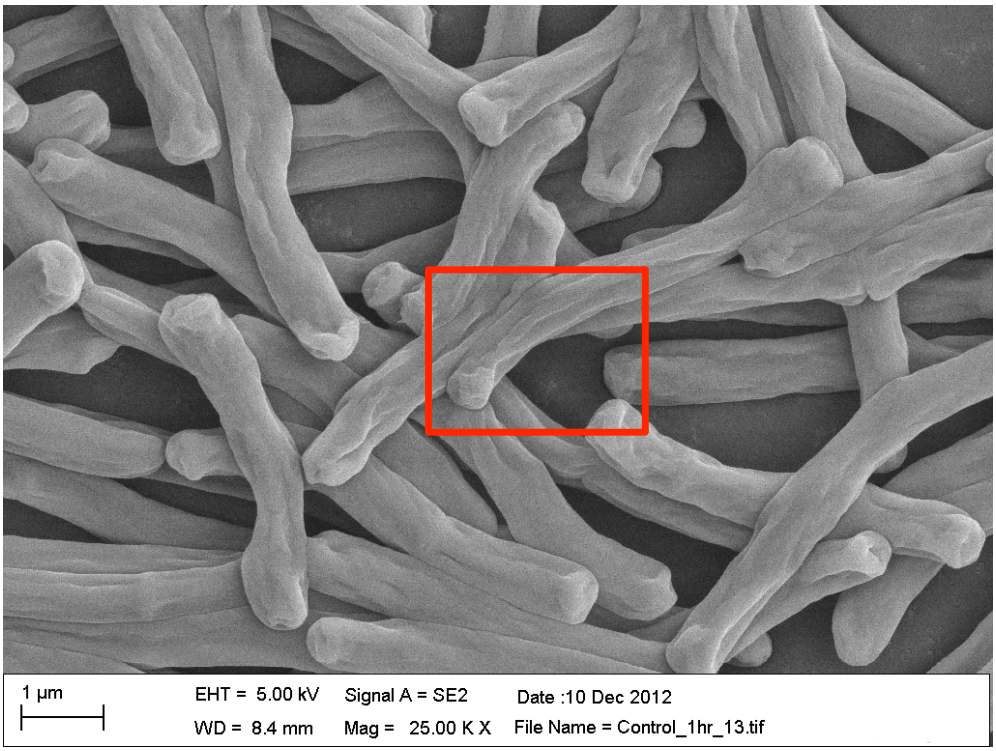
The appearance of antagonism would appear to be strain specific, although it is unclear how this would occur. Previously, it was found that strain *R22888* was less susceptible to the effects of tea and catechins than other strains. It is possible that this attribute may be linked to the antagonism seen in combination with antibiotics. However, this phenomenon was not observed with other less susceptible strains. Further investigation would be needed to clarify the nature of this antagonism.

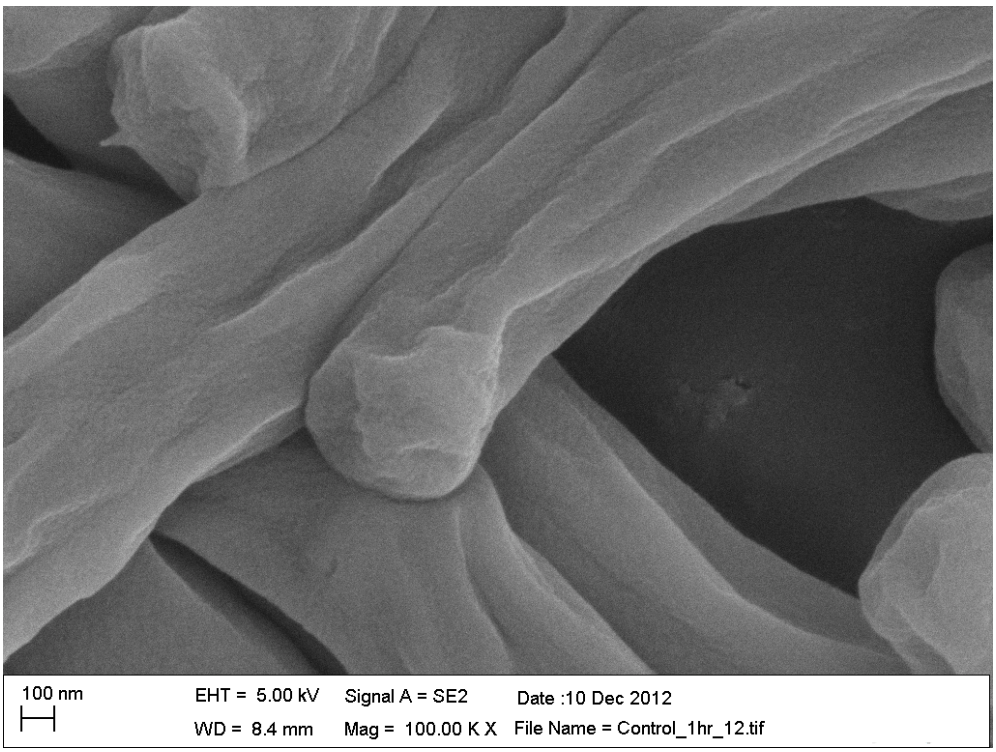
In summary, the interaction of combinations of green tea (GP-2) and the antibiotics to treat *C. difficile* appear to be predominately indifferent, suggesting that potential dosing of tea *in vivo* would not be affected by co-administration with these antibiotics. Yet, it does not rule out other potential interactions that might be found in an *in vivo* model.

5.4.3 Evaluation of the Effects of Tea GP-2 and EGCG Against *C. difficile* Using Scanning Electron Microscopy

In figures 5.10a-h the effects of water, green tea (GP-2) and EGCG against *C. difficile DS2335* can be seen over contact times of 1, 5 and 10 hrs. This strain was chosen as it represents one of the most prevalent clinical ribotypes (027) and its sensitivity to tea and EGCG was representative of all the strains in the study.

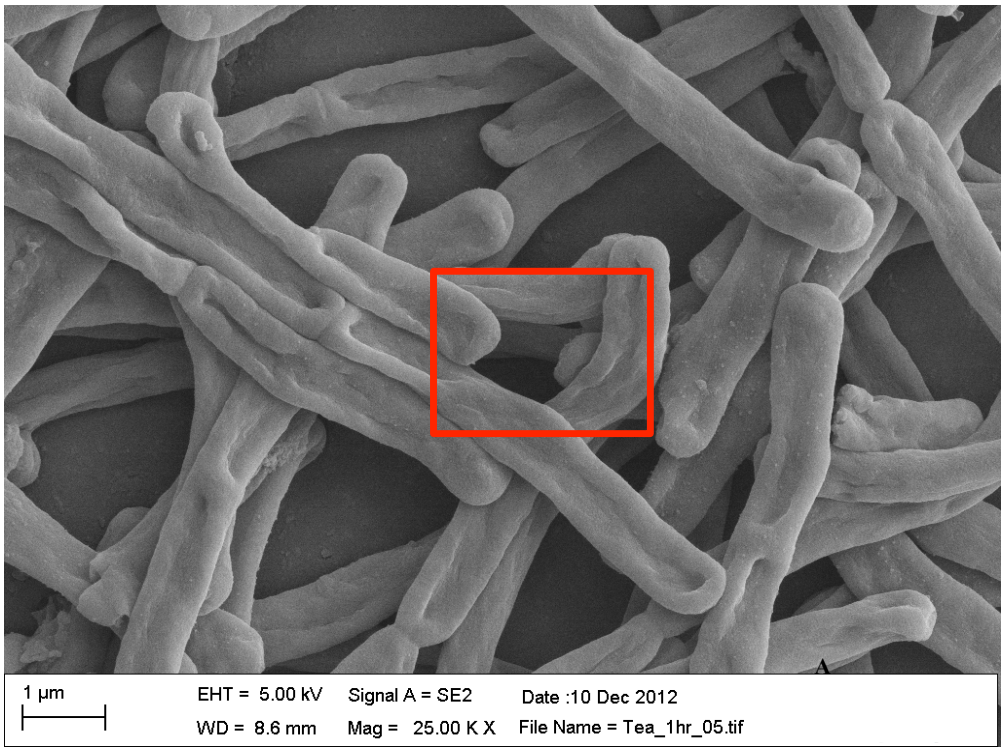
Fig 5.10a. *Scanning electron images of C. difficile DS2335 treated with water (control) for 1h at 25 k and 100 k magnification. Red box indicates area zoomed in at 100 k.*

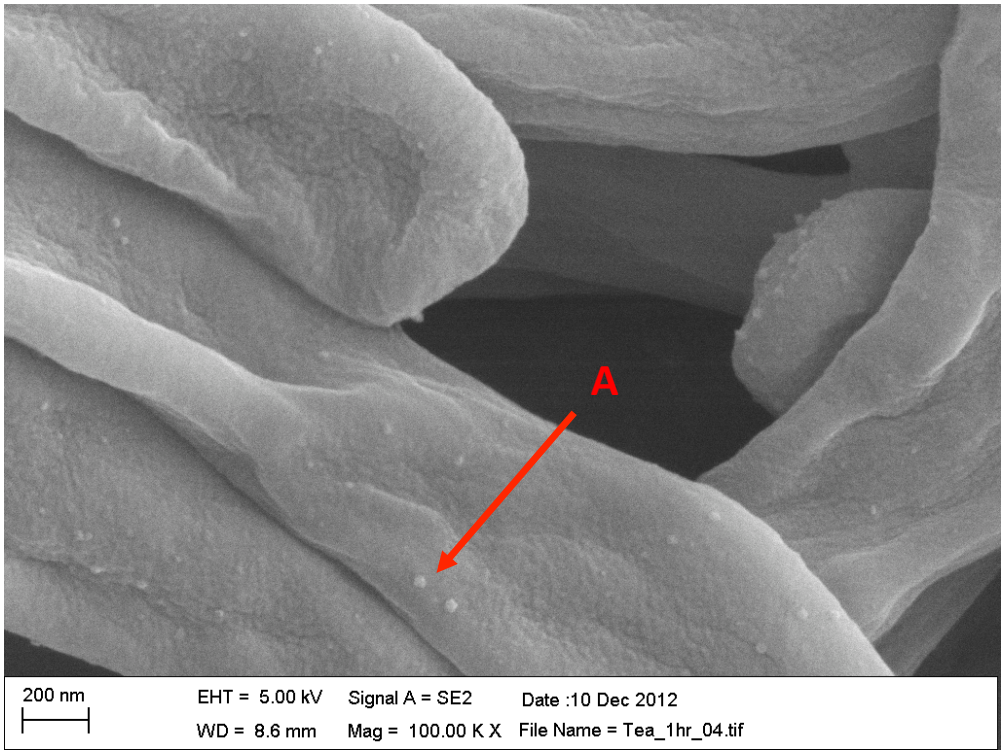




Water 1 h

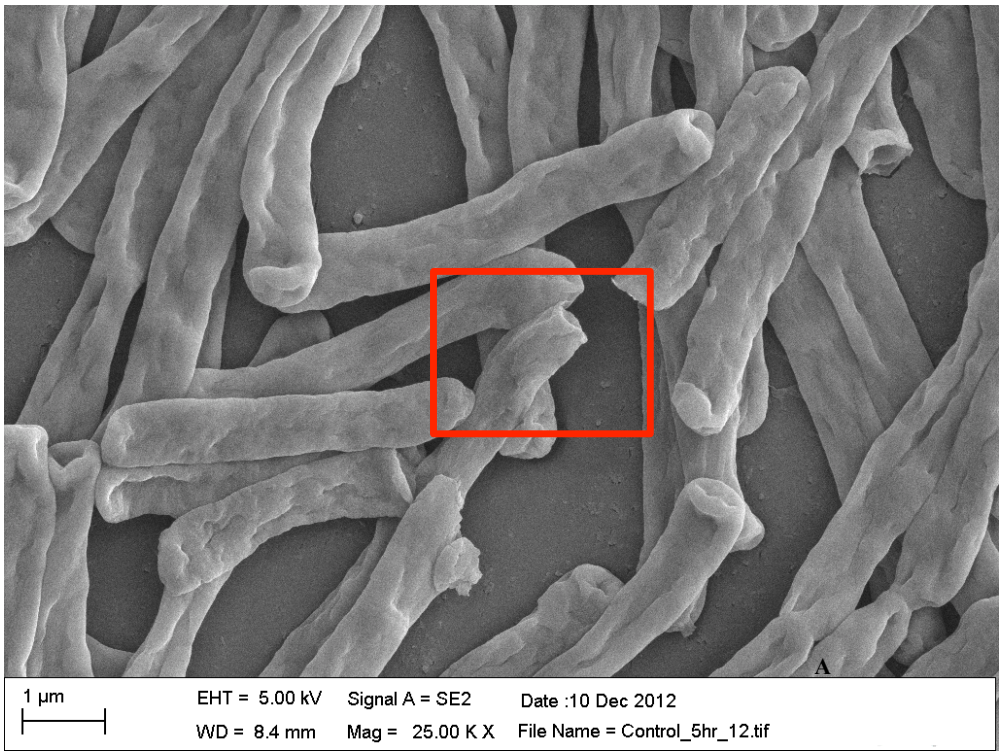
Fig 5.10b. *Scanning electron images of C. difficile DS2335 treated with green tea (GP-2l) for 1h at 25 k and 100 k magnification. Red box indicates area zoomed in at 100 k. 'A' = potential blebbing*

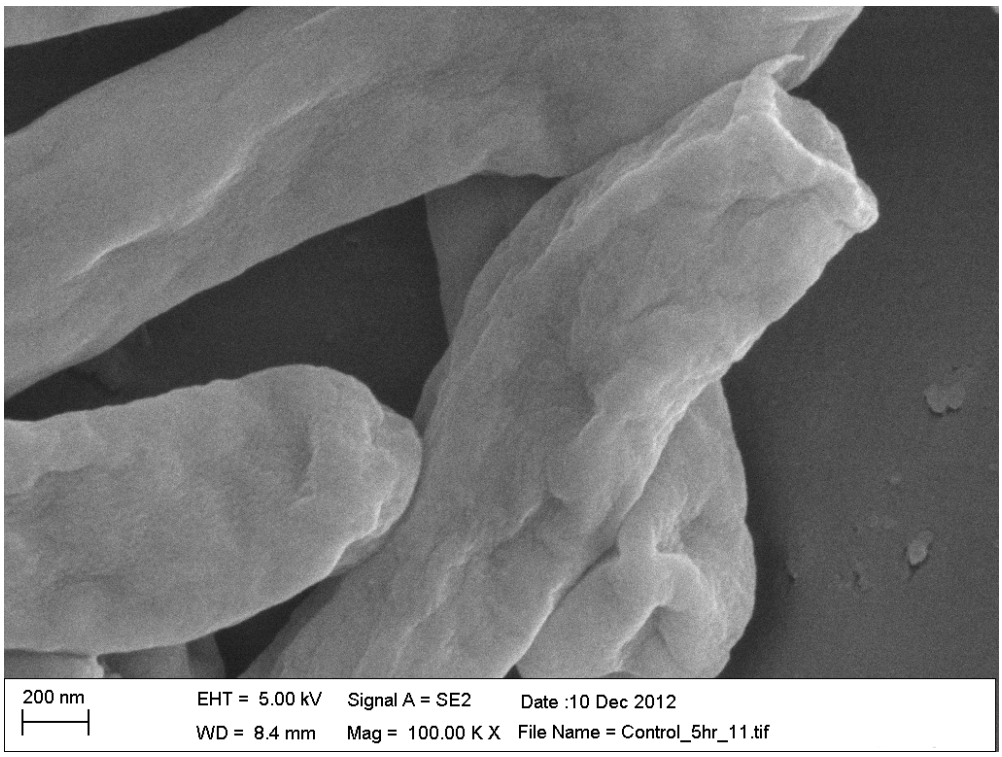




Tea 1 h

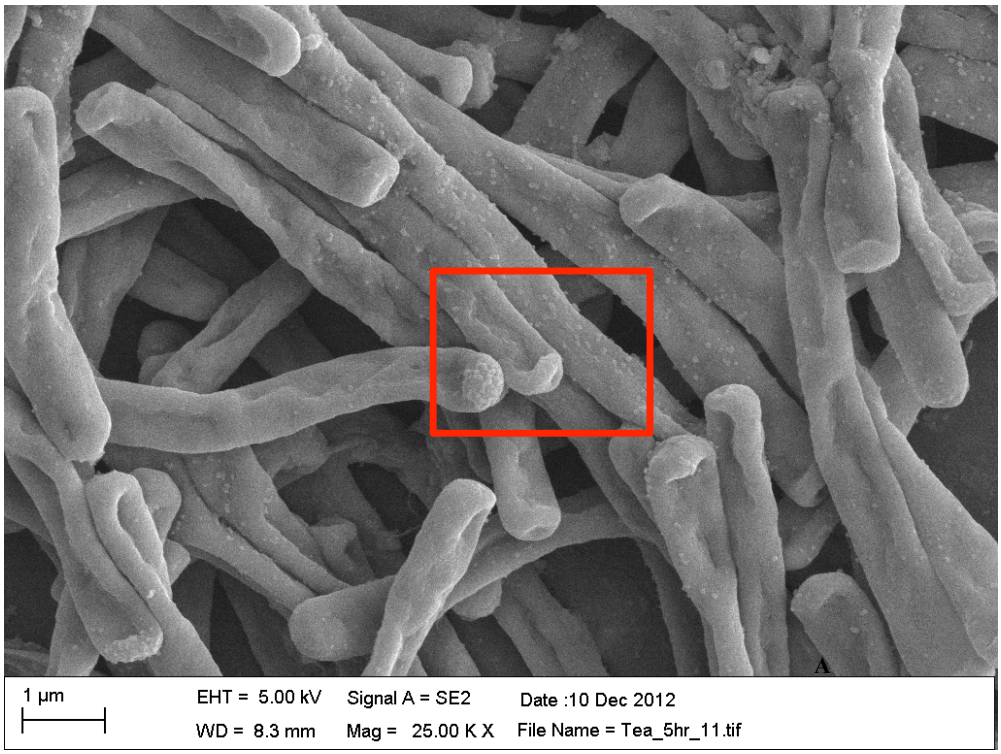
Fig 5.10c. *Scanning electron images of C. difficile DS2335 treated with water (control) for 5 h at 25 k and 100 k magnification. Red box indicates area zoomed in at 100 k.*

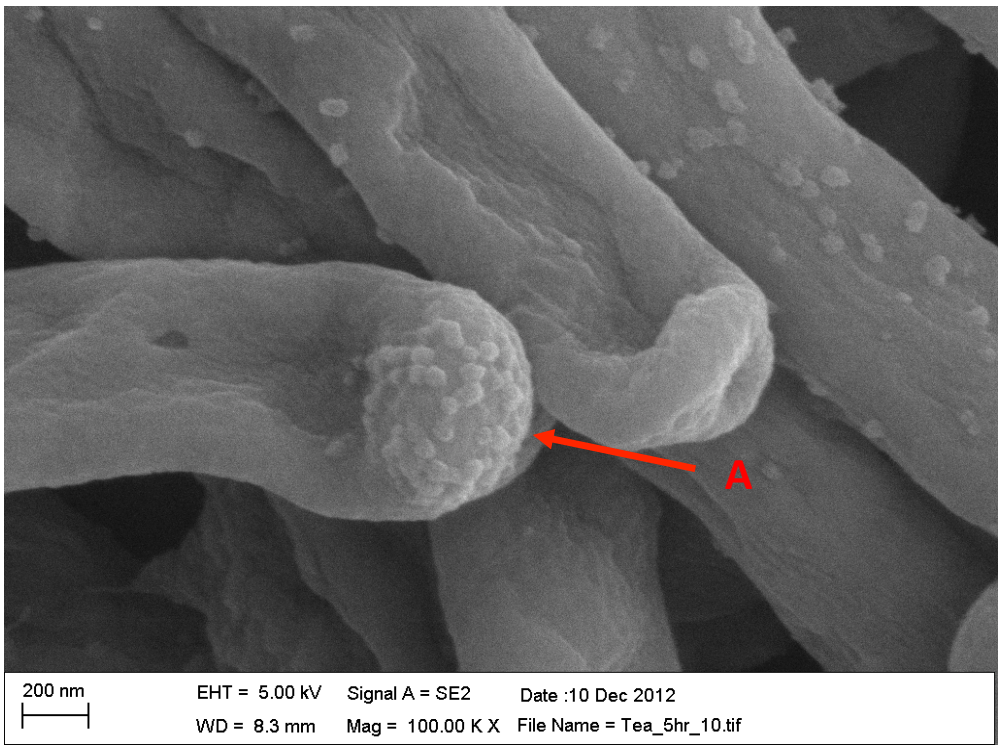




Water 5 h

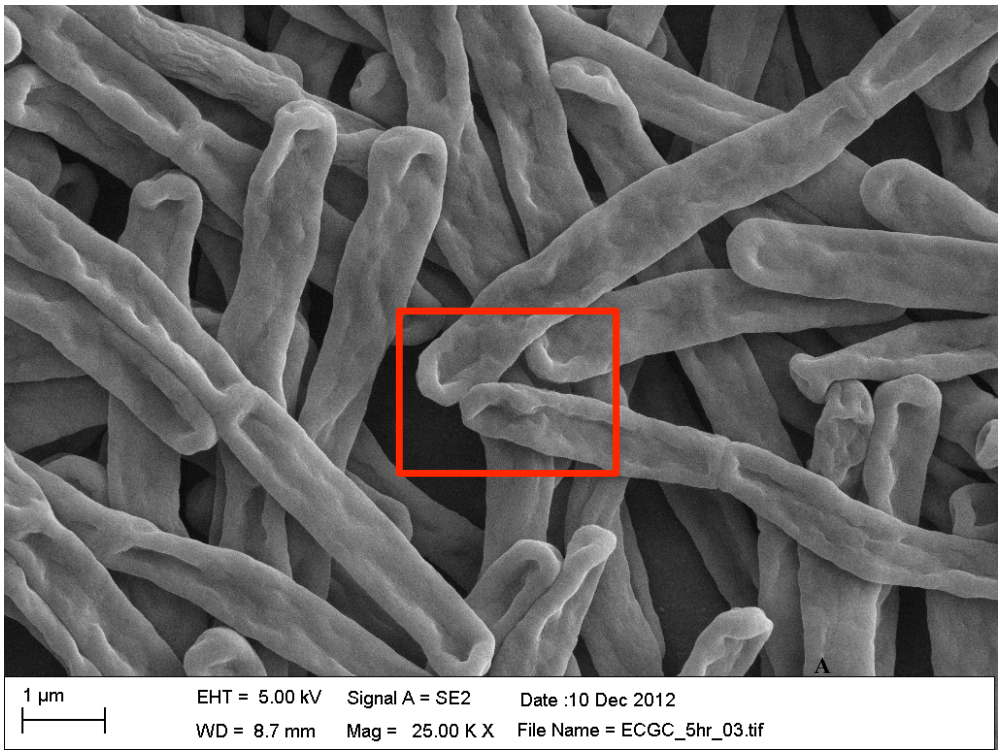
Fig 5.10d. *Scanning electron images of C. difficile DS2335 treated with Tea (GP-2) for 5 h at 25 k and 100 k magnification. Red box indicates area zoomed in at 100 k. A' = potential blebbing*

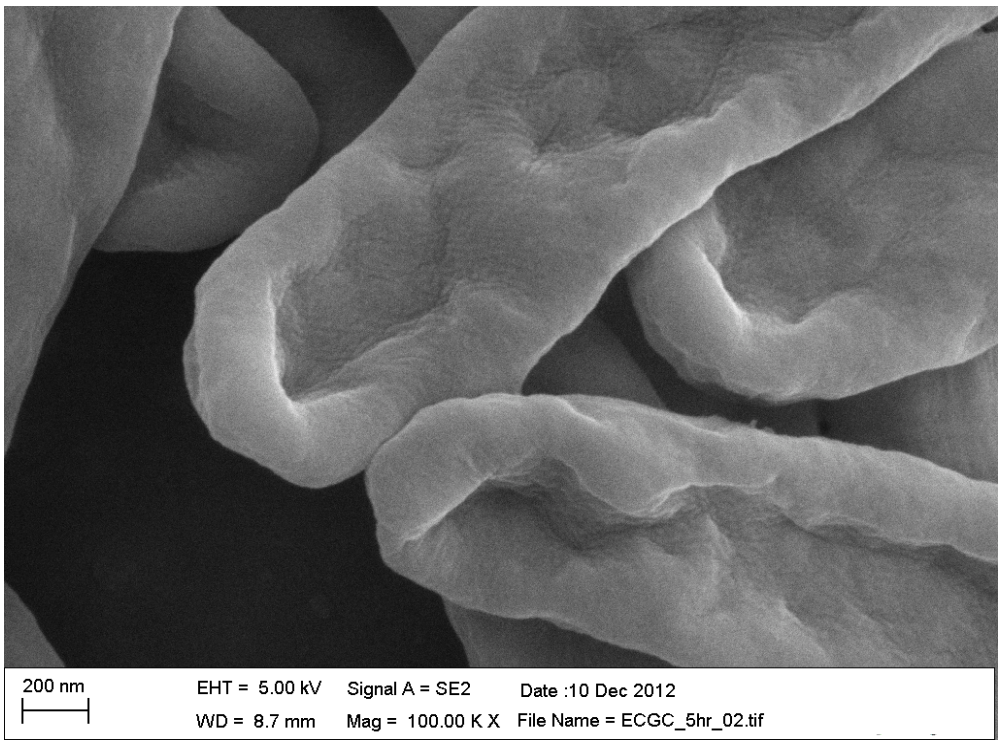




Tea 5 h

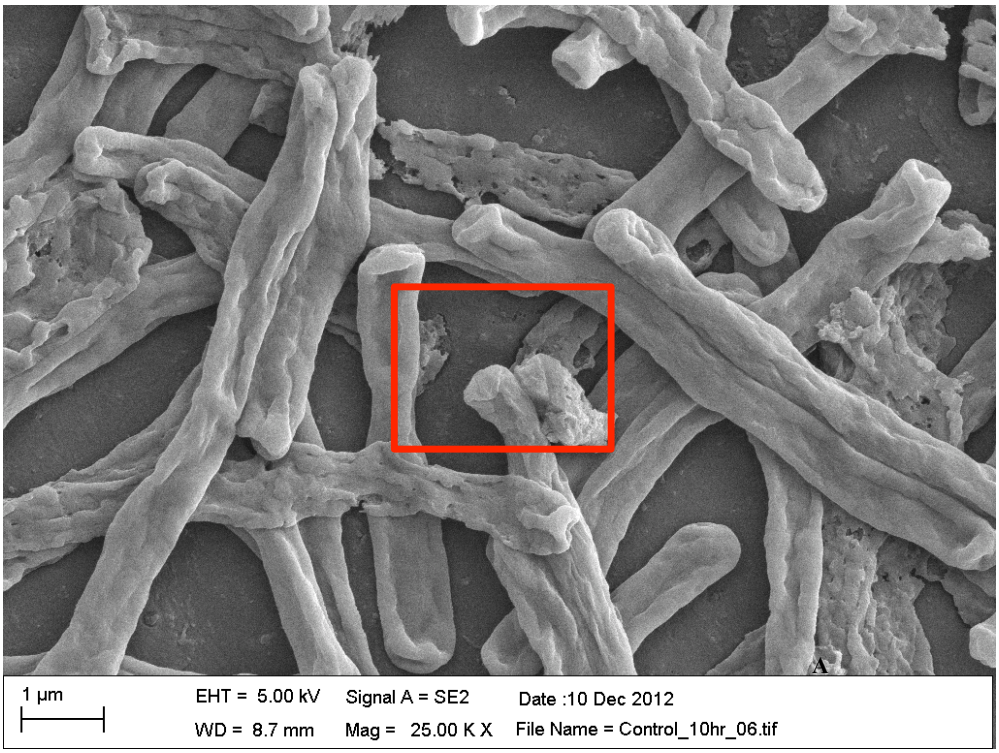
Fig 5.10e. *Scanning electron images of C. difficile DS2335 treated with EGCG (0.5 mg/mL) for 5 h at 25 k and 100 k magnification. Red box indicates area zoomed in at 100 k.*

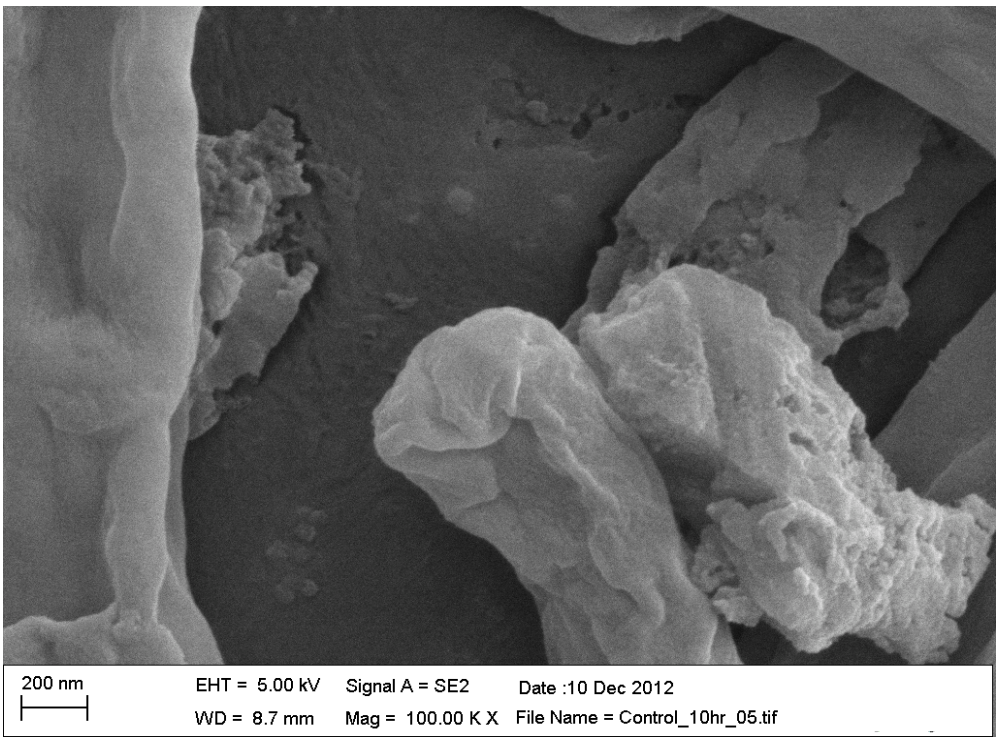




EGCG 5 h

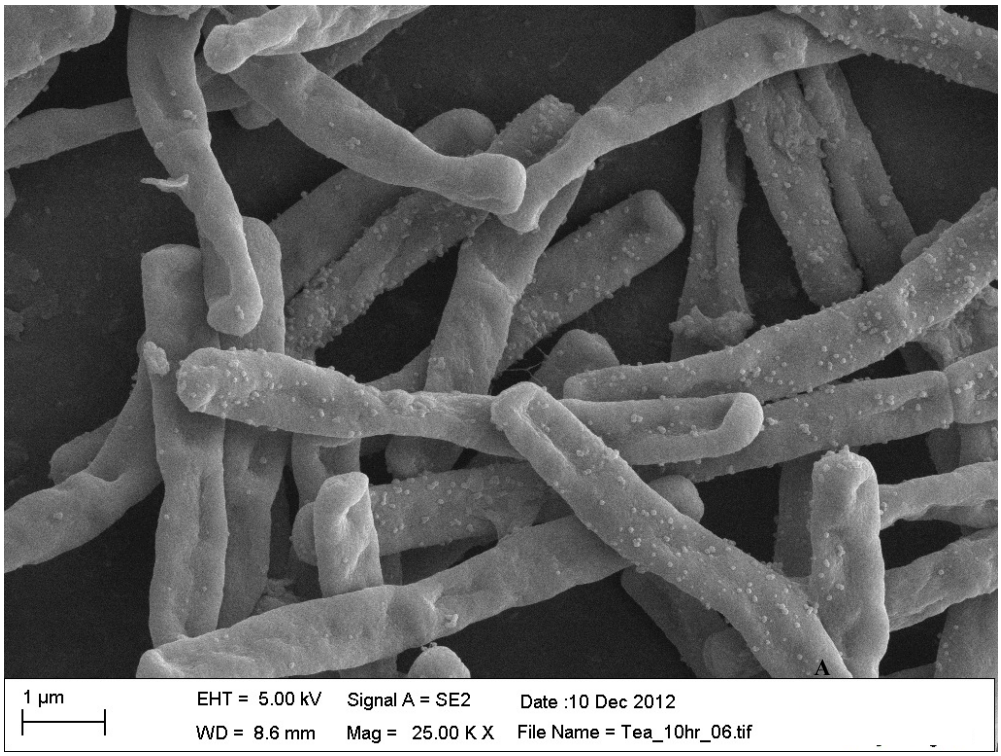
Fig 5.10f. *Scanning electron images of C. difficile DS2335 treated with water for 10 h at 25 k and 100 k magnification. Red box indicates area zoomed in at 100 k.*

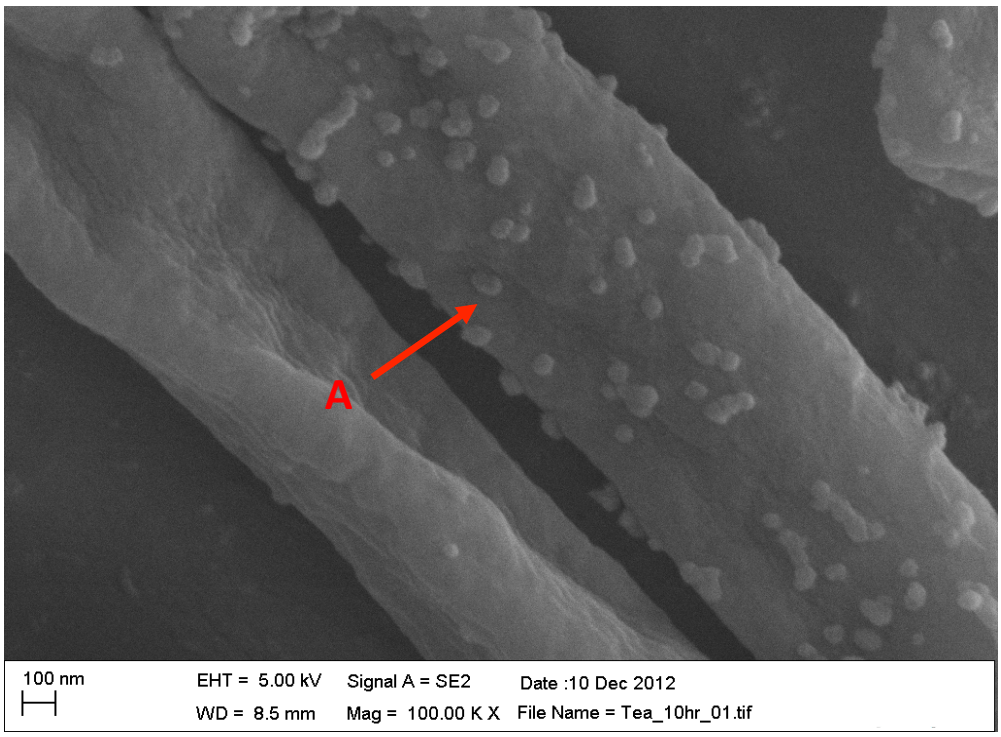




Water 10 h

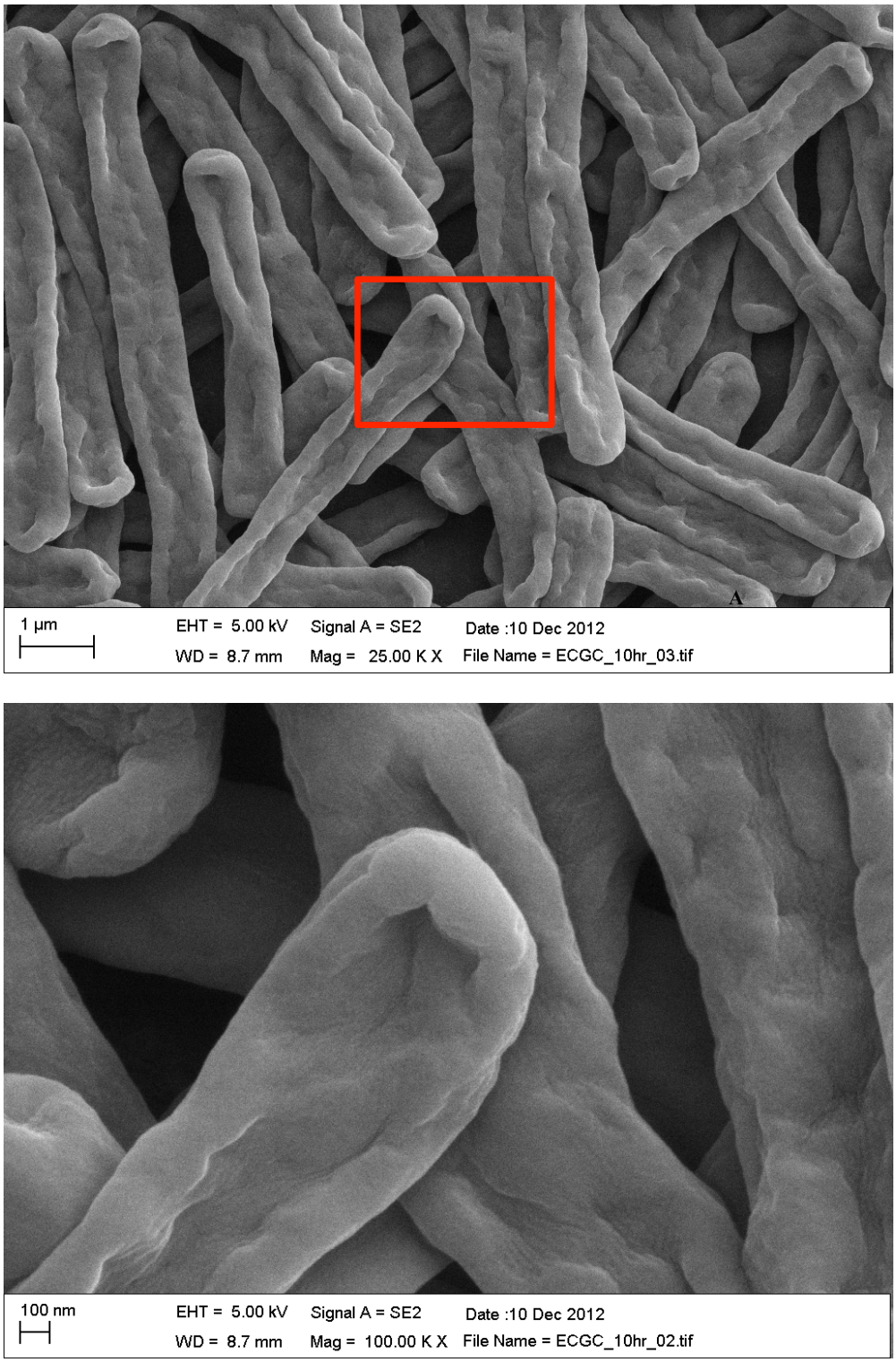
Fig 5.10g. *Scanning electron images of C. difficile DS2335 treated with tea (GP-2) for 10 h at 25 k and 100 k magnification. A' = potential blebbing*





Tea 10 h

Fig 5.10h. *Scanning electron images of C. difficile DS2335 treated with EGCG (0.5 mg/mL) for 10 h at 25 k and 100 k magnification. Red box indicates area zoomed in at 100 k.*



EGCG 10 h

It is evident that in all SEM images that the bacterial cells have collapsed, which is most likely an artefact of the air-drying process. Nonetheless, the integrity of the cell surface has been preserved, which allows comparison of treated and untreated cell surfaces without ambiguity.

After one hour in contact with water the surface of the bacterial cells appear to be smooth (fig 5.10a). In contrast, round structures approximately 30 – 40 nm in diameter appeared on the surface of cells treated with tea (fig. 5.10b). These round structures were also present in the cells treated with tea after 5 h, where they were more abundant and larger in size (50 – 60 nm) than those present at 1 h (fig 5.10d).

The presence of these round structures was even more extensive after exposure to tea for 10 h (fig 5.10g), although their size was similar to that observed at 5 h (fig 5.10d). In contrast the surface of EGCG treated bacteria after 5h and 10h remained smooth (fig 5.10e + 5.10h).

Although the surface of the cells remained smooth after treatment in water for 10 h it is evident that lysis of some cells has occurred (fig 5.10f). This is not apparent in cells treated with tea or EGCG, possibly due to the hypertonic nature of these solutions.

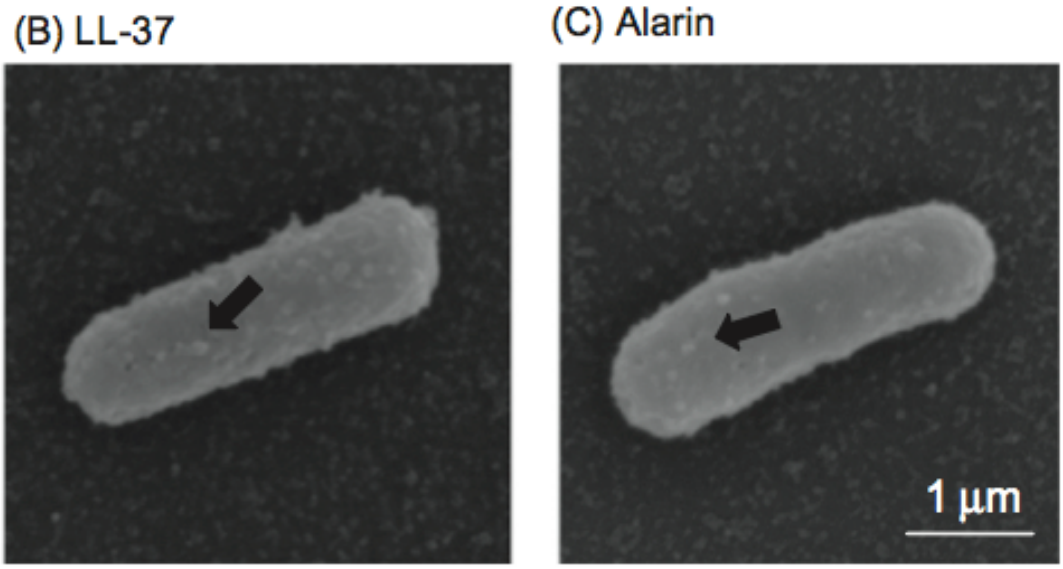


Figure 5.11. *Membrane blebbing on E. coli cells after treatment with antimicrobial peptides LL-37 and Alarin (Wada et al., 2013).*

Current evidence suggests that tea polyphenols interact with bacterial cell membranes and cell walls (Yoda *et al*., 2004) (Ikigai *et al* 1993; Hashimoto *et al*., 1999; Taylor *et al*., 2009). Therefore, it is possible that these round structures represent blebbing of the cell membrane. Blebbing is thought to occur when the integrity of the outer cell structures are compromised causing the cell membrane to protrude through the cell wall due to the high internal pressure (Daly *et al.,* 2011), as seen in some bacteria after treatment with antimicrobial peptides (Wada *et al*., 2013; fig 5.11) and the biguanide chlorhexidine (Vitkov *et al.,* 2005). This is also similar to what was seen when *S. aureus* was treated with ECG (Taylor *et al*. 2009; fig 5.1).

These images of *C. difficile* treated with green tea support the current theory that the antimicrobial mechanism of tea may involve some form of interaction with the bacterial cell wall and/or membrane. Indeed, this activity has been linked to ECG and EGCG as it has been reported that exposure of *S. aureus* to these compounds also causes blebbing (Taylor *et al*. 2009; Cui *et al.,* 2012; fig 5.1). Interestingly we observed no visible changes when *C. difficile* was exposed to EGCG suggesting that that the mode of action of the compound may differ between the two bacteria. Assessing the viability of the cultures post treatment may help to determine if these structural changes have had an adverse effects on the viability of the bacteria.

5.5. Conclusions

This study has shown that both green and black tea demonstrate inhibitory activity against a wide range of clinical isolates of *C. difficile* at concentrations lower than that found in a full cup of tea (200 mL). It has also shown that the common *C. difficile* ribotypes, 027, 001 and 106 are equally susceptible to the antibacterial activity of tea. Furthermore, in the 10 year period between 2000 – 2011, clinical isolates of the pathogen appear to have become more susceptible to the effects of tea highlighting the potential advantages of its use in the treatment of CDI. The inhibitory activity of green tea appears to be closely related to catechin content while this relationship is not as clear for black tea.

This chapter demonstrates that *in vitro*, tea infusions may be potentially beneficial in the treatment of CDI. Furthermore, as the interactions between tea and the antibiotics used to treat *C. difficile* are predominately indifferent, tea could be potentially used in combination with these antibiotics.

A recent study has shown that *C. difficile* is particularly susceptible to the effect of antibiotics that target the cell membrane (Wu *et al*., 2012). This may explain why tea is effective against a wide range of *C. difficile* isolates and would support the findings of the SEM performed in this chapter, which suggest that tea interacts with this structure in in a manner that is yet to be determined. To help further understanding in this area, Transmission Electron Microscopy (TEM) may provide useful information into the structural changes to *C. difficile* after exposure to tea. In addition, further investigation of the strains in this study, which were consistently more or less susceptible to the effects of tea, may help in determining the mechanism of action of tea.

To date, tea has been shown to selectively inhibit the *Clostridia* family of bacteria in the gut (Ahn *et al*., 1991; Lee *et al*., 2006), promote the growth of *lactobacillus* (Axling *et al*., 2012) and concentrate in the small and large intestine after consumption (Lee *et al.,* 1995). In combination with these attributes, this chapter has provided evidence to suggest that the consumption of tea or its components could make a positive impact on the treatment or prevention of CDI and provides a rational for extending this work to an *in vivo* model.

CHAPTER 6

GENERAL DISCUSSION

6. General Discussion

6.1 Discussion

While the antimicrobial activity of tea polyphenols, particularly catechins, has been extensively studied (Ahn *et al*., 1995; Hamilton-Miller *et al*., 2005; Lee *et al*., 2006; Freidman *et al*., 2006; Betts *et al.,* 2011), a comparable and comprehensive characterisation of the activity of tea infusions has yet to be undertaken. Thus far, published studies investigating the antimicrobial activity of tea infusions employ a range of non-standardised methods, which make the comparison of the results from different studies almost impossible (Ahn *et al*., 1991; Yi *et al*., 2010; Tariq and Reyaz 2012).

To address this issue we have quantified the inhibitory activity of a comprehensive collection of green and black tea infusions using an internationally recognised antimicrobial activity assay (CLSI., 2003).

During the optimisation of this assay it was discovered that different culture medium components such as casein hydrolysate and metal rich medias such as Iso-Sensitest® interfered with the antibacterial activity of both tea infusions and catechins. Other media supplements such as those used for testing anaerobic bacteria have also been shown to be incompatible when assessing the antibacterial activity of tea (CLSI. 2003). These issues highlight the need to consider the role of the culture media when seeking to assess the antibacterial activity of tea and its derivatives.

While we have not been able to define the precise mechanism by which tea inhibits bacteria, we have identified a link between the polyphenol content and antioxidant/pro-oxidant properties of tea infusions and their antimicrobial properties. Teas infusions with higher catechin content were shown to be the most potent inhibitors of *S. aureus* and *C. difficile* achieving inhibition at concentrations as low as 3.1 %(v/v) of a cup of tea (1.25 %w/v, 5 min brew; green tea GP-2). However, it was observed that catechin content was not the best predictor of the antimicrobial activity of black teas, highlighting the need to further explore the antimicrobial activities of the black tea polyphenols.

In an attempt to determine if there was a direct correlation between antibacterial activity and a specific component present in tea we undertook a comprehensive chemical analysis of each tea sample using HPLC and HPCCC. Indeed, to our knowledge this is the first study, which has attempted to correlate the results of individual HPLC profiles of tea samples with antibacterial activity.

Previous studies have shown that the inhibitory activity of catechins can be enhanced as a consequence of synergistic interactions with other catechins and polyphenols (Sasaki *et al*., 2004; Betts *et al.,* 2004). However, it was unknown if these interactions contributed to the antimicrobial activity of tea infusions. Our analysis has revealed that the inhibitory activity of tea is indeed the result of the synergistic and/or additive effects of polyphenols rather than the action of individual antimicrobial compounds. Regrettably, statistical analysis failed to provide insight into the compounds that directly contribute to these interactions. However, by emulating the composition of green tea we have evidence to suggest that the components involved in these interactions are likely to be the 6 major catechins, EGCG, EC, C, GCG, ECG, EGC and gallic acid. In combination with the long-standing safety profile of tea consumption, these results provide a rational for exploiting the natural synergy of tea as a potential treatment for antimicrobial infections as apposed to employing individual catechins.

In addition to employing HPLC to identify the major catechins, we also used HPCCC to exclude the possibility that we had overlooked other antimicrobial factors. Although our findings are limited to a single green tea, the data from our HPCCC analysis suggests that the vast majority of the detectable antibacterial activity is due to the catechins. However, future studies of multiple tea samples would be required to reinforce these findings.

Moreover, this study has provided the first example of the use of antimicrobial screening as a tool for solvent selection in HPCCC. At present, solvent selection is the rate-limiting step when this technology is used to extract bioactive compounds (Ignatova *et al*., 2011). The inclusion of an antimicrobial screening step enabled us to rationally select the most appropriate solvent system and thus eliminate the trial and error elements of this process. This provided a novel and high-throughput solvent selection method for separating antimicrobial compounds from crude mixtures.

Considerable variations in the catechin content and associated antimicrobial activity of individual teas appear to be influenced by the country where the tea has been cultivated . This was demonstrated by the potent inhibitory activity of African green teas and the poor activity of both green and black teas from China. On reflection, it is clear that multiple factors could contribute to these differences such as variations in the local environment (Yao *et al*., 2005), the use of different variants of *Camellia sinensis* (Sabhapondit *et al*., 2012) and variations in the manner in which the tea leaves were processed once they have been harvested (Chou *et al*., 1999). Moving forward, these variables could be manipulated to create conditions that optimise the production of catechins and thus maximise the antimicrobial activity of tea.

While catechins have been identified as the principal antibacterial compounds in tea their mode of action has yet to be fully understood. To date membrane disruption and the release of H_2O_2 are the two most heavily investigated theories (Ikigai *et al*., 2003; Nakayama *et al.,* 2002; Arakawa *et al.,* 2004; *Bernal et al.,* 2010). Clearly tea has a pronounced effect on the outer structure of Gram-positive bacteria as shown against *C. difficile* in this study, however it is uncertain if these morphological changes are indicative of cell wall/membrane damage, other structural interactions or an unrelated stress response.

Interestingly no evidence of cell wall damage/membrane perturbation was observed when *C. difficile* was exposed to EGCG suggesting that other compounds in tea acting alone or in combination with EGCG are required to disrupt the membrane. With *S. aureus*, it was shown that antimicrobial activity of tea infusions is most likely due to the sum of the synergistic/additive effects of the catechin polyphenols. It is possible that the antimicrobial mechanism of EGCG is different to the combined effect of the catechins in the tea infusion. This may explain the difference in the SEM observations, highlighting the need for further investigation in this area.

With regards the role of hydrogen peroxide, we have shown a direct correlation between the levels of H_2O_2 produced and the inhibitory activity of tea infusions. We have also found that the order of antibacterial activity of catechins and gallic acid is similar to the order of H_2O_2 production of these compounds also reported by Arakawa *et al*. (2004). This supports the hypothesis that catechins, which are more readily converted to

benzoquinones and thus produce the highest levels of H_2O_2, are the most potent antimicrobials (Sahu *et al.,* 2003). All withstanding, it is still uncertain if H_2O_2 is directly involved in the inhibitory activity or is simply intermediate in a more complex pathway.

It is unfortunate that specific tea infusions did not show synergy with the antibiotics vancomycin and metronidazole against *C. difficile,* as tea synergistic properties with β-lactams has proved to be an expanding area of interest for treating MRSA infections (Yiannakopoulou, 2012). Synergy with antibiotics used to treat *C. difficile* would undoubtedly increase the potential of taking tea forward as a complimentary treatment of CDI. Nevertheless, this study has shown that the interactions were largely indifferent which is an important outcome in its own right when considering combined therapy.

Whilst the antibacterial properties of tea have been known for many years, its widespread use as an antimicrobial agent has yet to be realised. In part, this is due to the poor systemic bioavailability of tea (Lee *et al.,* 1995) and the relatively modest inhibitory activities of individual catechins compared to traditional antibiotics. In spite of these concerns, this study provides a strong argument that tea infusions could potentially be used for the treatment of CDI. We have shown that tea has uniform inhibitory activity against a collection of clinical isolates of *C. difficile* representing the major ribotypes seen over the last 11 years in the UK. In addition, tea compounds have been shown to have a positive effect on the gut flora (Lee *et al*., 2006; Axling *et al*., 2012). Therefore they may also have potential use as supplements for prevention of *C. difficile* and maintenance of healthy gut function, much like that of probiotics. This could possibly be achieved by regular consumption of infusions with optimised catechin content. In addition, it is not subjected to the limitations of bioavailability faced when treating systemic infections, as it has been demonstrated that the green tea polyphenol EGCG, and most likely other tea catechins, concentrate in the small intestine and colon after consumption, which is where the *C. difficile* is found to colonise (Lee *et al.,* 1995). Granted that there is evidence of partial biotransformation and metabolism of some catechins within the gut (Sang *et al*., 2011), it remains a valid hypothesis that tea infusions could potently be useful in treating CDI due to their low inhibitory concentrations.

6.2 Limitations and Future Work

It is clear from this investigation that tea infusions, particularly green tea infusions, may be of benefit in the treatment of CDI. Our *in vitro* testing has demonstrated that tea is inhibitory across a wide range of clinical isolates of the pathogen. Therefore, the next logical progression of this work would be to determine the effects of tea *in vivo*. Currently, probiotics such as *lactobacillus* are used in the treatment of *C. difficile* as it thought that they help replenish the gut flora (Lawrence *et al.,* 2005). Recently it has been shown that green tea can help the growth of *Lactobacillus* in the gut (Axling *et al*., 2012), which may provide an opportunity for tea to be employed in combined with probiotics in the treatment of CDI.

Although this investigation has provided an extensive overview of the catechin content and antimicrobial activities of tea, there is still some ambiguity over the antimicrobial activities of black tea. It would appear that residual catechins in black tea contribute to the antimicrobial activity, although as demonstrated in this study, other interactions are clearly present. An investigation comparing the theaflavin and thearubigin content of black teas to their antimicrobial activity may help to provide a better understanding of how these groups contribute to activity. However, this endeavor is likely to be challenging due to the complex nature of these compounds.

This study has shown that a synthetic tea was able to successfully replicate the antimicrobial activity of the green tea it was based on. Yet, it is unknown if any of the components of this mixture are superfluous. To determine if each catechin is critical to this mixture, a subtractive approach should be taken to rule out redundant compounds and to quantify the contribution of each active component. Ultimately, combining this approach for multiple tea samples may provide a powerful tool for selecting potent antimicrobial teas based on their HPLC profile or catechin ratio alone and will help further the understanding of the combinational effect of catechins. The use of HPCCC may also prove useful in this investigation, as with the right solvent selection it has the ability to pull out pure compounds from crude mixtures. Since this process is non-destructive, both the isolated compounds and remaining mixture can be tested for activity and therefore help to determine synergy. Furthermore, study into the nature of the synergistic interactions of the catechin polyphenols may help to elucidate their

antimicrobial mechanism or provide insight into other compatible antimicrobials that catechins may have a synergetic relationship with.

Although the role of hydrogen peroxide production in the antibacterial action of tea infusions is clearly important, its specific function is as yet unknown, warranting further investigation into this area. Previously it has been reported that the production of H_2O_2 by catechins is dependent on the pH of the testing environment (Arakawa *et al*., 2004) and that the antibacterial activity of tea increases with increasing pH (Liu *et al.,* 2012). The pH of the small and large intestine is reported to be 7.5 and 6.4 – 7.0, respectively (Evans *et al*., 1988). Therefore, it is important that future studies determine if the inhibitory activity of tea infusions against *C. difficile* varies over a broader pH range or in conditions simulating these environments. Not only will this help predict the potential activity of these compounds *in vivo*, but may provide further insight into the role of H_2O_2 in the antimicrobial mechanism of tea. It has been observed that only small amounts of H_2O_2 are produced by tea polyphenols at ranges between pH 5.5 – 6.5 (Nakayama *et al.,* 2002). Therefore, if reduced antimicrobial activity of tea infusions were observed at these lower pH ranges then it would strengthen the hypothesis of H_2O_2 involvement in teas antimicrobial mechanism.

The use of SEM in this study revealed gross structural changes to *C. difficile* when exposed to green tea. However, the observation of these changes was restricted to the outermost structures of the bacterial cell. The use of TEM, which looks at cross sections of cells, would provide much more detail into specific parts of the cell (i.e. cell wall, membrane, etc.) that have been affected and thus further understanding of the interaction. Moreover, since no obvious changes were apparent with bacteria treated with EGCG, it would be of interest to conduct SEM on cells treated with other tea catechins. In addition, as H_2O_2 is implied in the antimicrobial mechanism of tea, it would be of interest to compare its affect on the surface of *C. difficile* in comparison to tea infusions and tea polyphenols.

Finally green teas from Kenya were found to possess potent inhibitory activity compared to other green teas. As previously mentioned, the diversity of tea plants may be caused by multiple variables. Environment (Yao *et al*., 2005), processing (Chou *et al*., 1999) and type of tea bush (Sabhapondit *et al*., 2012) are just some of factors shown

to affect the levels of polyphenols in tea leaves. Future work to identify these variables may provide a novel method for producing tea blends with exemplarity antimicrobial activity.

6.3 Concluding Remarks

In summary, this work has shown that tea infusions have inhibitory activity against one of the most prevalent hospital acquired infections in the UK. We have also provided evidence that green tea polyphenols, namely catechins are the dominant antimicrobial factors in green tea and they exert their antibacterial activity through synergetic or additive interactions. Albeit inconclusive, the work presented in this thesis supports the current hypothesis that the antimicrobial mechanism of tea involves H_2O_2 and that exposure to tea alters the structure of the bacterial cell in a manner which is yet to be determined.

In conclusion, tea a drink consumed by millions worldwide has been shown to be a versatile antimicrobial against *C. difficile in vitro* and may prove to be a useful natural antimicrobial in an era were antibiotic resistance is becoming ever more prevalent.

CHAPTER 7

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7. References

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APPENDIX

Composition of catechins and caffeine (mg/200mL) in single plantation green tea infusions (n=15) determined by HPLC

Tea	GA	EGC	C	Caffeine	EGCG	EC	GCG	ECG
Single Plantation								
GP-1	15.33 ± 0.19	93.08 ± 1.12	17.23 ± 0.13	76.96 ± 0.86	120.81 ± 1.52	25.43 ± 0.21	3.96 ± 0.05	26.07 ± 0.36
GP-2	17.32 ± 0.17	92.12 ± 1.35	19.29 ± 0.12	90.70 ± 0.47	132.77 ± 0.86	26.14 ± 0.18	5.51 ± 0.06	28.56 ± 0.21
GP-3	10.24 ± 0.19	42.97 ± 0.67	15.43 ± 0.27	74.04 ± 1.30	70.79 ± 1.06	23.33 ± 0.43	5.72 ± 0.08	23.33 ± 0.45
GP-4	15.33 ± 0.13	45.41 ± 0.54	13.66 ± 0.18	65.95 ± 0.77	89.28 ± 0.78	17.62 ± 0.16	4.58 ± 0.03	21.86 ± 0.32
GP-5	12.77 ± 0.31	80.67 ± 1.13	16.30 ± 0.26	70.48 ± 1.09	105.17 ± 1.94	24.32 ± 0.37	3.25 ± 0.07	23.36 ± 0.51
GP-6	11.52 ± 0.15	47.47 ± 0.36	12.41 ± 0.11	58.72 ± 0.63	76.42 ± 0.82	15.35 ± 0.20	3.44 ± 0.04	15.58 ± 0.16
GP-7	8.47 ± 0.09	34.33 ± 0.19	11.26 ± 0.16	3.80 ± 0.02	60.68 ± 0.55	13.86 ± 0.15	3.99 ± 0.01	12.23 ± 0.06
GP-8	8.80 ± 0.05	42.89 ± 0.33	11.40 ± 0.11	47.09 ± 0.37	54.35 ± 0.34	16.53 ± 0.12	3.33 ± 0.05	11.58 ± 0.07
GP-9	12.69 ± 0.15	39.07 ± 0.18	12.25 ± 0.12	61.39 ± 0.54	79.41 ± 0.96	15.43 ± 0.11	3.43 ± 0.01	17.61 ± 0.17
GP-10	4.58 ± 0.02	8.31 ± 0.10	12.60 ± 0.18	44.47 ± 0.31	50.75 ± 0.66	9.17 ± 0.06	3.34 ± 0.00	18.45 ± 0.06
GP-11	8.29 ± 0.18	34.90 ± 0.51	11.64 ± 0.18	65.90 ± 0.88	69.61 ± 0.92	13.93 ± 0.31	3.79 ± 0.07	14.15 ± 0.21
GP-12	6.30 ± 0.09	35.95 ± 0.53	11.12 ± 0.14	50.41 ± 0.55	54.10 ± 0.49	13.72 ± 0.11	3.23 ± 0.01	10.57 ± 0.05
GP-13	5.61 ± 0.14	28.56 ± 0.69	11.07 ± 0.19	26.41 ± 0.65	35.57 ± 0.71	12.21 ± 0.21	3.08 ± 0.05	8.12 ± 0.13
GP-14	4.88 ± 0.06	33.10 ± 0.24	11.12 ± 0.15	55.22 ± 0.45	56.22 ± 0.72	13.09 ± 0.11	3.65 ± 0.04	10.00 ± 0.01
GP-15	2.91 ± 0.01	17.43 ± 0.15	11.01 ± 0.16	44.09 ± 0.54	31.86 ± 0.38	8.41 ± 0.04	3.42 ± 0.03	5.17 ± 0.04

Results are the mean of 3 repeats (error = SE)

Composition of catechins and caffeine (mg/200mL) in commercially sourced green tea infusions (n=23) determined by HPLC

Tea	GA	EGC	C	Caffeine	EGCG	EC	GCG	ECG
Commercial Blend								
GC-1	11.42 ± 0.16	77.50 ± 0.72	12.70 ± 0.12	61.03 ± 0.76	101.73 ± 1.12	23.04 ± 0.19	3.60 ± 0.03	22.30 ± 0.31
GC-2	10.61 ± 0.13	46.01 ± 0.41	14.63 ± 0.10	77.12 ± 0.92	71.20 ± 0.42	21.05 ± 0.29	4.97 ± 0.04	18.98 ± 0.13
GC-3	9.66 ± 0.12	53.47 ± 0.62	11.86 ± 0.09	54.95 ± 0.68	74.12 ± 0.92	18.94 ± 0.27	4.05 ± 0.04	2.73 ± 0.01
GC-4	9.74 ± 0.18	39.80 ± 0.62	13.70 ± 0.24	1.75 ± 0.03	77.94 ± 1.16	15.83 ± 0.32	5.01 ± 0.11	16.95 ± 0.26
GC-5	5.51 ± 0.06	33.27 ± 0.36	14.09 ± 0.11	62.71 ± 0.39	61.36 ± 0.75	17.91 ± 0.15	3.91 ± 0.03	19.24 ± 0.17
GC-6	8.62 ± 0.09	59.76 ± 0.34	14.05 ± 0.13	55.60 ± 0.59	74.02 ± 0.68	21.34 ± 0.32	3.87 ± 0.04	18.62 ± 0.16
GC-7	5.79 ± 0.13	26.45 ± 0.18	18.34 ± 0.21	81.43 ± 0.53	58.50 ± 0.22	29.37 ± 0.38	3.57 ± 0.04	36.81 ± 0.39
GC-8	3.85 ± 0.07	78.96 ± 1.26	10.93 ± 0.26	51.91 ± 0.78	72.61 ± 1.32	18.16 ± 0.32	4.13 ± 0.09	10.77 ± 0.17
GC-9	3.16 ± 0.01	59.13 ± 0.66	11.44 ± 0.12	66.26 ± 0.69	62.27 ± 0.84	20.44 ± 0.26	4.00 ± 0.02	12.10 ± 0.16
GC-10	3.53 ± 0.05	24.01 ± 0.29	12.07 ± 0.13	80.75 ± 0.99	77.85 ± 0.58	11.30 ± 0.08	7.00 ± 0.04	15.05 ± 0.11
GC-11	4.65 ± 0.06	84.55 ± 0.75	10.89 ± 0.15	49.36 ± 0.69	84.25 ± 0.98	22.24 ± 0.21	3.93 ± 0.01	13.83 ± 0.18
GC-12	8.15 ± 0.06	33.64 ± 0.25	12.32 ± 0.11	60.33 ± 0.61	61.50 ± 0.41	12.78 ± 0.26	3.75 ± 0.02	13.21 ± 0.09
GC-13	6.74 ± 0.08	36.28 ± 0.24	11.48 ± 0.14	50.50 ± 0.52	59.22 ± 0.31	16.33 ± 0.11	3.86 ± 0.03	14.47 ± 0.21
GC-14	4.07 ± 0.07	23.88 ± 0.42	11.83 ± 0.18	65.65 ± 1.13	39.58 ± 0.60	15.40 ± 0.36	3.38 ± 0.08	11.55 ± 0.28
GC-15	3.17 ± 0.01	56.35 ± 0.42	11.75 ± 0.11	54.60 ± 0.51	55.79 ± 0.71	19.34 ± 0.15	4.13 ± 0.03	8.82 ± 0.08
GC-16	2.67 ± 0.05	54.25 ± 0.51	11.30 ± 0.13	86.96 ± 0.44	84.43 ± 0.78	16.55 ± 0.18	4.91 ± 0.03	12.49 ± 0.15
GC-17	4.55 ± 0.09	6.06 ± 0.13	12.57 ± 0.29	50.72 ± 0.98	23.61 ± 0.38	11.80 ± 0.24	3.42 ± 0.06	13.97 ± 0.33
GC-18	4.08 ± 0.03	22.58 ± 0.19	13.79 ± 0.12	60.72 ± 0.21	44.20 ± 0.24	15.85 ± 0.18	3.47 ± 0.01	15.23 ± 0.19
GC-19	5.96 ± 0.06	6.71 ± 0.07	12.88 ± 0.16	59.81 ± 0.54	33.28 ± 0.48	12.07 ± 0.19	3.25 ± 0.02	20.24 ± 0.22
GC-20	6.54 ± 0.13	3.74 ± 0.04	12.28 ± 0.19	47.10 ± 1.01	18.08 ± 0.42	9.40 ± 0.16	3.11 ± 0.06	11.00 ± 0.23
GC-21	2.19 ± 0.04	5.35 ± 0.01	33.89 ± 0.25	22.50 ± 0.11	6.32 ± 0.01	5.02 ± 0.01	6.02 ± 0.06	1.25 ± 0.01
GC-22	2.02 ± 0.03	13.60 ± 0.19	10.90 ± 0.11	44.22 ± 0.32	15.78 ± 0.09	9.82 ± 0.10	3.00 ± 0.03	4.48 ± 0.03
GC-23	2.32 ± 0.01	9.39 ± 0.11	10.77 ± 0.15	41.47 ± 0.62	17.16 ± 0.15	8.14 ± 0.11	2.98 ± 0.02	4.20 ± 0.05

Results are the mean of 3 repeats (error = SE)

Composition of catechins and caffeine (mg/200mL) in single plantation black tea infusions (n=18) determined by HPLC

Tea	GA	EGC	C	Caffeine	EGCG	EC	GCG	ECG
Single Plantation								
BP-1	12.54 ± 0.22	1.70 ± 0.04	11.30 ± 0.19	52.71 ± 0.89	7.98 ± 0.13	5.90 ± 0.12	nd	4.09 ± 0.09
BP-2	12.54 ± 0.11	10.01 ± 0.06	11.53 ± 0.15	66.92 ± 0.79	49.09 ± 0.39	9.28 ± 0.05	3.51 ± 0.01	17.71 ± 0.09
BP-3	7.95 ± 0.03	6.53 ± 0.04	11.33 ± 0.16	81.10 ± 0.85	47.68 ± 0.24	7.15 ± 0.02	3.54 ± 0.01	15.91 ± 0.14
BP-4	8.05 ± 0.01	0.10 ± 0.00	10.75 ± 0.15	50.17 ± 0.33	4.05 ± 0.05	4.76 ± 0.03	nd	2.08 ± 0.02
BP-5	13.35 ± 0.17	0.92 ± 0.01	11.07 ± 0.07	66.90 ± 0.36	6.69 ± 0.05	5.74 ± 0.04	nd	4.49 ± 0.00
BP-6	9.96 ± 0.11	0.49 ± 0.00	10.88 ± 0.15	70.18 ± 0.79	4.97 ± 0.06	4.79 ± 0.01	nd	1.70 ± 0.00
BP-7	12.84 ± 0.32	0.24 ± 0.00	11.09 ± 0.18	77.49 ± 1.10	6.27 ± 0.10	4.74 ± 0.07	nd	2.22 ± 0.05
BP-8	9.13 ± 0.04	0.24 ± 0.00	10.65 ± 0.12	49.10 ± 0.59	4.88 ± 0.03	4.80 ± 0.02	nd	1.98 ± 0.01
BP-9	8.45 ± 0.03	1.07 ± 0.01	11.12 ± 0.09	43.95 ± 0.58	9.15 ± 0.06	6.42 ± 0.04	nd	4.89 ± 0.06
BP-10	9.27 ± 0.16	0.62 ± 0.01	10.75 ± 0.19	46.34 ± 0.71	6.92 ± 0.10	5.74 ± 0.09	nd	4.29 ± 0.09
BP-11	11.54 ± 0.10	0.10 ± 0.00	11.02 ± 0.11	76.20 ± 0.99	5.71 ± 0.03	4.67 ± 0.01	nd	1.92 ± 0.02
BP-12	15.80 ± 0.18	1.35 ± 0.02	11.13 ± 0.14	76.37 ± 0.92	8.35 ± 0.12	6.42 ± 0.04	nd	5.97 ± 0.12
BP-13	7.99 ± 0.09	0.29 ± 0.01	10.63 ± 0.15	36.74 ± 0.32	5.09 ± 0.12	5.21 ± 0.02	nd	2.77 ± 0.01
BP-14	9.32 ± 0.11	0.49 ± 0.00	11.00 ± 0.09	53.23 ± 0.36	5.21 ± 0.07	5.58 ± 0.02	nd	4.40 ± 0.05
BP-15	7.21 ± 0.03	0.08 ± 0.00	10.67 ± 0.13	40.02 ± 0.25	4.59 ± 0.04	4.96 ± 0.00	nd	3.11 ± 0.03
BP-16	7.38 ± 0.04	0.76 ± 0.00	10.53 ± 0.10	62.83 ± 0.58	4.69 ± 0.02	5.04 ± 0.06	nd	2.25 ± 0.01
BP-17	12.81 ± 0.26	0.89 ± 0.02	10.53 ± 0.19	39.42 ± 0.56	4.20 ± 0.08	4.84 ± 0.06	3.05 ± 0.02	1.80 ± 0.03
BP-18	4.95 ± 0.03	0.11 ± 0.00	10.72 ± 0.08	40.26 ± 0.37	3.02 ± 0.02	4.69 ± 0.00	nd	1.65 ± 0.01

Results are the mean of 3 repeats (error = SE)

Composition of catechins and caffeine (mg/200mL) in commercially sourced black tea infusions (n=16) determined by HPLC

Tea	GA	EGC	C	Caffeine	EGCG	EC	GCG	ECG
Commercial Blend								
BC-1	13.34 ± 0.16	1.7 ± 0.01	11.67 ± 0.15	87.87 ± 0.90	8.98 ± 0.07	5.65 ± 0.06	nd	5.25 ± 0.02
BC-2	15.13 ± 0.17	1.94 ± 0.01	11.89 ± 0.11	82.04 ± 0.65	10.91 ± 0.14	6.65 ± 0.05	nd	6.49 ± 0.04
BC-3	2.26 ± 0.03	1.06 ± 0.02	11.34 ± 0.11	77.02 ± 0.71	8.95 ± 0.02	7.45 ± 0.05	nd	7.52 ± 0.02
BC-4	5.30 ± 0.03	1.00 ± 0.02	10.90 ± 0.11	45.91 ± 0.49	8.00 ± 0.01	5.38 ± 0.01	3.72 ± 0.03	3.17 ± 0.01
BC-5	13.08 ± 0.11	6.43 ± 0.03	11.57 ± 0.13	76.73 ± 0.69	43.27 ± 0.21	8.05 ± 0.10	3.32 ± 0.03	17.32 ± 0.11
BC-6	6.73 ± 0.14	8.85 ± 0.19	11.12 ± 0.20	70.48 ± 1.17	105.17 ± 1.73	24.32 ± 0.37	3.25 ± 0.05	23.36 ± 0.52
BC-7	10.21 ± 0.08	0.57 ± 0.00	10.95 ± 0.10	77.20 ± 0.69	7.27 ± 0.04	5.37 ± 0.03	nd	4.04 ± 0.02
BC-8	9.25 ± 0.12	0.48 ± 0.00	10.83 ± 0.11	67.58 ± 0.55	7.72 ± 0.03	5.50 ± 0.02	2.67 ± 0.05	4.80 ± 0.06
BC-9	10.73 ± 0.15	1.77 ± 0.03	11.04 ± 0.07	49.42 ± 0.36	12.02 ± 0.23	8.10 ± 0.02	2.86 ± 0.01	8.04 ± 0.04
BC-10	9.83 ± 0.13	0.18 ± 0.00	11.40 ± 0.11	60.59 ± 0.54	6.18 ± 0.01	6.42 ± 0.04	nd	5.74 ± 0.06
BC-11	9.11 ± 0.09	0.10 ± 0.00	10.98 ± 0.08	55.44 ± 0.57	3.97 ± 0.02	5.21 ± 0.03	nd	2.58 ± 0.04
BC-12	11.31 ± 0.15	3.08 ± 0.04	11.05 ± 0.12	65.51 ± 0.66	27.28 ± 0.33	6.40 ± 0.05	3.04 ± 0.02	9.65 ± 0.06
BC-13	2.90 ± 0.05	7.21 ± 0.05	10.78 ± 0.09	42.67 ± 0.45	16.80 ± 0.11	7.87 ± 0.09	2.97 ± 0.04	5.09 ± 0.09
BC-14	3.23 ± 0.04	9.27 ± 0.10	10.70 ± 0.12	51.80 ± 0.44	17.40 ± 0.32	7.29 ± 0.04	2.90 ± 0.01	4.24 ± 0.01
BC-15	2.60 ± 0.06	nd	nd	55.67 ± 0.86	nd	4.68 ± 0.10	nd	1.39 ± 0.03
BC-16	13.11 ± 0.24	1.86 ± 0.03	11.78 ± 0.26	88.30 ± 1.24	10.53 ± 0.27	6.44 ± 0.16	2.91 ± 0.05	7.18 ± 0.12

Results are the mean of 3 repeats (error = SE)