Antimicrobial effects of white tea extracts in combination with putative adjuncts against *Staphylococcus aureus* and other microbes of importance

Andrew Charles Holloway

A thesis submitted in partial fulfilment of the requirements of Kingston University for the degree of Doctor of Philosophy

Kingston University, School of Life Sciences

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Declaration

I declare that the work reputed in this thesis is entirely my own and has been carried out at Kingston University, UK.

This thesis has not been submitted, in whole or in part, for any other degree at this or any other University

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Abstract

Previous studies have investigated biological activities of plant extracts and their subcomponent compounds as antimicrobial treatments. The addition of adjunct substances in varying combinations to such agents has also been shown to enhance their antimicrobial activities – a desirable outcome in the development of novel treatment substances to combat pathogens. Whole extracts of tea leaves processed in different ways to produce black, green and white tea types (BT, GT,WT) show different activities. In addition, the effects on pathogens of highly active GT extract rich in flavan-3-ols which are catechins have also been investigated alone and with additives and these can compare favourably with the activities of conventional antibiotics such as oxacillin and ciprofloxacin. However, relatively little attention has been paid to enhancing the activity of whole WT or its weak antimicrobial subcomponents.

The activities of GT and BT were compared to the less researched white tea (WT). Principal tea flavan-3-ols were also screened for activity alone, and combined with a known antimicrobial agent copper(II)sulphate against *Staphylococcus aureus* NCTC 06751. WT, a sub-fraction, (WTF<1kDa) and the weak antimicrobial catechin were further tested with another antimicrobial agent vitamin C. A catechin-copper(II) combination was initially tested against a panel of 4 bacterial species and then investigated further against the least susceptible *S. aureus* using the antimicrobial agents: caffeine, and iron(II), as well as vitamin C additions. *Escherichia coli* NCTC 14441 was also tested for comparison. Reaction mechanisms of catechin enhanced with additives were investigated using stoichiometry, crystallisation, UV-vis, solubility, heat treatment, pH, EDTA, H_2O_2 generation, catalase, kinetics and the effects of storage on antimicrobial activity.

In 30 minute exposures and within the other conditions tested, the following were seen: Copper(II) alone, and combined with WT produced similar antimicrobial activities. Other teas reduced the copper(II) activity indicating the presence of inhibitory components. Copper(II) combined with vitamin C or WTF increased activity. Iron(II) enhanced catechin against *S. aureus* but not against *E. coli*; subsequent addition of copper(II) or vitamin C did not raise activity further. Caffeine, and further antimicrobial agent zinc, and manganese had no effect against *S. aureus* whereas heat treatment of catechin raised activity of catechin-copper(II) against *S. aureus*. A catechin-copper(II) solution, stored at 20 °C for 1 day, revealed enhanced activity against *Proteus mirabilis* NCTC 7827 and *E. coli*. Vitamin C added to catechin-copper(II) did not enhance activity of stored solutions, but caused losses in activity. However, freeze-drying preserved mixture activities. WTF; freshly-made as well as stored catechin solution; and iron(II) combined with copper(II) generated bactericidal H_2O_2 whose formation was prevented by EDTA and removed by catalase. Addition of

copper(II) to catechin caused a fall in pH, a rise in solubility and in UV-vis absorption indicating a complex which showed rapid effects against *S. aureus*. Catechin heat treatment raised both speed and level of effect against *S. aureus* probably due to the production of novel substances.

This is the first time, we believe, that an enhancement of a weak antimicrobial such as catechin by adjuncts and by heating has been reported and the findings suggest new ways to develop antimicrobials. An additional finding was that catechin was enhanced most by copper(II) in its activity against *Pseudomonas aeruginosa* NCTC 950, a finding which may find importance in agricultural as well as other settings. The addition of catechin may facilitate a reduction in the use and footprint of copper(II) in the environment.

Dedication

This thesis is dedicated to all those persons who, mindful of their esteemed accomplishments and stations in life, manage to keep a sense of perspective about themselves with respect to others – as Sir Colin Davis (1927-2013) said, 'to put aside elitism and become a decent human being.'

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In the end I seem to have reined in my child-like speculation and developed a sense of scepticism. Hopefully, I can live up to the words of Francis Bacon (1561-1626) who said that 'If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts he shall end in certainties'.

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Abbreviations

BC	before the birth of Christ
BT	black tea extract
cfu	colony forming unit
C	catechin, (+)-catechin
CG	catechin gallate, (+)-catechin gallate
EC	epicatechin, (-)-epicatechin
ECG	epicatechin gallate, (-)-epicatechin gallate
EDTA	ethylenediaminetetraacetic acid
EGC	epigallocatchin, (-)-epigallocatechin)
EGCG	epigallocatchin gallate, (-)-epigallocatechin gallate
EM	electron microscopy
f.c.	final concentration
ent	enantiomer, *i.e.* stereo-isomer, either: (+)-dextro, D; or (-)-laevo- rotatory, L
g	acceleration due to gravity
GC	gallocatechin, (+)-gallocatechin
GCG	gallocatechin gallate, (-)-gallocatechin gallate
GSE	grape seed extract
GT	green tea extract
HIV	human immunodeficiency virus
H_2O_2	hydrogen peroxide
ICP-AES	inductively coupled plasma atomic emission spectroscopy
L	Linnaeus
LM	light microscopy
NCTC	National Collection of Type Cultures
MDR	multidrug resistant
MIC	minimum inhibitory concentration
MRSA	Methicillin resistant *Staphyloccus aureus*
MSSA	Methicillin sensitive *Staphyloccus aureus*

MWCO	Molecular weight cut-off
NMR	Nuclear magnetic resonance
PBS	phosphate buffered saline
PRE	pomegranate rind extract
p	p value indicating a probability of less than 0.05 or greater than 0.05 to find out statistical differences ($p<0.05$) between test samples at a level of 5% confidence (*i.e.*, a 95% confidence level of a significant, or a non-significant difference between the average means of two compared samples) within the protocol of the Student independent 2 sample, two tailed, t-test with parametric dispersion of data
ROS	reactive oxygen species
SD	standard deviation
SEM	scanning electron microscope
SEM	standard error of the mean
UV-vis.	ultraviolet-visible spectrophotometry
v/v	volume fraction
w/v	weight per volume
WT	white tea extract
WTF	white tea extract sub-fraction
WTSN	white tea supernatant

Παντες ανθρωποι του ειδεναι ορεγονται φυσει

'All men naturally desire knowledge'

ARISTOTLE, *Metaphysics*

"Aristotle wrote thousands of sentences. But one, the first in his first book of *Metaphysics* defines him. 'All men', he says, 'desire to know'. But, he continues, 'Not all forms of knowledge are equal - the best is the pure and disinterested search for the causes of things'. He has no doubt that 'searching for them is the best way to spend a life'. It's a claim for the beauty... and worth... of science."

Armand Marie Leroi, *'Aristotle's Lagoon'*

God gives great thereon to look,
But not to look, but understand
For learning is better than either house or land

Charles Soosby from 'His book' *Ann. dom.* 1678

Χαλεπα τα καλα

The good/beautiful things are difficult to attain

PLATO, *Republic*

Chapter 1: Introduction

1.1 Introduction to infection control

Microbes that cause infections are referred to as pathogens, and these can generate the signs and symptoms of disease. Despite considerable progress in treating infections over millenia the challenge of infection control remains and is likely to continue, particularly with the rise in human numbers and as a consequence increasing proximity to other humans as potential sources of infection. The same arguments also apply to large economically important monocultures of farmed animals and plants as well as bees that live as social insects.

In contemporary times considerable efforts are being made worldwide to combat infections that affect humans as well as animals and plants of economic importance. These efforts include investigations into a very broad range of different types of pathogenic microbes that can cause or predispose individual people, and other organisms, to important endemic and emerging infections and conditions, *e.g.* malaria, human immunodeficiency virus (HIV), hepatitis C, *Clostridium difficile,* vaginosis, gonorrhoea, chlamydia, turkey clostridial dermatitis and cellulitis, and pumpkin fungal blight (Moonen *et al.*, 2010; Levitt *et al.*, 2011; Lambers *et al.*, 2011; Lo Vecchio and Zacur, 2012; Mitchell *et al.*, 2011; Clark *et al.*, 2010; Grube *et al.*, 2011).

Efforts to combat infections include developing novel disinfective agents and procedures for use in hospitals and community health settings, food preparation areas, veterinary and agricultural settings, aquaculture and water treatment systems (Nakai *et al.*, 2000; Winward *et al.*, 2008; Purcaro *et al.*, 2009; Graça *et al.*, 2011; Fleurat-Lessard *et al.*, 2011).

Measures of infection control include surveys of numerous species of pathogens. This is to identify and monitor developing trends and patterns of disease within specific regions, *e.g.* European censuses of medically important human pathogens (Antimicrobial resistance surveillance in Europe 2010, revised December, 2011), and global trends in zoonotic infections (Greger, 2007).

1.2 Some currently prominent infections and their traditional treatment

Different and varying types of pathogens become more or less prominent within infection control due to reasons such as changing world climate, translocation of species, natural and artificial selection, changing health practices, life style choices, and political upheavals such as war (Garratt, 1996; Ansart *et al.*, 2007; Mavroidi, 2008: Wingfield *et al.*, 2010; Alvarez *et al.*, 2011; Munro and Wallace, 2011). However, certain pathogens such as *Staphylococcus aureus* impact particularly on

western health policy due to their growing effects on human health as well as economic consequences (Shorr *et al.*, 2010). *S. aureus* is a facultative anaerobic Gram-positive coccal bacterium that colonises the skin and nostrils of about 30% of healthy humans. Although mainly harmless, *S. aureus* can cause severe infection. An antibiotic-resistant form that is resistant to methicillin (methicillin-resistant *S. aureus*, MRSA) is 'the most important cause of antibiotic-resistant healthcare-associated infections worldwide' (Antimicrobial resistance surveillance in Europe, 2010). Infections with MRSA may result in prolonged hospital stays and in higher mortality rates, owing mainly to limited effectiveness of treatment regimens. MRSA is currently the most commonly identified antibiotic-resistant pathogen in hospitals in many parts of the world, including Europe, the Americas, North Africa and the Middle- and Far East (Antimicrobial resistance surveillance in Europe, 2010).

In Europe the proportion of *S. aureus* isolates found to be MRSA is stabilising or decreasing in most countries. Between 1996 and 2010, seven countries reported decreasing trends while four countries reported an increasing trend (Antimicrobial resistance surveillance in Europe, 2010). Countries showing a more evident and sustained decrease of MRSA are Austria, Cyprus, Estonia, France, Ireland, Latvia and the UK (Antimicrobial resistance surveillance in Europe, 2010). Although these observations provide reasons for optimism, MRSA remains a public health priority, as the proportion of MRSA strains isolated from samples of *S. aureus* is still above 25% in eight out of 28 European countries - mainly in southern and eastern Europe (Antimicrobial resistance surveillance in Europe, 2010). *S. aureus* in humans can also cause food poisoning and certain skin infections as well as toxic shock syndrome (Floret, 2001; Franco *et al.*, 2010; Parsonnet *et al.*, 2010). *S. aureus* has also been shown to reduce colonisation resistance by predominant indigenous gut bacteria (Sannasiddappa *et al.*, 2011).

Whatever means may be found to control MRSA in humans, these means may also find application in controlling animal infections caused by *S. aureus*. This species can, for example, cause mastitis in cows and bumblefoot in poultry and waterfowl (Cooper and Needham, 1981; Keefe, 2012). The use of antibiotics as treatment agents in farmed animals to treat infections caused by *S. aureus* and other bacterial species is however controversial, partly due to residues of antibiotics found in meat and other products. As a consequence animal infections pose special control problems (Donkor *et al.*, 2011).

In humans, another opportunistic, nosocomial pathogen is *Pseudomonas aeruginosa*. This is a Gram-negative, aerobic, cocco-bacillus bacterium with unipolar motility. *P. aeruginosa* is an opportunistic pathogen causing infections of the pulmonary and urinary tracts, burns and other wounds, and can cause blood sepsis, which can be fatal. In hospital and clinic burn units, it is very

difficult to eradicate colonising strains of *P. aeruginosa* with classic infection control procedures. Because it thrives on most surfaces, this bacterium can be found on and in medical equipment, including catheters, causing cross-infections in hospitals and clinics which have also proved difficult to control (Antimicrobial resistance surveillance in Europe, 2010). In the community, *P. aeruginosa* is a frequent cause of skin infections such as folliculitis and *otitis externa* among recreational and competitive swimmers. In patients with cystic fibrosis, *P. aeruginosa* causes severe bacterial complication leading to chronic colonisation and intermittent exacerbation of the condition with, for example, bronchiolitis and acute respiratory distress syndrome (Antimicrobial resistance surveillance in Europe, 2010). In agricultural settings, *Ps. aeruginosa* and other species of *Pseudomonas,* being part of the soil flora, have also become opportunistic pathogens of plants (Wagner *et al.*, 2008). *Pseudomonas* is ubiquitous in aquatic environments in nature and is resistant to many detergents, disinfectants and antimicrobial compounds.

Another species, *Escherichia coli,* has become more prominent in the UK popular media due to a number of serious human food poisoning cases caused by particular strains which have the capacity to act pathogenically, *e.g.*, *E. coli* O157:H7. This species is a rod-shaped Gram-negative, facultative anaerobe, that is commonly found in the lower intestine of warm-blooded organisms. It is the most frequent cause of bacteraemia, community and hospital-acquired urinary tract infections, and is associated with spontaneous and surgical peritonitis and with skin and soft tissue infections. *E. coli* is the most frequent Gram-negative rod isolated from blood cultures in clinical settings. It also causes neonatal meningitis and is one of the leading causative agents in food-borne infections worldwide (Antimicrobial resistance surveillance in Europe, 2010). Although part of the normal gut flora, the species can also cause urinary tract infections such as cystitis (Clements *et al.*, 2012). Similar urinary tract infections can also be caused by *Proteus mirabilis,* which is also commonly found in human intestines (Alamuri *et al.*, 2010). *P. mirabilis* is also a Gram-negative, rod-shaped facultative anaerobe. However, unlike *E. coli,* it shows swarming motility and urease activity.

The performance of many conventional antibiotics, such as penicillin and streptomycin in common use since the 1940's, is now under review due to rising resistance to antibiotics within bacterial populations (Hseuh *et al.*, 2005). Part of the response to failing conventional antibiotic treatments has been to enhance a given antibiotic with other substances, such as other antibiotics, vitamin C and honey (Salaman, 2004; Mandal *et al.*, 2010).

Whilst research into the control of infection of humans and other organisms of importance is paramount, the development of new products to control infections needs to take into account side effects that can arise from their use (Volcheck, 2004). Such side effects, *e.g.* raised blood pressure

and anaphylatic shock can be potentially serious in susceptible patients (Navas, 2002). In the control of infection within agricultural settings the side effects of anti-infective treatments, may be extensive on collateral, non-target species, as well as on whole ecological communities and ecosystems, *e.g.* due to 'run off' from fields (Dagostin *et al.*, 2011). This has proved to be the case historically with agents such as copper-based anti-fungal products used on crops in bulk amounts and which affect operatives and the environment (Montuelle *et al.*, 2010; El Hadri *et al.*, 2012). Replacing the use of harmful substances such as copper, with other substances to control pathogenic fungi in field crops and in aquaculture would reduce the footprint of these substances on the environment (Schrader *et al.*, 2003; Darwish *et al.*, 2012).

1.3 Historical perspectives on anti-infective agents

Many traditional practices used in folk medicines have received little evaluation and consequently are ignored in the development of antimicrobials. Recently however, some products such as herbs, spices, wine products and honey have been scientifically evaluated (Bekele *et al.*, 2006; Aween *et al.*, 2012; Chan-Rodriguez *et al.*, 2012). Throughout history humans have used animal, plant and mineral products, singly or in combination, to combat infection. Many plant products are convenient to obtain and economical to use. Substances used for wound dressings such as alcohol, wine, honey and moss have also contributed to the therapeutic effect of other applied plant products since ancient times.

The therapeutic properties of plant products have been realised following extraction procedures such as the slow heating of woody stems such as pine or birch to manufacture tree tar (Stockholm tar), or brewing tea leaves in hot water to produce extracts with enhanced antimicrobial activity (Asaka *et al.*, 2000). Heat-treated plant products stored as geological deposits such as coal tar or bitumen have also long been used in human history as antimicrobial treatments (Kopteva *et al.*, 1988). Many chemical derivatives formed during thermo-geological processes such as cresol or xylol show considerably greater antimicrobial activity than the original plant products from which they were derived. Curing harvested plant organs for food at much lower temperatures of around 40 to 60 °C to produce, *e.g.*, black tea, vanilla or other products can result in substances with higher antimicrobial activities than those of the parent substances (Fitzgerald *et al.*, 2004; Kim *et al.*, 2011).

Plants which have evolved to produce high concentrations of antimicrobial compounds to defend themselves from pathogens have often been selected by humans as a source of agents to treat infections and to preserve food. These include tea, garlic, grapes, cloves, olives and pomegranate fruits (Arora and Kaur, 1999; Voravuthikunchai and Mitchell, 2008).

The antimicrobial properties of metals particularly copper have long been recognised (Baker *et al.*, 2010). For example, the ancient Eygyptians used powdered meteorites containing iron, and mined malachite containing copper, as anti-infection agents dusted onto wounds (Sipos *et al.*, 2004). Combinations of minerals from such metals used in ceramic glazes have also proven to be antimicrobial (Kim *et al.*, 1998; Dowling *et al.*, 2003). Other combinations have applications in agriculture, *e.g.* Varro (116 - 27 B.C.), an ancient Roman scholar and writer, noted in the first century B.C. that crushed olives boiled with salt in copper pans showed good pesticidal and herbicidal qualities. In Roman agriculture, wild cucumber leaves and roots were boiled together and sprinkled on vines to prevent mildew (Niaounakis, 2011).

Throughout history people have used acidic plant extracts such as wine, vinegar and citrus fruits as disinfective and antiseptic agents (Imade *et al.*, 2008; Niaounakis, 2011). Short term exposure of pathogens to high or low pH alone may however have little effect on their survival. In Captain Cook's use of sauerkraut as a compress to sailors' wounds to prevent the onset of gangrene, the therapeutic effect it has been stated was probably due to vitamin C and lactate rather than any pH effect. However, in recent studies the antimicrobial effects of lactic and acetic acids may in part, be possibly due to pH (Tejero-Sarina *et al.*, 2012).

Novel agents and combinations with each other and with adjuncts as potential therapeutic antimicrobial treatments need to be effective, fit for purpose, and not show unacceptable or unreasonable levels of secondary effects. There is a growing belief that plant products can fulfil these criteria, whether used alone or in combination with each other, or with synthetic drugs (Takahashi *et al.*, 1995; Bikels-Goshen *et al.*, 2010).

1.4 Plant extracts as new antimicrobial agents

Plants produce a very large number and variety of chemical compounds to defend themselves against the harmful actions of animals, fungi, bacteria, viruses, and other plants (Cowan, 1999; Kim *et al.*, 1998; Borochov-Neori *et al.*, 2011). If novel compounds are to be sourced from nature for use as antimicrobials and other purposes, it is useful to know in which plant organs they are most highly concentrated. Other factors affect their concentration levels such as seasonal variations, growing conditions and soil types (Wang *et al.*, 2011). Further that relationships of plants with other species and environmental conditions can also affect levels of antimicrobial substances, *e.g.*, colonisation of the tea bush by mycorrhiza can stimulate increases in host plant polyphenols and alkaloids and stressing tea bushes by drought, temperature and pH results in similar effects as well as inoculating bushes with selected colonising organisms (Singh *et al.*, 2010). These findings may throw light on alternative ways to investigate tea polyphenol effects on microbes (Ghassempour *et al.*, 2011; Luna *et al.*, 2012).

Plant root systems within soil offer a noteworthy potential source of antimicrobials as the mixture of mineral particles, water, mineral ions, and humus support extensive numbers of pathogenic bacteria which challenge plant roots to manufacture antimicrobials as a defence (Challice, 1973). An example has been shown by Dziri *et al.* (2012) who investigated the phenolic content and antimicrobial activity of flower, leaf, stem and bulb extracts of rosy garlic (*Allium roseum* var. *odoratissimum*). They showed that only the stem extract was effective against *S. aureus* and *E. coli* whereas all extracts were effective against *Enterococcus faecium*. None of the extracts were effective against *Ps. aeruginosa*. The activity of extracts depended on the plant subspecies, season and cultivation region.

Plants respond to the type and numbers of attacking organisms as well as other environmental factors by synthesising appropriate types and concentrations of defensive compounds (El Modafar *et al.*, 1996; Feucht *et al.*, 1997; Punyasiri *et al.*, 2005; Cheruiyot *et al.*, 2008; Choudary *et al.*, 2008) Defensive, antifeedant and antimicrobial compounds are often phenolics, including tannins and flavanols such as catechins, as well as odorous volatiles such as resins and essential oils; alkaloids including glycosides; and carotenoids. Individual antimicrobial compounds often have additional roles, *e.g.* as pigments, odours and antioxidants (Cevallos-Casals *et al.*, 2006; Mimica-Dukic and Bozin, 2007). Some tannins are effective against certain species of bacteria whereas others are not, *e.g.* in a study by Costabile *et al.* (2011) gallotannins were found to be particularly effective against *Salmonella Typhimurium* whereas other types of tannin had little or no effect. In order to develop the commercial potential of a product sourced from a organism it is useful to know which stressors can increase the concentration of these substances as well as which antimicrobial candidates have other useful properties for example as anti-inflammatories. Examples of plant product uses include: as medicines (*e.g.* quinine against skin pathogens, Wolf *et al.* 2006), as preservatives (*e.g.* turmeric and garlic, Paramasivam *et al.*, 2007), as disinfectants (*e.g.* essential oils added to grey water, Winward *et al.*, 2008), and as pesticides (*e.g.* black tea against phytopathogenic microbes, Nuri, 2004).

Catechins (flavan-3-ols) are a group of tea phenolics and belong to the general group of plant flavanoids, many of which provide partial protection against human diseases (Ververidis *et al.*, 2007; Cushnie and Lamb, 2011). There is considerable interest in tea catechins due to their wide ranging biological effects including antimicrobial activity (Ferrazzano *et al.*, 2009). Tea catechins are found mainly in white and green teas (WT and GT) with only trace amounts in black teas (BT) (Scoparo *et al.*, 2012). Catechins are also present in many food products derived from plants such as berries, apples, wine and cocoa (Auger *et al.*, 2004). Catechins are mainly ingested by humans in tea beverage (Friedman *et al.*, 2006). The main tea catechins in terms of abundance are: catechin* (C), epicatechin (EC), epicatechingallate (ECG), and epigallocatechingallate (EGCG) (Gradisar *et*

al., 2007). Various isomers are also found in smaller quantities. Besides growing conditions the amounts and relative proportions of catechins depend on cultivar and post-harvesting treatments which result in black, green, white, oolong, puer and other kinds of teas (Chou *et al.,* 1999; Ferrara *et al.,* 2001; Du *et al.,* 2006; Wang *et al.*, 2011). Some main catechins and derivatives are shown below (Figure. 1.1).

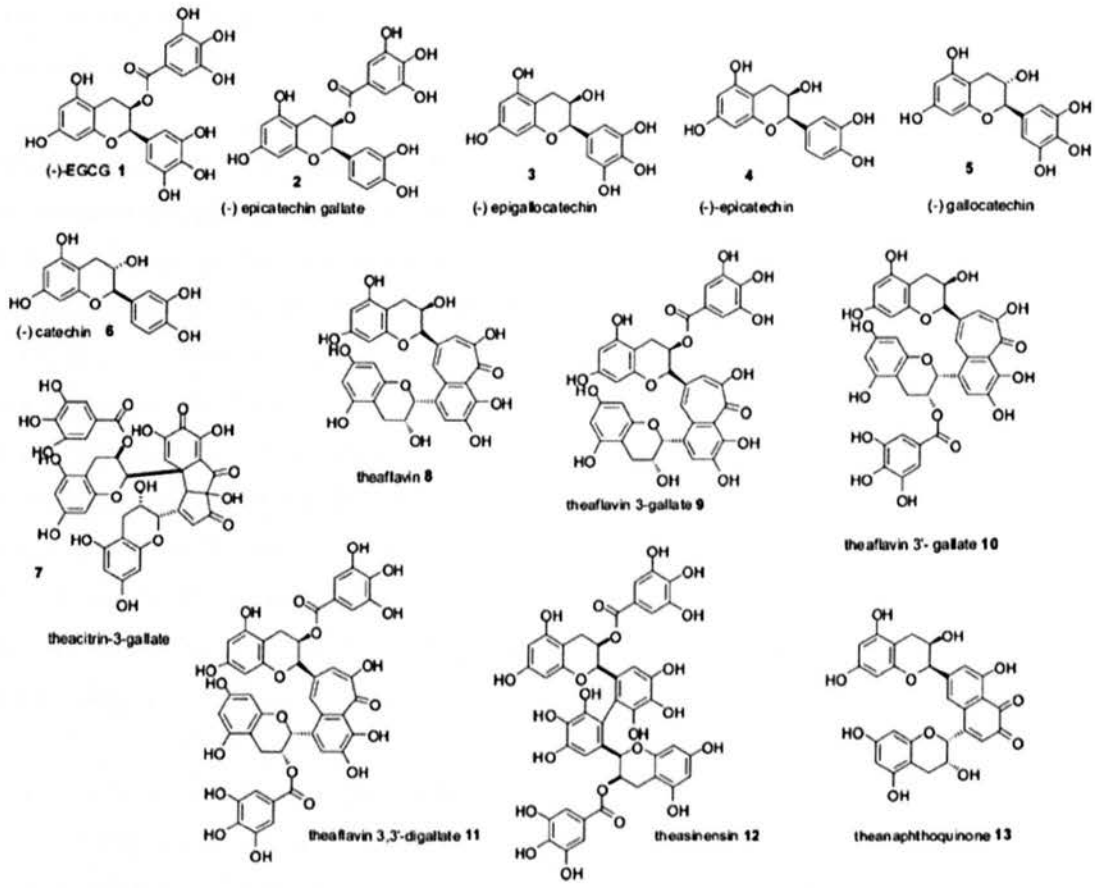


Figure 1.1 Examples of catechins found in green tea and their derivatives formed during the 'fermentation' process to make black tea. EGCG =epigallocatchin gallate; **1-6** Structures of green tea catechins and **7-13** structures of derivatives formed in black tea (taken from Kuhnert *et al.*, 2010).

***Note**: In a recent revision of chemical nomenclature the use of (+) and (-) to denote specific isomers is no longer recommended and the alternative use of the prefix term *enantiomer* is now preferred for the less abundant isomer existing in nature, *e.g.* (+)-catechin becomes 'catechin' whilst the less abundant (-)-catechin becomes '*ent*-catechin', similarly (+)-epicatechin becomes *ent*-epicatechin and the more abundant (-)-epicatechin becomes 'epicatechin' (Mueller-Harvey, 2011). Other flavan-3-ols not found in tea such as robinetinidol and guibourtinidol show further variations in the number of OH groups in the A and B rings.

Tea catechins have wide ranging therapeutic effects including anticancer, antidementia, antihypertensive, antiobesity, antidiarrhoeal, antidiabetic, antiatherosclerotic, antiviral, antiinflammatory, and antilipophilic effects on the brain and pancreas (Suzuki *et al.*, 2012; Schuier *et al.*, 2005; Maurizio *et al.*, 2008; Mitsumoto *et al.*, 2005; Abib *et al.*, 2008; Wolfram 2007; Chen *et al.*, 2008; Unno *et al.*, 2009; Cavet *et al.*, 2011; Relja *et al.*, 2011; de Moraes *et al.*, 2012). Catechins have also been studied extensively for their antimicrobial effects (Auger *et al.*, 2004; Hara-Kudo *et al.*, 2005; Taguri *et al.*, 2006; Friedman *et al.*, 2006; Si *et al.*, 2006; Wu *et al.*, 2007; Song and Seong, 2007; Isaacs *et al.*, 2008; Gordon, and Wareham, 2010; Yi *et al.*, 2010; Cushnie, and Lamb, 2011).

Catechins can have wide ranging effects against different microbes. Numerous studies have investigated the antimicrobial actions of individual catechins which show them to be active against a range of Gram-positive and Gram-negative bacteria such as *S. aureus* and *E. coli.* Generally, catechins are more active against Gram-negatives than Gram-positives (Ikigai *et al.*, 1993). Catechins can even be more active, in some *in vitro* cases, than antibiotics at comparable concentrations (Friedman *et al.*, 2006). Against bacteria, Si *et al.* (2006) showed that epigallocatechin (EGC) and epigallocatechingallate (EGCG) were the most effective of the tea catechins tested against MRSA. Taguri *et al.* (2006) showed that in a study of plant phenolics against a range of bacteria, *E. coli* was particularly sensitive to EGCG; *P. mirabilis* was as sensitive to EGC and EGCG. By comparison, in another study by Theobald *et al.* (2008) EGCG was shown to have stimulating or inhibitory effects depending on concentration against the wine fermenting *Oenococcus oeni.*

Topical catechin actions on an animal body are longer lasting than transient actions in the gut and this fact offers potential in using them as external antimicrobials, where absorption into the blood is of less importance (Leber, 2006; Stockfith *et al.*, 2008). Crop plants might also be sprayed with phenolics, or submerged, for sustained disinfective action (Schrader *et al.*, 2003; Darwish *et al.*, 2012). Sustained antimicrobial action also points to a potential role for catechins as food preservatives and disinfectants and since they are easily broken down, within the manufacture of biodegradable products (Kim *et al.*, 2006; Sun *et al.*, 2011).

1.5 Combining plant products and with adjuncts to enhance antimicrobial activity

It is now generally accepted that plants synthesise a battery of antimicrobial compounds which act collectively against invasive pathogens (Lewis and Ausubel, 2006). This theory argues that individual types of compounds act on different bacterial cell systems such as cell wall synthesis, efflux pump activity and nucleic acid mechanisms simultaneously, so reducing the chances of

recovery by the cell. Combinations of substances to combat bacteria therefore may be a rational way to raise the antimicrobial activity of treatments. This has been supported by studies which have compared the antimicrobial activity of whole plant extracts with those of individual sub-components (Hsieh *et al.*, 2001; Pretorius *et al.*, 2003; Perumalla and Hettiarachchy, 2011; Wang *et al.*, 2011). As an example, in an investigation on goldenseal (*Hydrastis canadensis*), Junio *et al.* (2011) showed that leaf extracts and root extracts produced different antimicrobial activities against *Staphlococcus aureus*. The leaves were found to contain more flavonoids than the roots whilst the roots contained more of the alkaloid berberine. The flavonoids, containing three newly identified sideroxylins, were shown to be able to act in synergy with berberine by inhibiting the NorA multidrug resistance pump of *S. aureus*. A combination of the roots and leaves of this plant, the authors suggest, may provide a mixture with optimal activity against *S. aureus*. These findings can be compared with the ancient practice of combining of wild cucumber leaves and roots boiled together described above to produce a liquor to control fungi.

Extracts from different species, particularly from plants have been mixed together to investigate antimicrobial activity, *e.g.*, Hsieh *et al.* (2001) tested the effect of additives, including metal ion salts, on the antimicrobial activity of a food seasoning mixture of corni fructus (*Cornus officinalis*), cinnamon (*Cinnamomum cassia*) and Chinese chives (*Allium tuberosum*). They tested the activity of the mixture against *S. aureus, E. coli, Ps. aeruginosa* and other food spoilage bacteria, yeasts and moulds. The effect of adding a range of different metal ions to the mixture showed varying effects of enhancement or inhibition, or even no effect. McCarrell *et al.* (2008) showed that antimicrobial pomegranate rind extract (PRE) could be enhanced by transition metal (II) ions and further with vitamin C against certain species such as *S. aureus, P. mirabilis* and *E. coli* but not against *Ps. aeruginosa or Bacillus subtilis*. Further investigations by Gould *et al.* (2009a, 2009b), showed that copper (II) considerably enhanced antimicrobial activity of PRE against the clinical isolates of *S. aureus*, MRSA and Panton-Valentine Leukocidin positive community-acquired methicillin sensitive *S. aureus* (MSSA).

1.6 Tea as a rich source of antimicrobial agents and adjuncts

Certain species of plants, particularly ones with a rich folkloric history, have attracted considerable interest as potential sources of novel treatment agents, *e.g.* chamomile, aloe vera, neem, and tea (Cinco *et al.*, 1983; Mehrotra *et al.*, 2010). For millennia tea beverage made from the leaves of the tea bush (*Camellia sinensis*) has been used as a treatment against a range of conditions, *e.g.* as a demulcifying astringent in catarrh (Wu *et al.*, 2012). Other tea actions include activity against:

oxidative stress (Almajano *et al.*, 2008); inflammation (Wu *et al.*, 2012); the malaria causing protozoan (Sannella, 2006) and infections caused by microbes (Yam *et al.*, 1997; Friedman, 2006); and cancer (Lim and Cha, 2011).

Tea leaves are used to make white and green teas (WT, GT) and contain high levels of antimicrobial compounds which diffuse into the water during the process of brewing including phenolics (Weisburger and Chung, 2002). Other anti-microbial compounds such as condensed tannins, theaflavins and theasinensins are formed by oxidation in the 'fermentation' process when GT leaves are converted into black tea (BT) (Ferrara *et al.*, 2001; Karori *et al.*, 2007). Previous investigations have reported that teas show antimicrobial activity against a range of Gram-positive and Gram- negative bacteria such as *S. aureus, S. epidermidis, Streptococcus mutans* and *E. coli* (Almajano *et al.*, 2008; Neves *et al.*, 2010; Sharma *et al.*, 2012). Comparisons have been made of the activity of different tea extracts against target bacteria; *e.g.* Taguri *et al.* (2006) showed that fresh GT extracts had higher activity than fermented teas. Different tea extracts also vary in their specificity against bacteria. In one study, Ankolekar *et al.* (2011) showed that tea extracts were active against *Helicobacter pylori* whilst beneficial lactic acid bacteria were unaffected. In another example, Su *et al.* (2008) found that GT extract minimum inhibitory concentrations (MICs) against probiotic strains of *Lactobacillus* and *Bifidobacterium* were considerably higher than the MICs of *S. aureus* and *Streptococcus pyogenes*. This finding suggests that GT extract may be used to inhibit or kill susceptible species in certain mixed populations such as may be found on food or within animal biofilms, *e.g.* the mouth and vagina. WT containing unique glycosides has been shown to contain particularly active components which may form part of the basis of its antimicrobial action (Schiffenbauer *et al.*, 2004; Ozcelik *et al.*, 2006; Lin *et al.* 2008).

Tea has recently also found use as an antibacterial component in surgical dressings and as a synergist with antibiotics against enteropathogens (Leber, 2006; Tiwari *et al.*, 2005). Tea extracts are a rich source of a range of different antimicrobial substances which can be exploited in a potentially wide range of settings.

1.7 Antimicrobial activity of tea with different adjuncts

Whole tea extract can be combined with other agents in an attempt to raise antimicrobial activity or to raise the antimicrobial activity of other agents within a mixture. The antimicrobial and anti-oxidant properties of GT extracts suggest that it may be used as a food preservation agent in combination with other extracts, such as from garlic and grape seed (Hsieh *et al.*, 2001; Perumalla, and Hettiarachchy, 2011). By comparison, in an investigation Tabasco *et al.* (2011) showed that flavanol (catechin and epicatechin) enriched grape seed extract was remarkably active against

certain species of *Lactobacillus* whilst other types of *Lactobacillus* were unaffected. Perumalla and Hettiarachchy (2011) reported that a GT with grape seed extract combination was further enhanced synergistically with organic acids including malic, tartaric, and benzoic acids; bacteriocins, *e.g.* nisin; and the chelating agent ethylenediamine tetra-acetic acid (EDTA) – the latter suggesting the presence of deactivating metal ions. Conversely von Staszewski *et al.* (2011) showed that GT activity against *S. aureus* and *E. coli,* was reduced by adding whey protein in a dose dependent manner. Hsieh *et al.* (2001) tested the effect of adding GT and BT extracts to a food preservation mixture of corni fructus, cinnamon, and Chinese chives described above. They found that GT enhanced the seasoning mixture more than BT against food pathogens.

Tea extracts can show synergy when combined with conventional antibiotics, *e.g.* Isogai *et al.* (2001) showed that GT fed to mice increased their survival rate when challenged orally with *E. coli* O157:H7 and that this effect was enhanced synergistically with levofloxacin. Yam *et al.* (1998) similarly showed synergy between different BTs, Oolong tea and GT with β-lactam antibiotics against MRSA. In another investigation GT and the yeast, *Hanseniaspora uvarum,* were shown to both act against the grey mould infection of grape caused by *Botrytis cinera.* Applied in combination the tea and yeast enhanced each other's effects against the mould (Liu *et al.*, 2010). However, not all antimicrobial plant extracts show enhancement effects when combined with other antimicrobials, *e.g.* pannarin isolated from lichen species of the genus *Pannaria* showed minimal effect, no effect, or antagonism of different antibiotics against MRSA isolates (Celenza *et al.*, 2012). In comparison to tea studies it is noteworthy that other antimicrobials, such as bacteriocins are also being combined with classical antibiotics to combat antibacterial resistant microbes (Montalban-Lopez *et al.*, 2011).

1.8 Antimicrobial effects of metal ions and vitamin C adjuncts

It is known that the addition of particular transition metal ions can enhance the activity of many biological agents such as enzymes and other proteins as well as some conventional and novel therapeutic agents, *e.g.*, antibiotics and polyphenols (Zago *et al.*, 2000; Ming and Epperson, 2002; McCarrell *et al.*, 2008). In addition, oxido-reduction agents such as vitamin C can further enhance the activity of biological compounds. Both transition metal ions and vitamin C have been investigated as potential additives to increase the potency of a range of substances (Murata and Yano, 1990; McCarrell *et al.*, 2008). However, if the concentration of certain micronutrients such as copper, iron, zinc, and manganese is increased beyond normal requirements this can become harmful to the life of a cell. Aarestrup and Hasman (2004) demonstrated large variations in susceptibility to copper (II) and zinc (II) ions in different species of bacteria. Other investigations have explored the effects of combined as well as single transition metal ions with other substances

(Hassan *et al.*, 2001; Aarestrup, and Hasman, 2004; Cavaco *et al.*, 2011). As examples, metal ions such as copper (II) and iron (II) ions above particular concentrations are harmful to bacteria and their harmful actions may be enhanced further by silver (I) or zinc (II) ions (Goncharuk, 1995).

Findings from many investigations suggest that metal ions are able to act as enhancers or antagonists to antimicrobial compounds. Ming and Epperson (2002) showed that the antimicrobial peptide bacitracin requires activation via a divalent metal cation which could be zinc, manganese, cobalt, nickel or copper. Podlesek and Comino (1994) found that bacitracin was inhibited by magnesium (II) ions, calcium (II), citrate and pyrophosphate. Interestingly, Sultana *et al.* (2005) reported erythromycin synergism with a range of essential and trace elements and conversely that cephalosporins were inhibited by essential and trace elements (Sultana *et al.*, 2007).

Vitamin C has also been shown to be ineffective against a wide range of bacteria as well as viruses without any evident harmful effect at high dosages (Jungeblut, 1939; Murata and Yano, 1990). In certain cases vitamin C can activate a compound which contains transitional metal ions, *e.g.* Van Asbeck *et al.* (2005) showed that vitamin C enhanced the bacterial inhibitory action of the iron chelating deferoxamine. If, however, reducing agents such as vitamin C are present even trace amounts of certain metal ions such as copper (II) ions are sufficient to show bactericidal effects. This enhancement effect is believed to considerably increase the amount of hydrogen peroxide generated by the copper (II) ions by Fenton chemistry. In other contexts, naturally occurring or artificial compounds may decrease or increase the antimicrobial effects of transition metal ions by chelation or enhancing chemical attraction to cellular structures, respectively (Weber, 1998; Hoshino *et al.*, 2000; Braud *et al.*, 2010).

1.9 Antimicrobial activities of whole tea combined with metal ions and vitamin C

Some studies have shown that tea can interfere with the biological activity of certain ions such as iron (II) and tin (II) but not with others such as calcium (II), zinc (II) or magnesium (II) (Addy *et al.*, 1997; Perez-Llamas *et al.*, 2011). Other investigations found varying enhancing or inhibitory effects from different metal ions with whole plant extract combinations, including tea, dependent on the concentrations used and the target bacterial species (Hsieh *et al.*, 2001). In other investigations combinations have been tested, *e.g.* GT, vitamin C and grape seed extract (GSE) were shown to be mutually enhancing and increase the shelf life of beef patties reducing the need for sulphite preservative (Banon *et al.*, 2007).

1.10. Antimicrobial activity of tea sub-fractions with adjuncts

Plant extracts can be fractionated to investigate which type of components show the greatest levels of antimicrobial activity (Ebi *et al.*, 1999; Nakai *et al.*, 2001; Sokmen *et al.*, 2003). It is possible however, that whole plant extracts contain substances which inhibit the activity of antimicrobial substances found within the same extract. It is useful, therefore, to consider whether a low activity whole extract, therefore, contains such inhibitory substances or whether the whole extract simply contains no, or very few, active antimicrobial agents (Akhavan *et al.*, 2012).

1.11 Alternative methods of enhancing the antimicrobial activity of whole tea extracts

In ancient times combinations of plant products and other additives were used to combat plant diseases and infestations. The activity of these mixtures was sometimes raised by heating (see section 1.3). It is useful to consider whether different physical and chemical methods might be used to modify antimicrobial agents and their adjuncts to raise antimicrobial activity. Beside heating other means could include: the inoculation of harvested tea leaves with specific fungi or bacteria as in the manufacture of Pu-erh tea or Kombucha beverage (Battikh *et al.*, 2012); adjusting the acidity or alkalinity of tea extracts in solution (Hsieh *et al.*, 2001); altering the chemical structure of specific tea components (Park and Cho, 2010); and to prepare cold tea extracts to reduce loss of active substances through oxidation (Nishiyama *et al.*, 2010).

Heating extract substances may lead to an increase, or a decrease, or no change in their antimicrobial activity. Heating GT and BT had no effect on inhibitory activity against *Campylobacter* spp. and *Helicobacter pylori* (Diker *et al.*, 1991, 1994). Asaka *et al.* (2000) found that *Bacillus subtilis* and *B. stearothermophilus* spores germinated in GT previously heated to 95 °C but not in GT infusion heated to 120 °C. Kim *et al.* (2011) investigated the effect of heating fresh tree tannic acid, and whilst this is not found in teas, it is a useful polyphenol for comparison and was found following heating between 65 °C and 200 °C to result in an increase in antimicrobial activity particularly against the tested Gram-positive strains, *S. aureus* compared to Gram-negative strains. Thermal treatment of tea products may raise the antimicrobial activity of tea products against target bacteria (Asaka *et al.*, 2000).

The pH value of a tea extract may also affect its antimicrobial activity. Sreeramula *et al.* (2000) reported that BT extract samples adjusted to pH 5 and 7 had no effect on the growth of *S. aureus*, *E. coli*, or *Ps. aeruginosa*. Li *et al.* (2009) in a later investigation of tea saponins against test species of bacteria found the highest antimicrobial activity at pH 4.8 and the minimum antimicrobial activity at pH 8.0. It appears however, that while tea contains substances whose

individual subcomponent antimicrobial activities are affected by pH that, overall, activities of the whole tea extract are apparently not affected by pH.

Tea catechin solutions may show greater antimicrobial activity if spontaneous auto-oxidation occurs following exposure to atmospheric oxygen (Nakai *et al.*, 2000, 2001). Photochemically induced changes also take place if catechins are exposed to light. Such processes may be accelerated by the presence of copper (II) ions and by heating (Hathway and Seakins, 1957; Es-Safi *et al.*, 2003). The oxidation of flavanols within foods has received considerable attention due to its effects on food quality, *e.g.* colour changes, taste, and texture (Hsieh, 2000; Es-Safi *et al.*, 2003; Kim *et al.*, 2011). Investigations have shown that the degree of oxidation of tea leaf flavanols during the 'fermentation' stage of the manufacture of black tea affects antimicrobial activity (Yam *et al.*, 1997, 1998; Chou *et al.*, 1999). Besides flavanols, tea extracts also contain glycosides which show antimicrobial activity (Engelhardt *et al.*,1993; Lin *et al.*, 2008). In non tea sourced glycosides, oxidation via heating in solution with certain metal salts, has been shown to release the constituent residues of aglycones such as alkaloids which have themselves been shown to be antimicrobial (Jackson and Hudson, 1937; Tran *et al.*, 2012). Glycosides from other plants such as β-D glycoside of vanillin found principally in the vanilla orchid (*Vanilla planifolia*) can be oxidised by slow curing to release the antimicrobial vanillin (Dignum *et al.*, 2001; Fitzgerald *et al.*, 2004).

1.12 Therapeutic applications of whole tea extracts and sub-components

Besides the pharmacological effects of tea it has also been used to treat bowel upsets and diarrhoea. More recently the action of tea against different types of cancer has been recognised (Butler and Wu, 2011). Cold tea bags are used to soothe wounds and reduce inflammation and may also reduce pathogen numbers causing vaginal thrush, although their effectiveness against pathogens has not been proven. Certain tea metal ions may show antimicrobial activity at typical tea beverage concentrations although this has also not been investigated (Karak and Bhagat, 2010). Some metals and their compounds particularly those from the transition elements show antimicrobial activity. Copper, iron, manganese and zinc, are all found in tea although their antimicrobial activity appears not to have been investigated at tea concentrations. Some of these metals particularly copper are now being incorporated into antiseptics and into garments, wound dressings and surfaces to act as disinfective agents to reduce bacterial loads (Borkow *et al.*, 2010; Espirito Santo *et al.*, 2011). Vitamin C found in tea shows antimicrobial activity within combinations and is also used in wound dressings and in toothpaste (Wolinsky *et al.*, 2000; Salaman, 2004; Szabo and Nemeth, 2010). Caffeine found in tea is being investigated for its antimicrobial activity in preserving chicken products (Maletta and Were, 2012). Tea flavanols and

other polyphenols are increasingly being incorporated into potential treatments with other antimicrobial and antifouling substances, *e.g.* with copper (II) ions in paints, with antibiotics such as oxacillin, with antifungal agents and are also being investigated as potential enhancers of disinfective agents (Kaur *et al.*, 2005; Leber, 2006; Bellotti *et al.*, 2010; Perez *et al.*, 2006; Takabayashi *et al.*, 2004; Wolska *et al.*, 2012). Tea polyphenols have recently been recognised as prebiotic substances along with other food substances, as diverse as cheese and globe artichokes, to manage the profile of bacterial species within biomes (Khalil, 2010; Costabile *et al.*, 2010; Oliveira *et al.*, 2012). Tea catechins specifically have been investigated and recommended for treatments against oesophageal cancer, breast cancer, prostate cancer, Alzheimer's disease; and for use as a longevity agent, and as antioxidants in food and wine preservation.

1.13 Mechanisms of action of whole tea extracts and with additives

When exposed to the atmosphere, heating of tea extract may evaporate water, essential oils and other volatile substances, which may result in changes of their antimicrobial activities. This may possibly be due to an increase in concentration of antimicrobial compounds; evaporation of inhibitory substances; degradation of inhibitory substances; or the production of more active antimicrobial substances via condensation or polymerisation of polyphenols such theaflavins and theasinensins produced in the manufacture of BT during within the heat treatment stages of 'fermentation' (Hsieh *et al.*, 2000, 2001). Asaka *et al.* (2000) found that *B. subtilis* and *B. stearothermophilus* spores germinated in GT extract previously heated to 95 °C but not in GT extract heated to 120 °C. However, spores germinated and outgrew in the 120 °C GT extract following removal of tannin components by polyvinylpolypyrrolidone (PVPP). Heating the GT extract at 120 °C produced a lower molecular weight catechin polymer, which was antimicrobial. Other effects of heating GT resulted in the conversion of catechins (flavan-3-ols) to their epimers although this did not affect bactericidal activity on spores. Friedman *et al.* (2006) showed that freshly-made tea infusions (7 types of GTs and 4 BTs) showed higher antimicrobial activity than 1 day old teas against *Bacillus cereus*.

GT extract is enhanced by NaCl when tested against *S. aureus* and *E. coli* (Nakayama *et al.*, 2012). This combination forms high MW complexes on the surface of the cells. A possible mechanism of action on the cells is an effect on the uptake and secretion of substrates as well as an inhibition of enzyme activities (Nakayama *et al.*, 2012).

GT extract has been shown to sensitise *Listeria innocua* cells to the antimicrobial effects of lactic and citric acid stress on cell membrane structures, causing a more hydrophobic cell surface and a

less negative ζ- (zeta) potential (Lehrke *et al.*, 2011). A further example of cell sensitisation by other natural substances was reported by Perumalla and Hettiarachchy (2011) who showed that organic acids, *e.g.*, malic and lactic acids, as well as nisin increased the antimicrobial activity of a combination of GT and GSE. Similar enhancement effects of tea extracts against *S. aureus*, *E. coli* and other microbes were seen with acetic acid produced in the fermentation of a sweetened tea beverage 'Kombucha' (Battikh *et al.*, 2012). It is possible that these other acids and bacteriocins work by interfering with the cell membrane as in the Lehrke *et al.* (2011) study above, and possibly by affecting metal ion availability, since EDTA had the same effect on the GT/GSE mixture as the other test substances. Sivarooban *et al.* (2008) showed that GT extract combined with nisin against *Listeria monoctogenes* resulted in an altered cell membrane and condensed cytoplasm.

Goodman *et al.* (2012) used electron paramagnetic resonance (EPR) and found that both BT and GT extracts produced at least 6 types of complexes with copper (II) ions, the formation of each of which was pH dependent. GT and BT also showed several common complexes. This evidence supports the notion that by forming metal ion complexes, tea components may reduce the availability of copper (II), iron (II) and zinc (II) transition metal ions, which are required for adequate nutrition by many bacteria. Another possible consequence of this could be a reduction in the effects of free, non-complexed tea agents. Conversely complexation between tea agents and metal ions may raise the biological activity of either or both.

1.14 Mechanisms of action of tea fractions and with additives

Chosa *et al.* (1992) compared the antimicrobial activities of GT and BT to their components, EGCG and theaflavin digallate (TF3), against *Mycoplasma* spp. Both components were active against *Mycoplasma salivarum* whilst neither of the whole teas were. It is possible that other factors within the teas inhibited the effects of EGCG and theaflavin digallate.

Mehrotra *et al.* (2010) tested the antimicrobial activity of two thin layer chromatography fractions of Assam tea (BT) adjusted to pH 2.0, 7.0, and 8.0 against *S. aureus*. The antimicrobial activity of the lower fraction was reduced at pH 2.0 and pH 8.0 whereas the upper fraction was unaffected by pH. In another investigation GT extract caused cell perforations, cell aggregates and the leakage of material from cells (Cho *et al.*, 2010).

Scanning electron microscopy (SEM) has revealed that MRSA, but not MSSA, grown with a pure component of GT, showed that cell division and separation of daughter cells was disrupted with nodular packets of unseparated cocci being seen (Hamilton-Miller and Shah, 1999). The cell walls dividing individual cells within each packet showed abnormal thickening whilst the external wall

of the cluster showed normal thickness. This observation is consistent with selective inhibition of the synthesis of penicillin-binding proteins, affecting membrane structure and properties as described by Hamilton-Miller and Shah (1999). In another investigation, BT proanthocyanidins were shown to bind with lipopolysaccharide on the outer membrane of Gram-negative species, affecting their functional characteristics (Johnson *et al.*, 2008). A pure component extract of GT has been shown to reverse methicillin resistance in MRSA and to reduce penicillin resistance by *S. aureus*. This was explained by the GT component preventing synthesis of the penicillin binding protein, PBP2', and the inhibition of β-lactamase* (Yam *et al.*, 1998).

***Footnote:** Penicillin, perhaps the best known antibiotic, is a β-lactam antibiotic which is naturally secreted by the ascomycete fungus *Penicillium chrysogenum* [formerly, *notatum*] to eliminate competitive bacteria. Bacteria respond to this fungal defence by secreting β-lactamases to deactivate the penicillin. Practioners respond to this bacterial defence by administering β-lactamase inhibitors to reduce bacterial defences when treating infections.

The above findings support the notion that tea extracts work against bacteria by binding to and interfering with cell membrane structure. This in turn negatively affects the ability of a cell to control and regulate the transport of substances in and out of the cell.

1.15 Antimicrobial activity of tea catechins in the presence of other substances

(i) Polyphenols: Catechins have been shown to enhance the activity of other antimicrobial polyphenols within tea. In an investigation to combat the antimicrobial resistance of the nosocomial pathogens *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*, Betts *et al.* (2011) showed that epicatechin (EC) synergistically enhanced the action of BT theaflavin against both species.

(ii) Enzymes: ECG has been found to suppress the secretion of coagulase and alpha toxin by *S. aureus* (Shah *et al.*, 2008). EGCG has also been shown to inhibit the enzyme dihydrofolate reductase from *Stenotrophomonas maltophilia* (Betts *et al.*, 2011). Generally, green tea polyphenols such as catechins interact with added proteins *in vitro* forming complexes which reduce the biological activity of both the protein and the polyphenol although the particular type of polyphenol determines the degree of mutual inhibition between the two (von Staszewski *et al.*, 2011). Nirmal and Benjakul (2012) found that catechin could inhibit melanosis in Pacific white shrimp, and they suggested that the catechin inhibited different types of polyphenoloxidase enzymes on account of the catechin binding with copper (II) ions which feature in the active site of this enzyme.

(iii) Transition metal ions alone and with added vitamin C: Initially in considering copper (II) ions alone, these ions are often required for activating enzymes and other proteins (Basu *et al.*, 2003; Yang *et al.*, 2007). Copper metal and its salts, however, beyond trace amounts, are usually antimicrobial against a wide range of bacterial species. Zevenhuizen *et al.* (1979) found that different bacterial species showed considerable differences in copper (II) ion tolerance, *e.g. Klebsiella aerogenes* was inhibited in the range 0.01μM – 1.0 μM. However, viable strains of *Pseudomonas* spp. could be isolated from a stock solution of 100 μM copper (II) sulphate, and could tolerate levels up to 1 mM. Bacterial susceptibility to copper (II) ions depended on copper (II) ion bioavailability as EDTA removed the harmful effects (Zevenhuizen *et al.*, 1979). McCarrell *et al.* (2008) found copper (II) ions were more active against Gram-negative than Gram-positive species.

Lebedev *et al.* (2005) working on *E. coli* described a model of cell permeability to show that copper (II) could exert its effects by binding to membrane protein disulphide groups and opening non-specific cation channels for other chemicals to enter or leave the cell. More recently Bondarenko *et al.* (2012) showed that in *E. coli* the effects of copper (II) oxide nanoparticles once solubilised, generated reactive oxygen species (ROS) as superoxide anions (O_2^-) and H_2O_2, which are known to damage nucleic acids, proteins and lipids (including those in the cell membrane) and to produce single strands of DNA. Non-solubilised copper (II) oxide had no effect on *E. coli.*

In consideration of copper (II) combined with catechins, Kimura *et al.* (1998) studied the effects of different tea catechins with copper (II) ions on *E. coli* and showed that EGC but not EC killed the organisms in the presence of 1 μM copper (II) salt. Similar results were found by Hoshino *et al.* (2000) against *S. aureus* as well as *E. coli.*

The above investigations on the antimicrobial activities of copper (II) ions suggest they can enhance the activities of at least, some but perhaps not all of tea catechins. The details of the extent of the effect of a catechin-copper (II) ion combination on cell viability is lacking detail. In addition the combination's precise mechanism of action with regard to the structure of less active catechins such as catechin itself and epicatechin are not yet fully known or understood.

Iron is required by many bacteria for normal growth and development. However, above normal requirements iron can be antimicrobial since it can like copper (II) ions enter Fenton chemistry and produce harmful ROS (Schmidt *et al.*, 2012; Ndounla *et al.*, 2013). Within the cell harmful ROS such as H_2O_2 can be deactivated with catalase or their production reduced by various deactivation mechanisms of Fenton reactants like iron (II/III) (Chiancone *et al.*, 2004). Externally, however, iron (II/III) ions could be used as antimicrobial agents to generate damaging ROS. McCarrell *et al.* (2008) found that following cell exposure to iron (II) ions when tested alone were antimicrobial to a similar degree against a range of Gram-positive and Gram-negative species.

It is possible that such iron (II/III) action above may be increased by combining iron (II/III) ions with other antimicrobials such as catechins to produce an enhanced effect. Mira *et al.* (2002) showed that flavonoids activated with iron (II/III) and copper (II), showed a greater reducing capacity for copper (II) compared to iron (III) and this characteristic may be important in explaining the additional potency of flavonoid-copper (II) combinations compared to iron (II/III). The extent of reduction of copper (II) seemed to be dependent on the number of phenolic OH groups. Combinations of metal ions may be more effective as antimicrobials than single metal treatments at comparable concentrations. It would also be interesting to know whether copper (II) and iron (II/III) ions together can enhance one another's antimicrobial actions and if so, whether the mixture added to a tea catechin could enhance its antimicrobial activity more than a single type of metal ion addition. Other effective antimicrobial transition metal ion combinations have been investigated and may be useful under certain circumstances in order to use lower concentrations of, *e.g.* copper on antimicrobial surfaces (Vargas-Reus *et al.*, 2012).

Zinc (II) and manganese (II) ions are both micronutrients required for the activation of certain enzymes, and can both show antimicrobial activity with varying degrees of effect on different species, (Hagerman *et al.*, 2003; McCarrell *et al.*, 2008; Hassan *et al.*, 2012). Zinc (II) and manganese (II) ions could therefore, be used as potential antimicrobial adjuncts with untested tea catechins.

In comparison to the above it is worth noting that antimicrobials may be activated or inhibited by various ions or other substances. Ming and Epperson (2002) showed that a bacterial secretion, bacitracin, requires activation via a divalent metal cation which could be zinc, manganese, cobalt, nickel or copper. This and other studies suggest that metal ions are able to act as enhancers or antagonists to antimicrobial compounds (Podlesek and Comino, 1994). Other substances which can also activate or inhibit antimicrobial components include non-metallic elemental ions as well as compounds such as Cl^- and vitamin C. Vitamin C on its own has little effect against bacteria. However, it can enhance the antimicrobial activity of other antimicrobial substances (Ericsson, 1954; Hatano *et al.*, 2008; McCarrell *et al.*, 2008). For example, Ragab-Depre (1982) and later Murata and Yano (1990) showed that vitamin C acted as reducing agent to copper (II) ions, which enhanced the bactericidal effect of copper (II) against Gram-negative species, but less so against Gram-positive species. Further addition of hydrogen peroxide enhanced the antimicrobial effect of the vitamin C-copper (II) combination. However, the further addition of iron (II) added no extra effect. It was suggested that copper (II) combined with dehydro-ascorbic acid or an auto-oxidised intermediate produced a lethal agent capable of penetrating the cell membrane and that that free radicals were involved. Overall it appears that Gram-positive species are less susceptible to

antimicrobial transition metal effects than Gram-negative species. This is possibly due to the greater thickness of the cell wall of Gram-positive compared to Gram-negative species.

(iv) Alkaloids: The antimicrobial activity of catechin may be raised by the addition of alkaloids. Bacteria and plants as well as fungi and animals synthesise alkaloids which are often harmful to other organisms (Fardiaz, 1995). Plant alkaloids including those derived from purine such as caffeine as well as its derivatives including xanthine, theobromine and theophylline have been investigated for antimicrobial effects (Raj and Dhala, 1965; Lin *et al.*, 2008). Raj and Dhala, 1965 demonstrated synergy between caffeine and tetracycline, but less so with streptomycin, suggesting inhibition of the effluxing multidrug resistant pump (MDR pump), a structural feature of cell membranes which expels harmful substances from the cell protoplasm, rather than inhibition of protein synthesis.

(v) Antibiotics: Akiyama *et al.* (2001) investigated the effects of different tea catechins in combination with the β-lactamase inhibiting antibiotic, oxacillin, against *S. aureus*. They found that catechins with gallic acid side chains (ECG, EGCG) were more effective than other polyphenols (tannins) with EGCG being more effective than ECG. Further to this investigation, Anderson *et al.* (2011) showed that deletion of the hydroxyl groups from the A and B rings of ECG reduced the MIC of oxacillin in this combination.

1.16 Structure-activity relationships of tea catechins as antimicrobials

Investigations with synthesised analogues of GT catechins have shown that substitutions and stereochemistry of functional groups at specific positions within the molecular structure affects antimicrobial activities (Figure. 1.1) (Stapleton *et al.*, 2004a, 2004b; Park and Cho, 2010). The molecular structure of the common tea catechins affects the different MICs found against *S. aureus* (Figure. 1.2). (Takahashi *et al.*, 1995; Akiyama *et al.*, 2001; Stapleton *et al.*, 2004a, 2004b; Gibbons *et al.*, 2004; Taguri *et al.*, 2004; Roccaro *et al.*, 2004). In contrast to *S. aureus,* Friedman *et al.* (2006) found that *Bacillus cereus* was not affected by a catechin without a gallate side chain.

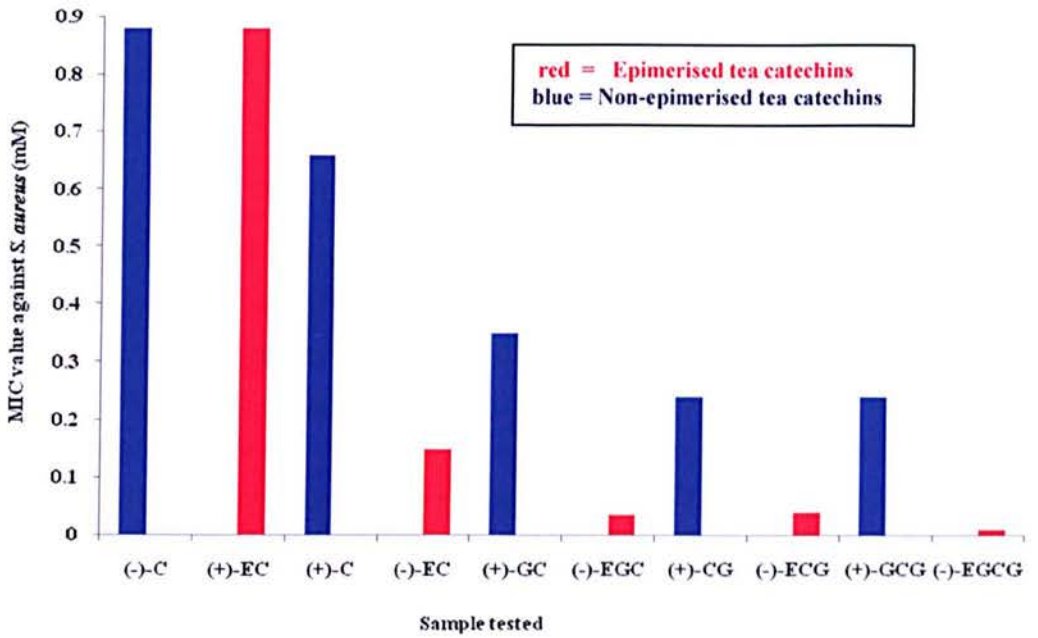


Figure 1.2 Average MICs expressed as molarities of 10 different tea catechins against various strains of *Staphylococcus aureus*. '(+)' and '(-)' formerly recommended conventions still in present use are added here to emphasise the different isometric forms of the enantiomeric and epimeric tea catechins. Non-epimeric catechins are shown in blue, epicatechins are shown in red. Ent (-)-CAT = enantiomer of catechin; ent (+)-EC = enantiomer of epicatechin; (+)-CAT = catechin; (-)-EC = epicatechin; (+)-GC = gallocatechin; (-)-EGC = epigallocatechin; (+)-CG = catechin gallate; (-)- ECG = epicatechin gallate; (+)-GCG = gallocatechin gallate; (-)-EGCG = epigallocatechin gallate . Data based on 7 published studies.

1.17 Effects of pH on tea catechin and adjunct antimicrobial activity

In some cases very low or high pH values may affect antimicrobial activity (Hsieh *et al.*, 2001). The knowledge of any pH value or range that optimises antimicrobial activity of agent-adjunct mixtures, *e.g.* in the case of food preservation or to maximise disinfective action on surfaces or in aquaculture would be useful to know. Yoshida *et al.* (1999) showed that pH could affect the binding of tea catechins to bacterial cells and consequently their antimicrobial activity. Mabe *et al.* (1999) showed that EGCG had bactericidal activity at pH 7, but not at pH 5 or below against *Helicobacter pylori*. Clearly it is necessary to know whether changes in antimicrobial activity can be brought about by pH alone rather than other possible actions of antimicrobial agents or adjuncts.

1.18 Effects of heat and oxidation on catechin antimicrobial activity

Structural changes or transformations of catechins molecular structure may offer a means of altering antimicrobial activities. In the production of black tea, compounds produced by the

'fermentation' process such as theaflavins and theasinensins have been to shown to possess novel antimicrobial activity. This finding suggests that heat treatment may be a way of enhancing the antimicrobial activity of catechins (Chosa *et al.*, 1992; Betts *et al.*, 2011) (see Figure 1.1). Auto-oxidation of catechin solutions also offers an additional mechanism for altering their antimicrobial activities.

1.19 Binding studies and stoichiometric effects of catechins combined with putative adjuncts

Investigating the stoichiometry of reacting chemical species against bacterial cells can lead to the development of optimal mixtures and a better understanding of the chemistry involved in reaction mechanisms. This in turn can lead to development of a combination treatment. Studies by Mira *et al.* (2002), and Esparza *et al.* (2005) suggested that a 1:1 stoichiometry was likely between catechin hydrate and copper (II) ions. It would be informative to know whether the antimicobial activity of a catechin-copper (II) mixture was optimal at this ratio.

1.20 Effects of catechins on cell wall structure and function

Animal and *in vitro* studies can also provide useful information for investigating responses of bacterial cell mechanisms (Ikigai *et al.*, 1993; Williams *et al.*, 2003; Tamba *et al.*, 2006; Sun *et al.*, 2009; Sun *et al.*, 2011). For example, structure-activity relationships of catechins with membrane systems may generate insights for developing substances with increased activity against bacteria. Bacterial cell wall composition and its electrical charge are important factors to consider when studying mechanisms of action as the bacterial cell membrane is exposed to many harmful agents. In animal studies, variations in molecular structure of cell membranes determine the extent of catechin binding (Uekusa *et al.*, 2008; Sirk *et al.*, 2008, 2009).

Catechins can interact with cell walls via intercalation (Stapleton *et al.*, 2006, 2007) and modify cell aggregation, cell morphology and architecture as well as β-lactam antibiotic susceptibility of *S. aureus* (Hamilton-Miller and Shah, 1999; Shah *et al.*, 2008). Bernal *et al.* (2009) showed that *S. aureus* grown in the presence of ECG had thickened cell walls due to a disruption of D-alanyl esterification of cell wall teichoic acids, which changed teichoic acid chain flexibility into a more random coil conformation. Cui *et al.* (2012) found that EGCG generated H_2O_2 and this affected cell walls via aggregation and lysis in *S. aureus* and *Streptococcus mutans,* and as perforations and grooves in *E. coli.*

Catechins also damage living and model cell membranes by causing cell contents to leak out. Leakages include metal ions (*e.g.* such as potassium), proteinacious substances and fluorescent and

dye indicators which had been previously introduced artificially. Cushnie *et al.* (2008) found that in both β-lactam sensitive and resistant forms of *S. aureus*, exposure to 3-*O*-octanoyl-epicatechin resulted in a loss of 50% of internal potassium within 10 minutes. The authors suggested that further structural modifications of this epicatechin derivative may lead to enhanced antimicrobial activity. Caturia *et al.* (2003) showed that galloylated catechins penetrated phospholipid model membranes by intercalating between the hydrocarbon chains. However, the non-galloylated catechins gave only shallow penetration and were located close to the phospholipid/water interface. Catechins promoted carboxyfluorescein leakage from cells only if phosphotidylethanolamine formed part of the membrane suggesting that this component could be responsible for cell leakage in bacteria that are exposed to catechins.

Catechins that are complexed with copper (II) ions are positively charged (Weber, 1988). This may explain the greater antimicrobial activity of positively charged catechin-copper (II) complexes on negatively charged Gram-negative *E. coli* cells compared to Gram-positive *S. aureus* cells (Hoshino *et al.*, 2000). Pirker *et al.* (2012) showed that the speciation of EGCG with copper (II) depended on pH. Therefore, pH may also affect the antimicrobial activity of catechin-copper (II) ion combinations.

1.21 Economic and ecological perspectives on development of new antimicrobial agents

Considerable efforts are being made to combat pathogens using novel disinfective agents for use in various settings, in order to meet the on-going challenges of antibiotic resistance of pathogens such as methicillin-resistant *S. aureus* (MRSA). Clearly, there is also a need for low cost, environmentally friendly preparations for use in diverse geographical locations (Simões *et al.*, 2009; Graça *et al.*, 2011; Roekel and Fernandez, 2012). Developing new antimicrobials from waste products is also of particular interest.

Plants provide a convenient and economical source of substances that can act alone or in combination with other substances as agents. Plant extracts can be used whole or fractionated to concentrate active components, or to remove inhibitory substances, and to isolate individual substances for particular uses. Plant extracts can be heat-treated to raise antimicrobial activity. Studies of the mechanism of plant antimicrobials are needed in order to find substances that can mimic effective antimicrobial agents.

Tea leaf extracts offer promise as they possess therapeutic effects for the treatment of various infections. Polyphenols from tea, particularly the catechins are an area of active research in finding

new treatments and combinations of treatments to respond to the continuing problem of antimicrobial resistance.

1.22 Aims and objectives of this study

The principal aim of this project was to probe catechin activities under a range of conditions to generate antimicrobial agents. Of specific interest were tea extracts, which may have the potential to overcome the increasingly encountered resistance of pathogens such as methicillin-resistant *S. aureus* (MRSA). Enhancement of antimicrobial activity was studied by combining the most active tea extracts with putative adjuncts and to reduce the effect of any inhibitory substances that may be present.

This study first surveyed the literature for minimum inhibitory concentrations of different tea catechins against *S. aureus* with a view to selecting the weakest in order to enhance antimicrobial action with adjuncts and by using heat treatments. Following preliminary screening of 5 main tea catechins alone and with copper (II) sulphate, subsequent experiments investigated the kinetics and reaction mechanisms of enhanced mixtures and their activity during storage.

Objectives

1. To measure the antimicrobial activity at different pH values of white tea (WT), green tea (GT), and black tea (BT) extracts against laboratory strains of *S. aureus, Ps. aeruginosa, P. mirabilis* and *E. coli* using diffusion assays and viable cell counts.

2. To measure the activity of tea extracts combined with the putative adjunct copper (II) sulphate. Controls included copper (II) concentrations as found in the most active teas. Activity enhancement was attempted by the addition of vitamin C. Thereafter, sub-fractions with the same combinations were tested to establish the most active molecular weight range against *S. aureus.*

3. To investigate the antimicrobial activity of the weakest tea catechin tested alone and with adjuncts against *S. aureus* and to compare these activities with those of related isomers with adjuncts against *S. aureus.* This part focused on identifying molecular features that affected antimicrobial activity most.

4. To investigate any effect from physically altering the molecular structure of catechin by heat treatment of catechin solutions at different temperatures and for different lengths of time. To test such treated solutions with adjuncts against target bacterial species.

5. To investigate mechanisms of activity by the use of metabolic inhibitors in order to investigate the possibility that active mixtures acted via ROS generation and if so to suggest possible mechanisms of action. These studies included: UV-vis spectrophotometry, solubility, crystallisation, pH measurements and antimicrobial kinetics to investigate the formation of chemical complexes between catechin and various adjuncts as well as examination of mixture effects on cells using by microscopy.

6. To investigate the stability of active mixtures over time during storage under different conditions and to attempt to account for any observed changes in activity.

2.1 Materials

All substances below were purchased from Sigma-Aldrich, UK, unless stated otherwise and were of analytical grade.

2.1.1 Organisms and strains used

The organisms were sourced in disc form from Pro-Lab Diagnostics, UK and stored at -20 °C until revival with brain heart infusion, and thereafter were stored at -80 °C (see section 2.2.5).

Staphylococcus aureus, a facultative anaerobic, Gram-positive coccal bacterium, was the principal target species used to test susceptibility towards novel combinations of putative antimicrobial mixtures. For comparison, mixtures were tested against organisms selected from the following Gram-negative pathogenic types: *Pseudomonas aeruginosa* (aerobic coccobacillus with unipolar motility), *Escherichia coli* (facultative anaerobic bacillus, non-motile), and *Proteus mirabilis* (facultative anaerobic bacillus, motile) all of which are causative agents for several disease states.

Staphylococcus aureus NCTC 06571
Pseudomonas aeruginosa NCTC 950
Escherichia coli NCTC 14441
Proteus mirabilis NCTC 7827

NCTC = National Collection of Type Cultures

2.1.2 Microbiological media and other reagents

All solutions and extracts were made up using sterile deionised water unless specified otherwise. Growth and cell suspension media were manufactured by Oxoid, Basingstoke, UK and were prepared using deionised water prior to autoclaving.

Growth media were prepared in half or whole litre volumes using deionised water prior to autoclaving at 121 °C for 15 minutes. Brain heart infusion broth was prepared in volumes of half of a litre. Nutrient agar was prepared generally, 3 or 4 litres at a time and following sterilisation left to cool at room temperature until *ca.* 60 °C when the glass Shott bottle containing the medium could be handled and the contents poured. Following gentling swirling to homogenise the contents, the medium was poured out into sterile plastic Petri dishes to a depth of *ca.* 4 mm for diffusion assays and *ca.* 5mm for other purposes, and the lid replaced immediately to reduce contamination from

aerial spores. Plates were then left until the contents set into a solid and usually stored on a bench surface for 1 day to allow some drying before use. After 2 days any remaining plates were placed into polythene sleeve-shaped bags to maintain hydration levels. Any unused plates thereafter were discarded.

(i) Growth medium for initial cell revival

Brain heart infusion broth (product code CM1135)	**g L^{-1}**
Brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
pH in solution 6.8±0.2	

(ii) Growth medium for further cell revival and suspension assays

Nutrient agar (product code CM003B)	**g L^{-1}**
Lab-Lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
NaCl	5.0
Agar	15.0
pH in solution 6.8±0.2	

(iii) Growth medium for diffusion assays

Mueller-Hinton agar (product code CM337)	**g L^{-1}**
Beef infusion	300
Casein hydrolysates	17.5
Starch	1.5
Agar	17
pH in solution 7.3±0.1	

(iv) Suspension medium for preparation of inocula and controls in some suspension assays

Ringer's solution (product code BR0052)	**g L^{-1}**
Sodium chloride	2.25
Potassium chloride	0.105
Calcium chloride heptahydrate	0.12
Sodium bicarbonate	0.05
pH in solution 7.0	

Ringer's solution was prepared following manufacturer's instructions prior to autoclaving.

(v) Suspension medium to wash cells for electron microscopy

Phosphate buffered saline (Sigma)	g L^{-1}
Sodium chloride	8.0
Potassium chloride	0.2
di-Potassium hydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2
pH 7.3	

(vi) Lambda buffer adjusted to pH 7.2 used as a control in cell suspension assays (Stewart *et al.*, 1998)

Lambda buffer adjusted to pH 7.2	g L^{-1}
Tris buffer (British Drug Houses, BDH)	0.729
Magnesium sulphate (Mg (II) $SO_4.7H_2O$)	1.204
Gelatin	0.05

Key: Tris = 2-Amino-2-hydroxymethyl-propane-1,3-diol

(vii) Suspension assay stop solution

Tween®-80 was used to neutralise any inhibitory activity on cells from test sample mixtures following timed exposures. A 2% (v/v) Tween®-80 in in Lambda buffer was freshly-made for each cell suspension assay experiment. Unused stop solution was discarded.

2.1.3 Antimicrobial test agents (whole teas and their sub-components)

Leaves from commercial cultivars of the tea bush (*Camellia sinensis*, Linnaeus) were obtained from local commercial sources as fresh products within sell by dates. Product descriptions indicated that the teas were grown and harvested by traditional means in their country of origin (Darjeeling, India) and subsequently processed by further traditional methods such as drying and oxidative 'fermentation'.

The investigated teas and suppliers were as follows:

White tea 'Silver Tips' (WT) Fortnum & Mason Ltd., London, UK
Green tea 'Sencha' (GT) Waitrose Ltd., Kingston-upon-Thames, UK
Black tea 'Yorkshire' (BT) Waitrose Ltd., Kingston-upon-Thames, UK

These three types of tea product represent teas, firstly, with little or no post harvesting processing (WT); secondly with wilting and drying (GT); and thirdly with extensive curing of the leaves to

producing 'Indian tea' (BT) via the oxidative 'fermentation' process. These three types of tea leaf are believed to contain varying profiles of different antimicrobial agents.

For antimicrobial activity investigations, aqueous tea extracts were prepared using the methods described below (see section 2.2.2). Individual tea sub-components were also purchased commercially as pure substances and investigated as antimicrobial agents or as putative adjuncts (Sigma-Aldrich, UK). The sub-components investigated were: green tea flavan-3-ols; *catechin, enantiomer of catechin, epicatechin, *ent*-epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate; vitamin C and caffeine. Flavan-3-ols ('catechins') were stored in powder form at 5 °C, whilst vitamin C and caffeine were stored at room temperature at *ca.* 20 °C.

2.1.4 Transition metal ions investigated as putative antimicrobial adjuncts in suspension assays

Stock solutions of salts of the transition metal ions below were made up in sterilised deionised water and stored at 5 °C for subsequent use directly or following dilution with sterile deinonsed water. Iron (II) sulphate was freshly-made for each assay experiment (see 2.2.1 b below for further details).

Copper (II) sulphate pentahydrate
Iron (II) sulphate heptahydrate
Iron (III) chloride
Zinc (II) sulphate monohydrate
Manganese (II) sulphate tetrahydrate

2.1.5 Substances used to investigate antimicrobial mechanisms of reaction

Following the method of Brown *et al.* (1998) disodium ethylenediamine tetra-acetic acid (EDTA), a transition metal ion chelating agent, was added to samples containing added copper (II) and iron (II) ions and tested against *S. aureus* NCTC 06751 and *E. coli* NCTC 14441 to remove the bioavailability of transition metal ions from test solutions.

Tested samples which showed antimicrobial activity as well as relevant controls were also tested by adding fixed or progressive concentrations of commercially available bovine liver catalase solution (50 mg mL^{-1} with an activity of *ca.* 47 kU mg^{-1} protein, stored at 5 °C) to remove hydrogen peroxide from extracellular solution in suspension assays testing the effect of samples against *S. aureus* NCTC 06751 and *E. coli* NCTC 14441.

***Note:** Prior to recent conventions the above principal tea catechins, were and are generally known as: (+)-catechin, (-)-catechin, (-)-epicatechin, (+)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, (-)-epigallocatechin gallate

Hydrogen peroxide generation by active samples was also investigated by a colormetric method (modified after Lespinas *et al.*, 1989) to measure hydrogen peroxide present in suspension assay samples where the inoculum was replaced by Ringer's solution. The reagent was prepared as per the table below and was used to measure levels of test sample hydrogen peroxide with which, the components develop a magenta chromophore (see 2.2.9 (v)). A solution of hydrogen peroxide 5% (w/v) was serially diluted and used as assay control as well as to construct a calibration curve.

Modified reagent after Lespinas	Quantities per litre
HEPES buffer adjusted to pH 7.2	50 mM
Horseradish peroxidase	3 kU
Phenol	16 mM
4-aminophenazone	0.4 mM
Triton® X-100	1g
Not autoclaved, stored at 5° C and used within 3 weeks	

Key: HEPES = N-2-Hydroxyethylpiperazine-N′-2-Ethanesulfonic acid. Triton® X-100, a non-ionic surfactant detergent used for solubilising protein

2.1.6 pH adjustment reagents

Commercially available pH 4 and 7 buffer solutions (Thermo Electron Corporation, UK) were used to calibrate a Corning pH meter. This was used to measure the pH of aqueous solutions and mixtures, and to adjust similar solutions and mixtures to specified pH values. Alkali and acid, 1M NaOH and 1M HCl, were used to adjust the pH of copper (II) sulphate, Ringer's, N-2-Hydroxyethylpiperazine-N′-2-Ethanesulfonic acid (HEPES), and lambda buffer solutions, as well as whole tea extracts.

2.2 Methods

All media were made up following manufacturer's instructions in deionised water and sterilised using an autoclave at 121 °C for 15 minutes at a pressure of 15 lbs. per sq. inch, unless specified otherwise.

Extracts and solutions stored at -20 °C were aliquotted for convenience and tested periodically for any changes in antimicrobial activity for up to 3 years to monitor stability (see section 5.2).

2.2.1 Preparation and maintenance of stock and test solutions

All fresh and thawed solutions and extracts; reagents and buffer were tested periodically for contamination by streaking onto nutrient agar plates and incubated aerobically overnight at 37 °C

since this is an optimal temperature for the growth of human pathogens. Any solutions showing colony growth were discarded.

(i) Buffer: Lambda buffer adjusted to pH 7.2* using 0.1 M NaOH was made according to Stewart *et al.* (1998) prior to autoclaving for subsequent use in cell suspension assays and in a cell assay stop solution described below.

*A Corning glass pH electrode and meter was used to measure the pH of test samples and for prior adjustment of reagents and extracts to a standardised pH using 1 M NaOH or 1M HCl as necessary. Readings of pH were taken following repeated calibrations with pH 4 and 7 buffers, (Thermo Electron Corporation, UK).

(ii) Untreated fresh solutions (not autoclaved): Stock solutions of 19.2 mM copper(II) sulphate pentahydrate; 472 μM zinc (II) sulphate; 472 μM manganese (II) sulphate; 2140 μM disodium ethylenediaminetetra-acetic acid (EDTA); and modified Lespinas H_2O_2 assay reagent were each made up with sterile deionised water and stored in dark conditions at 5 °C, and diluted with sterile water as necessary prior to use. Freshly-made 1888 mM iron (II) sulphate heptahydrate; 472 μM iron (III) chloride; and a range of concentrations of caffeine between 0.01% and 1% (w/v), were made up and serially diluted as required prior to aliquotting and storage at -20 °C until use. The tea catechins: (+)-catechin, (-)-catechin, (-)-epicatechin, (+)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin, (-)-epigallocatechin gallate were made up to 1 mM in sterile deionised water and serially diluted as required and used fresh or subsequently stored at -20 °C until use.

Commercially available bovine liver catalase solution was stored at 5° C prior to use and diluted with Ringer's solution as necessary. Freshly-made 1712 μM vitamin C was made up and diluted with sterile water as necessary prior to immediate use with any remaining unused solution discarded. Freshly-made and thawed reagent solutions were stored in the dark and used at room temperature within 20 minutes. For further details of dilutions of the above reagents see Appendices IIA and IIB. A stop solution used to neutralise any inhibitory activity of cell viability as well as cell growth was freshly-made for each experiment using 2% (v/v) Tween-80 in Lambda buffer previously prepared as above.

(iii) Heat-treated solutions: Purchased bovine liver catalase solution and freshly prepared solutions of: 1mM catechin, 1888 μM copper (II) sulphate, iron (II) sulphate, and iron (III) chloride solutions were rapidly brought to 100 °C and held at this temperature for 10 minutes. An additional sample of catechin solution was also similarly heat-treated for 30 minutes. Original volumes were restored using sterile deionised water at *ca.* 20 °C, before use or aliquotting prior to storage at -20 °C.

The following solutions were pretreated by autoclaving at 121 °C for 15 minutes before use or prior

to storage at -20 °C: 4.8 mM copper (II) sulphate; 1888 μM iron (II) sulphate; iron (III) chloride; 500 μM catechin, and 500 μM catechin previously heated at 100°C for 10 minutes (prepared as described immediately above). In addition combinations of 500 μM catechin and 500 μM previously heated catechin (100 °C for 10 minutes) each with 4.8 mM copper (II) sulphate using suspension assay volumes were autoclaved before use or prior to storage at -20 °C.

(iv) Aged solutions: To investigate the antimicrobial activity of test solutions over time the solutions described below were made up and stored exposed to the atmosphere in dark conditions. Freshly prepared 1mM catechin was made up and stored at room temperature for 14 days. A solution of freshly-made combined equimolar 321μM catechin plus copper (II) sulphate was made up and subsequently divided into 3 portions; the first stored portion was stored for 7 days at room temperature; a second portion was stored at 5°C; and the third portion added to 1284 mM vitamin C and mixed. This third portion was then subdivided, with one half stored at room temperature and the other half in stored in at 5 °C.

(v) Freeze-dried solutions: In order to test the feasibility of storing solutions of antimicrobial mixtures without loss of antimicrobial activity, freeze-dried preparations were made and assayed using suspension assays. A freshly prepared 10 mL mixed solution of combined catechin, copper (II) sulphate, vitamin C, in a molar ratio of 1 catechin : 1 copper (II) sulphate : 4 vitamin C was prepared, this ratio having been investigated previously in suspension assays. A separate 10 mL solution of each of the 3 components was also prepared as controls. The amounts of dry components weighed out for each 10 mL mixture was equivalent to 500 X the amount needed for a single cell suspension assay of 1 mL prior to the addition of inoculua were as follows:

0.0465 g catechin + 0.04 g copper (II) sulphate + 0.1129 g vitamin C

Immediately following mixing, the 4 solutions were each placed into a round bottom flask and placed onto dry ice for rapid chilling and freezing. The frozen mixtures were freeze-dried using a Telstar Cryodos 50 Freeze Drier, refrigerated at -50 °C and connected to a Varian DS 102 vacuum pump for the sublimation of ice for 6 hours. Following drying the residue was removed and placed into open tubes with cotton wool bungs and stored in a desiccator at 5 °C in the dark.

2.2.2 Preparation and maintenance of tea extracts and sub-fractions

Teas were prepared using boiling water extraction from 'Silver tips' WT, 'Sencha' GT and Yorkshire' BT obtained from commercial outlets. An 8 g sample of WT, GT, or BT loose tea leaves was added to 100 mL boiling, deionised water and maintained at boiling for a period of 10 minutes. Initial volumes were restored with cold deionised water after leaves were removed using a

sterilised tea strainer prior to extracts being adjusted to pH 7 using 1 M NaOH (for pH method, see below). All solutions were kept in the dark and used fresh or stored at -20 °C.

WT extract was also centrifuged for 15 minutes at 3,220 g using a Sigma 2/16 Centrifuge (Model no. 1360472) to produce a white tea supernatant (WTSN) which was removed for use or for storage at -20 °C. Further WT sub-fractions were prepared from WTSN by syringe filtration (0.45 μM and 0.1 μM Whatman) to remove particulate matter prior to ultra-filtration through a series of decreasing molecular weight cut-off ultra-centrifuge tubes (Amicon Centriprep: 30 kDa, 10 kDa, 5 kDa, 3 kDa, and Pall: 1 kDa) at 3,220 g for 60 minutes. The final filtrate was used fresh or stored in 1 mL aliquots at -20 °C.

2.2.3 Measurement of pH

Measurements of various test solutions were taken following repeated calibrations using buffers as described above (see section 2.1.6). Lambda buffer was freshly-made and adjusted to pH 7.2 as described by Stewart *et al.* (1998) using 1M HCl and 1M NaOH prior to autoclaving. HEPES buffer was similarly freshly-made and also adjusted to pH 7.2 for use in preparing hydrogen peroxide assay reagent (Lespinas *et al.*, 1989). Tea extracts and copper (II) sulphate solutions were adjusted in pH values to 3.0, 5.5, 6.0, 7.0, 7.2, and 9 using 1M HCl and 1M NaOH prior to the addition of inoculum or substituted Ringer's solution for use in various assays (*e.g.*, Tables 3.1, 4.1)

The pH levels of 4.8 mM copper (II) sulphate, WT extract and white tea sub-fraction (WTF) solutions were measured before and following combination together as WT-copper (II) ions, WTF-copper (II) ions using the same volumes as in the suspension assays, substituting Ringer's solution for the bacterial suspension. Mixtures were allowed to stand for 10 minutes prior to pH measurements. Fresh and heat-treated 214 μM catechin (final concentration) solutions were similarly tested prior to and following combination with equimolar and with 4.8 mM copper (II) sulphate.

Suspension assay concentrations of 214 μM (final concentration, f.c.) catechin were combined in molar ratios with copper (II) sulphate (0.25, 0.5, 0.75, 1.0, 2.0, 4.0, and 8.0) and after 10 minutes tested for resulting pH value (Figure 4.10).

2.2.4 Quantification of levels of copper in WT samples by ICP-AES

In order to investigate the antimicrobial activity of any endogenous copper found in tea extracts, aliquots of 1mL samples of freshly prepared whole WT and WTSN were prepared as above on three separate occasions. Using Inductively Coupled Plasma – Atomic Emission Spectrometry

(Jobin Yvon Ultima 2C ICP atomic emission spectrometer) a total of seventeen samples from batches prepared on different days were analysed to investigate their levels of copper.

2.2.5 Preparation of cells

Pure uncontaminated sources of *Staphylococcus aureus* NCTC 06571, *Pseudomonas aeruginosa* NCTC 950, *Proteus mirabilis* NCTC 7827 and *Escherichia coli* NCTC 14441 purchased in disc form (Pro-Lab Diagnostics, UK) were each suspended in 5 mL of sterilised brain heart infusion broth and grown aerobically overnight at 37 °C. The resulting culture was poured into cryogenic tubes and stored at -80 °C.

2.2.6 Preparation of inocula for cell suspension assays

Cells were revived following storage at -80 °C by passaging twice onto nutrient agar plates and incubated aerobically overnight at 37 °C. Such cultures were then stored at 5 °C and used within 7 days. In all assays growth phase inocula were prepared from these plates by overnight culture on nutrient agar and a sample suspended in Ringer's solution to produce a turbid suspension equivalent by eye to a 0.5 McFarland standard*. Bacterial suspensions were used within 15 minutes of preparation

*Note: These standards are used to produce bacterial suspensions with cell numbers within a given range (Zamora and Perez-Gracia, 2012). A traditional McFarland standard of 0.5 is a barium sulphate aqueous suspension similar in appearance to a bacterial equivalent with a cell density of ~1.5×10^8 colony forming units (cfu) mL^{-1}.

2.2.7 Antimicrobial assays using diffusion assays

To investigate whether adjusting the pH of tea extracts would affect subsequent antimicrobial activity, samples of each tea were individually adjusted to a specific pH; either pH 5, or 6, or 7 using the method described above (2.2.3) and tested against three species of bacteria: *Staphylococcus aureus* NCTC 06751, *Pseudomonas aeruginosa* NCTC 950 and *Proteus mirabilis* NCTC 7827.

Protocol: All assays were carried out following equilibration of test samples to room temperature. Aliquots (10 μL) of each tea sample were spotted on to individual plates of Mueller-Hinton agar previously seeded with 100 μL of each species at a concentration comparable to a 0.5 McFarland suspension. The plates were incubated at 37 °C for 24 hours. Thereafter, any zones of inhibition not showing any growth (usually, approximately circular) were measured using callipers. Average zone diamaters were calculated based on at least four meansurements.

2.2.8 Antimicrobial assays using suspension assays and viable cell counts

Comparisons were made of the antimicrobial activities of WT, GT, BT and WTFs. Tea sub-components including catechin and its flavan-3-ol isomers were also tested along with oxidised and heat-treated catechin solution.

The methods described here follow the design of those of Stewart *et al.* (1998) and McCarrell *et al.* (2008). Whole tea extracts and WT sub-fractions were assayed followed by 5 main tea flavan-3-ols and thereafter catechin and its flavan-3-ol isomers found in tea. All tea substances were tested with the putative adjunct copper (II) sulphate and further with additional vitamin C in the case of WT and WTF (< 1000 Da). Catechin was also further tested with other putative adjuncts as well as following heat treatment. Initial assays as well as controls investigated the antimicrobial activity of adjuncts tested alone. A summary of the protocol is given first with individual experiments detailed afterwards.

(i) Protocol

All assays were carried out following equilibration of test samples to room temperature.

Initially, 330 μL aliquots of tea extract or sub-component, or Ringer's solution as a control, were added to 700 μL samples of putative adjunct (typically copper (II) sulphate solution plus other additions in some cases) or a control of Ringer's solution placed into a sterile Eppendorf micro-centrifuge tube. Following mixing, the volume was adjusted from 1030 μL to 1 mL in order to follow the Stewart *et al.* (1989) protocol, and a further control of 1 mL Lambda buffer adjusted to pH 7.2 was used in this and all future experiments. All mixtures were allowed to stand in the dark for 10 minutes prior to the addition of bacterial suspensions. A 0.5 McFarland inoculum of a single species of bacterium was prepared as described above and, following gentle agitation, a 500 μL aliquot added to the mixture. Following mixing of contents the tube was incubated at room temperature in dark conditions for 30 minutes unless otherwise specified. Following the exposure period and mixing, 150 μL was removed from each tube and added to an equal volume of 2% (v/v) Tween-80 made up in Lambda buffer pH 7.2 and mixed to stop any antimicrobial activity against the cells.

Serial dilutions were undertaken using a 96-well microtitreplate with 20 μL transferred into 180 μL of Ringer's solution. Dilutions down to 10^{-6} were taken followed by 20 μL aliquots transferred and spread onto quarter nutrient agar plates. Where necessary, volumes of 100 – 1000 μL were plated to reduce the cell detection limit. As a guide if the number of cfus grown from the lowest dilution in the first experiment was less than 40 per sampling area (*e.g.* a within a quarter plate) sampling areas and sample volumes were increased in subsequent repeat experiments. This procedure was based on the premise that the cell detection limit might not have been reached with the initial spread volume of 20 μL.

In order to adequately increase cell detection it was found that as a rule of thumb that cell viabilities of between 3 – 4 $\log_{10}$ units typically required half plates with 100 μL of spread sample; for between 2-3 $\log_{10}$ units required whole plates with 200 μL spread sample; between 1-2 $\log_{10}$ units required whole plates with 500 μL sample volumes; and < 1 $\log_{10}$ units required whole plates with 1000 μL spread sample. Dry plates were selected for the larger sample volumes and, if necessary, extended drying time prior to incubation.

Plates were incubated at 37 °C aerobically for 24 hours when they were read. Observations of plates were made during a further 3 days incubation in order to note any recovery of injured cells which resulted in the appearance of new colonies. Each assay was conducted in triplicate as a minimum.

(ii) Assays of putative adjuncts and tea extracts (copper (II) sulphate and vitamin C), tea extracts and combinations of the two) See Appendix I.

(iii) Assays of putative adjuncts and tea catechins (fresh and heat pre-treated catechin; putative adjuncts: iron (II/III) salts, zinc (II), manganese (II), and copper (II) sulphate, vitamin C, caffeine; catechin and other flavanols with molar ratios of copper (II) sulphate) See Appendix II.

(iv) Assays of stored mixtures (stored solutions and freeze-dried mixtures) See Appendix III.

2.2.9 Investigations of mechanisms of action

Experiments to explain the observed enhancement effect of copper (II) sulphate on the antimicrobial activity of catechin were carried out using suspension assays, pH measurements, crystallisation studies, and measurements of solubility, and of UV-vis absorbance.

(i) Putative chemical complex formation with copper (II) ions:

(i-a) WT and WTF plus 4.8 mM copper (II) sulphate using suspension assays

The effect of subdividing WT into lower molecular weight fractions with subsequent combination with 4.8 mM copper (II) sulphate was investigated following the suspension assay protocol described above and investigated for their effects against *S. aureus* NCTC 06751 (see section 2.2.8). See section 3.8 for results of experiments.

(i-b) Fresh catechin plus copper (II) sulphate in molar ratios using suspension assays (Figure 4.1)

To investigate stoichiometry and using standard assay volumes described above (see section 2.2.8) an aliquot of 472 μM catechin was combined with each of the following copper (II) sulphate

concentrations: 118, 236, 354, 472, 944, 1888, 3776, and 7552 μM resulting in molar ratios of catechin 1: 0.25, 0.5, 0.75, 1, 2, 4, 8, and 16. These mixtures were then investigated for their effects on the viability of *S. aureus* NCTC 06751.

(i-c) WT and WTF plus copper (II) sulphate using pH (Figure 3.15)

pH measurements using the methods described above (see section 2.2.3) were used to investigate potential chemical changes within mixtures due to the possible formation of complexes between copper (II) ions and whole WT and sub-components. Investigations used suspension assay volumes substituting Ringer's solution for inoculum (see section 2.2.8). WT extract and WTF (< 1000 Da) mixtures were tested with both 4.8 mM copper (II) sulphate, and with Ringer's solution substituted for copper (II) sulphate solution as a control. The pH levels of 4.8 mM copper (II) sulphate with Ringer's solution substituted for tea extract was used as a control (See Appendix I: Table L for further details of mixture combinations, solution strengths and volumes).

(i-d) Fresh and heat-treated catechin plus copper (II) sulphate using pH (Figures 4.10, 4.12, Table 4.2)

Combinations of freshly-made commercial 1000 μM catechin combined with 472 μM copper (II) sulphate resulted in equimolar 214 μM final concentrations for each of the two solutes in the samples which were tested as above and similarly used suspension assay volumes substituting Ringer's solution for inoculum (see section 2.2.8). Thereafter varying molar ratios of copper (II) sulphate were tested with 1000 μM catechin at suspension assay concentrations previously tested (see section (i-b) above). Controls included 330 μL Ringer's solution plus the same molar ratios of copper (II) sulphate; 330 μL 1000 μM catechin solution diluted with deionised water; 330 μL 1000 μM catechin solution plus water acidified with 1M HCl to result in the same pH as 472 μM copper (II) sulphate solution (*i.e.*, pH 6.6) and diluted in proportions to that of the catechin: varying copper (II) sulphate ratios (See Appendix I: Table L for further details of mixture combinations, solution strengths and volumes).

(i-e) Fresh catechin plus copper (II) sulphate using crystallisation

If a crystal from catechin and copper (II) sulphate mixed in solution could be grown at a particular ratio of component concentrations, it may suggest an optimal antimicrobial activity ratio of these components for investigations using suspension assays against test organisms. Catechin and copper (II) sulphate were combined in catechin : copper (II) sulphate ratios of 1 : 0.75, 1 : 1, and 1 : 1.5 at two strengths of catechin solution (1 mL of 10 mM or 100 mM) for each of the 3 ratios tested with copper (II) sulphate. Copper (II) sulphate was added as 1 mL 7.5 mM or 75 mM copper (II) solution for the 1 : 0.75 ratio; 1 mL 10 mM or 100 mM copper (II) solution for the 1 : 1 ratio; and 1 mL 15 mM or 150 mM copper (II) solution for the 1 : 1.5 ratio. The degree of precipitation and

flocculation, colour of precipitate, shape, pattern and colour of protocrystals and other deposits were noted during the solvent evaporation process which occured at room temperature in the dark at *ca.* 20 °C.

(i-f) Fresh catechin plus copper (II) sulphate using solubility (Table 4.4)

Experiments were carried out to investigate whether the solubility of catechin in water could be raised by the addition of copper (II) sulphate. The solubilities of catechin combined with copper (II) sulphate in deionised water were compared to catechin, and copper (II) sulphate tested alone. A dry mixture with a molar ratio of 1:1 of catechin and copper (II) sulphate was made up using 0.29 g catechin with 0.233 g copper (II) sulphate. Approximately 0.01 g of the mixture was added to 1 mL deionised sterile water. Catechin (0.5g) was tested alone in a similar way and copper (II) sulphate (0.5g) was also tested alone. Following agitation the remaining undissolved mixture was gradually brought into solution with 100 μL additions of water shaking in between each addition until a clear solution was observed. The total amount of water used was noted and the solubility of the sample calculated. This procedure was repeated ten times and a mean taken. All solubilities were investigated at 25°C.

(i-g) WT plus copper (II) sulphate using UV–vis (Figures 3.12, 3.13)

Whole tea extracts and WT sub-components as well as fresh and heat-treated catechin (heated for 10 minutes at 100 °C) prepared as above were investigated using UV-vis as a possible means to reveal any absorption differences between them as well as a means to investigate the possible formation of chemical complexes between the substances and added copper (II) ions. The addition of EDTA to tea component solutions mixed with copper (II) ions was also investigated at 2.5 X the final concentration of copper (II) sulphate in the test sample (Brown *et al.*, 1998). Samples were made up as per suspension assays with 1000 μM catechin to a volume of 1500 μL, substituting Ringer's solution for inoculum. Mixtures were allowed to stand in the dark for 10 minutes before dilution to 50 % and 25 % strength using Ringer's solution in order to increase reliability of absorbance readings using a Varian Cary 300 Bio UV-Visible Spectrophotometer scanning between 190 – 900 nm. All cuvettes containing whole tea extracts were gently agitated immediately prior to UV-vis exposure to ensure homogeneity of the sample.

(i-h) Fresh and heat-treated catechin plus copper (II) sulphate using UV-vis (Figure 4.11)

Solutions of fresh and heat-treated catechin (heated for 10 minutes at 100 °C) were investigated using UV-vis following similar methods as decribed above. Samples were made up as per suspension assays with 1000 μM catechin to a volume of 1500 μL, substituting Ringer's solution for inoculum.

(ii) Kinetic studies to compare the rate of fall of viability of *S. aureus* NCTC 06751 when exposed to fresh and heat-treated catechin each combined with copper (II) ions (Figures 4.13, 4.14). See section 4.9 and Appendix II: Table G for further details.

(iii) Exogenous catalase to remove effects of putative hydrogen peroxide production (Figures 4.15, 4.16, 4.17). To test the hypothesis that antimicrobial mixtures investigated using suspension assays generated harmful hydrogen peroxide, exogenous catalase was added to combinations of both WT and WTF (< 1000 Da, the most antimicrobial sub-component of WT) with copper (II) sulphate. Further, catalase was also added to a combination of WTF (< 1000 Da) plus copper (II) sulphate and vitamin C. In each case the additions were made to suspension assays tested against *S. aureus* NCTC 06751 (see Appendix I: Table K for further details) (Figure 3.14).

Following the tea studies catalase was also added to combinations of catechin with adjuncts tested against *S. aureus* NCTC 06751 and *E. coli* NCTC 14441. Catalase was tested by adding fixed or progressive concentrations of the enzyme to: catechin alone; to catechin plus copper (II) sulphate, and plus iron (II) sulphate, separately, as well as combined; and the same combinations with the further addition of vitamin C (For further details see Appendix II: Table A and 'Notes on Methodology' Main Treatment (MT) 8, 9, & 10). In order to investigate whether catalase simply removed copper (II) ions from assay mixtures by forming a complex and thus removing the copper ions ability to generate hydrogen peroxide, the enzyme was combined at 50 and at 500 $\mu g\ mL^{-1}$ with 214 μM (f.c.) copper (II) sulphate and investigated using UV-vis (Figure 4.16).

(iv) EDTA to remove effects of putative antimicrobial transition metal ions

Following the method of Brown *et al.* (1998) EDTA was added to samples to remove the biological effects of transition metal ions by chelation. EDTA was added to solutions containing added copper (II) and iron (II) sulphate using a concentration of EDTA 2.5 X the final concentration of each added transition metal (II) ion, and were tested using suspension assays against *S. aureus* NCTC 06751 and *E. coli* NCTC 14441 (For further details see Appendix II: Table A and 'Notes on Methodology' Main Treatment (MT) 6 & 7).

(v) Hydrogen peroxide generation by test sample mixtures (Figure 4.19)

Sample mixtures tested against cells were also tested for the presence of hydrogen peroxide because in previous experiments when catalase was added to such mixtures antimicrobial effects were removed. In these experiments antimicrobial mixtures of flavan-3-ol isomers of catechin as well as heat-treated and room stored catechin were tested for hydrogen peroxide generation when combined with equimolar copper (II) sulphate.

The method used to measure H_2O_2 followed that of Lespinas *et al.* (1989) with modifications which removed the first four reaction stages of a urea assay, starting with a reaction mixture of phenol and 4-aminophenazone to test for the presence of H_2O_2 which was assayed colorimetrically via the generation of a magenta chromophore which absorbed UV-vis radiation maximally at 500 nm. The assay reagent was made up as described above (see section 2.1.5) and 1 mL aliquots were used to test each 5 μL sample of test substance. Following mixing, the sample was left to stand at 37 °C for 15 minutes and the absorbance measured against a reagent blank at 500 nm (Lespinas *et al.*, 1989). Initially a calibration curve was constructed using serial dilutions of stock 5% (v/v) hydrogen peroxide solution (stored at 5 °C) between 0.01% and 0.5% which was found to be reliably linear between 0.05 and 0.5% (Figure 2.1). The curve was subsequently used to convert absorbance measurements into percentage concentrations of hydrogen peroxide generated by antimicrobial test samples.

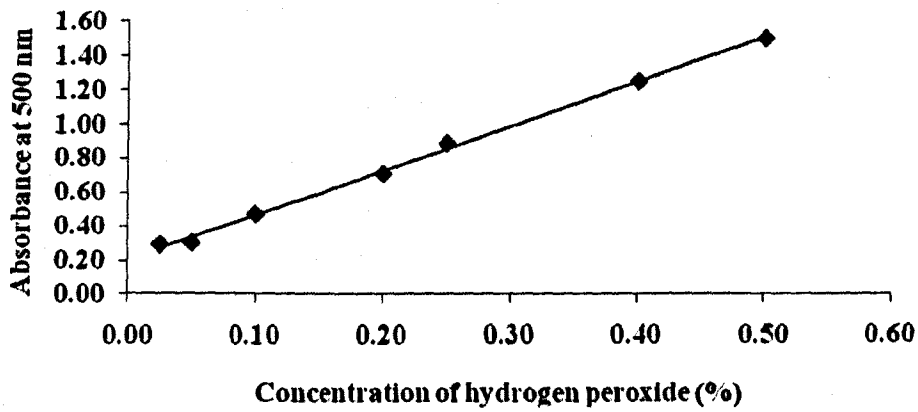


Figure 2.1 Calibration curve showing the UV-vis absorbances at 500 nm of different concentrations of hydrogen peroxide measured following a method by Lespinas *et al.* (1989). UV-vis measurements taken between 450 and 550 nm with maxima measured at 500 nm. Serial dilutions of 5% H_2O_2 were made in deionised water. Non-linear region below 0.025% H_2O_2 not shown. Result based on 3 replicates.

Following construction of the curve, 5 μL samples of the different catechin isomers and treated catechin suspension mixtures, where Ringer's solution replaced inoculum, were assayed. Mixtures with and without added copper (II) sulphate were also sampled and assayed.

(vi) Effects of mixtures on cells using microscopy

(vi-a) *Light microscopy*: In order to explore the possible visible effects of sample mixtures on cell structures which could be related to reductions seen in viability, 20 μL samples from stopped solutions were spotted on to glass microscope slides for examination following Gram staining

(Beveridge, 2001). Spotting was repeated five times to increase the density of cells seen in the visual field at X 1000 magnification. The slide was allowed to dry between each spotting to minimise spreading of the sample to maintain a small spot diameter. The method used was as follows: Slides with specimens dried at room temperature were heat fixed and a primary stain of 70% crystal violet applied. Following rinsing with deionised water, slides were flooded with the mordant iodine and rinsed again. Thereafter specimens were rapidly decolourised with 70% alcohol for a few seconds before rinsing again and counterstaining with 70% carbol fuchsin. After final rinsing slides were patted dry and examined by oil immersion light microscopy at x 1000 magnification (Leica ATC 2000). Cells were examined for changes in Gram staining characteristics and cell morphology, cellular adhesion, and the presence of pronounced cellular extrusions.

(vi-b) *Electron microscopy* (Figure 4.20): Aliquots of 3 mL of stopped sample mixtures were centrifuged at 9000 g for 15 minutes using an SLS SciQuip Centrifuge. Following removal of the supernatant, samples were re-suspended in PBS, to reduce any cell adhesion due to particulate matter, and re-centrifuged at 9000 g for 15 minutes. Samples were then used or stored at -20 °C for up to 6 weeks. Thereafter, following thawing at room temperature if necessary, samples were filtered through 0.1 μm polycarbonate membrane filters (Millipore). For primary fixing samples were suspended in 2.5% gluteraldehyde in 0.1M phosphate buffer for 1 hour. The samples were then washed in 0.1M phosphate buffer for 2 hours. Following a secondary fix in 1% osmium tetroxide in phosphate buffer for 1 hour, the samples were dehydrated through a graded series of ethanol (50%, 70%, 80%, 90%, and 95%) each for 20 minutes and then 3 changes of 100% ethanol. Following dehydration samples were dried using hexamethyledisilizane and air drying for a further 24 hours. Samples were splutter coated with gold and palladium using a Polaron SC7640 splutter coater and viewed using a Zeiss EVO 50 scanning electron microscope.

2.2.10 Statistical analysis

All data were processed using Microsoft Excel statistical software and expressed as means and ranges as appropriate following experiments conducted at least in triplicate (n=3 up to n=10). Further MS Excel statistical processing was used to calculate standard deviation, and standard error of the mean (SEM) for experimental groups. The Student independent 2 sample, two tailed, t-test with symmetrical parametric dispersion of data was used to determine between which groups statistical significance occurred ($p<0.05$).

Chapter 3: Antimicrobial action of teas against different bacteria

3.1 Introduction

The therapeutic effects of plant extracts dissolved in water and/or alcohol have been known in folklore since ancient times and some of these effects may be due to the presence of antimicrobial substances within the extracts (Sharangi, 2009). Tea beverage made from the leaves of the tea bush (*Camellia sinensis*) has been drunk for millennia as an invigorating and medicinal substance mainly in the form of a hot water infusion of dried leaves, popularly known as a brew (Weisburger, 1997). Tea extracts once cooled can be drunk or applied topically to prevent and treat a range of different conditions including microbial infections, cancer, inflammation, catarrh, sunburn and insect bites (Dufresne and Farnworth, 2001; Chen *et al.*, 2008; Butler and Wu, 2011). Different types and degrees of post-harvest tea leaf processing methods produce leaves of varied appearance and contents, from the simple dried leaf buds of white tea (WT) to the 'fermented' leaves of black teas (Chung *et al.*, 1998; Kim *et al.*, 2011). White, green, and black teas (GT, BT) typically contain varying amounts of substances common to all three types of tea as well as substances specific to each (Zhao *et al.*, 2011; Wang *et al.*, 2011; Aksuner *et al.*, 2012). Tea types differ in their food qualities and therapeutic effects such as mineral content and varying effects on microbes (Sharangi *et al.*, 2009; Aksuner *et al.*, 2012).

WT, GT, and BT, on account of their different profiles of agents with varying antimicrobial activities, are suitable candidates to investigate against *Staphylococcus aureus* and other microorganisms of medical importance and within other applications, and this choice of teas is investigated in this study. Following the traditional method of preparation to make a 'strong brew' cup of tea, with hot water, commercially available WT, GT, and BT leaves were used to make infusions and then subsequently strained. These teas were then allowed to cool and their antimicrobial activities investigated. Commercial tea leaf products, where possible, were sourced from similar geographical locations in order to reduce variation in leaf constituents such as polyphenols and alkaloids due to different soils and other variations in environmental growing conditions.

The extracts of the tea bush leaves, rich in antimicrobial polyphenols, can be enhanced with additives or by physico-chemical processes that can increase the antimicrobial effects of the tea (Yam *et al.*, 1997; Chou *et al.*, 1999; An *et al.*, 2004; Takabayashi *et al.*, 2004; Si *et al.*, 2006; Tiwari *et al.*, 2005; Wu *et al.*, 2007; Lin *et al.*, 2008; Yi *et al.*, 2010). Other plant extracts such as pomegranate fruit rind extract have been investigated in a similar manner (McCarrell *et al.*, 2008; Gould *et al.*, 2009a, 2009b). In the present study the effects of adding copper (II) sulphate and vitamin C on tea were investigated as these adjuncts had previously been shown to enhance the

antimicrobial activity of pomegranate rind extract (McCarrell *et al.*, 2008; Gould *et al.*, 2009a, 2009b). Factors that may affect the antimicrobial activity of different teas such as pH and sequencing of adjunct additions, including copper (II) sulphate, vitamin C, and the length of exposure time were also investigated in an attempt to improve antimicrobial activity.

Various mechanisms have been suggested for the action of teas on microbes including their roles as agglutination agents as well as substances which interfere with the cell envelope structure and function (Johnson *et al.*, 2008; Cho *et al.*, 2010; Zhang *et al.*, 2010). Therefore, investigations were made in the present study to elucidate possible mechanisms of action using fractionation, pH measurements, UV-vis spectroscopy, removal of generated hydrogen peroxide by exogenous catalase, the metabolic inhibitor EDTA, and investigations of the relationship between molecular structure of the principal tea *catechins and their antimicrobial activities.

Catechins sourced from the tea bush and other plants have attracted considerable interest due to their effects on microbes as well as other biological actions and inductrial applications. Analysis of both WT and GT show they are both rich in flavan-3-ol polyphenols and contain small quantities of other antimicrobial compounds (Engelhardt *et al.*, 1993; Lin *et al.*, 2008). These tea flavanols show a varying abundance and a spectrum of antimicrobial activity dependent on their molecular structure with epimerised and galloyl derivatives of catechin showing the highest abundances and antimicrobial activities (Gibbons *et al.*, 2004; Stapleton *et al.*, 2004b). Tea catechins (flavan-3-ols) were investigated for antimicrobial activity in the present study. These were tested alone as well as combined with the putative adjuncts copper (II) sulphate and vitamin C by measuring zones of inhibition in diffusion assays as well as using suspension assays to establish viable cell counts. Comparisons were made between different species of bacteria and possible mechanisms of action were investigated.

*Commonly used alternative terms for catechins, *viz.*, flavan-3-ols and flavanols are used interchangeably throughout this and other chapters. Catechin used in the singular only means (+)-catechin unless the context indicates otherwise, *e.g.* 'a type of tea flavanol'. The stereoisomer (-)-catechin is usually referred to by present current conventions using an abbreviated prefix *ent*-catechin, for enantiomer of catechin. Similarly, the diastereoisomer or epimer, epicatechin only refers to (-)-epicatechin unless the context indicates otherwise. The stereoisomer (+)-epicatechin is referred to usually as *ent*-epicatechin.

3.2 Antimicrobial activities of teas at different pH values on *S. aureus* NCTC 06751

It is possible that tea leaf extracts produced from different types of processed leaves could show varied pH values in aqueous solution, partly due to chemical differences produced by different post harvesting process methods and partly due to different brewing methods (Chou *et al.*, 1999). Different pH levels of tea extracts and levels of ionisation of antimicrobial components within extracts may affect any antimicrobial activity. In general, solutions and suspensions of antimicrobial treatments, wherever possible, are adjusted to a neutral pH in order to avoid acidity or alkalinity effects causing harm to animal or plant tissues on which they may be used (Nas, 2004). In addition, a neutral pH may reduce the likelihood that mixtures when used in applied settings could cause adverse effects due to acidity or alkalinity on personnel. Treatments which retain antimicrobial activity across a wide range of pH values are adjusted to pH 7 for these reasons, and this enables comparison with other similarly pH adjusted substances.

Growth inhibition zones

The background to this work includes consideration of established facts such as the effects of pH on biological dependent variables – in this case antimicrobial activities of different substances. In addition, antimicrobial substances such as tea extracts are likely to show different levels of activities following different post-harvesting processing methods (Chou *et al.*, 1999). Consequently, the hypotheses being tested in this experiment are that both pH and type of post-harvesting treatment of tea leaves affects subsequent antimicrobial activity when tested against different types of bacteria.

In these experiments in order to investigate whether adjusting the pH of tea extracts would affect subsequent antimicrobial activity, samples of WT, GT, and BT were individually adjusted to a specific pH, either pH 5, or 6 or 7. Subsequently, each adjusted tea sample was investigated using the broadly qualitative diffusion assay method which produced zones of inhibition where extracts were seen to be active. The activities of the pH adjusted WT, GT, and BT were investigated against three species of bacteria: *Staphylococcus aureus* NCTC 06751, *Pseudomonas aeruginosa* NCTC 950 and *Proteus mirabilis* NCTC 7827 (Table 3.1).

Table 3.1 The inhibitory effects of pH adjusted white, green and black teas on the growth of three laboratory species of bacteria

Species tested	Diameter of zone of inhibition produced by different types of tea adjusted to different pH values on bacterial growth (mm)								
	White Tea			Green Tea			Black Tea		
	pH: 5	pH: 6	pH: 7	pH: 5	pH: 6	pH: 7	pH: 5	pH: 6	pH: 7
S. aureus	16 (±0.58)	15 (±0.33)	15 (±0.22)	20 (±0.74)	22 (±1.96)	21 (±0.59)	22 (±0.58)	21 (±1.50)	23 (±0.43)
Ps. aeruginosa	8 (±0.38)	8 (±0.60)	11 (±0.22)	9 (±0.29)	8 (±0.42)	8 (±0.17)	9 (±0.01)	7 (±0.17)	9 (±0.98)
P. mirabilis	8 (±0.10)	8 (±0.10)	8 (±1.20)	15 (±2.00)	15 (±0.1.5)	15 (±1.6)	13 (±0.10)	15 (±1.25)	15 (±2.0)

Key: *S. aureus* = *Staphylococcus aureus* NCTC 06751; *Ps. aeruginosa* = *Pseudomonas aeruginosa* NCTC 950; *P. mirabilis* = *Proteus mirabilis* NCTC 7827. Aliquots of 10 µL of each tea sample were spotted onto Mueller-Hinton agar plates previously seeded with 100 µL inocula of each species at a concentration comparable to a 0.5 McFarland suspension. The plates were incubated at 37°C for 24 hours. Resulting measurements from triplicate tests were averaged from 3 separate experiments and adjusted to nearest whole millimetre (SEMs in brackets).

The results with *S. aureus* NCTC 06751 showed the following results: WT adjusted to pH 5 or 6 or 7 units produced similar circular zones of inhibition of *ca.* 15 mm diameter; GT and BT each adjusted to pH 5 or 6 or 7 units produced zones of *ca.* 20 mm in diameter. In summary against *S. aureus* NCTC 06751, WT had an effect of approximately 25% less ($p<0.05$) than that of either the GT or BT, which themselves had similar effects to each other. The results with *Pseudomonas aeruginosa* NCTC 950 showed that WT, GT, and BT, each adjusted to pH 5 or 6 or 7 units, produced similar circular zones of exclusion of *ca.* 9 mm in diameter. These results show that the different teas are approximately, equally effective against *Ps. aeruginosa* NCTC 950 irrespective of adjusted pH. The results with *Proteus mirabilis* NCTC 7827 showed that WT adjusted to pH 5, 6 or 7 units produced similar inhibition zones of *ca.* 8 mm diameter whilst, GT and BT adjusted to same pH's produced zones of *ca.* 15 mm (Table 3.1.). Against this species WT extract had around half the inhibitory effect of GT and BT, which had a broadly similar effect to one another.

The effect of WT at the three different pH's was greatest against *S. aureus* NCTC 06751 producing zones of *ca.* 15 mm irrespective of pH compared to the effects against the two Gram-negative species, *Ps. aeruginosa* NCTC 950 and *P. mirabilis* NCTC 7827 where zones of *ca.* 8 mm were produced. The results with the GT and BT showed a similar pattern with the greatest effects against *S. aureus* NCTC 06751 producing zones of *ca.* 21 mm compared to zones of *ca.* 8 mm against *Ps. aeruginosa* NCTC 950 and *ca.* 15 mm against *P. mirabilis* NCTC 7827. Overall the growth of *S. aureus* NCTC 06751 was affected most by the teas than the other two species.

A discussion of the results from the diffusion assay (zone of inhibition) experiments presents a possible issue of interpretation due to any possible physico-chemical interaction between the diffusing test substances and the solid medium supporting the growth of the target species of bacterium. This is despite that fact that Mueller-Hinton agar is a loose agar formulated to minimise diffusion limitations and interactions with bacteria (Pujol *et al.*, 2008). In order to obtain a more reliable assessment of the degree of inhibition, or death of cells, the three tea types were also tested and compared using viable cell counts (Papagianni *et al.*, 2006). WT, GT, and BT were adjusted to pH 7 and their activitites investigated using suspension assays followed by viable cell counts against *S. aureus* NCTC 06751 and *Ps. aeruginosa* NCTC 950 for comparison. The choice of *Ps. aeruginosa* NCTC 950 was based on its greater resistance to the inhibitory effects of all the teas compared to the other two species of bacteria within the diffusion assay experiments (Table 3.1).

The results with *Staphylococcus aureus* NCTC 06571 showed that in suspension assays all three teas at pH 7 showed similar viabilities against *S. aureus* NCTC 06751 ($p>0.05$) (Figure 3.1). The results with *Pseudomonas aeruginosa* NCTC 950 also showed no statistically significant differences in effect between the three types of tea ($p>0.05$).

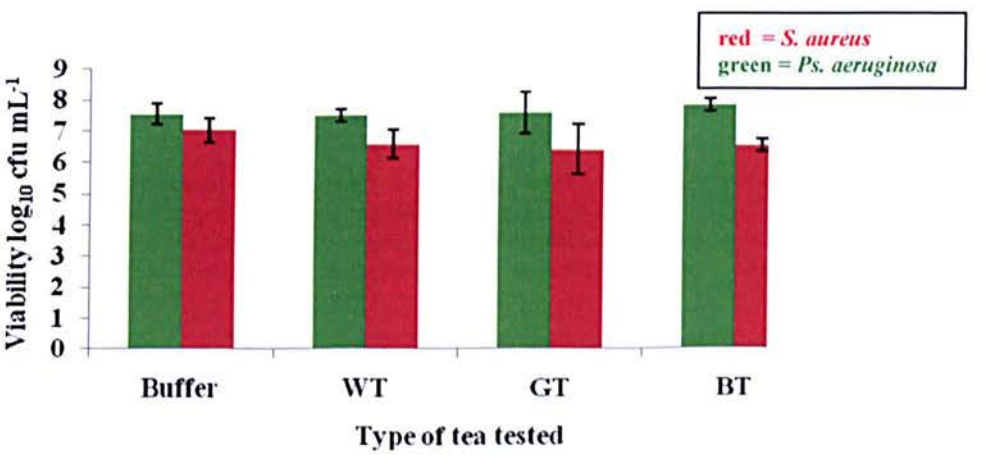


Figure 3.1 The effect of white, green and black tea extracts each adjusted to pH 7 on the viability of *Staphylococcus aureus* NCTC 06571 and *Pseudomonas aeruginosa* NCTC 950 Red bars = *S. aureus*, green bars = *Ps. aeruginosa*, Buffer = control, WT = white tea, GT = green tea, BT = black tea. Incubations were in the dark at room temperature for 30 minutes. The plates were incubated at 37°C for 24 hours. Results were based on triplicate tests from 3 separate experiments Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

In conclusion, the results from the zone experiments suggested that WT showed a greater antimicrobial activity against the principal target species in this study, *S. aureus* NCTC 06751, than the two comparison species, *Ps. aeruginosa* NCTC 950 and *P. mirabilis* NCTC 7827. Relatively few studies have addressed the antimicrobial activity of WT compared to GT and BT and for these reasons it was considered that further investigation into possible antimicrobial effects of WT extracts was justified (Schiffenbauer *et al.*, 2004).

White tea experiments

To further investigate the effects of white tea on *Staphylococcus aureus* NCTC 06571 further suspension assays followed by viable cell counts were carried out using WT extracts adjusted to greater range of pH values (from 3 to 9) than had been done in the zone experiments (pH values from 5 to 7). The rationale for this was to investigate whether more acidic or alkaline pH values would affect the antimicrobial activities shown by the WT.

Table 3.2 Effects of adjusting the pH value of Ringer's solution and white tea on the viability of *Staphylococcus aureus* NCTC 06571

Sample tested at pH indicated	Viability $\log_{10}$ cfu mL^{-1}
Lambda buffer 7.2	6.9 (±0.54)
Ringer's 3	6.8 (±0.44)
Ringer's 9	7.1 (±0.53)
WT 3	7.1 (±0.38)
WT 5	6.8 (±0.47)
WT 7	7.1 (±0.49)
WT 9	7.0 (±0.40)

Key: Ringer's solution was adjusted with 1M HCl or with 1M NaOH solution. Lambda buffer previously adjusted to pH 7.2 was used as a control as in all suspension assays. Test samples of 1000 µL were combined with 500 µL of inoculum and incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 3 separate experiments (SEMs in brackets).

The results suggest that neither Ringer's solution adjusted to pH 3 or 9, nor WT adjusted to pH 3, 5, 7 or 9 have any effect on the viability of *S. aureus* NCTC 06571, with no significant differences being shown between the buffer control and test samples ($p>0.05$) (Table 3.2). These more detailed findings based on suspension assays support the findings of the zone studies on *S. aureus* NCTC 06571 that pH does not affect the antimicrobial activity of this organism within the range tested. Furthermore these assays show that WT, contrary to the results from the zone experiments, does not have any antimicrobial activity towards *S. aureus* NCTC 06571, but may well inhibit growth within these conditions. Following these results the effects of copper (II) sulphate, a putative adjunct, were investigated against three laboratory strains to answer the question whether adjuncts tested alone would show any antimicrobial activity. Cells were exposed to the copper (II) salt for different periods for comparison.

3.3 Antimicrobial activity of copper (II) sulphate on different species of bacteria

Following the methodology of Stewart *et al.* (1998), 4.8, 9.6 and 19.2 mM copper (II) sulphate solutions were tested against *Staphylococcus aureus* NCTC 06751 (Gram-positive type); *Pseudomonas aeruginosa* NCTC 950 (Gram-negative type, motile in some conditions); and *Proteus mirabilis* NCTC 7827 (Gram-negative, highly motile in most conditions). Exposures to copper (II) sulphate were carried out at room temperature for 10 or 30 minutes. Lambda buffer adjusted to pH 7.2 acted as a control in each experiment (Stewart *et al.*, 1998) (Table 3.3).

Table 3.3 The effect of different concentrations of added copper (II) sulphate solution for two exposure times on the viability of three laboratory species of bacteria

Species tested	Buffer value (log_{10} cfu mL^{-1})	Log_{10} reduction in viability (final concentration and pH given in each case)					
		4.8 mM (2.17 mM; pH 4.5)		9.6 mM (4.35 mM; pH 3.1)		19.2 mM (8.70 mM; pH 3.0)	
		10 mins.	30 mins.	10 mins.	30 mins.	10 mins.	30 mins.
S. aureus	7.7 (±0.40)	2.2 (±0.18)	2.7 (±0.37)	3.03 (±0.24)	3.7 (±0.40)	3.3 (±0.66)	No cfus*
Ps. aeruginosa	7.5 (±0.50)	1.55 (±0.27)	2.05 (±0.40)	2.38 (±0.70)	No cfus	1.92 (±0.65)	No cfus
P. mirabilis	7.7 (±0.45)	2.5 (±0.30)	4.37 (±0.50)	4.11 (±0.58)	No cfus	4.0 (±0.45)	No cfus

Key: *S. aureus* = *Staphylococcus aureus* NCTC 06751; *Ps. aeruginosa* = *Pseudomonas aeruginosa* NCTC 950; *P. mirabilis* = *Proteus mirabilis* NCTC 7827. The plates were incubated at 37°C for 24 hours. Results were based on triplicate tests from 3 separate experiments (SEMs in brackets).*Absence of colonies may indicate 20 μL was an insufficient sample volume to detect cfus.

These results showed that the viability of *S. aureus* NCTC 06751 showed progressive reductions to 4.8 mM, 9.6 mM and 19.2 mM copper (II) sulphate after 10 and 30 minute exposures which were all statistically significant from the buffer value ($p<0.05$). *P. mirabilis* NCTC 7827 showed a greater susceptibility to the test conditions than *Ps. aeruginosa* NCTC 950. No colony forming units (cfus) were seen with certain assays which may have been due to any surviving viable cells being below the limit of detection of the sample volume.

Following these findings, the principal target organism, *S. aureus* NCTC 06751, was investigated for its susceptibility to a wider range of concentrations of the putative adjunct, copper (II) sulphate in 30 minute exposures based on progressive dilutions of typical MICs found for copper (II) sulphate against 43 isolates of *S. aureus* sourced from cattle in Denmark (Aarestrup and Hasman, 2004). This was done to facilitate observation of any possible enhancement effects following

combination with additional substances to the copper (II) sulphate in subsequent experiments. The range of concentrations used in this investigation, up to 10 mM, was similar to that used in the Danish study. In the Danish study, 22 of the 43 isolates, showed a MIC for copper (II) sulphate of 2 mM which is similar to the final concentration of 2.17 mM (4.8 mM added) used in this study, as well as in previous investigations by others (Stewart *et al.*, 1998; McCarrell *et al.*, 2008; Gould *et al.*, 2009a, 2009b).

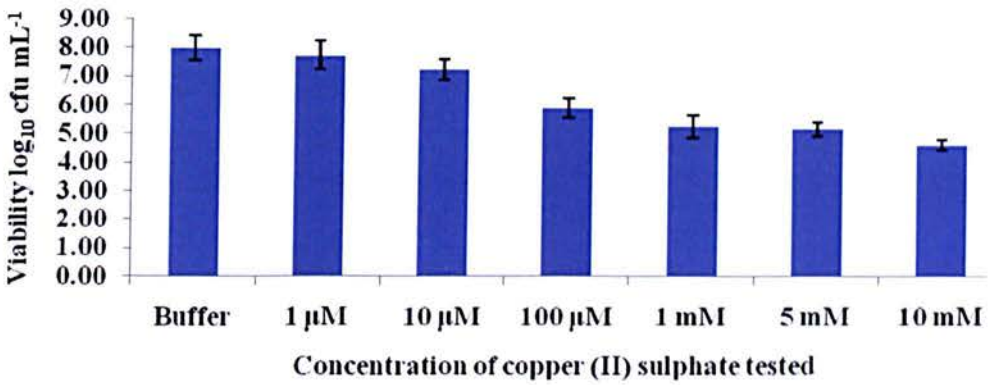


Figure 3.2 Effects of copper (II) sulphate added at increasing concentrations on *Staphylococcus aureus* NCTC 06751 viability Final concentrations of added copper (II) sulphate: 0.5 µm, 5 µm, 45 µm, 0.45 mM, 2.27 mM, 4.53 mM respectively). Incubations were at room temperature for 30 minutes. The plates were incubated at 37°C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

Increasing copper (II) sulphate concentration reduced the viability of *S. aureus* NCTC 06751, with a reduction of *ca.* 3.5 $\log_{10}$ units seen with 10 mM added copper (II) sulphate (Figure 3.2). Samples tested at or above 100 µM concentrations were significantly different from viabilities seen with the buffer ($p<0.05$). These findings supported the choice of 4.8 mM (final 2.17 mM) added copper (II) sulphate used by Stewart *et al.* (1998); McCarrell *et al.* (2008); Gould *et al.* (2009a, 2009b), as well as being near to the average MIC (5.45 mM) against *S. aureus* found by Aarestrup and Hasman, (2004). For further details see sections: 2.2.3, 2.2.5, and Appendix I, Table A. Following the above experiments, vitamin C, another putative adjunct was then tested alone for any independent antimicrobial activity against *S. aureus* NCTC 06751.

3.4 Antimicrobial activity of vitamin C on *S. aureus* NCTC 06751

Vitamin C has been reported as a substance that can enhance the antimicrobial activity of certain antimicrobial agents, *e.g.* transition metal ions; nitrite, antimicrobials such as curcumin; and siderophores such as deferoxamine (Carlsson *et al.*, 2001; Salaman, 2004; van Asbeck *et al.*, 2005; Braud *et al.*, 2010; Khalil *et al.*, 2012). However, vitamin C can often have little effect on bacterial cells when tested alone (Ericsson, 1954; Murata *et al.*, 1991).

A range of vitamin C concentrations (1 μM to 20 mM) were tested against *S. aureus* NCTC 06751 and the viabilities were similar to buffer values *ca.* 7 $\log_{10}$ units (data not shown, see Figures 3.4, 3.6, 3.10 for typical examples of independent vitamin C assays). In conclusion vitamin C tested alone had no apparent effect on the viability of *S. aureus* NCTC 06751, a finding similar found previously by Ericsson, (1954) and Murata and Yano (1990).

S. aureus NCTC 06751 previously showed intermediate susceptibility to copper (II) sulphate when compared to *Ps. aeruginosa* NCTC 950 and *P.mirabilis* NCTC 7827 (Table 3.2). The effect of adding vitamin C to copper (II) sulphate on the viability of *S. aureus* NCTC 06751 was then investigated in an attempt to enhance the antimicrobial activity of copper (II) sulphate. The same concentration of copper (II) sulphate (4.8 mM) was used as before, combined with equimolar (added 4.8 mM) and double concentrations of vitamin C. The results are shown below (Figure 3.3).

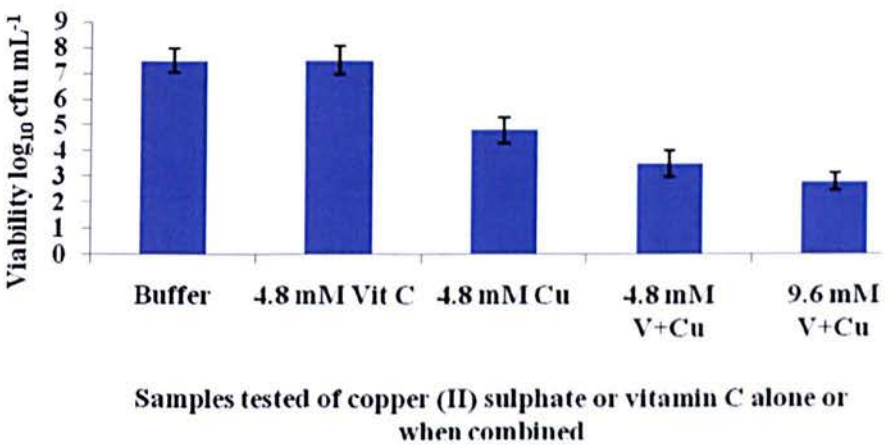


Figure 3.3 Effects of copper (II) sulphate combined with vitamin C on *Staphylococcus aureus* NCTC 06751 viability Cu = copper (II) sulphate, Vit C = vitamin C, Figures on x axis show concentrations of adjuncts added in mM (final concentrations: copper (II) sulphate, 2.17 μm; vitamin C, 2.17 μm and 4.34 μm respectively). Sample volumes were increased from 20 μL to 100 μL and 200 μL in the combined mixtures in order to maintain adequate detection). Incubations were at room temperature for 30 minutes. The plates were incubated at 37°C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results showed that the mean viabilities seen with the copper (II) sulphate tested alone and in the two combinations with vitamin C were significantly different from the values shown by the buffer and by the vitamin C tested alone ($p<0.05$). When combined with copper (II) sulphate, vitamin C enhances the antimicrobial activity by up to *ca.* 2 $\log_{10}$ units at the higher concentration. It is possible that vitamin C acts as a reducing agent with copper (II) leading to the formation of ROS via Fenton Chemistry (Murata *et al.*, 1991).

3.5 Activities of teas with copper (II) and vitamin C against *S. aureus* NCTC 06751

The findings above indicated that added 4.8 mM (2.17 mM final concentration) copper (II) sulphate tested alone produced antimicrobial activity against *S. aureus* NCTC 06751 within 10 minutes (Table 3.3). The effect of adding this concentration of copper (II) sulphate to WT, as well as GT and BT for comparison was investigated in two conditions. Firstly, following mixing of the reagents and prior to the introduction of inoculum, a standing time of 10 minutes was allowed for any chemical reactions to occur between the copper (II) sulphate and the teas. Secondly, a 30 minute standing time was allowed for any slower chemical reactions to occur before addition of the inoculum (Figure 3.4).

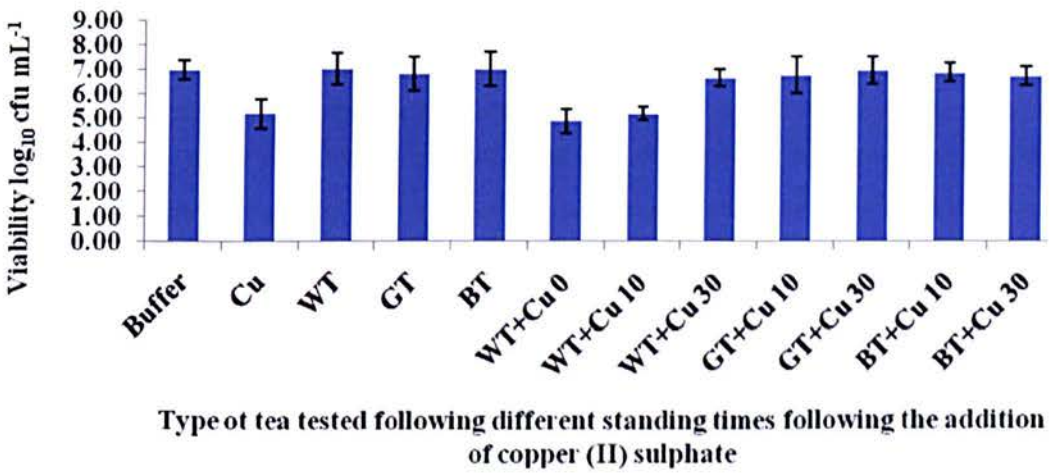


Figure 3.4 The effect of varying pre-incubation periods following mixing of tea extracts with copper (II) sulphate on the viability of *Staphylococcus aureus* NCTC 06571 Cu 4.8 mM, +Cu = added 4.8 mM copper (II) sulphate, WT = white tea, GT = green tea, BT = black tea. Tea extracts were adjusted to pH 7 prior to combination with copper (II) sulphate solution. Figures on horizontal axis show the period in minutes that mixtures were allowed to stand in the dark prior to the addition of the inoculum. Incubations were at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results showed that viability values for *S. aureus* NCTC 06751 following exposure to the copper (II) sulphate control and the WT plus copper (II) samples for 0, and for 10 minutes standing time were significantly different ($p<0.05$) from buffer values, whereas all other samples tested had means which were not signicantly different from the buffer viability ($p>0.05$). Viability values for *S. aureus* NCTC 06751 following exposure to WT plus copper (II) sulphate for 10 minutes standing time as well as copper (II) sulphate controls showed a viability of *ca.* 5.2 $\log_{10}$ units. Both GT and BT, when combined with copper (II) sulphate, removed the antimicrobial effects of copper (II) sulphate seen in the controls increasing the viability of *S. aureus* NCTC 06751 to *ca.* 6.8 – 7 $\log_{10}$ units which was similar to the viability of *S. aureus* NCTC 06751 seen with the buffer alone, *ca.* 7 $\log_{10}$ cfu mL^{-1}. However, WT combined with copper (II) sulphate for 10 minutes, or less prior

to the addition of inoculum, resulted in no loss of the antimicrobial effect of copper (II) sulphate. In contrast, with 30 minutes of standing time prior to the addition of inoculum, the WT had also removed the effect of the copper (II) sulphate (Figure 3.4).

The WT results suggested that when WT was combined with copper (II) and then allowed to stand for 0 or 10 minute standing time prior to the addition of inoculum, then the subsequent observed viabilities of *S. aureus* NCTC 06751 were the same as those seen with the copper (II) control. This indicates that the presence of WT had no negating effect on the harmful effects of the copper (II) ions on the cells when compared to the controls in the conditions tested. However, after 30 minutes of standing time prior to the addition of inoculum, the subsequent viabilityof *S. aureus* to the WT-copper (II) combination was greater than the copper (II) sulphate control suggesting an interference with the copper (II) availability by the tea. Following this experiment, all subsequent combinations of tea extracts with copper (II) sulphate were allowed to stand for only 10 minutes in the dark prior to the introduction of an inoculum unless otherwise stated. WT was then investigated further. Following the adopted protocol of allowing the WT to stand with added copper (II) sulphate in the dark for 10 minutes, prior to the addition of inocula, exposure times of such combined pre-treated WT with copper (II) sulphate to the target species of choice, *Staphylococcus aureus* NCTC 06571 was investigated to check an assumption that extended contact from 30 to 80 minutes would result in further falls in cell viability (Figure 3.5).

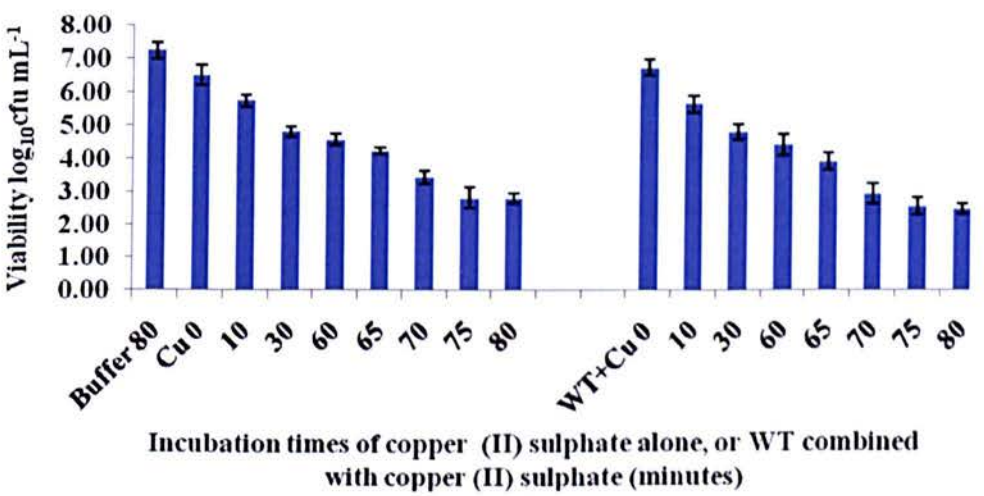


Figure 3.5 The effect of copper (II) sulphate alone and combined with white tea extract during an extended incubation of 80 minutes on the viability of *Staphylococcus aureus* NCTC 06571. Horizontal axis denotes time elapsed when sample was withdrawn, in minutes. Cu = added 4.8 mM copper (II) sulphate, WT = white tea, '+' indicates combination of substances. Tea extracts were adjusted to pH 7 prior to combination with copper (II) solution. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Incubations were at room temperature in the dark for up to 80 minutes. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

Statistical analysis of the results showed that the viability values of *S. aureus* NCTC 06751, in buffer alone, compared to the copper (II) sulphate alone samples were significantly higher for samples taken at 10 minutes and above ($p<0.05$). Comparing viabilities of *S. aureus* NCTC 06751 in buffer alone with the viabilities of *S. aureus* NCTC 06751 in combinations of WT and copper (II) sulphate taken at different times all showed significant differences ($p<0.05$). The extended exposure with added 4.8 mM copper (II) sulphate showed falls in *S. aureus* NCTC 06751 viability with increasing exposure time. The results with WT combined with copper (II) showed a broadly similar pattern indicating that the WT did not interfere with the antimicrobial activity of the copper (II) ions during the exposure time, nor enhance it.

Following the above assays of WT with copper (II) sulphate the effects of another adjunct, vitamin C, was combined with WT and investigated for antimicrobial activity. Vitamin C, acting as an anti-oxidant, could act as a tea preservative affecting antimicrobial activity indirectly by removing oxygen from an assay sample (Hatano *et al.*, 2008). Vitamin C was tested with WT alone and when combined with 4.8 mM copper (II) sulphate against *S. aureus* NCTC 06571 (Figure 3.6).

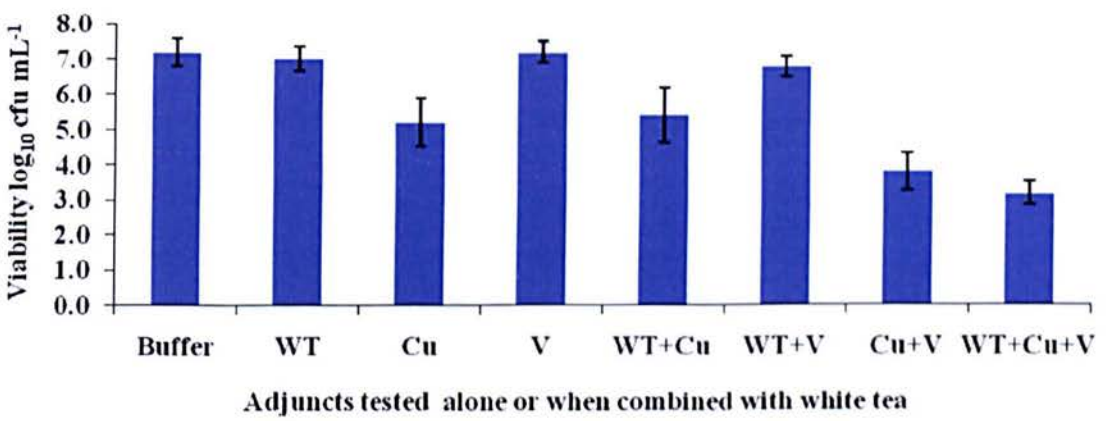


Figure 3.6 The effect of adding vitamin C to white tea alone and with copper (II) sulphate on the viability of *Staphylococcus aureus* NCTC 06571 WT = white tea, Cu = added 4.8 mM copper (II) sulphate, V = 4.8 mM vitamin C, '+' indicates combination of substances. WT was adjusted to pH 7 prior to combination with copper(II) solution. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of inoculum. Incubations were in the dark at room temperature for 30 minutes. The plates were incubated at 37°C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The viabilities of *S. aureus* when exposed to buffer, or WT, or vitamin C, and WT combined with vitamin C were similar (6.8 – 7.2 $\log_{10}$ cfu mL^{-1}) with $p>0.05$ between the means. With the other samples, 4.8 mM copper (II) sulphate alone, and combined with WT *S. aureus* NCTC 06751 showed similar viabilities of *ca.* 5.2 and 5.4 $\log_{10}$ units respectively with $p>0.05$ between the means. When exposed to vitamin C plus copper (II) sulphate *S. aureus* NCTC 06751 viability was

ca. 3.8 $\log_{10}$ cfu mL^{-1} and with added WT viability was *ca.* 3.2 $\log_{10}$ cfu mL^{-1} (Figure 3.6). As shown earlier, vitamin C enhanced the antimicrobial activity of copper (II) against *Staphylococcus aureus* NCTC 06571 (Figure 3.4) possibly by reducing the copper (II) to copper (I) and generating harmful reactive oxygen species (ROS) via Fenton chemistry. It appears from the results shown in Figure 3.6 that the WT did not add to these effects.

3.6 Activities of teas plus copper (II) at different pH values on the viability of *S. aureus* NCTC 06571

Earlier in the study, the effects of adjusting WT, GT, and BT to pH 5, 6, and 7 was investigated against test species using diffusion assays (section 3.1). Subsequently the pH of copper (II) sulphate solutions assayed against *S. aureus* NCTC 06571 was also measured at assay final concentrations (Figure 3.7). Later in the study, the pH values of each of the combined teas plus the copper (II) sulphate was considered to be possibly greater than the individual ranges previously tested for each component. In order to establish whether a wider range of pH values could affect the bacterial viability the pH of test samples was measured prior to the introduction of an inoculum (Table 3.4). These values were then subsequently used to adjust Ringer's solution to a range of pH values between 5.5 and 9, and subsequently assayed as independent controls.

Table 3.4 The pH of unadjusted teas tested alone and when combined with copper (II) sulphate

Samples tested: controls, teas alone, and in combination with copper (II) sulphate	pH value
Lambda buffer	7.2 (±0.2)
4.8 mM added copper (II) sulphate	4.5 (±0.2)
Ringer's solution	6.9 (±0.4)
WT alone	6.9 (±0.5)
GT alone	5.7 (±0.4)
BT alone	5.3 (±0.4)
WT + Cu(II)SO_4	4.0 (±0.5)
GT + Cu(II)SO_4	4.2 (±0.4)
BT + Cu(II)SO_4	4.0 (±0.2)

Key: WT = white tea, GT = green tea, BT = black tea; '+' denotes addition of substances. Cu(II)SO_4 = 4.8 mM copper (II) sulphate final concentration = 2.17 mM. Samples were allowed to stand for 10 minutes in the dark prior to the addition, where appropriate, of Ringer's solution to reach standard suspension assay volume. Results were based on triplicate tests from 3 separate experiments (SEMs in brackets).

In order to investigate this range of pH values for their effects on the viability of *Staphylococcus aureus* NCTC 06571, a range of *ca.* 3 to *ca.* 9 was chosen to allow for somewhat greater acidity

and alkalinity effects on *S. aureus* NCTC 06571. The choice of a slightly below and mid-range pH of *ca.* 5.5 was somewhat arbitrary as it was an average of the two more acidic GT and BT pH values. As an extension to this work, samples of the WT were combined with copper (II) sulphate and copper (II) sulphate adjusted to pH 3, 5.5 and 9 to investigate any effect of pH of the mixture on the viability of *S. aureus* NCTC 06571.

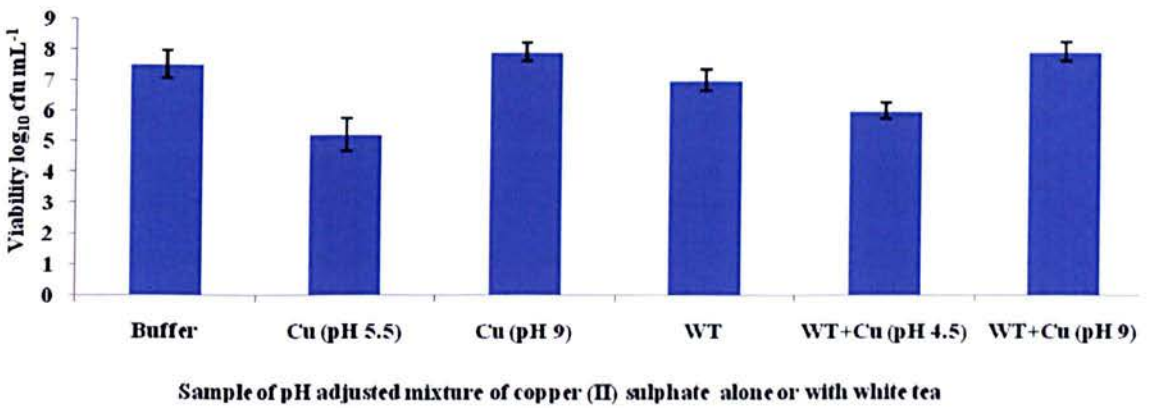


Figure 3.7 Effects of copper (II) sulphate alone and combined with white tea following pH adjustments on the viability of *Staphylococcus aureus* NCTC 06571 WT = white tea, Cu = added 4.8 mM copper (II) sulphate solution – pH figures indicate pH of samples prior to addition of inoculum. WT added to Cu (pH 5.5) caused an increase in acidification to pH 4.5. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of inoculum. Incubations were in the dark at room temperature for 30 minutes. The plates were incubated at 37°C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results show that 4.8 mM copper (II) sulphate solution adjusted to pH 5.5 showed a similar antimicrobial activity to that of a combined mixture of WT plus 4.8 mM copper (II) sulphate adjusted to pH 4.5 and subsequently tested against *S. aureus* NCTC 06571with no statistically significant difference seen between the two test samples ($p>0.05$) (Figure 3.7) (A comparable result was obtained with similar samples that were adjusted in pH and shown above in Figure 3.6) However, in the case of copper (II) sulphate adjusted to pH 9 antimicrobial activity was lost, both in the control and in the combination with WT when in each case viability values of *S. aureus* NCTC 06751 similar to buffer control values were seen with no statistically significant differences between the sample means ($p>0.05$) (Figure 3.7). This may have been due to the addition of NaOH solution, used to adjust the sample to pH 9, reacting with copper (II) ions to form copper (II) hydroxide which is insoluble in these conditions. As a consequence, the availability of free copper (II) ions was reduced producing the observed loss of viability of *S. aureus* NCTC 06751.

The results of suspension assays of pH adjusted Ringer's solution against *S. aureus* NCTC 06571 tested following 30 minute exposures and in the same conditions as other suspension assays produced *S. aureus* NCTC 06751 viabilities as follows: buffer *ca.* 8 $\log_{10}$ cfu mL^{-1}; Ringer's solution adjusted to pH 3, *ca.* 7 $\log_{10}$ units; Ringer's solution adjusted to pH 5.5 *ca.* 7.5 $\log_{10}$ units; and Ringer's solution adjusted to pH 9, *ca.* 7.6 $\log_{10}$ units. No statistically significant differences were seen between the means of the different samples tested ($p>0.05$). These results indicate that compared to buffer values, pH adjustment of Ringer's solution to values between pH 3 and 9 prior to the introduction of the inoculum has no apparent effect on the viability of *S. aureus* NCTC 06571.

In all the results described above, *S. aureus* NCTC 06751 when exposed to any whole tea combined with copper (II) sulphate only showed, at best, a similar cell viability to a copper (II) sulphate control - more often *S. aureus* NCTC 06751 showing higher viabilities similar to the buffer controls (see Figure 3.5). In order to test the possibility that tea extracts may contain antimicrobial substances, whether enhanced by added copper (II) ions or not, further analysis of tea components was undertaken. This was also done to investigate whether inhibitory substances could be present in the teas preventing enhancement by added copper (II) sulphate. WT sub-components were subsequently fractionated as it was considered a possibility that antimicrobial active and inhibitory substances could be separated from each other and such fractions were tested for antimicrobial activity using suspension assays with viable cell counts.

3.7 Activities of white tea sub-fractions against *S. aureus* NCTC 06571

Initially, WT extract was centrifuged for 15 minutes at 3,220 g using a Sigma 2/16 Centrifuge (model no. 1360472) which resulted in the formation of a pellet of dark brown matter at the bottom of the tube and a pale, fawn-coloured, slightly turbid, supernatant. This supernatant was removed and investigated for antimicrobial activity alone and combined with 4.8 mM copper (II) sulphate and compared to whole white tea combinations using diffusion assay (zone of inhibition) experiments.

Table 3.5 The inhibitory effects of whole white tea extract and a sub-fraction, each tested alone and combined with added 4.8 mM copper (II) sulphate on the growth of *Staphylococcus aureus* NCTC 06571.

Sample tested	Mean Diameter of Zone of Inhibition (mm) (SEMs)
WT	12 (±0.38)
WTSN	11 (±0.44)

Cu	9 (±0.50)
WT+Cu	9 (±0.45)
WTSN+Cu	9 (±0.39)

Key: Tea extracts were adjusted to pH 7 prior to combination with copper(II) solution. WT = white tea, WTSN = white tea supernatant, Cu = copper (II) sulphate, '+' indicates combination of substances. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of inoculum. A 10 µL aliquot of each tea sample was spotted onto Mueller-Hinton agar plates previously seeded with the bacteria at a concentration comparable to a 0.5 McFarland suspension. The plates were incubated at 37°C for 24 hours. Results were based on triplicate tests from 3 separate experiments (SEMs in brackets).

The results showed that inhibition diameters of *S. aureus* NCTC 06751 growth were as follows: whole WT *ca.* 12 mm; WT supernatant *ca.* 11 mm, copper (II) sulphate *ca.* 9 mm, whilst whole WT and WT supernatant both with added copper (II) saw *ca.* 9 mm inhibition (Table 3.4). These results indicate no significant difference in antimicrobial activity between the whole WT or the WT supernatant nor when combined with 4.8 mM copper (II) sulphate on the growth of *S. aureus* NCTC 06571 ($p<0.05$). Following this result similar samples were investigated using suspension assays with viable cell counts (Figure 3.8).

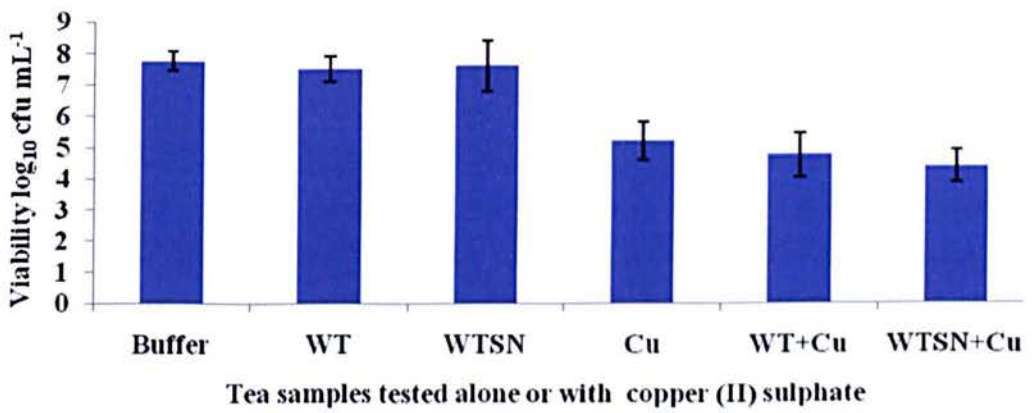


Figure 3.8 Effects of whole white tea and a white tea sub-fraction tested alone or combined with 4.8 mM copper (II) sulphate on the viability of *Staphylococcus aureus* NCTC 06571. Tea extracts were adjusted to pH 7 prior to combination with copper (II) solution. WT = white tea, WTSN = white tea supernatant, Cu = added 4.8 mM copper (II) sulphate solution, '+' indicates combination of substances. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of inoculum. Incubations were in the dark at room temperature for 30 minutes. The plates were incubated at 37°C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

There was no significant difference between the antimicrobial activities of the whole WT and the WTSN tested alone when compared to buffer values on *S. aureus* NCTC 06751($p>0.05$). The antimicrobial activities of similar samples combined with added 4.8 mM copper (II) sulphate produced similar *S. aureus* NCTC 06751 viabilities to the 4.8 mM copper (II) sulphate control following exposure.

3.8 Activities of endogenous levels of copper found within WT and WT sub-fractions against *S. aureus* NCTC 06571

Previously, WT combined with 4.8 mM copper (II) sulphate showed similar antimicrobial activity to 4.8 mM copper (II) sulphate tested alone against *S. aureus* NCTC 06571. This statement implies an assumption that WT contains little or no endogenous copper which, if actually present, could be inhibited in its effects by the possible presence of inhibitory substances within the tea. To test this assumption typical copper (II) levels of whole white tea and supernatant were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES). These concentrations of copper were then independently investigated using copper (II) sulphate against *S. aureus* NCTC 06571.

The results of analysis of WT showed the concentrations of copper were within a range of 0.12 – 0.17 mg L^{-1} with a mean of 0.14 mg L^{-1} (SD±0.03). These levels are similar to published findings (Karak and Bhagat, 2010). The concentrations of copper (II) found in the WTSN were within a range of 0.07 – 0.24 mg L^{-1} with a mean of 0.12 mg L^{-1} (SD ±0.11). The difference in the size of the two ranges is curious and may relect slight differences in preparation of the WTSN *e.g.*, through variations in the centrifugation process. The difference between the mean for the whole WT and the supernatant may be due to copper (II) being adsorbed onto the pellet during centrifugation. If so, *ca.* 12% was evidently lost in this way.

When copper (II) sulphate was tested against *S. aureus* NCTC 06751 in suspension assays at the average copper level found in whole WT and WTSN (0.14 and 0.12 mg L^{-1}, being equivalent to 0.56 and 0.48 μM added copper (II) sulphate respectively) there was no loss in viability with such samples showing the same levels as the buffer control with no significant differences ($p>0.05$) (data not shown). Following further sub-fractionation of the WTSN using a series of decreasing molecular weight cut-off (MWCO) ultra-centrifuge tubes, the sub-fractions appeared as clear pale yellow solutions which became paler with each successive ultra-filtration. The different sub-fractions showed the following levels of copper (II): MWCO < 10 kDa: 0.8 (SD± 0.02) mg L^{-1}; MWCO < 3 kDa: 0.52 (SD± 0.04) mg L^{-1}; MWCO < 1 kDa: 0.08 (SD± 0.04) mg L^{-1}. An explanation for the decreasing concentrations of copper (II) found in the filtrate of the ultra-filtration tubes may be a retention of copper (II) ions adsorbed onto the residue as well as surface of the filter in each case. Earlier suspension assay results indicated that when tested independently the endogenous concentrations of copper (II) in whole WT and WTSN did not produce any increase in antimicrobial activity when compared to the buffer against *Staphylococcus aureus* NCTC 06571 (data not shown).

In order to investigate any increase in antimicrobial activity of the WT extracts due to an ultra filtration concentration effect, two of the above sub-fractions; one with a molecular weight cut off

of < 5 kDa and a second with a MWCO < 1 kDa were assayed separately for antimicrobial activity, alone and combined separately with 4.8 mM vitamin C and with 4.8 mM copper (II) sulphate, or together as a joint combination.

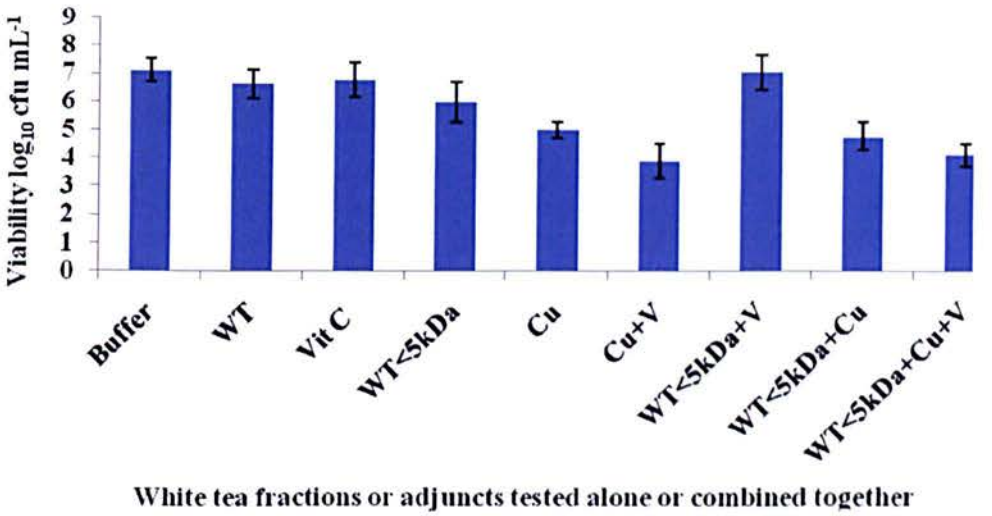


Figure 3.9 Effects of a white tea sub-fraction (< 5 kDa) alone and combined with vitamin C or copper (II) sulphate on the viability of *Staphylococcus aureus* NCTC 06571. WT=white tea, Vit C, or V = 4.8 mM vitamin C, Cu= 4.8 mM copper (II) sulphate solution, Tea extracts were adjusted to pH 7 prior to use, '+' indicates combination of substances. Mixtures were allowed to stand for 10 minutes in the dark prior to the addition of inoculum. Incubations were in the dark at room temperature for 30 minutes. The plates were incubated at 37°C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The viabilities shown by *S. aureus* to the buffer, WT, vitamin C, WT < 5 kDa, WT < 5 kDa plus vitamin C were all similar with no significant differences between them ($p>0.05$). In addition, copper (II) sulphate alone; the WT sub-fraction (< 5 kDa) combined with copper (II) sulphate and further with added vitamin C; and the vitamin C plus copper (II) sulphate control also all showed similar activities against *S. aureus* with no statistical differences between them ($p>0.05$) (Figure 3.9). In summary, at the < 5 kDa level of sub-fractionation the antimicrobial activity of WT is not enhanced by the addition of the copper (II) sulphate and vitamin C adjuncts whether separately or when combined against *S. aureus* NCTC 06571. The experiment was then repeated with the < 1 kDa sub-fraction.

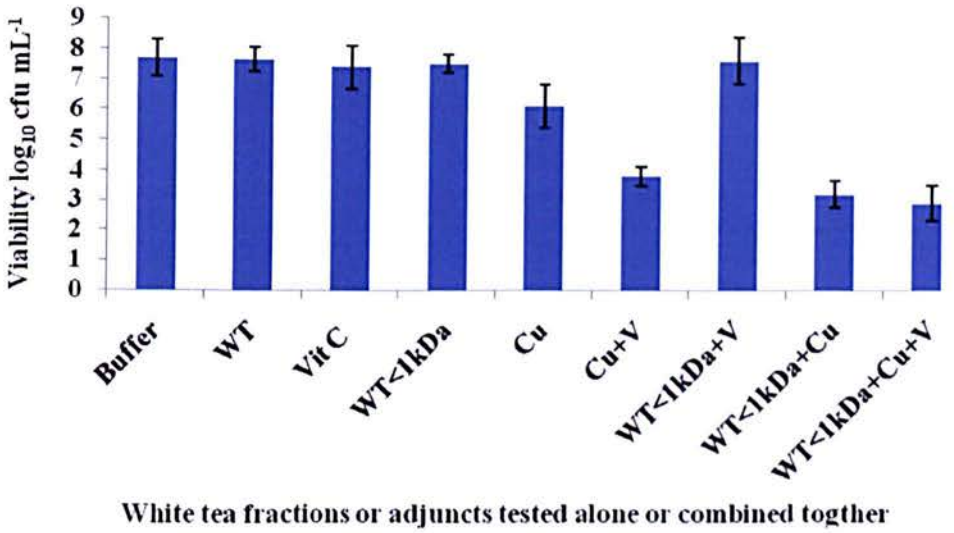


Figure 3.10 Activities of white tea sub-fraction (< 1 kDa) alone and in different combinations with 4.8 mM copper (II) sulphate and 4.8 mM vitamin C against *Staphylococcus aureus* NCTC 06751 WT = white tea, WTF = white tea fraction, Vit C, or V = vitamin C, Cu = copper (II) sulphate, '+' = combination specified. Mixtures were allowed to stand for 10 minutes in the dark before addition of culture. Sample volumes of 500 µL of Cu+V it, WTF + Cu and WTF+Cu +Vit were used to reduce the detection limit. Incubations were in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results show that when tested and compared to the viability for *S. aureus* NCTC 06751 in the buffer, added WT sub-fraction < 1 kDa to copper (II) sulphate significantly reduced the antimicrobial activity of copper (II) sulphate by *ca.* 3 $\log_{10}$ units when compared to the copper (II) sulphate control ($p<0.05$). The further addition of vitamin C to this combination did not result in any significant further increase in antimicrobial activity against *S. aureus* NCTC 06571($p>0.05$) (Figure 3.10). To investigate whether different tea fractions contained different amounts of components that could be responsible for the greater or lesser antimicrobial activities of the fractions the UV-vis absorption of the WT sub- fractions were compared to the whole WT to investigate any differences between them. Any spectra obtained could then be compared and contrasted for absorption characteristics such as the number, position and relative sizes of any absorbance peaks shown by this technique. Such information could give clues as to the relative amounts and types of phenols within the whole WT compared to the different fractions (Hung and Yen, 2002; Chen *et al.*, 2008; Joubert *et al.*, 2008).

3.9 UV-vis spectrophotometry of WT and sub-fractions

Three WT fractions were prepared using molecular weight cut off (MWCO) filters of less than 10 kDa, < 5 kDa and < 1 kDa. To investigate any differences in the chemical content between the various fractions which may be related to differences in their antimicrobial activity, absorption spectroscopy was used as a guide to indicate varying polyphenolic contents of the fractions since these generally appear coloured. Using UV-vis absorption spectroscopy, the absorption spectra of

the whole white tea and each of the sub-fractions was measured between 200 – 900 nm to investigate any similarities or differences between them.

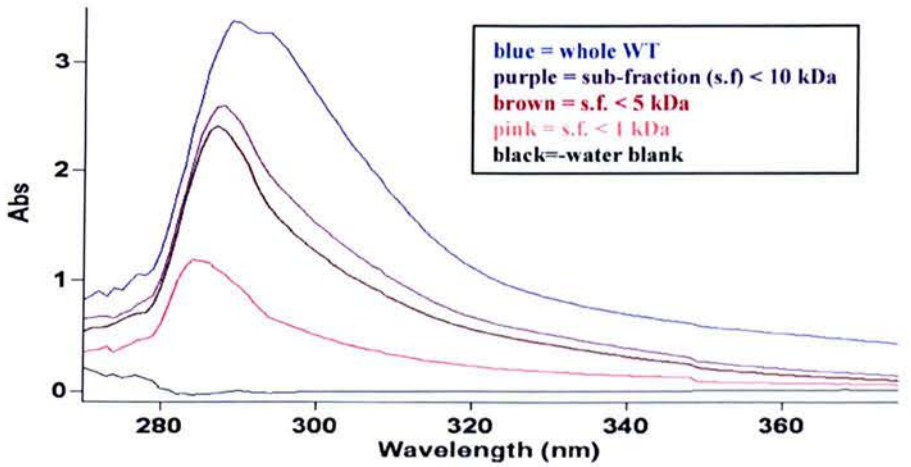


Figure 3.11 UV-vis absorption spectra between 270 nm and 375 nm of whole freshly-made whole white tea extract and sub-fractions prepared using ultra-filtration. Spectra shown downwards in the following order: whole WT (blue line), sub-fraction (s.f.) < 10 kDa (purple line), s.f. < 5 kDa (brown line), s.f. < 1 kDa (pink line), water blank (black line). Tea and fraction samples diluted to 25% of final concentrations used in bactericidal assays (equivalent to 5.5% of whole tea extract). Absorbance traces below 270 nm and above 375 nm not shown. The peak absorbances of < 5 kDa , < 10 kDa, and whole WT though similar in repeated experiments may be unreliable. Results were based on triplicate tests from 3 separate experiments.

The results showed that whole WT had the greatest absorbance at 289 nm with progressively smaller sub-fractions showing smaller absorbances at 288 nm, 286 nm and 284 nm (Figure 3.11). Polyphenols are known to be in high concentrations in tea, and since phenols absorb strongly at *ca.* 290 nm it is likely that the absorbance at *ca.* 290 nm is due to polyphenols. The successive reduction in absorbance of the spectra suggested a loss of material at each of the filtration stages. This may be explained by each finer, smaller gauged filter used in the process removing successively smaller sizes of polyphenol molecules. Since the colour of aqueous solutions of individual polyphenols is related to their molecular weight with larger ones being a dark brown and smaller ones being yellowish or colourless in appearance, the increasingly paler filtrates found in the ultra-filtration process may broadly relate to smaller sized pigmented components (Hathway and Seakins, 1957; Joubert *et al.*, 2008). These observations are unlikely to be due to a simple successive increase in dilution of only one component since the peak positions for each fraction vary.The above findings suggest that WT, and its fractions vary in their UV-vis absorption characteristics. In addition, WT and a sub-fraction of < 1 kDa vary in their antimicrobial activities when combined with copper (II) sulphate against *S. aureus* NCTC 06751. To explore the hypothesis that WT and its sub-fractions may contain active components complexed with copper

(II) ions as well as inactivating/inhibitory substances, WT was investigated alone and with copper (II) sulphate using UV-vis spectrophotometry to look for any differences in absorption that could be caused by the addition of copper (II) ions forming chemical complexes (Goodman *et al.*, 2012). WT was also compared to GT and BT.

3.10 Investigations into the possible formation of chemical complexes between teas and added copper (II) ions

To investigate whether the teas formed complexes with copper (II) sulphate, combinations of WT, GT, and BT each with added copper (II) sulphate were examined visually and analysed using UV-vis spectroscopy. The results showed that on addition of copper (II) sulphate to the teas, their appearance changed from a semi-opaque, turbid pale brown colour to a clearer, less turbid, deeper shade of pale brown, suggesting a possible increase in tea component solubility (van Koningsveld *et al.*, 2002).

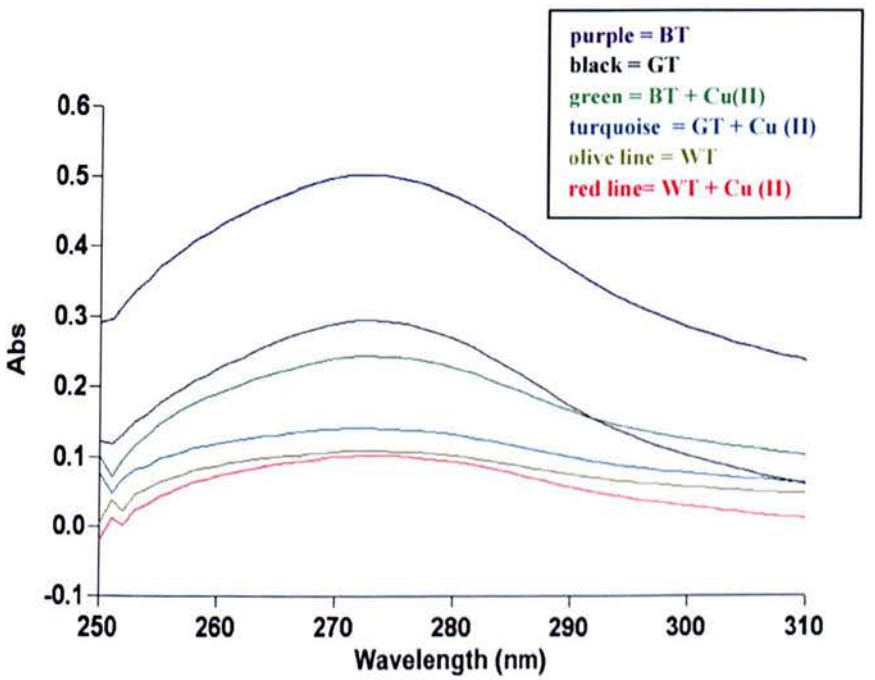


Figure 3.12 UV-vis absorption spectra between 250 nm and 310 nm of freshly-made tea extracts alone, and with added 4.8 mM copper (II) sulphate Spectra shown downwards in the following order: BT alone (purple line); GT alone (black line); BT plus copper (II) (green line); GT plus copper (II) (turquoise line); WT alone (yellow line); WT plus copper (II) (red line). Following 10 minutes of standing time, samples were diluted to 25% of final concentrations used in bactericidal assays (equivalent to 5.5% of whole tea extract).. Absorbance traces below 250 nm and above 310 nm not shown. Results were based on triplicate tests from 3 separate experiments.

The results for absorbances shown by the teas alone and combined with copper (II) sulphate for 10 minutes were compared at their peak absorbances at *ca.* 273 nm. The results showed the following order of level of absorbances: BT > GT > WT; and BT > BT+Cu(II), GT > GT+Cu(II), and WT >

WT+Cu(II) (Figure 3.12). No change in WTF (< 1k Da) absorbance was seen following addition of copper (II) sulphate between 190 – 900 nm (results not shown).

The absorbance values for the individual teas plus copper (II) sulphate were all lower than the absorbance for the whole teas tested alone (BT + Cu(II), *ca.* 50% lower than BT; GT + Cu(II) also *ca.*, 50% lower than GT; and WT + Cu(II), *ca.* 20% lower than WT). Lower absorbance values of the teas with copper (II) ions suggested that the tea components could have become more soluble in the presence of the copper (II) ions possibly by weak interactions with tea components increasing their solubility or by the formation of chelate complexes.

The increasing absorbance values of WT *cf.* GT *cf.* BT suggested that the individual types of teas contained greater amounts of a substance, or substances that absorbed at *ca.* 273 nm. Such substance/s may be polyphenols which are higher in concentration in GT than WT (Chen *et al.*, 2008; Si *et al.*, 2006). In BT, such polyphenols become condensed during the tea 'fermentation' process into higher molecular weight (MW) structures which could absorb UV-vis radiation more strongly than the low molecular weight flavanols in the GT and WT (Xie *et al.*, 1993; Chou *et al.*, 1999; Kim *et al.*, 2011; Hatano *et al.*, 2003, 2005).

The possibility of physico-chemical interactions between the teas and added copper (II) ions leading to the formation of antimicrobial active complexes may explain the difference in antimicrobial activity between whole WT with copper (II) compared to WTF < 1 kDa with copper (II). This possibility was investigated using experiments to elucidate possible mechanisms of actions using enzymes, pH studies and molecular structure-activity experiments on tea components.

3.11 Effects of adding catalase to whole WT and WT sub-fractions (< 1 kDa) with adjuncts against *S. aureus* NCTC 06571

Previous investigations found that flavan-3-ols isolated from GT tea showed antimicrobial activity against microbes including typical laboratory strains of bacteria such as *S. aureus* and *E. coli* (Mabe *et al.*, 1999; Taguri *et al.*, 2004; Meltzer *et al.*, 2008). In addition flavanol activities were enhanced by other substances such as copper (II) sulphate (Murata *et al.*,1990; Ikigai *et al.*,1993; Hoshino *et al.*, 2000; Arakawa *et al.*, 2004). One suggested hypothesis is that flavanols, when tested alone, and in combination with copper (II) sulphate generate harmful levels of reactive oxygen species (ROS) including hydrogen peroxide (Hoshino *et al.*, 2000; Arakawa *et al.*, 2004). This hypothesis was tested in the present study by adding bovine catalase in sufficient quantities to test mixtures to remove any extracellular hydrogen peroxide that may have been harming the *S. aureus* NCTC 06751 cells and reducing viability levels. Catalase additions were done with both whole white tea and white tea sub-fractions < 1 kDa each tested alone as well as combined with copper (II) sulphate, and further with vitamin C added to the same combination (Figure 3.13).

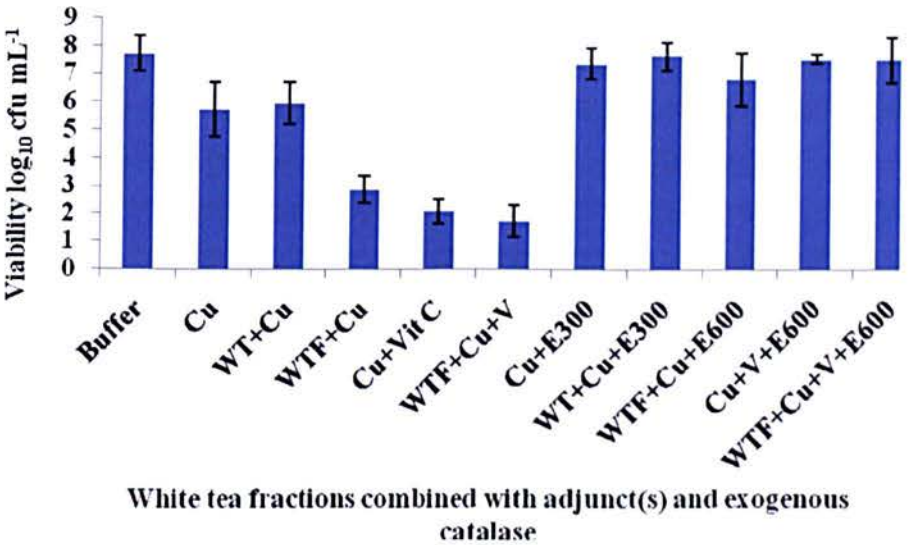


Figure 3.13 Concentrations of catalase required to reverse the antimicrobial effects of white tea sub-fraction combined with copper (II) sulphate and vitamin C against *Staphylococcus aureus* NCTC 06751. Cu = added 4.8 mM copper (II) sulphate, WT = white tea, '+' denotes combination of agents specified, Vit C, or V = added 4.8 mM vitamin C, WTF = white tea fraction (< 1 kDa), E300, E600 = added level of enzyme, 300 or 600 μg/mL (f.c.), Mixtures were allowed to stand for 10 minutes in the dark before the addition of an inoculum and bovine catalase. Incubations were in the dark at room temperature for 30 minutes. The plates were incubated at 37°C for 24 hours. Sample volumes of 500 μL of Cu+V, WTF+Cu and WTF+Cu+V were used to reduce the detection limit. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

Exogenous catalase at a final concentration (f.c.) of 300 μg mL^{-1} was required to remove the antimicrobial activity of copper (II) alone as well as the WT plus copper (II) combination. In comparison 600 μg mL^{-1} (f.c.) was required to remove both the antimicrobial activity of the WTF plus copper (II) alone as well as the same combination with added vitamin C. In the data shown the differences between corresponding samples with and without the added catalase were significant ($p<0.05$). This suggests that the main effect of the copper (II) sulphate tested alone, or when combined with WTF (< 1 kDa) and further with added vitamin C, the principal antimicrobial effect was via the production of harmful hydrogen peroxide.

Other investigations have also shown that WT contains flavanols, some of which are known to generate hydrogen peroxide in aqueous solution, and more so when copper (II) ions are present (Hoshino *et al.*, 2000, Arakawa *et al.*, 2004). In this investigation hydrogen peroxide and other reactive oxygen species (ROS) could have been produced by the re-oxidation of copper (I) following reduction of the added copper (II) by chemical species present in the mixtures such as water, vitamin C and other compounds within the WTF (< 1 kDa) such as flavanols (Hoshino *et al.*, 2000; Arakawa *et al.*, 2004). In this investigation, as shown in Figure 3.13 above, WTF (< 1 kDa) tested in the absence of copper (II) ions had no apparent antimicrobial effect on *S. aureus* NCTC 06751. In comparison, copper (II) sulphate tested alone in the absence of added catalase

produced a significant level of antimicrobial activity against *S. aureus* as seen in a fall in viability of the organism. However, when 300 μg mL^{-1} catalase was added to a similar level of copper (II) sulphate the added enzyme resulted in *S. aureus* showing buffer levels of viability. This observation suggested that the antimicrobial effects of the copper (II) sulphate had been brought about by harmful levels of hydrogen peroxide which were removed by the addition of the catalase. In further tests when copper (II) sulphate was combined with white tea fraction (WTF) < 1 kDa, a greater amount of 600 μg mL^{-1} of catalase was needed to negate the antimicrobial effects of the WTF-Cu(II) mixture to a level comparable to the buffer control. This suggested that the WTF (< 1 kDa) had enhanced the production of hydrogen peroxide by the copper (II) ions, this mixture requiring double the concentration of added catalase to negate the combined antimicrobial effects. Addition of vitamin C to the WTF (< 1 kDa) and copper (II) sulphate did not require further amounts of added catalase for reversal suggesting the vitamin C had contributed little to the generation of hydrogen peroxide when WTF (< 1 kDa) was present. In comparison when WTF (< 1 kDa) was absent, a combination of copper (II) sulphate and vitamin C required the same level of catalase (600 μg mL^{-1} f.c.) suggesting that the vitamin C had contributed to the generation of hydrogen peroxide. Possibly the WTF (< 1 kDa) and the vitamin C both interacted with the copper (II) ions in a similar way such as by reducing them to copper (I) and may both have worked at the same reaction site. If this were so then it could explain why either agent enhanced the copper (II) ion production of hydrogen peroxide and yet did not show any summative effect on the viability of *S. aureus* NCTC 06751 when both were present. Such a mechanism is analogous to that of competitive inhibition in enzyme kinetics.

Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide can cause the collapse of cell viability (Arakawa, 2004; Delbes-Paus *et al.*, 2010). One way *S. aureus* NCTC 06571 defends itself against the harmful effects of ROS such as hydrogen peroxide, is by synthesising enzymes such as catalase to deactivate ROS usually by catabolic breakdown (Das and Bishayi, 2009). The amount of hydrogen peroxide released by the agents and adjuncts in this investigation apparently exceeded the ability of the bacterial cells to adequately defend themselves by their levels of endogenous cellular catalase.

A change in the pH value of solutions of chemicals added together can imply that a chemical reaction has taken place. In order to further investigate possible mechanism of reactions of the interaction of copper (II) ions with WT components pH measurements were taken of WT and WTF (< 1 kDa) before and after the addition of 4.8 mM copper (II) sulphate (Figure 3.14).

3.12 Investigation of any pH changes following the addition of whole WT and WTF < 1 kDa to copper (II) ions

The measurement of pH values were used to investigate possible chemical changes within WT, and WTF (< 1 kDa) with added copper (II) sulphate following mixing to help elucidate a possible mechanism of action of mixtures against cells. Aqueous copper (II) sulphate which contains mainly positively charged free copper (II) ions would have a high affinity for the negatively charged oxygen ions within the ionised hydroxyl groups present in some tea components such as polyphenols. Possessing such high affinity, copper (II) ions would tend to displace the less positively charged hydrogen ions from the hydroxyl groups in aqueous solution resulting in a greater level of free protons and thus a lower pH.

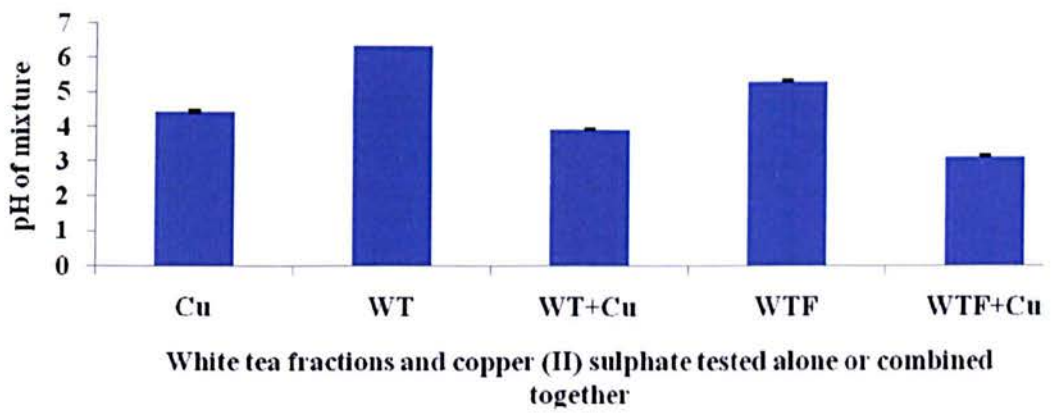


Figure 3.14 pH measurements of whole and sub-fractionated (< 1kDa) white tea alone and when added to 4.8 mM copper (II) sulphate tested at suspension assay concentrations Cu = 4.8 mM copper (II) sulphate, WT = whole white tea, '+' denotes combination of agents specified, WTF = white tea sub-fraction (< 1 kDa). Mixtures were allowed to stand for 10 minutes in the dark before pH measurements. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results show that WT, WTF (< 1 kDa), and copper (II) sulphate tested alone as well as combinations of WT+Cu(II), WTF+Cu(II) showed significant differences in pH values ($p<0.05$) (Figure 3.14). The falls in pH seen when WT or WTF were added to copper (II) sulphate were greater than expected from a simple dilution of copper (II) sulphate alone. This suggested that further protons had been added or had been released by the tea. However, the pH values of the WT and WTF added to the value of the copper (II) sulphate could not account for the pH change seen following mixing. The second possibility that protons had been released from the weakly acidic tea extracts by the copper (II) sulphate therefore seemed plausible supporting the rationale for this experiment.

A series of experiments was carried out to investigate the possibility that catechins were responsible for the activities of the WTF < 1kDa combined with copper (II) sulphate since flavanols having a molecular weight of *ca.* 300 to 450 are likely to have been present in the WTF <

1 kDa. If so, it was also possible that there were differences in the antimicrobial activities of the various types of catechin found in the WTF when combined with copper (II) sulphate, since the tea catechins vary in their molecular structures and antimicrobial activities (Hoshino *et al.*, 2000) (Figures 3.15, 3.16). Five principal types of flavan-3-ols ('catechins') commonly found in WT and GT, and available commercially, were investigated for antimicrobial effects against *S. aureus* NCTC 06751.

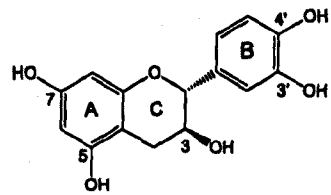


Figure 3.15 General structure of the tea catechins. Isometry occurs at positions 2 and 3 in the C ring where the 3',4'-dihydroxyphenyl and hydroxyl group respectively can each be found in two configurations giving rise to catechin and *ent*-catechin as well as epicatechin and *ent*-epicatechin. Substitutions of OH for H at 5' in the B ring and gallic acid for OH at 3 in the C ring give rise to other tea catechins.

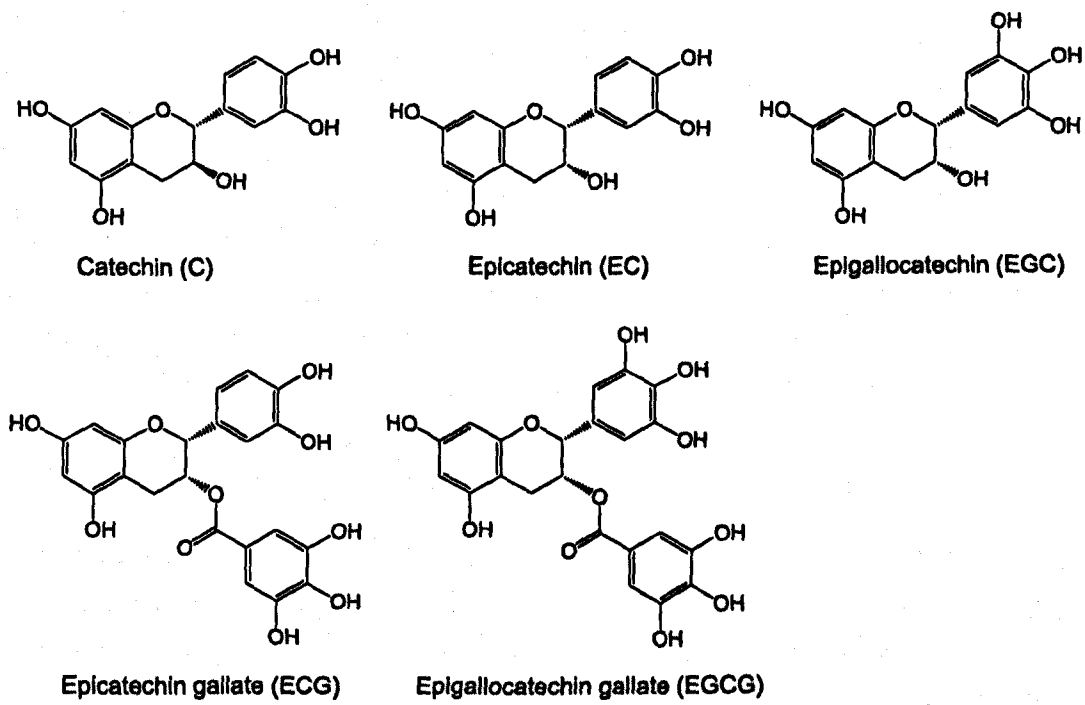


Figure 3.16 Chemical structures of the five common catechins used in this investigation. Catechin does not possess the features normally associated with higher antimicrobial activities seen with other tea flavanols *i.e.* 3 OH groups in the B ring, favourable epimerisation of the C ring OH group; a gallic acid substitution at the same position and with favourable epimerisation.

3.13 Activities of five common tea catechins tested alone and combined with copper (II) ions against *S. aureus* NCTC 06751

S. aureus NCTC 06571 was exposed to 1 μM, 10 μM, 100 μM, and 1000 μM added concentrations of the following flavanols: catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) (Figure 3.17).

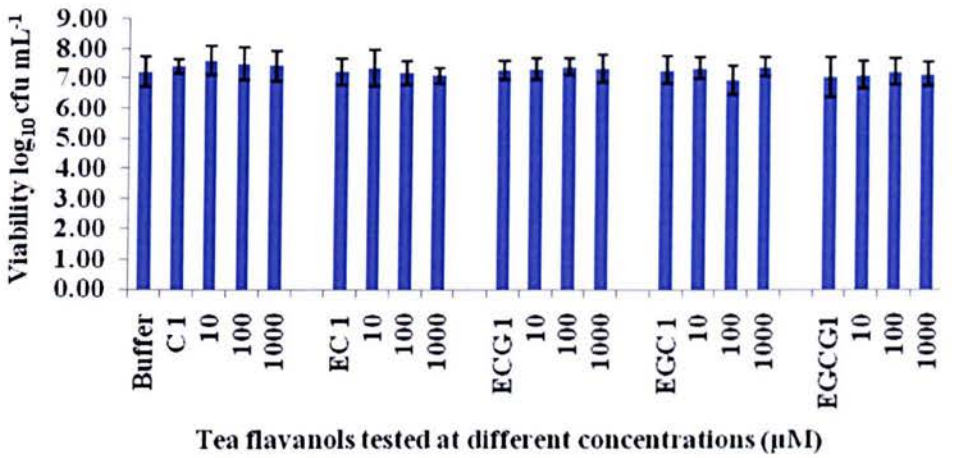


Figure 3.17 Effects of five common tea flavanols at different concentrations against *Staphylococcus aureus* NCTC 06751. C = catechin; 1, 10, 100, 1000 = added μM concentrations of each flavanol (final concentrations = 0.214, 2.14, 21.4 and 214 μM respectively), EC = epicatechin, ECG = epicatechin gallate, EGC = epigallocatechin, EGCG = epigallocatechin gallate. Mixtures were allowed to stand for 10 minutes in the dark before addition of culture. Incubations were at room temperature for 30 minutes in dark conditions. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results show that when exposed to *S. aureus* NCTC 06751 the different concentrations of the flavanols as well as the buffer control showed similar effects on cell viability with no significant differences between them ($p>0.05$). These results show that the flavanols, tested in these conditions, had no apparent effect on this strain of bacterium. The same concentration range, or extended if necessary, was used in the next set of experiments to investigate whether the addition of a putative adjunct, namely 4.8 mM copper (II) sulphate, could enhance the antimicrobial activity of these flavanols against *S. aureus* NCTC 06751.

The addition of 4.8 mM copper (II) sulphate as a putative enhancement agent was tested in combination with the five common previously investigated flavonols: catechin, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG). Each flavanol was tested at increasing concentrations to discover the amount of each flavanol required in the presence of added 4.8 mM copper (II) to reduce the viability of *S. aureus* to *ca.* 3 $\log_{10}$ cfu mL^{-1}. The results are showed in Table 3.6:

Table 3.6 Concentrations of five common tea flavanols combined at different concentrations with copper (II) sulphate to reduce *Staphylococcus aureus* NCTC 06751 viability to 3 $\log_{10}$ cfu mL^{-1}

Catechin tested	Concentration of catechin solution needed to reduce *S. aureus* NCTC 06751 viability to 3 $\log_{10}$ cfu mL^{-1} (µM)
Catechin (C)	1000
Epicatechin (EC)	500
Epicatechin gallate (ECG)	150
Epigallocatechin (EGC)	1
Epigallocatechin gallate (EGCG)	1

Key: Flavanols were each tested at progressive concentrations with added 4.8 mM copper (II) sulphate to reach a point where resulting viability was *ca.* 3 $\log_{10}$ cfu mL^{-1}. Mixtures were allowed to stand for 10 minutes in the dark before addition of culture. Incubations were at room temperature for 30 minutes in dark conditions. The plates were incubated at 37°C for 24 hours. Results were based on triplicate tests from 3 separate experiments.

Compared to the flavanols alone, where no antimicrobial activity was seen against *S. aureus* NCTC 06751 (Figure 3.17), adding 4.8 mM copper (II) sulphate enhanced the antimicrobial activity of the catechins. The results showed that combined with 4.8 mM copper (II) ions, the catechins produced different levels of antimicrobial activity. The order of enhancement by the different flavanols was in the order: catechin < epicatechin (EC) < epicatechin gallate (ECG) < epigallocatechin (EGC) = epigallocatechin gallate (EGCG). It may be possible to explain these results on the basis of the differing molecular features of the tested flavanols. The order of enhancement followed that of averaged published MICs for each of the catechins against *S. aureus,* except in the last case where typically EGC has a higher MIC than EGCG (Takahashi *et al.*, 1995, Akiyama *et al.*, 2001; Stapleton *et al.*, 2004a, 2004b; Gibbons *et al.*, 2004; Taguri *et al.*, 2004; Roccaro *et al.*, 2004).

Flavanols with trihydroxy benzyl groups (epigallocatechin, epicatechin gallate and epigallocatechin gallate) have lower MICs against *S. aureus* than the other flavanols (catechin and epicatechin) (Stapleton *et al.*, 2006). Stereochemistry at two chiral centres of the non-galloylated flavonols suggests an influence of epimerisation on antimicrobial activity. In this study broadly similar trends were seen with the flavanols combined with copper (II) sulphate against *S. aureus* and a similar argument can be applied as the one for MICs above. Such molecular variations are believed to be associated with the type and degree of flavanol to cell structure interaction such as intercalation effects on cell wall, membrane fluidity and ion transport (Stapleton *et al.*, 2006; Stapleton *et al.*,

2007; Sirk *et al.*, 2009). Other flavanol actions have been found to include affects on cell agglutination, cell division, quorum sensing, and generation of reactive oxygen species (ROS) such as H_2O_2, DNA structure and replication (Hayakawa *et al.*, 1999, Hoshino *et al.*, 2000, Stapleton *et al.*, 2004a; Gradisar *et al.*, 2007; Shimamura *et al.*, 2007; Vandeputte *et al.*, 2010). In order to investigate and develop ways of enhancing the antimicrobial activity of plant products such as flavanols, the weakly antimicrobial flavanol, catechin, was chosen and investigated as a suitable candidate to attempt further enhancement.

3. 14 Summary and conclusions

In suspension assays copper (II) sulphate tested at the level found in the whole white tea (0.56 μM added to assay, equivalent to level of 0.14 mg L^{-1}) was found to have same effect as a buffer control on the viability of *S. aureus* NCTC 06571 in the conditions tested. Copper (II) sulphate tested in similar conditions at higher concentrations up to 10 mM produced an antimicrobial effect. Vitamin C assayed in the absence of teas enhanced the antimicrobial activity of copper (II) sulphate against *S. aureus* NCTC 06751 although vitamin C itself showed no antimicrobial activity when tested alone. When exposed to whole WT added to 4.8 mM copper (II) sulphate *S. aureus* NCTC 06751 showed the same viability as seen following cell exposure to 4.8 mM copper (II) sulphate alone which suggested no additional effect on *S. aureus* NCTC 06751 viability from the WT. However, following exposure of *S. aureus* NCTC 06751 to GT and BT each combined with 4.8 mM copper (II) sulphate viabilities similar to buffer controls were seen which suggested that both GT and BT had removed the antimicrobial effect of the copper (II) sulphate. It is possible that GT and BT both reduced the bioavailability of the copper (II) ions by the formation of inactive chemical complexes, which showed no antimicrobial activity. Whole WT and WT sub-fraction < 5 kDa showed no antimicrobial activity in any of the combinations tested. WT sub-fraction < 1 kDa was enhanced with copper (II) sulphate and further with vitamin C, possibly via the formation of putative hydrogen peroxide as exogenous catalase removed antimicrobial effects on cells.

The higher copper (II) concentrations contained within WT when compared to WT supernatant, suggest whole WT contains higher MW components than WTSN which react with copper (II) ions more readily than WTSN components. However, any such adsorption of copper (II) ions does not affect the antimicrobial activity of the whole WT compared to the WTSN. Differences in the UV-vis absorbance of whole WT compared to the WT sub-fractions probably reflected differences in substance concentration brought about by the filtration processes.

Both whole WT and WT sub-fraction < 1 kDa increased the acidity of copper (II) sulphate solution possibly via the formation of chemical complexes. However, antimicrobial activities of pH adjusted Ringer's solution, or teas, or copper (II) sulphate had no effect on the subsequent antimicrobial activity of these substances against *S. aureus* NCTC 6571 except in the case of copper (II) sulphate

adjusted to pH 9 when antimicrobial activity was lost. This loss was probably due to the precipitation of the copper (II) ions by NaOH reducing their bioavailability and consequently their harmful effects. Such harmful effects were probably caused by the generation of hydrogen peroxide possibly via the formation of putative hydrogen peroxide generating chemical complexes, since the addition of exogenous catalase to harmful test mixtures removed antimicrobial effects on cells.

The antimicrobial activity of five common tea catechins; catechin, epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate, were compared to investigate any effect of the different catechin molecular structures on antimicrobial activity when combined with copper (II) ions. All catechins were tested individually alone and with combined copper (II) sulphate at concentrations below the published MIC values for *S. aureus*. None of the catechins tested alone showed any effect on the viability of *Staphylococcus aureus* NCTC 06571 at the concentrations tested when compared to a buffer control. In further experiments 4.8 mM copper (II) sulphate was added to each catechin which enhanced the antimicrobial activity of each of the catechins to a degree which could be related to their individual molecular structures. The order of antimicrobial activity seen with each catechin when combined with copper (II) sulphate was in the order EGCG = EGC > ECG > EC > C. This order follows a broadly similar pattern seen for the catechins tested alone at higher concentration to determine MIC values found in other investigations. Flavanol epimers which possess more hydroxyl groups show higher activities against *S. aureus* NCTC 06571.

4.1 Introduction

Polyphenols such as tea flavanols have been shown to work in cooperation with each other in living systems (Williams *et al.*, 2003; Lewis and Ausubel, 2006). Catechins are known to have wide ranging biological affects including as therapeutic and antimicrobial agents (see section 1.16). The possibility of artificially enhancing the antimicrobial activities of less active tea flavanols such as catechin with other additives and by pre-treatment, for example by heat, has however received less attention. In this study the hypothesis tested was that a weak antimicrobial agent could be enhanced by the addition of adjuncts in optimal molar ratios to reach a maximal antimicrobial effect within a 30 minute exposure period. In addition, that further enhancement could be reached through other methods of activation such as by physical means like heating or pressure. In this study, therefore, the improvement of the activity of catechin, by the addition of transition metal (II) ions, the reducing agent vitamin C, and the alkaloid caffeine, an anti-efflux MDR pump inhibitor as an additional putative adjunct, in specific molar ratios was attempted, partly to investigate any direct enhancement effects but also any effects due to stoichiometry. The results in Chapter 3 (Table 3.6) showed that copper (II) sulphate improved the antimicrobial activity of catechin. Plant polyphenols, including flavanols, can form complexes with transition and other metal ions with a range of stoichiometries (Weber, 1988, Kuo *et al.*, 1998, Fernandez *et al.*, 2002, Esparza *et al.*, 2005, Ghosh *et al.*, 2006). It has been suggested that such complexes are more active against microbes than free, *i.e.* uncomplexed, polyphenols or even antimicrobial metal ions such as copper (II) (Weber, 1988; Hoshino *et al.*, 2000). Others have argued that flavonoids can chelate metal ions which would reduce their ability to generate free radicals and thus could reduce their effect against microbes (Fernandez *et al.*, 2002). Catechin-copper(II) complexes may generate bactericidal substances or interfere with cell physiology and or metabolism (Hoshino *et al.*, 2000, Ghosh *et al.*, 2006). If a catechin-copper(II) complex is formed, this is likely to generate a precise stochiometric ratio between the two components. In addition these studies investigated whether catechin and its possible oxidation products enhanced antimicrobial activities. These investigations used suspension assays with cell viable counts to evaluate the effect of novel mixtures against target species of bacteria and investigations of reaction mechanisms using pH and H_2O_2 measurements as well as enzyme and metal chelating metabolic inhibitors. Since previous investigations by Hoshino *et al.* (2000) have shown that copper (II) metal ions enhance the activity of tea catechins similar investigations were carried out in this project. The mechanism of action of catechin combined with transition metal (II) ion against bacteria was investigated using EDTA to remove transition metal (II) ion availability from any reaction mechanisms. Mechanisms of action investigations also included interactions between catechin and copper(II) that could indicate the possible formation of

an active chemical complex using pH, solubility, crystallisation studies, structure activity relationships, and UV-vis absorption studies. Further mechanism studies included the activity kinetics of fresh and heat-treated catechin compared to another tea flavanol, epigallocatechin gallate (EGCG), combined with adjuncts against *S. aureus* and whether the activity of active mixtures was related to hydrogen peroxide generation as suggested in other investigations (Arakawa *et al.*, 2004). This was done by identifying and measuring hydrogen peroxide production and by investigating any effects of hydrogen peroxide against bacteria using exogenous catalase. The effect of active mixtures on bacterial morphology and Gram staining was also investigated by microscopy.

4.2 Effects of the weak antimicrobial flavanol, catechin, alone, and with copper (II) sulphate against the viability of *S. aureus* NCTC 06751 and other microbes of medical importance

To investigate whether a precise stoichiometry occurs between catechin and copper (II) different molar ratios of the copper (II) were combined with a fixed concentration of catechin and investigated for their effects against *S. aureus* NCTC 06751 (Figure 4.1).

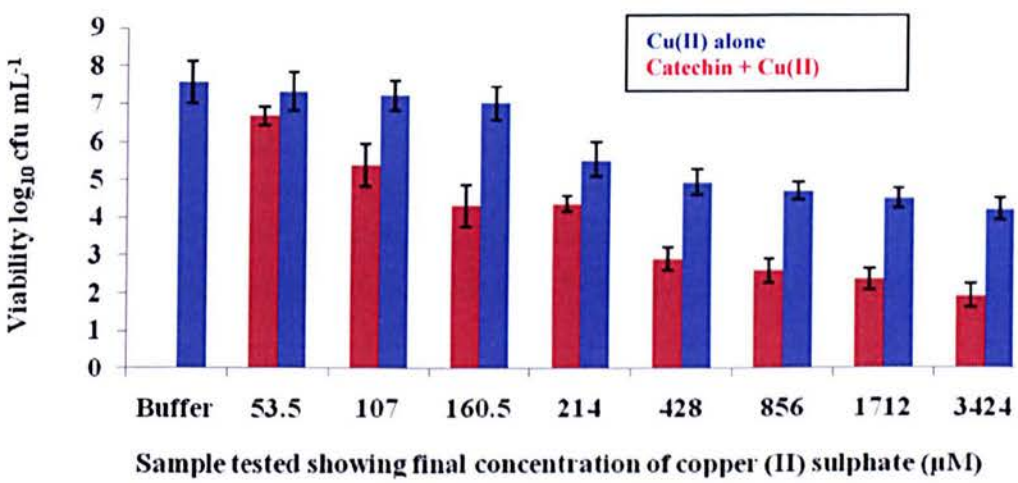


Figure 4.1 Effects of adding catechin to increasing molar ratios of copper (II) sulphate against *Staphylococcus aureus* NCTC 06751. Blue columns = copper (II) sulphate controls with added Ringer's solution; red columns = copper (II) with 214 µM (final concentration) catechin. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Incubations were at room temperature for 30 minutes in dark conditions. Sample volumes were increased for catechin plus copper (II) combinations from 20 µL to 100 µL for the 428, 856, & 1712 µM copper (II) sulphate additions, and to 500 µL for the 3424 µM copper (II) sulphate addition to maintain detection levels. The plates were incubated at 37 °C for 24 hours. Viabilities for catechin were similar to buffer values (Appendix II: Table A: Controls). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results show that catechin in the presence of increasing copper (II) sulphate concentrations progressively reduced the viability of *S. aureus* NCTC 06751 to a greater extent than did copper

(II) sulphate controls when exposed to *S. aureus* NCTC 06751. The differences in the means of the copper (II) sulphate samples tested alone against *S. aureus* NCTC 06751 compared to the catechin plus copper (II) sulphate combinations tested against *S. aureus* NCTC 06751 at final concentrations of 107 μM and above were all significant ($p<0.05$). Copper (II) sulphate enhanced catechin activity, particularly at concentrations of 160.5 μM which represented a ratio of 0.75 : 1 of 214 μM catechin ($p<0.05$). This ratio may correspond to the formation of a catechin-metal(II) ion complex as previously described and could in this ratio represent a stoichiometry of 3 copper (II) ions : 4 catechin molecules (Hoshino *et al.*, 2000; Fernandez *et al.*, 2002; Esparza *et al.*, 2005; Ghosh *et al.*, 2006). Such a complex could be more active against cells than separate catechin molecules and copper (II) ions. At greater ratios of 2 : 1 up to 16 : 1 of copper (II) : catechin, significant differences in antimicrobial activity compared to the corresponding copper (II) sulphate controls were also seen ($p<0.05$), although these differences were not as large as seen in the 0.75 : 1 ratio. It is curious that at the 1:1 ratio (214 μM f.c.) a decrease in differential antimicrobial activity was seen compared to the 0.75 : 1 and the 2 : 1 ratio (428 μM f.c.). It is possible that the further copper (II) sulphate, added to make up the 1:1 ratio, bound to the catechin molecules but in some way reduced their antimicrobial activity resulting in an activity similar to the 0.75 : 1 ratio ($p>0.05$). It is possible that an equivalent stoichiometry of 3 copper (II) : 4 catechin is less active than that of 4 : 4 ratio as the net charge and steric factors were altered making the complex less attracted to the cell envelope. At ratios of 2 : 1 and above there were no significant differences between the mean activities of the copper (II) sulphate controls ($p>0.05$), and the combined copper (II)-catechin samples ($p>0.05$). This suggested a maximal effect had been reached at the 2 : 1 ratio within the conditions tested. Complex formation between catechin and copper (II) sulphate was further investigated in subsequent experiments by pH, UV-vis spectroscopy and crystallisation studies (see section 4.8).

It is possible that the above activity of catechin-copper(II) combinations against *S. aureus* may be partly due to a pH effect. Therefore another experiment tested the effects of pH on the activities of *S. aureus* NCTC 06571 and other reference species of bacteria. Three pH values were chosen: 3.5, 5.5 and 7.2 since copper (II) sulphate solutions are acidic and the lambda buffer control used in the suspension assays was slightly alkaline (Table 4.1).

Table 4.1 Effects of adjusting the pH value of Ringer's solution on its antimicrobial activity against different test species of bacteria

Species tested	Viabilities of bacterial species following exposure to Ringer's solution adjusted to different values of pH, or to lambda buffer control at fixed pH			
	pH 3.5	pH 5.5	pH 7.2	Lambda buffer (pH 7.2)
S. aureus	7.63 (± 0.76)	7.61 (± 0.82)	7.65 (± 0.85)	7.66 (± 0.52)
E. coli	7.77 (± 0.40)	7.89 (± 0.83)	7.60 (± 1.03)	7.08 (± 0.21)
Ps. aeruginosa	7.92 (± 0.55)	7.59 (± 0.65)	8.01 (± 0.40)	7.83 (± 0.21)
P. mirabilis	7.87 (± 0.43)	7.79 (± 0.50)	7.53 (± 0.82)	7.18 (± 1.08)

Key: *S. aureus* = *Staphylococcus aureus* NCTC 06571, *E. coli* = *Escherichia coli* NCTC 14441, *Ps. aeruginosa* = *Pseudomonas aeruginosa* NCTC 950, *P. mirabilis* = *Proteus mirabilis* NCTC 7827. Ringer's solution was adjusted with 1M HCl or with 1M NaOH solution. Lambda buffer previously adjusted to pH 7.2 was used as a control as in all suspension assays. Test samples of 1000 µL were combined with 500 µL of inoculum and incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 3 separate experiments (SEMs in brackets).

These results show that pH over the range 3.5 - 7.2 has no statistically significant effect (p values all > 0.05) on the viability of *S. aureus* NCTC 06571, *E. coli* NCTC 14441, *Ps. aeruginosa* NCTC 950, or *P. mirabilis* NCTC 7827. The viabilities of *S. aureus* NCTC 06751 following exposures to Ringer's solution and to lambda buffer each adjusted to pH 7.2 were similar, indicating no specific viability effect on *S. aureus* NCTC 06751 from the compositions of the two solutions. Following this, an equimolar mixture of catechin with copper (II) sulphate was tested against *Ps. aeruginosa* NCTC 950, *P. mirabilis* NCTC 7827, *E. coli* NCTC 14441 and compared to *S. aureus* NCTC 06571. This was done to investigate which of these species would be the most susceptible (Figure 4.2), so that it could be used for comparison in subsequent experiments.

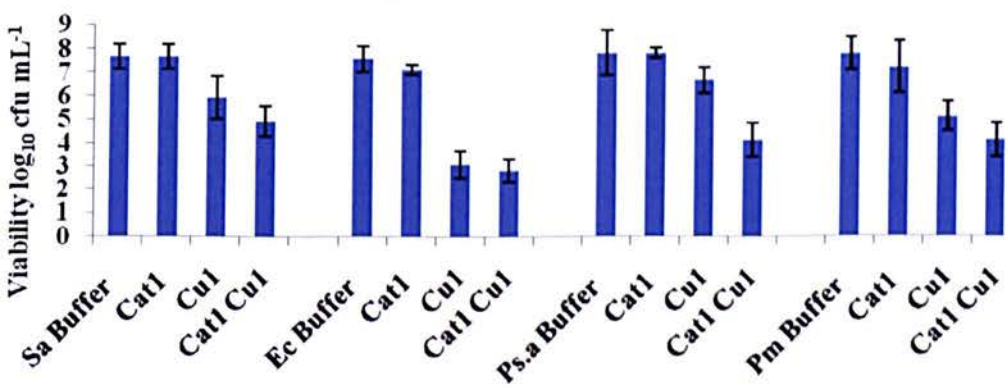


Figure 4.2 Effect of catechin and copper (II) sulphate mixtures against four bacterial strains. *Sa* = *Staphylococcus aureus* NCTC 06571, *Ec* = *Escherichia coli* NCTC 14441, *Ps.a* = *Pseudomonas aeruginosa* NCTC 950, *Pm* = *Proteus mirabilis* NCTC 7827, Cat1 = 214 µM (final concentration) catechin, Cu1 = 214 µM (f.c.) copper (II) sulphate, Cat1Cu1 = equimolar 214 µM (f.c.) catechin and copper (II) sulphate combined. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. Sample volumes for Cu1 and Cat1Cu1 combinations with *E. coli* were increased to 100 µL to maintain detection of colony forming units. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The catechin-copper(II) combination produced broadly similar effects on the viabilities of *S. aureus*, *Ps. aeruginosa*, and *P. mirabilis* with *E. coli* being the most susceptible although there were no significant differences between them ($p>0.05$). However, *Ps. aeruginosa* NCTC 950 was the least susceptible to copper (II) sulphate showing little difference in viability when compared to to the buffer viability following exposure ($p>0.05$). Strains of this species have previously been shown to be relatively tolerant to copper (Braud *et al.*, 2010). Of the four species of bacteria tested adding catechin to the copper (II) solution, caused the greatest reduction in *Ps. aeruginosa* viability when compared to the *Ps. aeruginosa* viability seen with the copper (II) sulphate alone sample ($p<0.05$). The other three test species, in contrast, showed no statistically significant differences between viabilities shown by the copper (II) sulphate alone sample compared to the catechin-copper (II) combination (all $p>0.05$). The least susceptible species to the combined mixture was *S. aureus* NCTC 06571 possibly on account of its thicker cell wall compared to the three Gram-negative species tested. *S. aureus* NCTC 06571 may also have some other more effective defence systems such a higher levels of protective substances, *e.g.* enzymes or chelating agents.

To investigate whether *S. aureus* NCTC 06571 would show lower viabilities if exposed to the catechin-copper mixture with other adjuncts, vitamin C and caffeine were evaluated. Similar samples were also tested against *E. coli* NCTC 14441. Initially, vitamin C was tested because it is a known reducing agent and believed to enhance the antimicrobial action of copper (II) ions. It is also possible that vitamin C may enhance the antimicrobial activity of a catechin-copper (II) sulphate solution (Ericsson, 1954; Murata and Yano, 1990; Hoshino *et al.*, 2000; McCarrell *et al.*, 2008).

4.3 Effects of combinations of equimolar catechin and copper (II) sulphate plus vitamin C added in molar ratios against *S. aureus* NCTC 06571 and *E. coli* NCTC 14441

Vitamin C was added in increasing amounts to an equimolar combination of catechin and copper (II) sulphate using a protocol similar to McCarrell *et al.*, 2008, (see section 2.2.5).

Table 4.2 The effect of catechin and copper (II) sulphate with vitamin C against *Staphylococcus aureus* NCTC 06751

Vitamin C level + 214 µM Cu(II)SO_4	Viability fall after 30 minutes (log_{10} cfu mL^{-1})	
	No catechin	+214 µM catechin
0 µM	1.31 (±0.40)	3.23 (±0.45)
428 µM	2.75 (±0.52)	3.83 (±0.82)
856 µM	3.23 (±0.73)	4.49 (±0.12)
1712 µM	5.19 (±0.79)	6.31 (±0.48)

Key: Catechin, copper (II) sulphate and vitamin C figures are final concentrations. Viability figures show typical fall compared to buffer values. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. Sample volumes for Cu and Cat-Cu combinations with 1712 µM vitamin C were increased to 500 µL to maintain cfu detection. The plates were incubated at 37 °C for 24 hours. Viabilities for catechin and vitamin C controls were similar to buffer values (Appendix II: Table 1, Controls; MT3). Viabilities for Cat +Cu controls were similar to those seen previously: 5.5 (±0.67). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

Table 4.2 demonstrates that vitamin C increased the antimicrobial activity of the catechin-copper (II) combination against *S. aureus* NCTC 06751 with all the samples showing significant differences in the viabilities of *S. aureus* NCTC 06751when compared to a control of catechin-copper (II) without vitamin C (all $p<0.05$). However, in controls without the presence of catechin, similar sized effects on the viability of *S. aureus* NCTC 06751 were seen with copper (II)-vitamin C combinations without catechin (all $p>0.05$). This suggested that the catechin contributed little to this mixture. There was also no difference between vitamin C and catechin (equimolar 214 µM f.c.) and buffer controls in both *S. aureus* NCTC 06751 and *E.coli* NCTC 14441 ($p>0.05$). The possible reaction mechanisms involving copper (II) ions are described in section 4.9.

4.4 Effects of addition of caffeine to combinations of equimolar catechin and copper (II) sulphate plus vitamin C added in molar ratios against *S. aureus* NCTC 06571

Following the findings with copper (II) and vitamin C as putative adjuncts to catechin, caffeine was added to a combination of catechin, copper (II), and vitamin C in an attempt to raise the antimicrobial activity of the mixture further. Caffeine is an alkaloid which is believed in nature to inhibit the MDR pump of bacteria leading to increases in effectiveness of antimicrobial plant products when present (Ball *et al.*, 2006; Couzinet-Mossion *et al.*, 2010). Caffeine is found in high

amounts in leaves of the tea bush and may thus work in conjunction with tea leaf flavonols against colonising pathogenic bacteria.

Caffeine, an additional putative adjunct, was initially tested on its own at concentrations between 0.01% and 1% (w/v), which is equivalent to 0.11 - 11.4 μM (final concentration), against *S. aureus* NCTC 06571 and *E. coli* NCTC 14441. There were no antimicrobial effects across the range of caffeine concentrations tested on the viability of either *S. aureus* NCTC 06571 or *E. coli* NCTC 14441 species (data not shown). Following this an intermediate concentration of 0.5% (w/v) caffeine was tested in several combination treatments against *S. aureus* NCTC 06571 (Figure 4.3). Vitamin C was tested at a concentration of 428 μM (f.c.) since this concentration had previously been found to add a statistically significant increase in antimicrobial acivity to both samples of 214 μM (f.c.) copper (II) sulphate and to an equimolar mixture of 214 μM (f.c.) catechin and copper (II) sulphate against *S. aureus* (both $p<0.05$) (Table 4.2). All tests indicated that caffeine did not enhance the antimicrobial activity of copper (II) sulphate on its own or with catechin/copper (II) sulphate and catechin/copper (II) sulphate/vitamin C mixtures. All the caffeine combination samples showed no significant differences in viability effects compared to combination controls without caffeine (all $p>0.05$) (Figure 4.3).

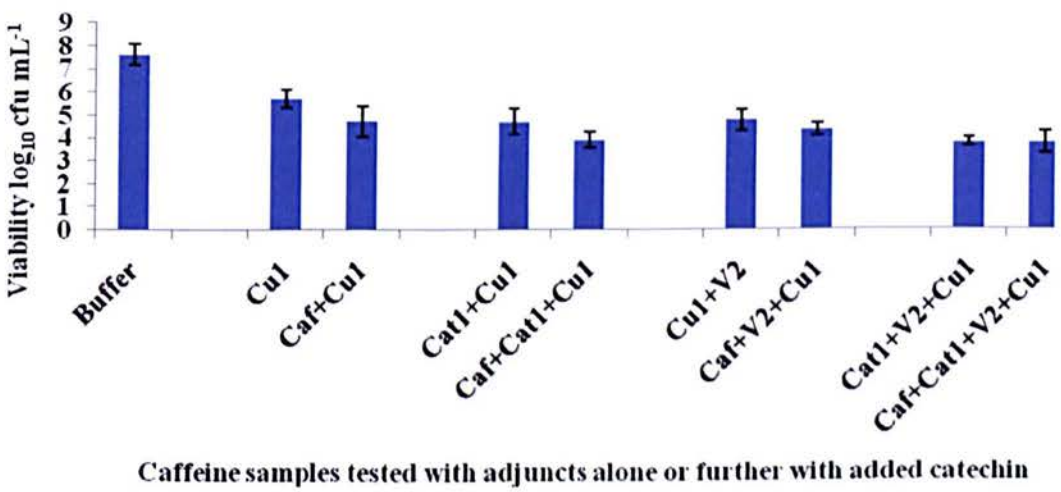


Figure 4.3 Effect of caffeine combined with catechin plus copper (II) sulphate and vitamin C against *Staphylococcus aureus* NCTC 06751. Horizontal axis: 1 = 214 μM (final concentration). Cu = copper (II) sulphate, Caf = 0.5% (f.c.) caffeine, V2 = 428 μM (f.c.) vitamin C. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. Sample volumes for catechin-containing samples were increased to 100 μL to maintain cfu detection. The plates were incubated at 37 °C for 24 hours .Viabilities for catechin and vitamin C controls; caffeine and plus vitamin C were similar to buffer values (results not shown). Viabilities for Cat+Cu controls were similar to those seen previously 5.1 (±0.49). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The next experiment investigated whether the same concentration of 0.5% (w/v, f.c.) caffeine could enhance the antimicrobial activity of a higher concentration of 4.8 mM copper (II) sulphate (f.c. 2.17 mM) against *S. aureus* NCTC 06751. This was to check whether copper (II) ions were in too low a concentration in the previous experiment to be affected by any hypothetical copper (II) induced MDR pump activity when tested in the experimental conditions of relatively short exposure periods of 30 minutes. In addition, for comparison purposes, results from previous experiments had indicated significant viability values shown by *S. aureus* NCTC 06751 in the presence of added 4.8 mM copper (II) sulphate compared to buffer values of viability (*e.g.*, Table 3.2, Figures 3.3, 3.4, 3.5, 3.6). Thus, this choice of copper (II) sulphate concentration was convenient.

Table 4.3 The effect of caffeine added to 4.8 mM copper (II) sulphate solution on *Staphylococcus aureus* NCTC 06751 viability

Sample tested	Log_{10} reduction in viability
Copper (II) sulphate	-2.5 (±0.44)
Caffeine + copper (II) sulphate	-3.3 (±0.52)

Key: Caffeine added at 0.5% (w/v, final concentration), copper (II) sulphate added at 4.8 mM (f.c. 2.17 mM). Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 3 separate experiments (SEMs in brackets).

The figures in Table 4.3 show reductions in viability of *S. aureus* NCTC 06571 when compared to buffer values of *ca.*7.6 (±0.45) log_{10} cfu mL^{-1}. These results show that 0.5% (w/v) (f.c.) caffeine had no additional effect to that of the added 4.8 mM copper (II) sulphate on the viability of *S. aureus* NCTC 06571 with no significance difference shown between the two samples ($p>0.05$). These results indicate that with this bacterium, the antimicrobial activity of this level of copper (II) sulphate was not enhanced by the addition of the caffeine. If caffeine really does inhibit cell membrane efflux pumps, any reduction in copper (II) ion or catechin-copper(II) combination efflux in this case is not sufficient to have any effect on cell viability within the conditions tested (Couzinet-Mossion *et al.*, 2010).

Since zinc and manganese also have antimicrobial effects which are enhanced by vitamin C (Inoue *et al.*, 1999; Aarestrup and Hasman, 2004; Iinumi and Tsuboi, 2012), both zinc and manganese were tested against *S. aureus* NCTC 06751.

4.5 Effects of combinations of catechin with zinc (II) and manganese (II) ions plus vitamin C against *S. aureus* NCTC 06571

This investigation explored whether zinc showed any antimicrobial activity against *S. aureus* NCTC 06571, and whether zinc would enhance the antimicrobial activity of catechin and copper

(II) sulphate mixtures. It is possible that zinc (II) and copper (II) may compete for the same flavanol binding sites, as both ions have two positive charges. This could thus reduce the antimicrobial activity of copper (II). Figure 4.5 shows that the effects of 214 μM (f.c.) zinc (II) sulphate and its combinations with 214 μM (f.c.) copper (II) sulphate, 214 μM (f.c.) catechin, and 428, 856, and 1284 μM (all f.c.s) vitamin C against *S. aureus* NCTC 06571. Zinc (II) sulphate alone and when combined with a control of 428 μM (f.c.) vitamin C tested alone and further with the addition of 214 μM (f.c.) catechin showed no difference in antimicrobial effect when compared to the control buffer on the cell viability of *S. aureus* NCTC 06571 ($p>0.05$). Additions of zinc (II) sulphate to previously demonstrated effects of copper (II) sulphate alone; equimolar catechin plus copper (II) sulphate; and the same combination plus the progressive molar ratios of vitamin C showed no further effect on *S. aureus* NCTC 06571above that shown by the controls in the absence of the zinc (II) sulphate (all values of $p>0.05$) (Figure 4.4).

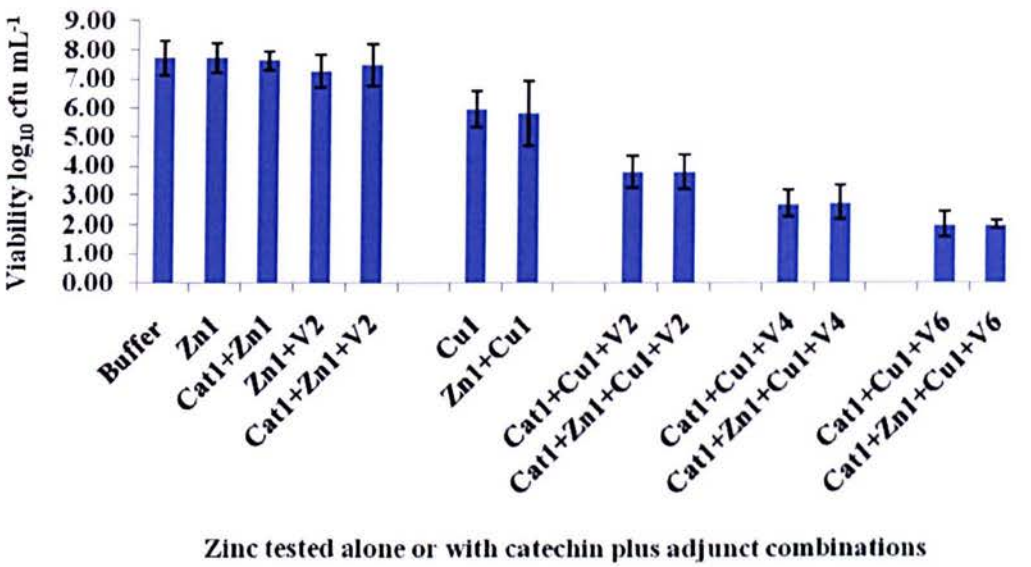


Figure 4.4 Effect of added zinc (II) sulphate on catechin, and copper (II) sulphate with vitamin C combinations against *Staphylococcus aureus* NCTC 06571. Cat = catechin, Cu = copper (II) sulphate, V = vitamin C, Zn = zinc (II) sulphate. Figures for Cat, Cu, V, Zn show final concentration of test reagent(s) in each sample where 1 = 214 μM (f.c.) and 2, 4, and 6 multiples thereof. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. Sample volumes of 500 μL were used to maintain detection of cfus from test samples with V4 and V6 levels of vitamin C. The plates were incubated at 37 °C for 24 hours. Viabilities following exposure to catechin and vitamin C controls were similar to buffer values (Appendix II: Table A Controls, MT3; Table D). Viabilities following exposure to Cat+Cu controls were similar to those seen previously 4.9 (±0.55). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

These results indicated that within the conditions tested zinc (II) sulphate had no effect on the antimicrobial activity of copper (II) sulphate nor on combinations of copper (II) sulphate plus vitamin C and further with added catechin against *S. aureus* NCTC 06751.

Other studies have shown that manganese can show antimicrobial activity against *S. aureus* (Inoue *et al.*, 1999).Therefore, in a shorter, initial experiment to investigate whether there might be any broad antimicrobial effects from manganese against *S. aureus* NCTC 06751 within the test conditions of the present protocol, a comparable experiment to the one described above, investigated whether manganese (II) sulphate could enhance the antimicrobial activity of catechin alone. Further, in an independent investigation of putative adjunct combinations the effect of vitamin C on any manganese (II) sulphate antimicrobial activity, as a putative adjunct, was also compared to that of vitamin C on copper (II) sulphate antimicrobial activity following a similar experiment described above (Table 4.2), although in this case using only two vitamin C concentrations ('medium' and 'high') to investigate any antimicrobial effects against *S. aureus* NCTC 06751.

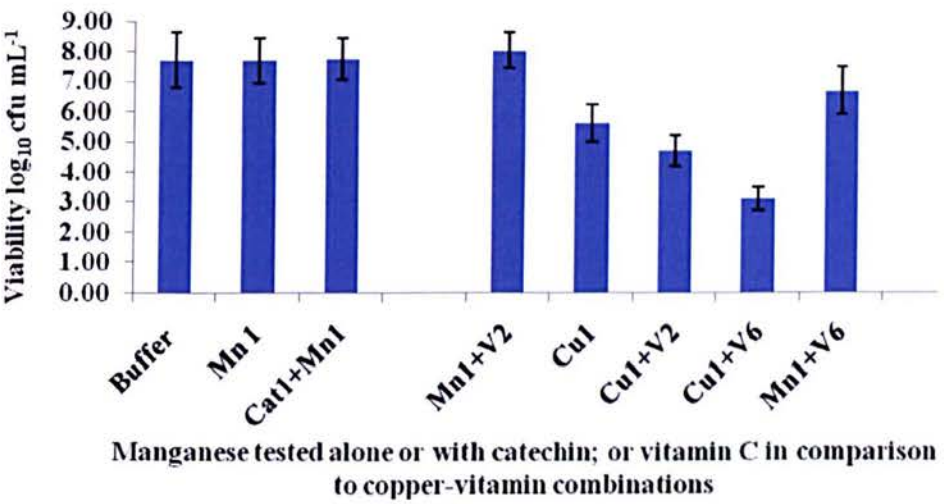


Figure 4.5 Effect of added manganese (II) sulphate added to catechin and to vitamin C compared to copper (II) ion-vitamin C combinations against *Staphylococcus aureus* NCTC 06571. Cu = copper (II) sulphate, V = vitamin C, Mn = manganese (II) sulphate, Cat = catechin,. Figures for Cat, Cu, V, Mn show final concentration of test reagent(s) in each sample where 1 = 214 μM (f.c.) and 2, and 6 multiples thereof. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. Sample volumes of 500 μL were used to maintain detection of cfus from the test samples with Cu 1 and V6 levels of vitamin C. The plates were incubated at 37 °C for 24 hours. Viabilities following exposure to catechin and vitamin C controls were similar to buffer values (Appendix II: Table A, MT3; Table B). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results showed that 214 μM (f.c.) manganese (II) sulphate on its own or in combination with 214 μM (f.c.) catechin or 428, 1284 μM (f.c.s) vitamin C had no significant effect on the viability of *S. aureus* NCTC 06751 when compared to that of the buffer control (all values of $p>0.05$). In the independent adjunct assay experiment 214 μM (f.c.) manganese (II) sulphate plus 428 μM (f.c.) vitamin C showed no antimicrobial activity and thus there was no significant difference when compared to the buffer effects on the viability of *S. aureus* NCTC 06751 ($p>0.05$). In contast, whilst 214 μM (f.c.) copper (II) sulphate tested alone showed a significant effect against *S. aureus*

NCTC 06751 with cell viabilities lower than those seen following exposure to the buffer ($p<0.05$), the addition of 428 μM (f.c.) vitamin C added no further antimicrobial effect ($p>0.05$). At the higher concentration of 1284 μM (f.c.) vitamin C significantly enhanced the antimicrobial effect of copper (II) ($p<0.05$) but not that of manganese (II) ($p>0.05$) (Figure 4.5). Manganese (II) sulphate was not considered to be an adjunct when tested in these conditions although it is possible that the concentration was too low to overcome the defences of *S. aureus* NCTC 06571.

Further to the studies above on the anitimicrobial effects of copper, zinc, and manganese ions, iron ions were then investigated for any effects on the viability of test organisms. Iron is an important nutritient for certain species of bacteria such as *E. coli* whereas other species require little or no iron such as *Lactobacillus* where manganese substitutes for the role of iron in cellular metabolic processes such as respiration. Other species and strains of bacteria may require lesser or greater amounts compared to the two above species *e.g., S. aureus* NCTC 06751. In contrast to its role as an important nutrient, iron, in its ionic forms of iron (II) and iron (III) ions, poses a threat to cell structure and metabolic integrity due to their ability to enter into Fenton chemistry and generate damaging ROS which oxidise diverse structural and metabolic molecular structures.

Furthermore, in investigations by McCarrell *et al.*, 2008, iron (II) was found to enhance the antimictobial activity of the natural phenolic containing pomegranate rind extract (PRE) against both *S. aureus* and *E. coli*. Thus, it is possible that within the conditions of this study iron ions could act either as possible growth agents or antimicrobial agents. In light of the findings in the study by McCarrell *et al.*, 2008, catechin, also a naturally occurring phenolic, was tested with both iron (II) and iron (III) salts against both *S. aureus* and *E.coli* to investigate their effects on cell viability.

4.6 Effects of combinations of equimolar catechin with combined copper (II), iron (II) and iron (III) ions plus vitamin C against *S. aureus* NCTC 06571 and *E. coli* NCTC 14441

Two iron (II) and (III) salts were investigated individually and in combinations with other adjuncts for their effects on catechin against *S. aureus* NCTC 06571 and *E. coli* NCTC 14441(see Appendix II: MT2, MT3, and MT5 for further details of assays). Heat-treated and freshly dissolved iron (II) salt solutions were also investigated to check whether any activity against cells was lost following oxidation to iron (III) salts (see section 4.10).

The results showed that neither freshly made iron (II) sulphate nor iron (III) chloride had no significant antimicrobial activity against either *S. aureus* or *E. coli* when compared to the effects of the buffer on cell viability ($p>0.05$) (Figure 4.6). A catechin-iron (II) combination proved more active than iron (II) against *S. aureus* NCTC 06571 ($p<0.05$) but not against *E. coli* NCTC

14441(p>0.05). Iron (II) added to copper (II) sulphate did not raise antimicrobial activity when compared to copper (II) tested alone against *S. aureus* or *E. coli* (p>0.05). Further, iron (II) combined with copper (II) mixture did not enhance catechin activity against either species (p>0.05). Vitamin C added to catechin plus copper (II), and to catechin-copper (II)-iron (II) mixture did not raise antimicrobial activity either (p>0.05). Subsequent experiments on mechanisms of action investigated the effect of exogenous catalase and EDTA on the activity of equimolar catechin with copper (II) and iron (II) salts, and with additional vitamin C against *S. aureus* NCTC 06751 and *E. coli* NCTC 14441 (see section 4.10) and whether active combinations produced different amounts of hydrogen peroxide (Hoshino *et al.*, 2000).

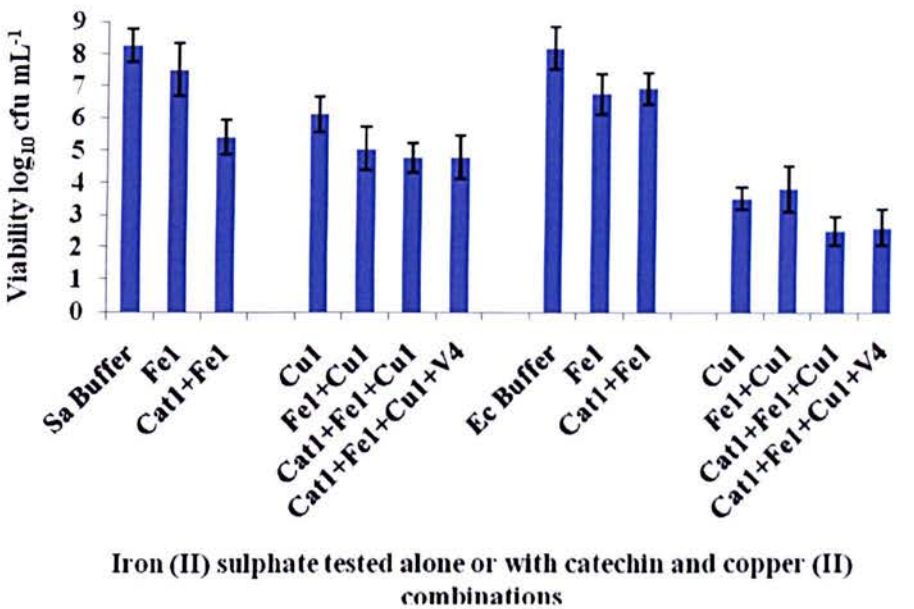


Figure 4.6 Effect of catechin plus copper (II) and iron (II) sulphate plus vitamin C combinations against *Staphylococcus aureus* NCTC 06571 and *Escherichia coli* NCTC 14441. Sa = S. *aureus*, Ec = *E. coli*, Cu = copper (II) sulphate, 1, 4 = 214 μM, 856 μM (f.c.) respectively. Fe = iron (II) sulphate, Cat = catechin, '+' denotes combinations. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. Sample volumes for *E. coli* Fe+Cu, Cat+Fe+Cu and Cat+Fe+Cu+V were increased to 1000 μL to maintain detection of cfus. The plates were incubated at 37 °C for 24 hours. Viabilities following exposure to catechin and vitamin C controls were similar to buffer values (Appendix II: Table A, MT3). Viabilities following exposure to Fe+Cu+V controls were similar to those following exposure to Fe+Cu (Appendix II: Table A, MT2, and MT 6). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The next set of experiments evaluated whether other treatments might enhance the antimicrobial activity of flavanols, as previous studies found that tannin activities can be increased by heating (Takahashi *et al.*, 1995; Asaka *et al.*, 2000; Ikeda *et al.*, 2003; Kim *et al.*, 2011). Heating may promote the formation of more active products via *e.g.*, hydrolysis, oxidation, epimerisation, or polymerisation.

4.7 Effects of heat treatment on catechin solution compared to storage for 14 days at room temperature on activity against *S. aureus* NCTC 06751

In the following experiments the activity of fresh 1000 μM catechin solution against *S. aureus* NCTC 06751 was compared to similar solutions that had been heated at 100 °C for 10 or for 30 minutes. In addition, the effects of storing catechin solution in the dark at room temperature, but exposed to the atmosphere was also tested for antimicrobial activity. The rationale for this experiment was to investigate the effect of atmospheric oxygen on catechin solution on subsequent antimicrobial activity avoiding possible effects of photo-oxidation.

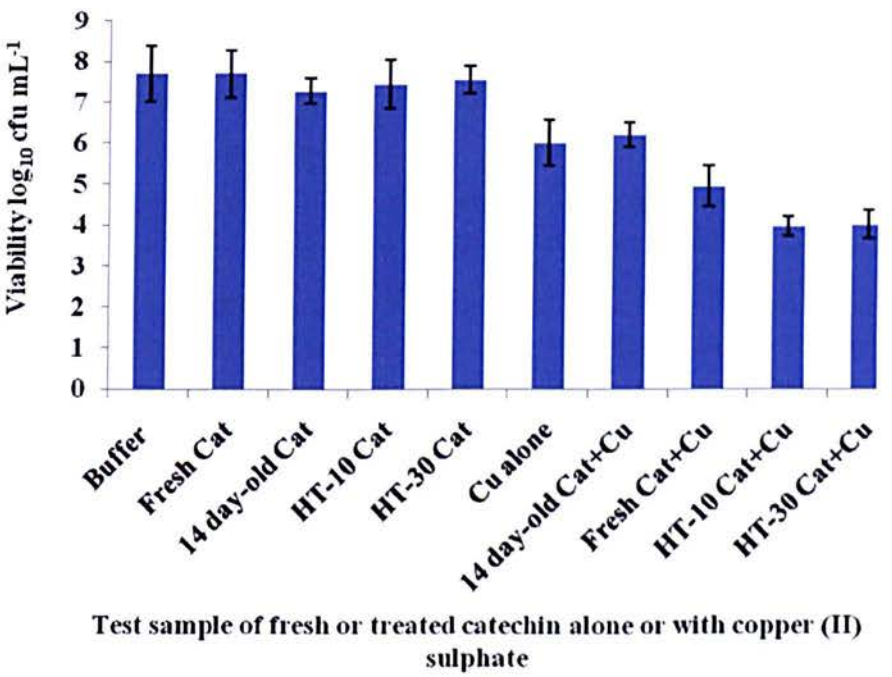


Figure 4.7 Effects of room temperature storage, and of heating catechin solution at 100 °C for 10 or for 30 minutes on its subsequent activity combined with equimolar copper (II) sulphate against *Staphylococcus aureus* NCTC 06751. Cat = 214 μM (f.c.) catechin used at this strength in all assays, 14 day-old catechin = solution stored for 14 days at room temperature in the dark and exposed to the atmosphere, HT-10 and HT-30 denote length of heating time in minutes at 100 °C when exposed to the atmosphere, Cu = 214 μM (f.c.) copper (II) sulphate used at this strength in all assays, '+' denotes combinations within test sample. Heat-treated solutions were restored to original volumes with deionised water before uses. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results showed that there were no significant differences between the viability of *S. aureus* NCTC 06571 following exposure to the buffer and following exposure to the freshly made, the stored and the heat-treated catechin solutions when tested alone ($p>0.05$). Stored catechin plus copper (II) solution reduced *S. aureus* NCTC 06571 viability by *ca.* 1.5 $\log_{10}$ units, this being

similar to and not significantly different from the reduction in viability shown following exposure to by the copper (II) solution control ($p>0.05$). In comparison, the freshly made catechin plus copper (II) combination produced a *ca.* 2 $\log_{10}$ unit reduction in the viability of *S. aureus* NCTC 06571 was significantly different from that following exposure to the buffer ($p>0.05$). Catechin solutions, that were first heat-treated for 10 and 30 minutes, and then combined with equimolar copper (II) sulphate reduced the viability of *S. aureus* NCTC 06571 by a further *ca.* 1 $\log_{10}$ cfu mL^{-1} compared to a fresh catechin with equimolar copper (II) solution, this difference in both cases being significantly different from that of the copper (II) tested alone ($p<0.05$) (Figure 4.7).

Storage of catechin solutions exposed to atmospheric oxygen in the dark at room temperature for 14 days and subsequently combined with fresh copper (II) sulphate and exposed to *S. aureus* NCTC 06571 resulted in no effects on viability when compared to the effects of fresh copper (II) sulphate tested alone against *S. aureus* NCTC 06571 (Figure 4.7). Freshly-made 1000 μM catechin solutions heated at 100 °C for 10 or 30 minutes produced yellowish solutions which were probably due to the formation of new chemical products. Previous studies identified yellow coloured substances as derivatives of catechin and found antimicrobial and antioxidant activity (Hathway and Seakins, 1957; Es-Safi *et al.*, 2003). Proposed structures include xanthylium salts and quinone methides. Xanthylium compounds are formed *e.g.*, in wine from the initial dimerisation of catechin molecules linked by carboxy-methine bridges. Following dehydration and further oxidation, yellowish pigments are formed which have been shown to possess antimicrobial and antioxidant properties (Es-Safi *et al.*, 2000). In other model solutions when subjected to heating, the dimerisation of catechin along with subsequent reaction products can result in the formation of quinone methides and other branched structures (Ferreira, 2012) (see p. 125). Quinone methides are related to quinones where one of the two carbonyl oxygens is replaced by a methylene group. This further polarizes the molecule making it more reactive. Quinone methides can show antimicrobial activity and are produced by certain bacteria such as *Chitinophagia sancti*, a myxobacterium, as an antibiotic (Jansen *et al.*, 2011). Quinones and quinone methides formed from epicatechin and copper (II) ions in solution have also been previously shown to possess anti-cancer activity (Azam *et al.*, 2004).

To investigate the possibility of raising the antimicrobial activity of catechin further, when subsequently combined with copper (II) sulphate, than that achieved by heating catechin solution at 100 °C, autoclaving catechin solution at 121 °C for 15 minutes, prior to combinination with copper (II) sulphate, was also tested. It was hypothesised that higher temperatures would generate more catechin derivatives and thus result in a higher antimicrobial activity when subsequently combined with copper (II) sulphate. Previous investigations showed that heat treatment of tannic acid could be used to produce gallic acid and pyrogallol which have higher antimicrobial activity

(Kim *et al.*, 2011). Autoclaving is of interest because if antimicrobial activity is retained by this typical sterilisation procedure, such mixtures could be used as a sterile treatment in applied settings.

In these experiments, the lower catechin concentration (500 μM, (107 μM f.c)) was used prior to autoclaving. This allowed for any autoclaved-enhanced activity to be easily detectable. At the same time copper (II) sulphate concentrations were increased to 4.8 mM (2174 μM f.c) to ensure that copper (II) would not be a limiting factor in any complex formation. A similar procedure was used previously when assaying different tea flavanols in the presence of excess copper (II) sulphate (see section 3.13).

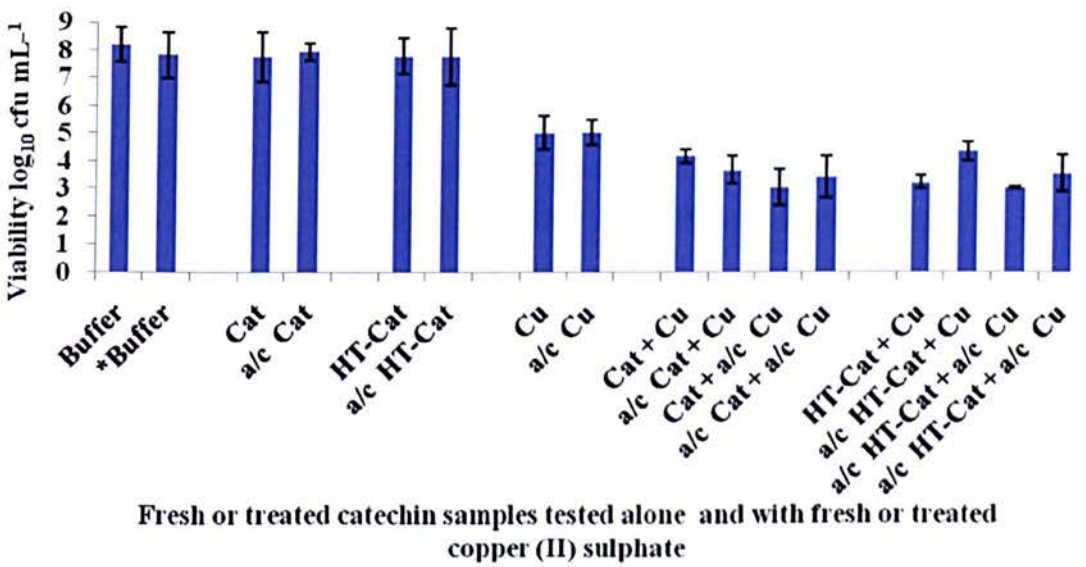


Figure 4.8 The effect of autoclaving fresh and boiled catechin, and copper (II) sulphate combinations on the viability of *Staphylococcus aureus* NCTC 06751. All catechin concentrations were 107 μM (f.c), a/c = test substance autoclaved at 121°C for 15 minutes, Cat = added catechin, HT-Cat = catechin previously heated at 100 °C for 10 minutes, Cu = added 4.8 mM copper (II) sulphate, '+' = mixture of antimicrobial components. Mixtures shown within brackets were combined prior to autoclaving. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. Sample volumes for catechin plus copper (II) combinations were increased to 1000 μL to maintain detection of cfus. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results show that autoclaved fresh, and previously heat-treated catechin, showed similar antimicrobial activities to both non-autoclaved fresh catechin and heat-treated catechin when tested alone and compared to buffer controls (all $p>0.05$). No significant differences were found between fresh and treated catechin solutions when combined with copper (II) sulphate and tested against *S.*

aureus NCTC 06751 (both $p>0.05$). Autoclaving catechin, or catechin heat-treated at 100 °C, combined together with copper (II) sulphate, resulted in the appearance of small amounts of dark brown precipitate seen at the bottom of the sample tube. Such precipitate was only seen in the tubes where catechin or boiled catechin were autoclaved together with copper (II) sulphate. This procedure reduced the antimicrobial activity of the combination to that shown by the buffer ($p>0.05$) and it is likely that an insoluble copper (II)-phenolic complex was formed which reduced the amount of active soluble components (data not shown). For the controls no significant differences were seen between the antimicrobial activities of fresh, compared to autoclaved copper (II) sulphate solution ($p>0.05$) In conclusion autoclaving at 121 °C for 15 minutes does not enhance the antimicrobial activity of catechin beyond the level seen by heating the solution at 100 °C for 10 or 30 minutes against *S. aureus* NCTC 06751. The next set of experiments were designed to investigate mechanisms of antimicrobial action. These included kinetic studies of catechin with adjuncts, the role of copper (II) and iron (II) ions in combination treatments, and the generation of bactericidal compounds.

4.8 Mechanisms of action I: Investigations of interactions of freshly dissolved and heat-treated catechin with copper (II) sulphate using pH, crystallisation, solubility, and UV-vis absorbance

Previous work showed that the pH of aqueous copper (II) sulphate solution was reduced upon the addition of white tea extracts (see section 3.12). Copper (II) can form a chelate complex with tea polyphenols which releases protons from phenolic groups and thus lowers the pH. In test samples each containing a fixed concentration of 214 μM catechin (f.c), increasing molar ratios of between 53.5 and 1712 μM (f.c) copper (II) sulphate were added which resulted in mixtures with molar ratios of catechin 1 : copper (II) sulphate 0.25 – 8.0. The measured pH values of the different ratio mixtures were each significantly different from the 214 μM catechin (f.c) control and from the 214 μM copper (II) sulphate control in all cases (all $p<0.05$) (Figure 4.9).

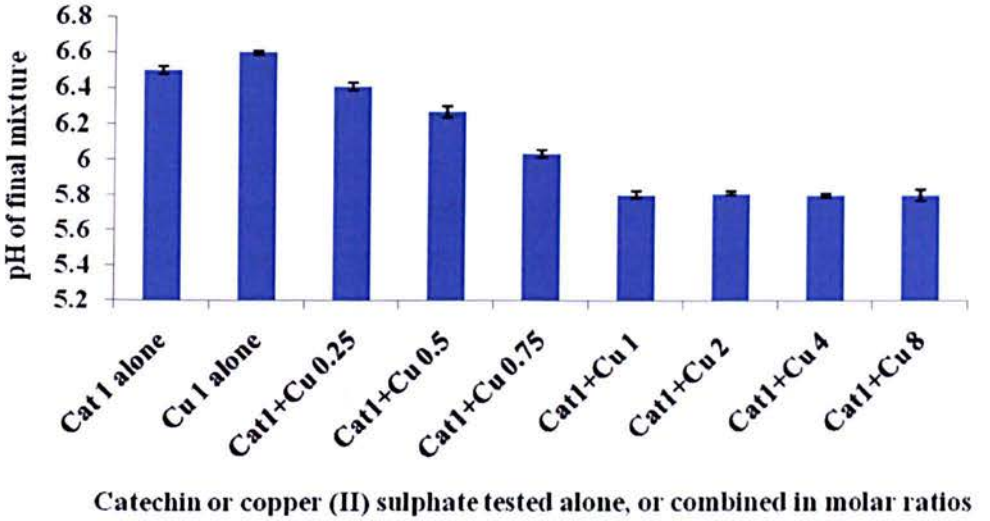


Figure 4.9 Effects of 214 μM catechin (final concentration) combined with increasing molar ratios of copper (II) sulphate on pH of final mixture. Cat = catechin, Cu = copper (II) sulphate, 1 = 214 μM (final concentration) present in each sample, '+' Cu denotes added copper (II) sulphate with figures expressing proportionate amounts of copper (II) sulphate present. Volumes and concentrations of solutions were used as in corresponding suspension assays with Ringer's solution replacing inoculum. Mixtures were allowed to stand in the dark for 10 minutes before testing. Samples were tested at *ca.* 22 °C. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results show that acidity increased with higher copper (II) sulphate concentrations and was maximal with a 1:1 catechin : copper (II) sulphate ratio (Figure 4.9). The increasing effect of adding molar ratios of 0.25, 0.5, and 0.75 copper (II) sulphate to 1.0 of catechin on its pH could be explained by a progressive loss of protons from catechin OH groups caused by positively-charged copper (II) ions displacing protons and in so doing forming an acidic catechin–copper (II) complex. To investigate the copper (II)-catechin complex formation further, crystallisation was attempted to ascertain whether certain molar ratios of these substances would result in crystals.

Previous investigations have shown that some plant products form biologically active complexes with transition metal ions *in vivo* and that these products are less active in the absence of the metal ions (Murata *et al.*, 1991, Shiono *et al.*, 2008). Such complexes are typically formed from stoichiometric ratios. Components in solution may spontaneously form crystals if conditions are suitable, *e.g.* concentration, solvent, pH, temperature, evaporation speed. Crystals can be examined by X-ray crystallography to determine the proportions and positions of added transition metal ions relative to the host molecule atoms with a view to suggesting a possible mechanism of action based on proximity of active functional groups acting via the metal ions to target substances.

If such a crystal can only be grown at a particular component ratio, it could suggest an optimal concentration for use in suspension assays against test organisms. Therefore, catechin and copper (II) sulphate were combined in different molar ratios between 1 : 0.75 and 1 : 1.5 and stored in the

dark under a range of conditions in order to facilitate crystal formation. Solution pH, extent of precipitation and flocculation, colour of precipitate, shape, pattern and colour of *protocrystals and other deposits were noted during the solvent evaporation process. However, none of these conditions resulted in catechin-copper (II) crystals. Instead, co-joined or separate crystals of catechin and copper (II) sulphate were formed. These findings do not support the hypothesis that an optimal antimicrobial ratio would necessarily lead to a crystallising complex.

Additional crystallisation tests at several molar ratios between 1: 0.75 and 1: 1.5 were conducted in polar and non-polar solvents at room temperature in the dark. During solvent evaporation, polar solutions changed from a very pale, clear yellowish green solution to a turbid, progressively darker brown colour which was probably due to chemical changes to the catechin. However, rapid evaporation produced microcrystals instead, particularly at the 1: 0.66 ratio of catechin : copper (II) sulphate. Two crystal types were identified. Firstly, separate crystals of catechin, seen as colourless needle like structures about 0.25 – 1.00 mm in length which grew in parallel or star-shaped clusters, or separately. Secondly, ortho-rhombic crystals of copper (II) sulphate crystals formed separately. However, in alkaline, polar solvents copper (II) sulphate microcrystals grew in a regular juxtaposed arrays down each side of single catechin crystals which could be compared to the arrangement of barbs joined to the rachis of a bird's feather, or the fletching on an arrow (Figure 4.10)

*Protocrystals are defined as the first microscopic crystals to appear during the evaporation of solvent, but which are not yet grown to macroscopic size.

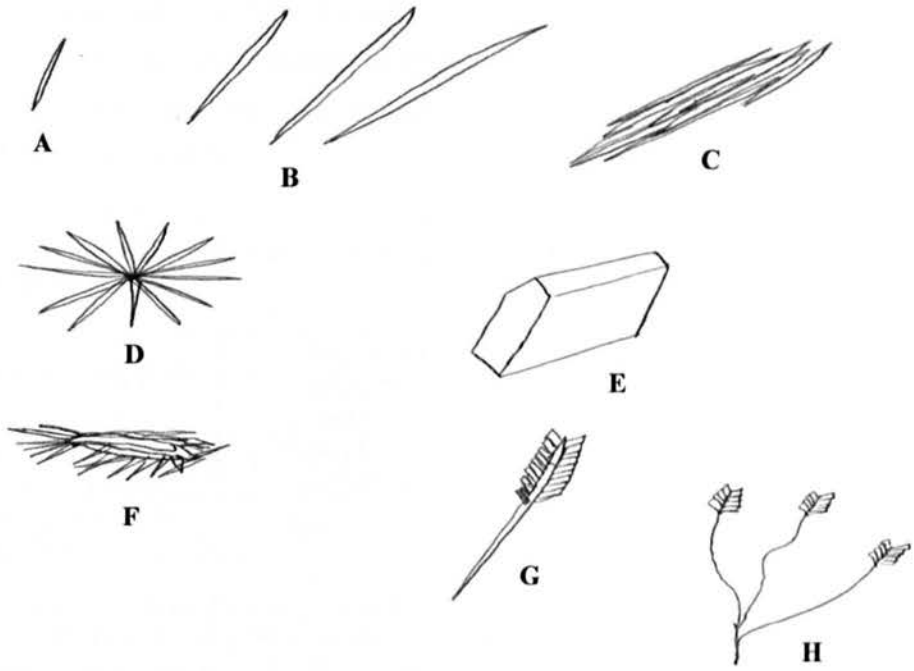


Figure 4.10 Appearance of crystals of catechin and copper (II) sulphate grown alone and in combination. Drawings made from light microscope images seen at x180 magnification. A = Small protocrystal of catechin, B = larger single crystals of catechin, C = catechin crystals aligned in a parallel cluster, D = Star-shaped cluster of catechin crystals, E = Ortho-rhombic crystal of copper (II) sulphate, F = mat of congealed substance derived from catechin? with protruding catechin crystals in non-alkaline conditions pH, G = single catechin crystal with feather-shaped array of copper (II) sulphate crystals, H = Branched catechin crystal array with terminal clusters of feather arrays of copper (II) sulphate crystals. G and H were both grown in alkaline conditions.

Other experiments measured the effect of added copper (II) ions on catechin solubility. In aqueous solution catechin loses a proton and becomes an anionic molecular ion carrying a monovalent negative charge. This catechin ion is thus then attracted to positively-charged cations such as that added large copper ions, which carry a divalent positive charge, and upon binding form a complex carrying a single positive charge (Weber, 1988). A complex of catechin-copper (II) ions could interact with more water molecules than uncomplexed free catechin molecules and thus increase the water solubility of the flavanol.

In this experiment catechin powder and copper (II) sulphate pentahydrate salt were mixed in equimolar quantities of constituent catechin and copper (II) sulphate molecules (1M quantities being 249.70g of $CuSO_4.5H_20$, and 290.26g of catechin, catechin making up therefore 54% of the total weight). Water was then added slowly and gently shaken, until all contents were dissolved. The volume of water required to do this was then noted as well as the temperature of the solution. From the weight of the mixture and the volume of water required to dissolve it, its solubility could be calculated in milligrammes per litre. Since the catechin-copper (II) sulphate dry mixture was

made up of equimolar amounts of catechin and copper (II), half of the final solubility figure calculated corresponds to the solubility of the catechin in the presence of copper (II) sulphate. The solubility of the catechin could then be compared to standard solubility values for catechin and copper (II) sulphate at 25 °C.

Table 4.4 Water solubilities of catechin in the absence and presence of added copper (II) sulphate at 25 °C.

Mixture investigated	Solubility of mixture (mg mL^{-1})	Solubility of catechin (mg mL^{-1})	Increase in solubility of catechin
Catechin alone	1.6 (±0.34)	1.6 (±0.34)	none
Cu (II) SO_4 alone	325 (±5.8)	N/A	N/A
***Catechin + Cu (II) SO_4**	5.8 (±1.3)	3.1	X 2 (approx.)

Key: Cu (II) SO_4 = copper (II) sulphate pentahydrate. *Catechin powder and copper (II) sulphate salt made up to equimolar amounts using 54% catechin and 46% copper (II) sulphate pentahydrate by weight thus correponding to their respective molecular weights: 290.26 and 249.70. N/A = not applicable, as no catechin was present in test sample. Each value given in the table is a mean based on 10 separate solubility investigations for each substance.

The results showed that catechin and copper (II) sulphate had solubilities of *ca.* 1.6 and *ca.* 325 mg mL^{-1}, respectively when tested at 25 °C. However, an equimolar mixture of catechin with copper (II) sulphate showed a solubility of *ca.* 5.8 mg mL^{-1}. This represents a catechin solubility within the mixture of *ca.* 3.2 mg mL^{-1}, which is a two-fold increase in solubility compared to catechin alone (Table 4.4). This observation adds further support to the theory that catechin forms a stable, soluble complex with copper (II) ions in aqueous solution (Weber, 1988).

Catechin and copper (II) sulphate interactions were also investigated using UV-vis spectroscopy. This technique can detect and measure the absorption of coloured compound such as those formed with transition metal (II) ions. The experiment below investigated the absorption of catechin and heat-treated catechin with different ratios of copper (II) sulphate. Similar samples were also tested in the presence of EDTA (ethylenediamine tetra-acetic acid) in order to test the metal chelating effect. The results are shown below (Figure 4.11).

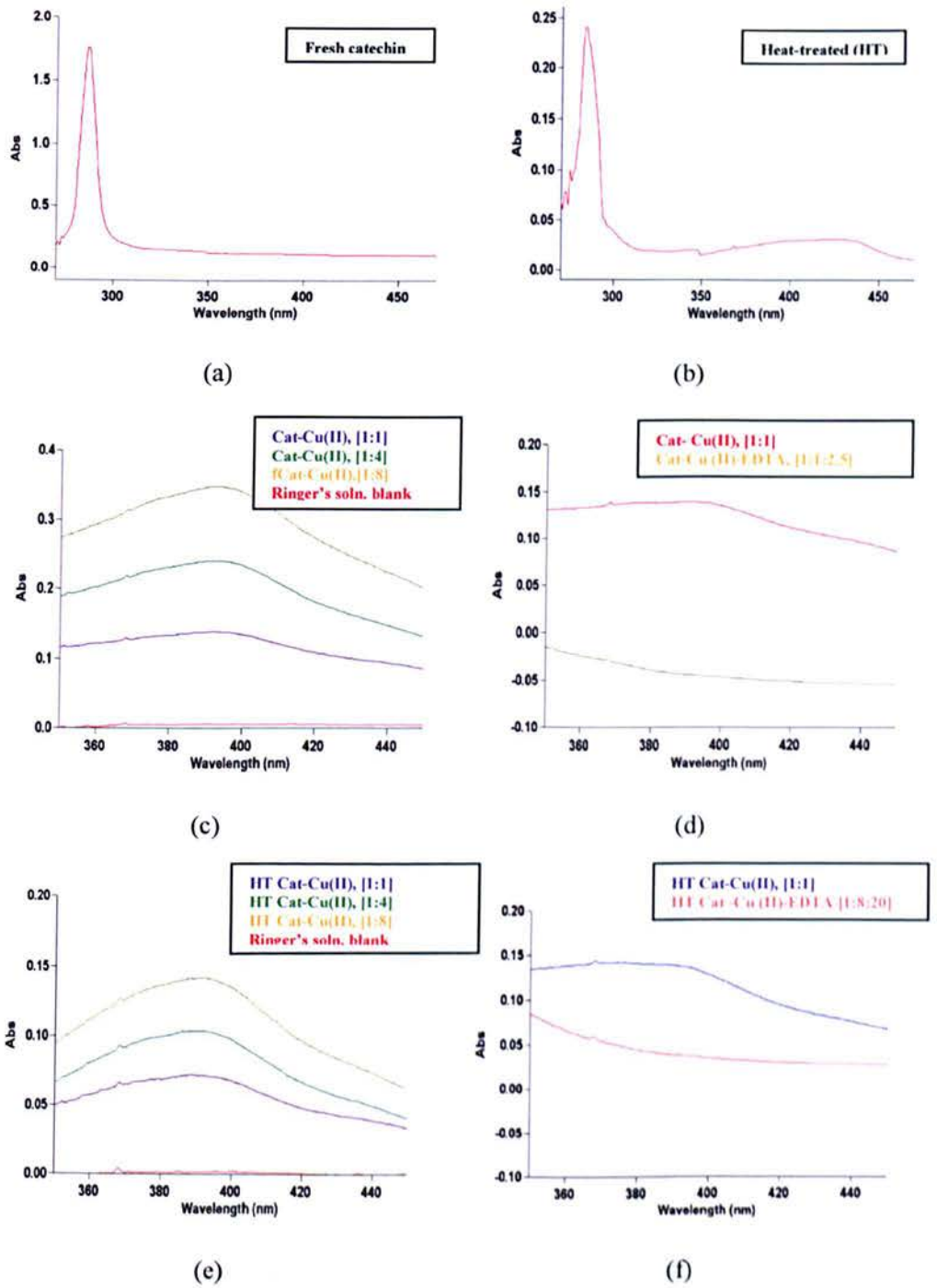


Figure 4.11 UV-vis spectra of fresh and heat-treated catechin alone and with combined copper (II) sulphate and with EDTA. All catechins tested at 214 μM (final concentration), (a) catechin, (b) catechin heated for 10 minutes at 100 °C, (c) catechin with copper (II) sulphate added in proportional concentrations, from bottom up: 214 μM (X1), 856 μM (x 4), 1712 μM (x 8), (d) fresh catechin combined with equimolar Cu (II) SO_4, lower trace the same with 535 μM EDTA, (e) heat-treated catechin (HT) combined with 214 μM, 856 μM, 1712 μM copper (II) sulphate, (f) heat-treated catechin with 1712 μM Cu (II) SO_4 and lower trace, the same with 4280 μM EDTA (x8). Mixtures were allowed to stand in the dark for 10 minutes before testing. Finally all samples were diluted to 25% of suspension assay level for reliable readings to be made. NB. Difference in scaling between (a) and (b), and (c) and (d) (Absorbance traces down to 200 nm and up to 900 nm not shown). Results were based on triplicate tests from 3 separate experiments.

Fresh and heat-treated catechin (214 μM solutions) showed similar profiles but the freshly dissolved solution gave a greater absorption at *ca.* 290 nm. Heat-treated catechin had an additional small peak at *ca.* 425 nm (see also section 4.6) (Hathway and Seakins, 1957; Es-Safi *et al.*, 2003), which is typical of yellow-brownish solutions (Bandara, 2012). Ringer's solution as well as deionised water had little or no absorbance (not shown). Peak heights at *ca.* 390 nm were positively linked to copper (II) sulphate concentrations whether added to fresh or heat-treated catechin solutions. It can be seen that freshly dissolved catechin with copper (II) sulphate gave higher absorbances than the heat-treated combinations. Such differences in absorption may in part be due to pH changes caused by the different concentrations of added copper (II) sulphate (Fisher and Naughton, 2004; Zhu *et al.*, 2008; Pirker *et al.*, 2012). Addition of EDTA completely removed the copper (II) sulphate effects. It is likely given the evidence from EDTA and from catalase studies (see section 4.9) that this chromophore may be linked to the generation of hydrogen peroxide (Hoshino *et al.*, 2000; Arakawa *et al.*, 2004).

Mechanisms of action of fresh and heat-treated catechin solution combined with copper (II) ions against test species were then investigated further by additional studies of putative complex formation using pH studies (Figure 4.12).

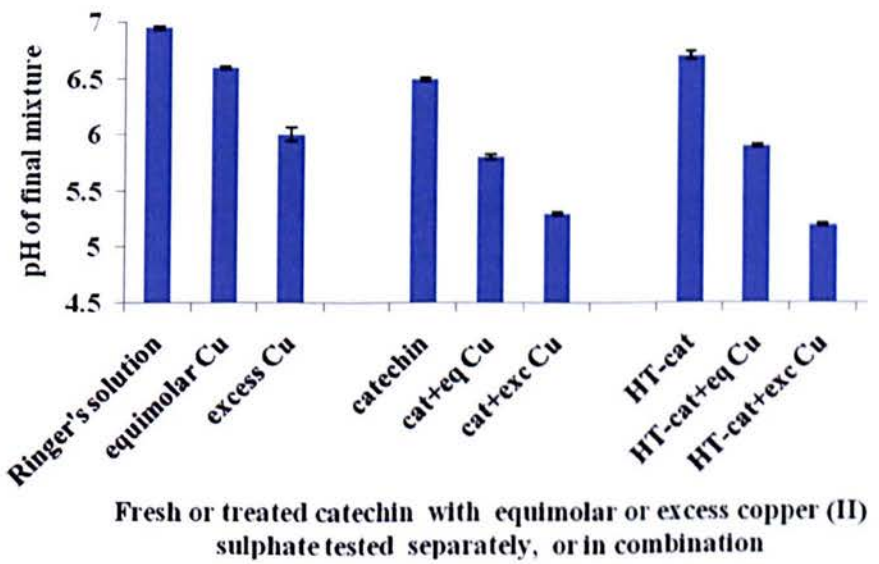


Figure 4.12 pH measurements of fresh and heat-treated catechin solutions combined with different concentrations of copper (II) sulphate. Cat, catechin = 214 μM (final concentration) catechin, HT-cat = 214 μM (f.c.) catechin heated at 100 °C for 10 minutes, equimolar, eq Cu = 214 μM (f.c.) copper (II) sulphate, excess, exc Cu = added 4.8 mM (2175 μM f.c.) copper (II) sulphate. Mixtures were allowed to stand in the dark for 10 minutes before testing. Samples were tested at *ca.* 22 °C. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results showed that an effect of heating catechin solution at 100 °C for 10 minutes caused the pH value of the catechin solution (214 μM f.c.) to increase by *ca.* 0.2 units which was significant

($p<0.05$) (Figure 4.12). This may be caused by the conversion of catechin to new products, which are less acidic, incorporating labile protons from the surrounding solution. Freshly-made as well as heat-treated catechin combined with either equimolar (214 μM f.c.) or excess copper (II) sulphate solution (2175 μM f.c.) both showed a similar significant increase in acidity of *ca.* 0.75 pH unit compared to that shown by the copper (II) sulphate solutions tested alone ($p<0.05$). This suggests a similar degree of proton loss from catechin and catechin derivatives in each sample. It also suggests that copper (II) ions were not a limiting factor since a similar magnitude was detected with the equimolar copper (II) sample as with the excess copper (II) sample.

To conclude, these studies showed that catechin reacts with copper (II) sulphate and releases protons in a dose dependent manner up to a ratio of 1:1. This ratio is also favourable in terms of antimicrobial activity against *S. aureus* NCTC 06571. Solubility studies and UV-vis measurements indicated that new chemical entities are formed, which absorb strongly at 390 nm.

4.9 Mechanisms of action II: Comparison of antimicrobial activity of fresh and heat-treated catechin compared to freshly-made epigallocatechin gallate (EGCG) in excess copper (II) sulphate on the viability of *S. aureus* NCTC 06751

Using kinetic studies comparisons were also made between the antimicrobial activity of fresh catechin and heat-treated catechin solutions on *S. aureus* NCTC 06751 viability. The rates of action against *S. aureus* NCTC 06751 were compared in the presence of excess copper (II) sulphate to ensure that bactericidal rates were not limited. In addition, the rates of change due to the fresh and heat-treated catechin solutions were also compared to EGCG solutions. EGCG is a well known tea flavanol and has been extensively researched. It is especially known for its highly efficacious action effects in numerous applications (Sun *et al.*, 2011; Singh *et al.*, 2011; Shigemune *et al.*, 2012).

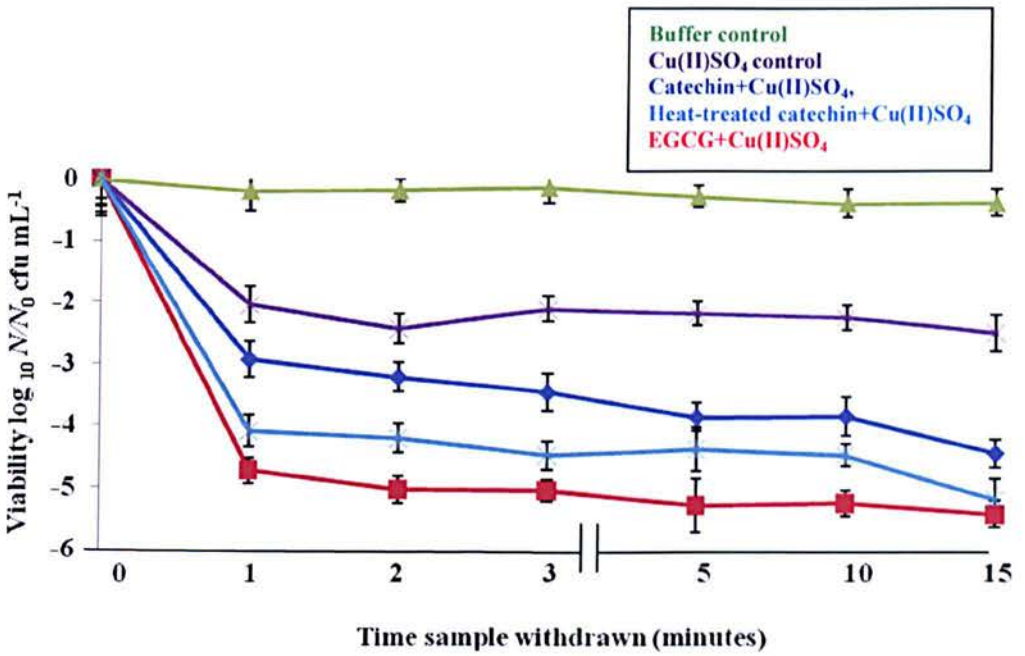


Figure 4.13 Reduction in viability of *S. aureus* NCTC 06571 on exposure to fresh and to heat-treated catechin solutions compared to epigallocatechin gallate each combined with excess copper (II) sulphate. Green line = Buffer, purple line = 4.8 mM added $Cu(II)SO_4$ tested alone, blue line = fresh 214 μM (final concentration) catechin with $Cu(II)SO_4$, sky blue line = catechin heated at 100 °C for 10 minutes plus $Cu(II)SO_4$, red line = 1 μM (f.c.) EGCG plus $Cu(II)SO_4$ (Concentrations of catechin and EGCG each gave similar levels of antimicrobial activity in independent assays with added 4.8 mM copper (II) sulphate, to reach a point where following exposure resulting viability of *S. aureus* was *ca.* 3 $\log_{10}$ cfu mL^{-1} after 30 minutes exposure). Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum and returned to the dark except when samples of 150 μL were removed for viable counts at the times indicated. Viability is expressed as a fraction of the numbers of cfus (N) seen at a time interval divided by the original number of buffer cfus at time zero (N_0). The plates were incubated at 37 °C for 24 hours. Viabilities seen following exposure to catechin controls were similar to buffer values sampled at 30 minutes (Appendix II: Table G). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The rates of *S. aureus* NCTC 06751 viability reduction seen following exposure to test solutions were as follows: EGCG + Cu (II) > heat-treated catechin + Cu (II) > fresh catechin + Cu (II) > Cu (II). All the rates were significantly different from each other after 1 minute's exposure and at all greater exposure periods tested (all $p<0.05$) (Figure 4.13). In each case most of the effect occurred within the first minute. Interestingly, heat-treated catechin had a faster effect than fresh catechin. Subsequent experiments investigated the viability reduction of *S. aureus* NCTC 06751 after 10 and 40 seconds of exposure from similar mixtures under the same conditions.

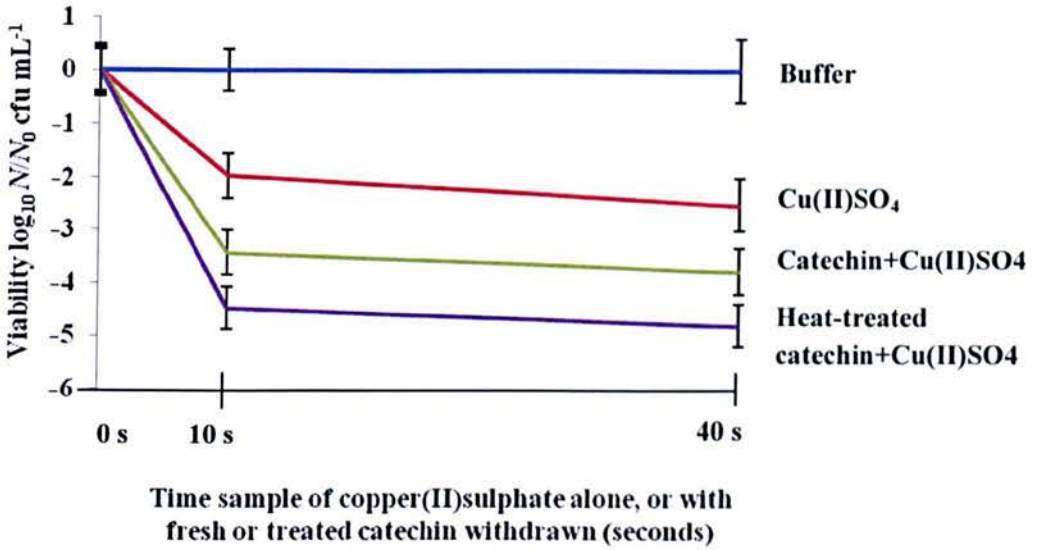


Figure 4.14 Reduction in viability of *Staphylococcus aureus* NCTC 06751 during timed exposure to fresh and to heat-treated catechins each combined with excess copper (II) sulphate. Cu (II) SO_4 = added 4.8 mM copper (II) sulphate. 214 μM (final concentration) freshly-made catechin as well as catechin heat-treated at 100 °C for 10 minutes was added to Cu (II) SO_4 in the two lower samples shown above . Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. Viability is expressed as a fraction of the numbers of cfus (N) seen at a time interval divided by the original number of buffer cfus at time zero (N_0). The plates were incubated at 37 °C for 24 hours. Viabilities following exposure to fresh and heat-treated catechin controls were similar to exposure to buffer sampled at 30 minutes (Appendix II: Table G). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The rates of *S. aureus* NCTC 06751 viability reduction seen following exposure to test solutions were again as follows: heat-treated catechin + Cu (II) > fresh catechin + Cu (II) > Cu (II) (Figure 4.14). Most of the effect occurred within the first 10 seconds (Figure 4.14). All the rates were significantly different from each other when measured following 10 seconds and following 40 seconds of exposure (all $p<0.05$). These results demonstrate that the antimicrobial effect of these samples was very rapid indeed.

In conclusion, the above rate experiments indicated that following exposure to excess copper (II) sulphate the heat-treated catechin produced a faster loss in *S. aureus* NCTC 06571 viability than did the fresh catechin combination with copper (II) sulphate. This greater effect may be due to a greater rate of action by the heat-treated catechin such as direct lysis of the cell envelope or the generation of harmful substances such as ROS; or the facilitation of copper (II) ion attachment to cell structures such as the cell membrane (Hoshino *et al.*, 2000; Lebedev *et al.*, 2005). The next set of experiments tested whether catechin plus copper (II) solutions would generate substances such as ROS that are harmful towards bacteria. Initial tests used exogenous catalase to investigate whether samples were generating hydrogen peroxide, a substance known to be used against

bacteria by competing bacteria, plants and animals (Tsan *et al.*, 1977; Delbes-Paus *et al.*, 2010; Tkalec *et al.*, 2012).

4.10 Mechanisms of action III: Investigation of the effect of exogenous catalase and EDTA on the activity of equimolar catechin with copper (II) and iron (II) salts, and with additional vitamin C against *S. aureus* NCTC 06751 and *E. coli* NCTC 14441

Results in Chapter 3 (see section 3.11) suggested that tea components plus copper (II) ions generated hydrogen peroxide as reported by Hoshino *et al.* (2000). Some authors have reported that catalase could negate this effect (Kimura *et al.*, 1998; Hoshino *et al.*, 2000). Catalase was therefore, added at different concentrations to copper (II) sulphate and tested against *S. aureus* NCTC 06571 and *E. coli* NCTC 14441. UV-vis spectroscopy was used to monitor whether catalase complexed copper (II) ions reducing their bioavailability and removing their harmful effects in this way rather than the breakdown of any harmful hydrogen peroxide. In addition, catalase was also tested on its own in the absence of added copper (II) sulphate for any direct effects on the viability of *S. aureus* NCTC 06571 and *E. coli* NCTC 14441.

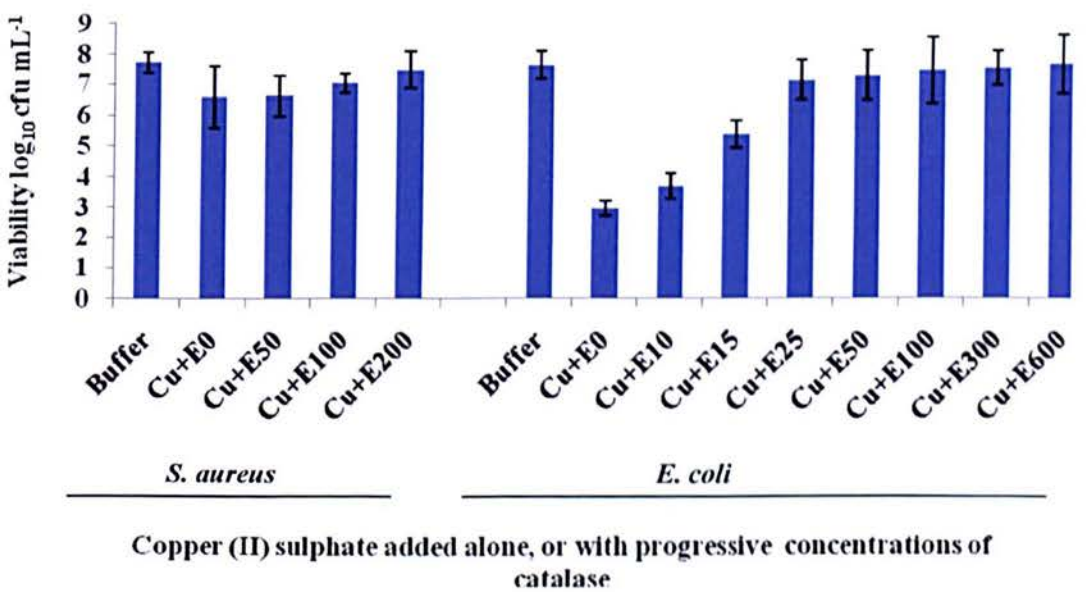


Figure 4.15 Effect of catalase added to copper (II) sulphate on the viability of *Staphylococcus aureus* NCTC 06571 and *Escherichia coli* NCTC 14441. Cu = 214 μM (final concentration) copper (II) sulphate, E + number = added catalase μg mL^{-1} (f.c.). Samples were incubated in the dark at room temperature for 30 minutes, the sample volume of *E. coli* + E0 (no added enzyme) was increased to 100 μL to maintain cfu detection. The plates were incubated at 37 °C for 24 hours. Viabilities following exposure to catechin controls were similar to buffer exposure values (Appendix II: Table A). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

Following exposures to combinations of copper (II) sulphate with different concentrations of added catalase little effect was observed on the viability of *S. aureus* NCTC 06751 when compared to viability effects of the buffer tested alone (all $p>0.05$). However, the effects of added catalase were

more pronounced on *E. coli* NCTC 14441 between 10 – 25 μg mL^{-1}. Samples containing 25 μg mL^{-1} or greater concentrations of added catalase did not show significant effects on viability when compared to the buffer control (all $p>0.05$) (Figure 4.15). The effects on *E. coli* followed a dose dependent manner. It appears that copper (II) sulphate produced sufficient H_2O_2 to have greater effects on the viability of *E. coli* than on *S. aureus*. This may reflect the different defence capacities of these two types of bacteria.

The UV-vis spectroscopy experiments showed that catalase at 50 and 500 μg mL^{-1} and the addition with 214 μM (f.c.) copper (II) sulphate made little difference to the catalase absorption spectrum and no new peaks appeared (Figure 4.16). This suggested that catalase did not form any complexes with copper (II) ions and thus it did not affect the capacity of copper (II) ions to generate H_2O_2. Catalase had no direct antimicrobial activity against *S. aureus* and *E. coli* compared to buffer controls (data not shown).

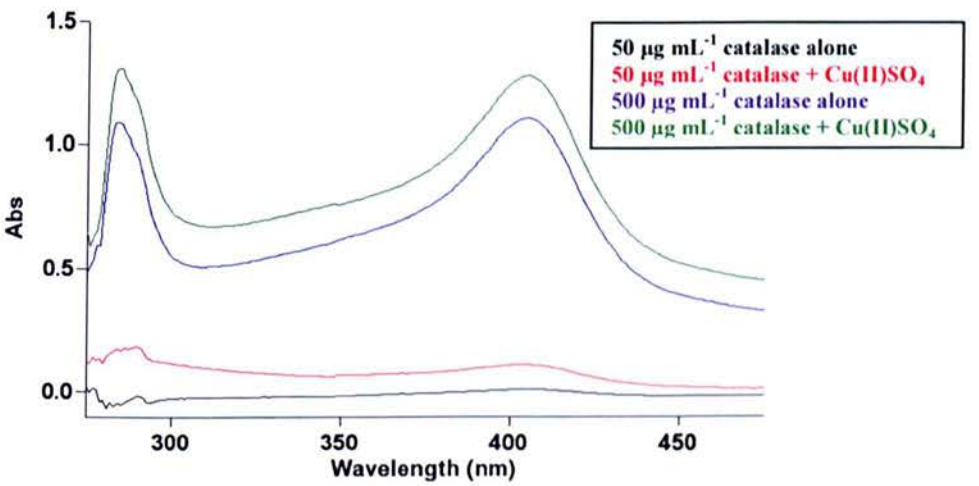


Figure 4.16 UV-vis absorbance spectra of bovine liver catalase alone and combined with added copper (II) sulphate. From bottom upwards: black line = 50 μg mL^{-1} (final concentration) catalase alone, red line = 50 μg mL^{-1} (f.c.) catalase plus 214 μM (f.c.) copper (II) sulphate, lilac line = 500 μg mL^{-1} (f.c.) catalase alone, green line = 500 μg mL^{-1} (f.c.) catalase plus 214 μM (f.c.) copper (II) sulphate. Assay volumes amd concentrations were similar to those used in suspension assays with inoculum replaced by Ringer's solution. Absorbance traces below 275 nm and above 475 nm not shown. Results were based on triplicate tests from 3 separate experiments.

In this investigation H_2O_2 and other reactive oxygen species (ROS) could have been produced by re-oxidation of copper (I) following reduction of the added copper (II) within Fenton-type chemistry. Following exposures to *S. aureus* NCTC 06571 a complete rather than a partial restoration of buffer values in the presence of copper (II) ion samples with added catalase was unexpected since other mechanisms besides the manufacture of hydrogen peroxide, such as

interference with cell membrane physiology, have been suggested for the antimicrobial action of copper (II) ions (Lebedev *et al.*, 2005; Braud *et al.*, 2010).

Since copper (II) sulphate can apparently generate H_2O_2 to levels beyond which the cells of *S. aureus* NCTC 06571 and *E. coli* NCTC 14441 can adequately defend themselves, it seemed reasonable to investigate the possibility that other test mixtures containing added transition metal (II) ions might also produce their bactericidal effects by generating H_2O_2. Catalase was therefore added to mixtures of catechin-copper (II) sulphate-vitamin C that had been shown to have antimicrobial activity against *S. aureus* NCTC 06571and *E. coli* NCTC 14441 (Figure 4.17).

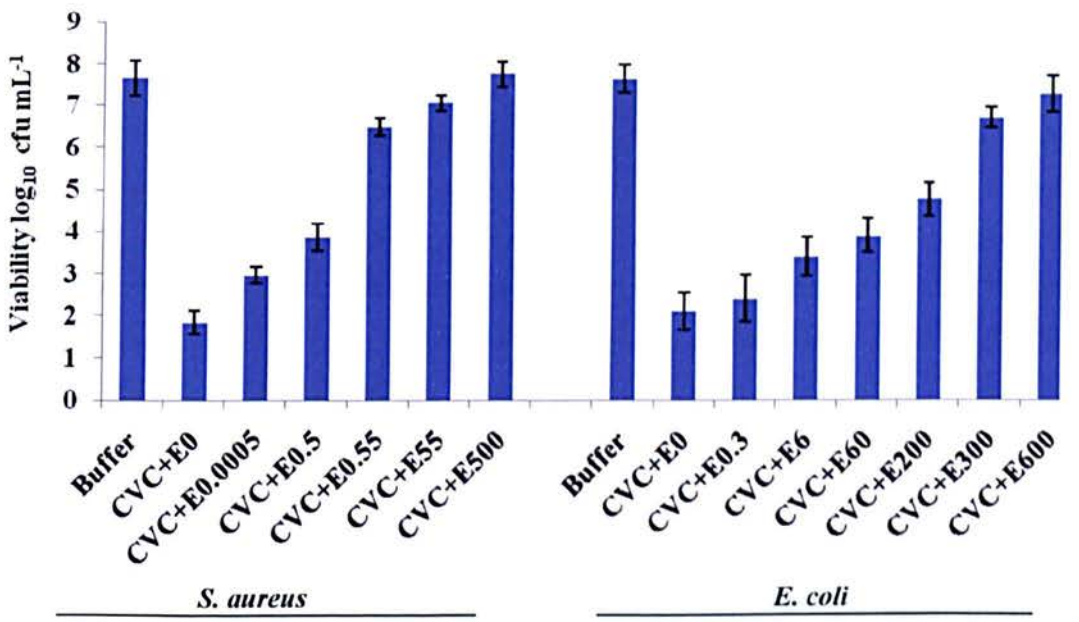


Figure 4.17 Effect of added catalase on catechin, copper (II) sulphate and vitamin C against *Staphylococcus aureus* NCTC 06571 and *Escherichia coli* NCTC 14441. CVC = 214 μM (f.c.) catechin plus 214 μM (f.c.) copper (II) sulphate plus 856 μM (f.c.) Vitamin C, '+' denotes combination, E + number = level of added catalase μg mL^{-1} (f.c.). Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. Sample volumes of 500 μL were used to maintain detection in two lowest concentrations of enzyme with each species. The plates were incubated at 37°C for 24 hours. Viabilities following exposure to catechin and vitamin C controls were similar to values following buffer exposure (Appendix II: Table A, controls, MT3). Copper (II) controls were similar to those previously shown, *S. aureus* = 5.92 (±0.89), *E. coli* = 3.09 (± 0.56) log_{10} cfu mL^{-1}. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results showed that with *S. aureus* test samples of less than 55 μg mL^{-1} showed significantly lower viability values when compared to the buffer viability value (all $p<0.05$). Samples containing

55 μg mL^{-1} or more of added catalase did not show significantly different viabilities compared to the buffer control (both $p>0.05$). The results showed that with *E. coli* there were significant differences seen between the buffer value compared to test samples of less than 300 μg mL^{-1} (all $p<0.05$). Samples containing 300 μg mL^{-1} or more of added catalase did not show significance compared to the buffer control (both $p>0.05$). These results showed that catalase eliminated the antimicrobial effect of the catechin-copper(II)-vitamin C mixture. Importantly, the extent of this reduction was directly related to the catalase concentration. A similar result was seen with *E. coli* NCTC 14441 but in this case, requiring higher concentrations of catalase to reduce the antimicrobial effects of the mixture to comparable levels seen with *S. aureus* NCTC 06571 (Figure 4.17).

These results clearly show that higher catalase concentrations negated the inhibitory effect of the catechin-copper (II) sulphate-vitamin C mixture against *S. aureus* NCTC 06571 and *E. coli* NCTC 14441 (Figure 4.17). It is possible that catechin and vitamin C are both able to reduce copper (II) ions to a greater extent and consequently generate H_2O_2 in greater quantities. Fairly high catalase concentrations 300 – 600 μg mL^{-1} are required to destroy any H_2O_2 that is generated by catechin or vitamin C and copper (II) ions. These findings raised the question whether less environmentally harmful transition metal (II) ions could be substituted for copper (II) in order to develop disinfective products based on flavanols.

Previous experiments (see section 4.6, Figure 4.6) investigated the effects of catechin with freshly-made iron (II) sulphate solution alone on the viabilities of *S. aureus* NCTC 06571 and *E. coli* NCTC 14441. Catechin was enhanced by iron (II) against *S. aureus* NCTC 06571 but not against *E. coli* NCTC 14441. Iron (II) controls were not active against *S. aureus* NCTC 06571 or *E. coli* NCTC 14441. These results suggested the possibility that catechin plus fresh iron (II) generated H_2O_2 via Fenton chemistry which was active against *S. aureus* NCTC 06571. To investigate this further the effect of oxidation of iron (II) to iron (III) on any H_2O_2 production and consequently on antimicrobial activity was tested against *S. aureus* NCTC 06571 and *E. coli* NCTC 14441. Iron (II) sulphate and iron (III) chloride solutions were either heated to 100 °C for 10 minutes or autoclaved for 15 minutes in the presence of atmospheric oxygen.

The results showed that heat-treated iron (II) and (III) salt solutions showed similar antimicrobial activities towards *S. aureus* NCTC 06571 and *E. coli* NCTC 14441 as freshly-made solutions and buffer controls with no significant differences seen between them (all $p>0.05$). These results indicate that any hydrogen peroxide generated by both iron salts was probably adequately defended by endogenous catalase from both bacteria species (Table 4.5a).

Next, EDTA was employed to test whether chelation of iron (II) and copper (II) sulphate would prevent hydrogen peroxide generation. A similar 214 μM (f.c.) copper (II) sulphate and iron (II) sulphate solution as used previously (section 4.5, Figure 4.6) and EDTA at 2.5 x the final concentration following Brown *et al.*, (1998) was added and tested against *S. aureus* NCTC 06571 and *E. coli* NCTC 14441. The results showed that combined iron (II) and copper (II) salt solutions alone and with the further addition of catechin showed similar antimicrobial activities towards *S. aureus* NCTC 06571 and *E. coli* NCTC 14441 as buffer and EDTA controls with no significant differences seen between them (all $p>0.05$).

Comparing the results shown in Table 4.5 a, b, and Figure 4.6 only free, un-chelated iron (II) and copper (II) ions produced antimicrobial activities greater than buffer controls or samples with EDTA. It is likely that the chelation of these ions prevented successive oxido-reduction reactions as required in Fenton chemistry to generate hydrogen peroxide. To further elucidate the mechanism of action of catechin acting on transition metal ions, EDTA was used to interfere with the bioavailability of the ions within UV-vis absorption spectra which indicated EDTA prevented the formation of catechin-copper(II) complexes (section 4.7, Figure 4.10).

Extending the above findings, catalase was added to 214 μM (f.c.) copper (II) sulphate and iron (II) sulphate solutions and tested against *S. aureus* NCTC 06571 and *E. coli* NCTC 14441. This showed that catalase reversed the metal(II) ion effects against both species by removing hydrogen peroxide with no significant differences seen between the antimicrobial activities of the test samples compared to those of the buffer controls (all $p>0.05$) (Table 4.5c). However, significant differences were seen between the results shown for the iron(II) and copper(II) salt combinations tested alone and with added catechin shown in Figure 4.6 and those shown in Table 4.5c with added 600 μg mL^{-1} catalase (all $p<0.05$).

Table 4.5 The antimicrobial effects of iron (II/III) ions alone and with added copper (II) ions, plus the effects of adding the metal ion chelator EDTA and catalases to remove any generated hydrogen peroxide on the viabilities of *Staphylococcus aureus* NCTC 06751 and *Escherichia coli* NCTC 14441.

Heat treatment:

Sample tested		Viabilities of tested species (log_{10} cfu mL^{-1})	
		S. aureus NCTC 06751	*E. coli* NCTC 14441
	Buffer	7.66 (± 0.52)	7.08 (± 0.21)
Fe(II) alone:	fresh	7.50 (± 0.82)	6.75 (± 0.62)
	heat-treated at 100 °C	6.99 (± 0.37)	7.44 (± 0.62)
	autoclaved at 121 °C	7.52 (± 0.55)	7.91 (± 0.76)
Fe(III) alone:	fresh	7.33 (± 0.48)	7.87 (± 0.61)
	heat-treated at 100 °C	7.49 (± 0.46)	7.77 (± 0.49)
	autoclaved at 121 °C	7.99 (± 0.44)	8.03 (± 0.41)
Catechin plus Fe(II):	fresh	5.38 (± 0.53)	6.93 (± 0.50)
	heat-treated at 100 °C	7.51 (± 0.50)	8.05 (± 0.38)
	autoclaved at 121 °C	7.92 (± 0.42)	7.89 (± 0.61)
Catechin plus Fe(III):	fresh	7.84 (± 0.68)	7.71 (± 0.48)
	heat-treated at 100 °C	7.22 (± 0.50)	8.64 (± 0.48)
	autoclaved at 121 °C	7.23 (± 0.67)	8.53 (± 0.65)

(b) EDTA chelation of metal ions:

Sample tested	*S. aureus* NCTC 06751	*E. coli* NCTC 14441
Buffer	7.66 (± 0.52)	7.08 (± 0.21)
EDTA alone	8.23 (± 0.41)	7.21 (± 0.51)
Fe(II)+Cu(II)+EDTA	8.02 (± 0.33)	7.12 (± 0.49)
Catechin+Fe(II)+Cu(II)+EDTA	7.97 (± 0.36)	7.50 (± 0.42)

(c) Catalase inactivation of hydrogen peroxide:

Sample tested	*S. aureus* NCTC 06751	*E. coli* NCTC 14441
Buffer	7.66 (± 0.52)	7.08 (± 0.21)
Fe(II)+Cu(II)+catalase	8.16 (± 0.70)	6.60 (± 0.44)
Catechin+Fe(II)+Cu(II)+catalase	8.02 (± 0.35)	7.77 (± 0.53)
Catechin+Fe(II)+catalase	7.23 (± 0.42)	8.01 (± 0.53)

Key: Buffer = lambda buffer adjusted to pH 7.2 as a control; Fe(II) = iron(II) sulphate, Fe(III) = iron(III) chloride; 214 μM (final concentration) Fe(II) & 214 μM (f.c.) Fe(III) alone samples were either freshly-made, or freshly-made and then heated to 100 °C for 10 minutes, or freshly-made and then autoclaved at 121 °C for 15 minutes; Cu(II) = 214 μM (f.c.) copper(II) sulphate; EDTA = 1070 μM (f.c.) ethylenediamine tetra-acetic acid; catechin = 214 μM (f.c.); Catalase = 600 μg mL^{-1} (f.c.) added just prior to inoculum. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The next set of experiments evaluated other flavanols for their hydrogen peroxide production in the presence of copper (II) sulphate. Certain flavanol isomers of catechin not previously investigated

viz. ent-catechin, and *ent*-epicatechin may show greater antimicrobial activity than catechin with added copper (II) sulphate. Other investigations have shown that variations in the molecular structure of catechin and other flavanols can result in enhanced antimicrobial activities (Park and Cho, 2010; Anderson *et al.*, 2011).

4.11 Mechanisms of action IV: Comparison of the antimicrobial activity and hydrogen peroxide generation of fresh, heat-treated, and oxidised catechin to other tea catechins when combined with equimolar copper (II) sulphate against *S. aureus* NCTC 06751

The following flavanol isomers were tested: catechin, *ent*-catechin, epicatechin, *ent*-epicatchin. Each was combined with equimolar 214 μM (f.c.) copper (II) sulphate and tested against *S. aureus* NCTC 06751 (Figure 4.18). In addition, the isomers were compared to catechin which was heated to 100 °C for 10 minutes as previously with catechin (see section 4.7, Figure 4.8) or exposed to the atmosphere and stored at room temperature for 14 days.

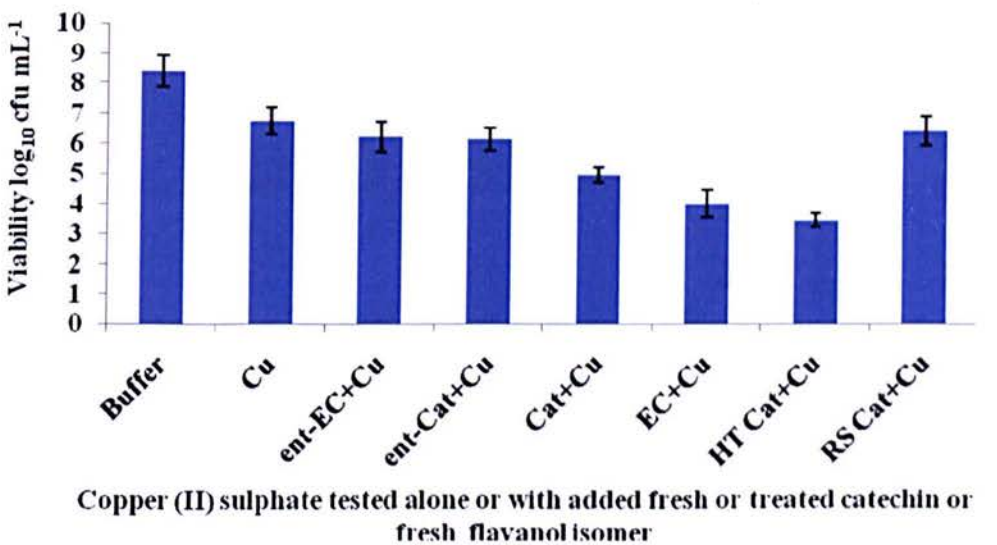


Figure 4.18 The effect of combined heat-treated catechin and copper (II) sulphate solution compared to fresh catechin and epicatechin with their enantiomers as well as stored catechin solution against *Staphylococcus aureus* NCTC 06571. Cu = 214 μM (final concentration), copper (II) sulphate, ent-EC = enantiomer of epicatechin, ent-Cat = enantiomer of catechin, Cat = catechin, EC = epicatechin, HT Cat = catechin heat-treated at 100 °C for 10 minutes, RS Cat = room stored catechin solution kept in the dark for 14 days at *ca.* 22 °C. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. Sample volumes for HT Cat+Cu combinations were increased to 100 μL to maintain detection of cfus. The plates were incubated at 37 °C for 24 hours. Viabilities following exposure to fresh, heat-treated, and room stored catechin controls were similar to values following buffer exposure (not shown). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results showed that the antimicrobial activities of all test samples containing copper (II) sulphate were significantly different from that shown by the buffer (all $p<0.05$). However, the fresh and heat-treated catechin as well as the flavanol isomer controls showed no significant differences

when compared to the buffer control (all $p>0.05$). The following order of antimicrobial activity was found against *S. aureus* NCTC 06571: heat-treated catechin+Cu > epicatechin+Cu > catechin+Cu > *ent*-catechin+Cu = *ent*-epicatechin+Cu > room stored catechin+Cu = Cu control (Figure 4.18). The mean value of each catechin control was significantly different from the corresponding catechin plus copper (II) sulphate combined sample ($p<0.05$) except for the room stored 14 day old catechin ($p>0.05$). Similar combinations of the same concentrations were also tested for hydrogen peroxide generation by substituting Ringer's solution for inoculum. H_2O_2 was measured with a modified colormetric method developed by Lespinas *et al.* (1989), was used which employed UV-vis spectroscopy at 500 nm and gave a linear curve (see section 2.2.15).

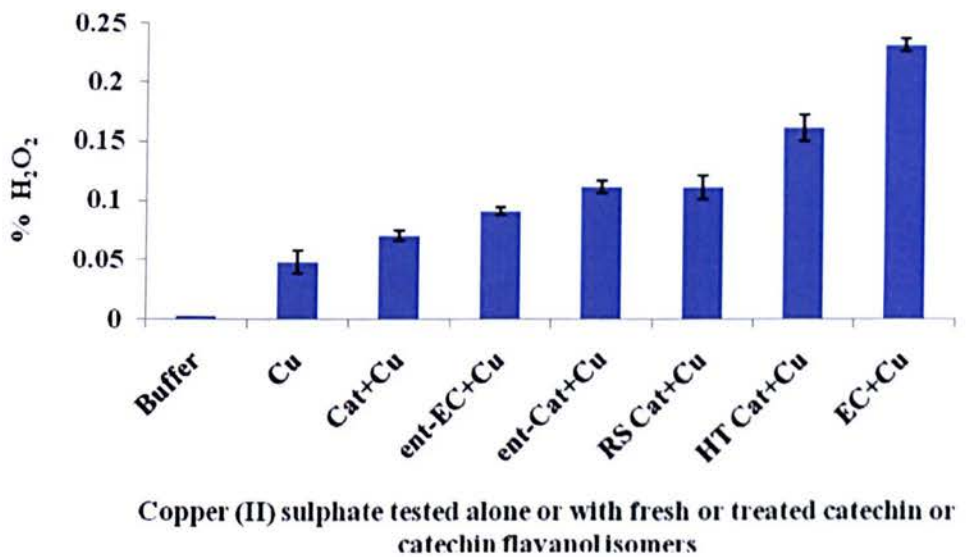


Figure 4.19 The generation of hydrogen peroxide by combined stored as well as heat-treated catechin compared to fresh catechin and epicatechin with their enantiomers each combined with equimolar copper (II) sulphate. Cu = 214 μM (final concentration) copper (II) sulphate tested alone, Cat = catechin, '+' denotes combination, ent-EC = enantiomer of epicatechin , ent-Cat = enantiomer of catechin, RS Cat = catechin solution stored in the dark at room temperature for 14 days exposed to the atmosphere, HT Cat = catechin solution heated at 100 °C for 10 minutes, EC = epicatechin. Samples were read for H_2O_2 generation after 15 minutes following initial mixing following a modified method of Lespinas *et al.* (1989). Buffer and fresh, treated, and stored catechin and flavanol isomers tested without added copper (II) showed no H_2O_2 generation (data not shown). Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

These solutions generated H_2O_2 in the following order: epicatechin+Cu > heat-treated catechin+Cu > room stored catechin+Cu > *ent*-catechin+Cu > *ent*-epicatechin+Cu > catechin+Cu > Cu tested alone (Figure 4.19). Control buffer and flavanols, on their own, did not produce H_2O_2. Figure 4.19 indicates that these flavanols in the presence of copper (II) sulphate generated different amounts of H_2O_2. These differences can possibly explain the inhibitory effects seen in the previous experiment if allowance is made for the different phenolic test substances acting with copper (II) ions to

possess varying abilities to interact with the cell envelope thus intensifying, more or less, the action of the generated hydrogen peroxide (Figure 4.18). This is discussed further in sections, 6.1.12, and 6.2 (iv).

Electron microscopy was used to check for any visible effects of antimicrobial mixtures on bacterial cells as other studies had shown that some flavanols caused cell aggregation, difficulties with cell separation during division, cell distortions and changes in cell wall thickness (Stapleton *et al.*, 2007, Shah *et al.*, 2008; Bernal *et al.*, 2009; Bikels-Goshen *et al.*, 2010).

4.12 Microscopic examination of effects of mixtures on cells using light and electron microscopy

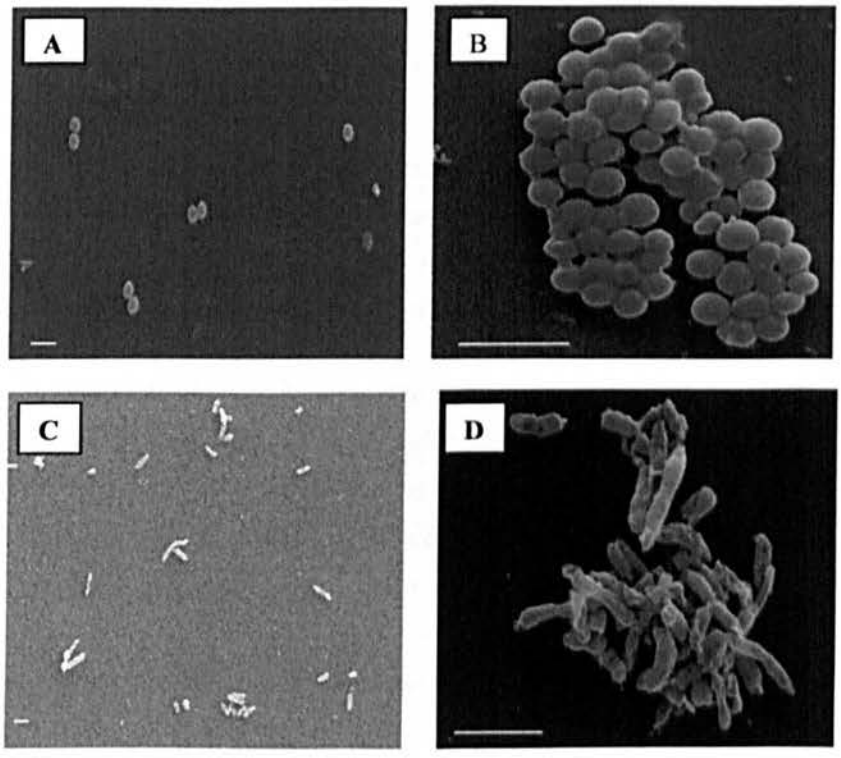


Figure 4.20 Scanning electron micrographs showing aggregation in *Staphylococcus aureus* NCTC 06571 and *Escherichia coli* NCTC 14441 cells following exposure to catechin plus copper (II) sulphate and vitamin C. (A) control cells of *S. aureus* following 30 minute incubation in lambda buffer and washed with phosphate buffered saline (x 4 300); (B) *S. aureus* cells following similar length of exposure to catechin-copper (II) ions-vitamin C (x 22 000); (C) control cells of *E. coli* following exposure to lambda buffer and PBS (x 1800); and (D) *E. coli* cells treated with catechin-copper(II) ions-vitamin C mixture (x 17 500) exposed to similar solutions as *S. aureus* . Scale bar in each image shows a distance of 1 μM.

Preliminary examination by oil immersion light microscopy showed that *S. aureus* NCTC 06571 and *E. coli* NCTC 14441 cells had retained their typical Gram staining characteristics compared to buffer control cells after exposure to antimicrobial samples. *S. aureus* NCTC 06571 appeared a deep purple colour (Gram-positive), and *E. coli* NCTC 14441 a reddish- pink colour (Gram-negative).

Scanning electron microscopy revealed effects of a combination of catechin-copper (II) ions–vitamin C on cells causing aggregation (Figure 4.20). The images show that cells treated with equimolar 214 μM (f.c.) catechin plus copper (II) ions plus 856 μM (f.c.) vitamin C and washed afterwards with phosphate buffered saline were aggregated into clumps. In comparison, cells exposed to lambda buffer and washed with PBS showed normal dispersion. This finding suggests that the combination of catechin plus copper (II) ions and vitamin C was responsible for the observed cell aggregation.

4.13 Summary and conclusions

Catechin proved to be a weak antimicrobial tea flavanol. However, when combined with copper (II) and iron (II) salts catechin activity was enhanced against *S. aureus* NCTC 06571. In other experiments *Ps. aeruginosa* NCTC 950, *P. mirabilis* NCTC 7827, and *E. coli* NCTC 14441 were also susceptible to catechin-copper (II) combinations.

The combinations with the metal ions lead to the formation of a chemical complex which generated hydrogen peroxide. The effects of the complex caused a loss in *S. aureus* NCTC 06571 viability. The stereochemistries of catechin and other principal tea flavan-3-ols were related to the antimicrobial activities and were ranked in the following order when combined with copper (II) ions: epicatechin > catechin > *ent*-catechin = *ent*-epicatechin.

Heat-treatment of catechin solutions increased the antimicrobial activity of catechin in the presence of copper (II) ions, possibly by the production of quinone methides which appear to be more active than fresh catechin. Such quinone methides could penetrate the cell envelope to a greater degree than catechin. If so, this may be due to the molecular structure of the quinone methides which conceivably contain a side chain which could act in a similar way to that of gallated flavanols, such as ECG and EGCG, by penetrating the cell membrane (Shrader *et al.*, 2003; Azam *et al.*, 2004).

Autoclaving either fresh catechin or previously heat-treated catechin did not affect antimicrobial activities unless autoclaved together with copper (II) ions which resulted in reduced antimicrobial activities. Oxidised catechin solution at room temperature combined with copper (II) ions showed the same activities as copper (II) controls.

Attempts to raise the activity of catechin against two unresponsive species, *S. aureus* NCTC 06571 and *E. coli* NCTC 14441, with iron (II) sulphate, iron (III) chloride, and vitamin C, showed that only iron (II) enhanced activity against *S. aureus* NCTC 06571, but not *E. coli* NCTC 14441; neither iron (III) nor an iron (II) and copper (II) mixture enhanced the activity against either species. Vitamin C enhanced copper (II) containing combinations against both species in the absence of catechin or of iron (II). Either catechin or vitamin C enhanced copper (II) ion activities, but a combination of these two ions did not produce a cumulative effect.

Catalase or EDTA added to active samples removed effects on bacterial viabilities suggesting that activity depended on H_2O_2 production generated by free transition metal (II) ions. H_2O_2 generation by catechin-copper (II) mixtures or by copper (II) ions on their own accounted, at least in part, for the principal effect of bacterial growth inhibition following 30 minute exposures as well as the antimicrobial effect of catechin-iron (II) against *S. aureus* NCTC 06571. Catechin solution, autoclaved or heat-treated at 100 °C for 10 or 30 minutes, had the same antimicrobial activity when combined with copper (II) sulphate against *S. aureus* NCTC 06751 which was higher than non heat-treated controls. Catechins and derivatives produced by heat treatment combined with copper (II) sulphate produced more rapid effects against *S. aureus* compared to copper (II) alone suggesting both increased hydrogen peroxide production and possible localisation effects at the cell membrane.

Hydrogen peroxide generation from polyphenol mixtures containing copper (II) ions with no added vitamin C was in the following order: epicatechin > heat-treated catechin > oxidised catechin = *ent*-catechin > *ent*-epicatechin > catechin > copper (II) controls. This order could be related to the antimicrobial activities of the mixtures except in the cases of the oxidised catechin and the two enantiomers where despite higher H_2O_2 generation than catechin, lower activities against *S. aureus* NCTC 06571 were found. This lower activity can be explained by a possible lower interaction and/or binding to the cell envelope of *S. aureus* NCTC 06571.

This research indicated that some plant compounds such as catechin may have their weak antimicrobial properties enhanced by additives, such as copper (II) ions to make them more active as antimicrobial agents. The findings from this study also suggested that catechin could partially replace copper (II) or iron (II) salts in formulations against *Ps. aeruginosa* NCTC 950 and *S. aureus* NCTC 06571. This finding is of practical importance as these microbes have recently attracted much scientific, public and media interest. Moreover, there may also be some economic and environmental benefits, as catechin may provide a means for lowering copper (II) or iron (II) contents in some crop protection products.The enhancement of catechin with adjuncts may also find application in disinfective or other antimicrobial formulations against *Staphylococcus aureus*

and possibly also *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus mirabilis*. This warrants further study and will require investigations into formulations that maintain antimicrobial activity over time, both in dry form and as an aqueous solution.

Chapter 5: Studies on the effects of the age and temperature of stored solutions of white tea extract, catechin, and adjuncts on antimicrobial activity

5.1 Introduction

The storage of pure as well as mixed substances for a wide range of uses, including as antimicrobials, is desirable for reasons of convenience and economy. However, a stored substance is subject to chemical and physical changes by processes that can lead to the degradation of the original substance as it changes into new chemical products that lack the desired activity of the original substance. Commonplace examples include bicarbonate of soda with various uses such as a food raising agent, an antacid, and as a weak disinfectant (Berry *et al.*, 2011). This substance loses activity as it becomes slowly converted into sodium carbonate during storage. Another example is iron (II) sulphate which has various uses such as a nutritional supplement, a moss killer and as a fungicide (Chowdary *et al.*, 2011). Iron (II) sulphate once in aqueous solution on contact with atmospheric oxygen quickly oxidises into iron (III) sulphate losing its activity. Such chemical changes which lead to losses in substance activity can occur within minutes as seen with freshly-made solutions of vitamin C or adrenaline, or may be very slow as with the oxidation of copper or iron metals occurring over a period of many years (Feng *et al.*, 2009; Hoellein and Holzgrabe, 2012).

In order to preserve the activity of substances consideration needs to be given to the conditions in which they are stored. Factors such as rises in ambient temperature, moisture, light and oxygen levels are known to reduce the stability of stored substances affecting their activities. Such factors are more pronounced when substances are stored in solution where solvents can facilitate degradative processes such as oxidation (Oms-Oliu *et al.*, 2012). Microbiological contamination may also be an additional problem in some cases such as with aqueous solutions of phenol and formaldehyde which can be colonised by halophilic bacteria and *Pseudomonas aeruginosa* respectively since these are relatively tolerant to these disinfectants (Hingst *et al.*, 1987; Peyton *et al.*, 2002).

Conversely, substances with low antimicrobial activity may increase their activity over time due to slow formation of more active products (Dignum *et al.*, 2001). Other changes may lead to decreased activity against some species of bacteria whereas no changes in activity are seen against other species (Bosch *et al.*, 2000). Changes to products caused by spontaneous degradation under specific conditions that lead to an increase in antimicrobial activity, can in some cases be increased to the scale of an industrial process such as the curing of vanilla pods to produce free aromatic vanillin. In another example various tea storage processes including the 'fermentation' of GT to produce BT lead to an increase in activity against certain types of microbes (Chou *et al.*, 1999, Kim

et al., 2011). New and existing formulations may be stored in certain conditions to maintain, or even increase, antimicrobial activity. Investigations are needed in each case to discover what conditions are optimal. Light, temperature, type of atmosphere and moisture levels are often investigated since these are known to affect stability and thus activity as mentioned above. Storage temperatures have been known for a long time to negatively affect the activity of some antimicrobial mixtures (Landry *et al.*, 2009). Other investigations have found that raising storage temperatures can have positive effects on activity (Kim *et al.*, 2011). Other types of approaches to maintain activity have included the addition of adjuvants such as iodide to stabilise the activity of antimicrobial mixtures as well as optimising activity by adjusting pH (Bosch *et al.*, 2000; Wood *et al.*, 2011). Some components may need to be stored separately and only mixed immediately prior to use in order to avoid loss of activity. For example, Fehling's reagent, used for detecting the presence of reducing sugars, is stored as two separate solutions, A and B which when mixed become active but unstable.

Mixtures stored in dry form are generally more stable than those in solution. In practice, dry mixtures also have the advantage of being easier and less costly to transport.

Physiological parameters of target organisms can be tested to assess the activity of stored fungal and antimicrobial substances (Tabib *et al.*, 1982). Often investigations evaluate the bacteriostatic and bactericidal activity of substances over time against target species of bacteria. In previous chapters catechin combined with adjuncts was shown in some cases to be effective against particular species of bacteria. In principle, such a mixture could be made up as a formulation to use in a variety of contexts such as on certain surfaces, in aquaculture, and in agricultural or veterinary settings.

The question posed here is which of the storage methods tested preserved overall antimicrobial activity best and whether any preserved degree of activity was specific to the species of bacteria tested: *Staphylococcus aureus* NCTC 06571, *Pseudomonas aeruginosa* NCTC 950, *Proteus mirabilis* NCTC 7827, and *Escherichia coli* NCTC 14441. In addition whether any of the preservation methods would result in an increase in antimicrobial activity compared to a freshly made mixture. Further, at what point during storage would activity begin to be lost or increased.

5.2 Effects of long term storage (3 years) of freshly made white tea extract at -20° C on the activity of *S. aureus* NCTC 06571

The question posed here was whether freshly made WT and WT frozen for extended periods would show similar levels of antimicrobial activity, measured as cell viability for *S. aureus* NCTC 06571 following storage in similar conditions. If this were so then this long term method of storage would

ensure that identical sources and methods of preparation for the WT could be used, removing a potential source of experimental variation. In order to investigate this, WT freshly made on the day, was assayed alone and in combination with copper (II) sulphate, following WT pH adjustment against *S. aureus* NCTC 06571, prior to aliquotting and storage at -20 °C. At regular intervals up to 172 weeks the similar samples and combinations of fresh and previously stored samples were compared for their effects on the viability of *S. aureus* NCTC 06571. The results shown in Figure 5.1 showed no significant statistical differences between the viability values of the following pairs of samples: fresh compared to stored WT ($p>0.05$); fresh compared to stored copper (II) sulphate ($p>0.05$); and fresh WT combined with fresh copper (II) sulphate solution compared to stored WT plus stored copper (II) sulphate solution ($p>0.05$). No differences in physical appearance or antimicrobial activity were seen between fresh and frozen teas up to 172 weeks following storage at -20 °C. Accordingly, aliquots of freshly made teas were frozen and stored at -20 °C for subsequent use.

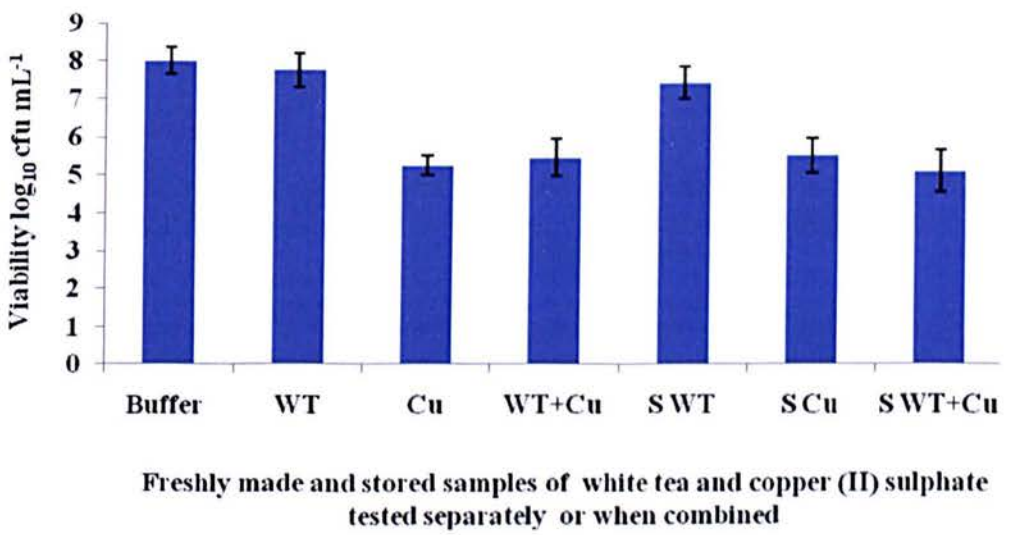


Figure 5.1 Effects of long term storage of whole white tea extract at -20 °C on the antimicrobial activity of *Staphylococcus aureus* NCTC 06571. WT = white tea extract freshly-made, adjusted to pH 7 and used immediately (or stored at -20 °C and kept in the dark = S WT), Cu = freshly made 4.8 mM added (2175 μM final concentration) copper (II) sulphate (or stored at 5 °C and tested periodically over a period of three years = S Cu), '+' denotes combinations. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Results were based on triplicate tests from 12 separate experiments. Bars show SEMs.

Note: As a precaution when new batches of teas were made up and adjusted to pH 7, they were assayed before freezing and assayed again after storage and subsequent thawing to check for any changes in antimicrobial activity against target organisms. Copper (II) sulphate reagent was made up by dilution from a 9.6 mM aqueous stock solution stored at 5 °C. Sterility tests performed from time to time on this stock showed this method of storage to be suitable since the stock remained

free from any apparent contamination by bacteria. Vitamin C solution when used was made up freshly to add to mixtures of catechin plus copper (II) sulphate.

5.3 Effects of storage, and temperature on a freshly made solution of catechin and copper (II) sulphate, with or without vitamin C on the antimicrobial activity of four different target species

***Staphylococcus aureus* NCTC 06571**

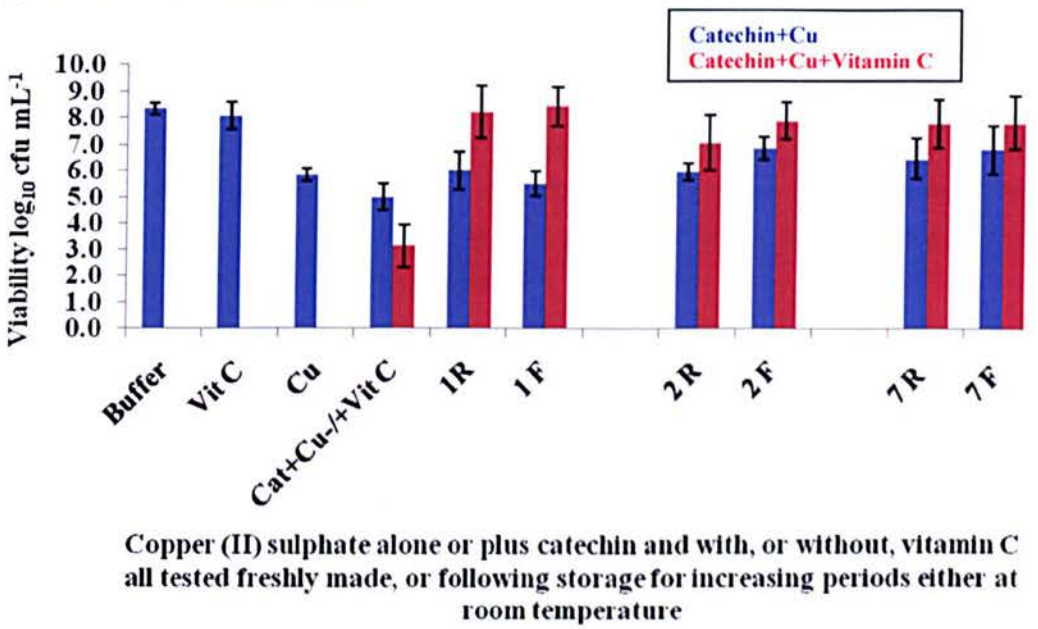
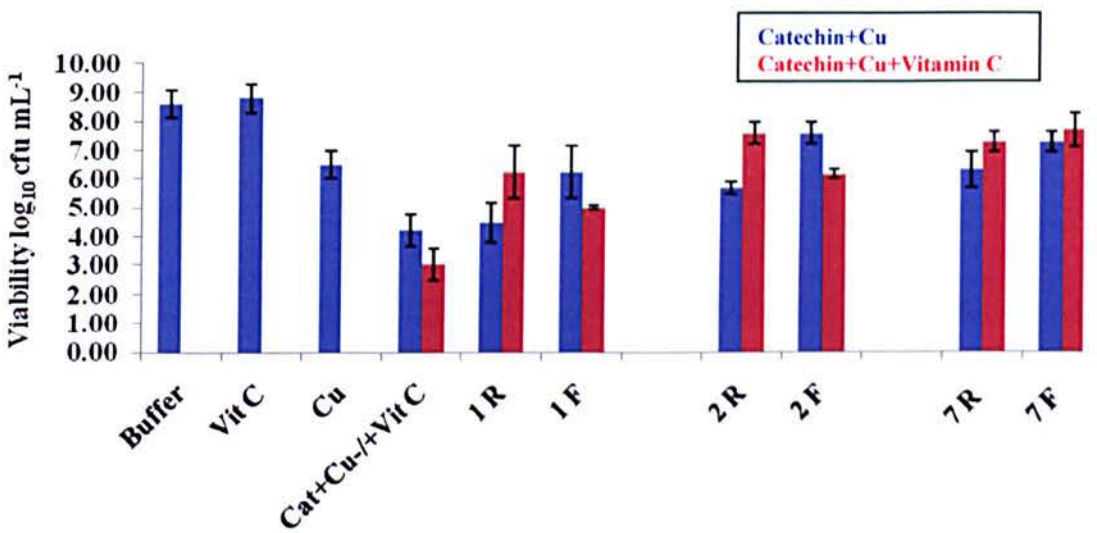


Figure 5.2 Effects of storage at two different temperatures on the antimicrobial activity of a freshly made solution of catechin combined with copper (II) sulphate as well as a similar solution with added vitamin C against *Staphylococcus aureus* NCTC 06571. Vit C = 856 μM (final concentration) vitamin C, Cu = 214 μM (f.c.) copper (II) sulphate, Cat = 214 μM (f.c.) catechin, '+' denotes combinations, '-/+' denotes absence or addition of 856 μM (f.c.) vitamin C (Vit C), blue bars denote mixtures of solutions without vitamin C stored at room temperature in the dark *ca.* 20 °C, red bars denote similar mixtures with added vitamin C stored in the dark in refrigerated conditions *ca.* 5 °C, R = room temperature, F = fridge temperature, 1,2,7 denote length of storage period in days prior to suspension assay. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Sample volumes for fresh mix catechin+Cu+vitamin C were increased to 500 μL to maintain detection of colony forming units. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results showed that the buffer control showed no significant difference in antimicrobial activity to that of the 856 μM (final concentration) vitamin C control ($p>0.05$) However, the results showed that the buffer control did show significant differences with the equimolar 214 μM (f.c.) catechin plus copper (II) sulphate samples tested without the presence of added vitamin C (all $p<0.05$). In the results of catechin plus copper (II) sulphate samples with added vitamin C no significant differences were seen when compared to the buffer (all $p>0.05$) - except for the freshly made mixture which was significantly different from the buffer ($p<0.05$) (Figure 5.2). The antimicrobial

activity of the mixture of catechin plus copper (II) sulphate solution lost *ca.* 1 $\log_{10}$ unit of activity during the seven day testing period when compared to the freshly made mixture against *S. aureus* NCTC 06751. This finding was irrespective of whether the catechin plus copper (II) sulphate solution was stored at room or fridge temperature. The activity of the catechin plus copper (II) sulphate mixture was lost, possibly in a similar manner to what was seen in Figure 4.18 when catechin was allowed to stand in dark conditions at room temperature for 14 days. A catechin plus copper (II) sulphate solution with 856 μM (f.c.) vitamin C showed a decrease in antimicrobial activity resulting in a fall of viability of *S. aureus* NCTC 06571 of *ca.* 5 $\log_{10}$ units after 1 day of storage, irrespective of storage temperature, to viability values similar to those following exposure to the buffer. This was somewhat surprising since the anti-oxidant vitamin C apparently exerted no preservation effect on the catechin plus copper (II) sulphate activity. It is possible that the vitamin C in solution quickly oxidised on contact with atmospheric oxygen forming products which complexed with the copper (II) ions removing their availability and at the same time as forming inactive vitamin C derivative-copper(II) complexes.

***Pseudomonas aeruginosa* NCTC 950**

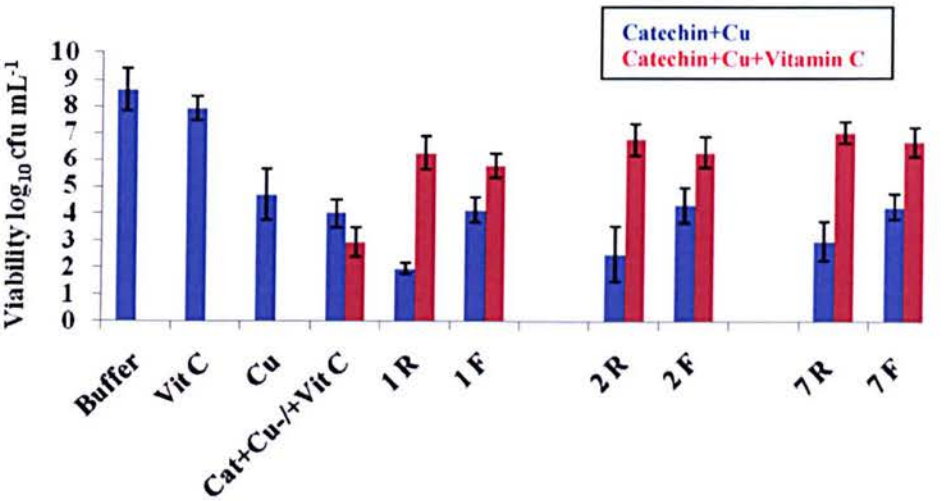


Copper (II) sulphate alone or plus catechin and with, or without, vitamin C all tested freshly made, or following storage for increasing periods either at room temperature or refrigerated

Figure 5.3 Effects of storage at two different temperatures on the antimicrobial activity of a freshly-made solution of catechin combined with copper (II) sulphate as well as a similar solution with added vitamin C against *Pseudomonas aeruginosa* NCTC 950. Cu = 214 μM (final concentration) copper (II) sulphate, Cat = 214 μM (f.c.) catechin, '+' denotes combinations, '-/+' denotes absence or addition of 856 μM (f.c.) vitamin C (Vit C), blue bars denote mixtures of solutions without vitamin C stored at room temperature in the dark *ca.* 20 °C, red bars denote similar mixtures with added vitamin C stored in the dark in refrigerated conditions *ca.* 5 °C, R = room temperature, F = fridge temperature, 1,2,7 denote length of storage period in days prior to suspension assay. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Sample volumes for fresh mix catechin+Cu+vitamin C were increased to 500 μL to maintain detection of colony forming units. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results showed that the buffer control showed no significant difference in antimicrobial activity compared to that of the 856 μM (final concentration) vitamin C control ($p>0.05$) However, the results showed that the buffer control showed significant differences in viability between the equimolar 214 μM (f.c.) catechin plus copper (II) sulphate samples tested without the presence of added vitamin C (all $p<0.05$), except for the two day old sample result ($p>0.05$). In the results similar samples with added vitamin C, when compared to the buffer control results, showed significant differences ($p<0.05$) except the two and seven day old room stored samples as well as the seven day old refrigerated sample which were not significant ($p>0.05$) when compared to the buffer. The results showed that when compared to the freshly made mixture, after 7 days of storage the catechin plus copper (II) sulphate solution had lost *ca.* 3 $\log_{10}$ units of antimicrobial activity against *Ps. aeruginosa* NCTC 950 irrespective of whether the solution was stored in the dark at room or fridge temperature with no significant difference between the two results ($p>0.05$). A similar solution of catechin plus copper (II) sulphate with added vitamin C showed a fall in antimicrobial activity of *ca.* 2 $\log_{10}$ units after 1 day of room temperature to values similar to those seen with the copper (II) sulphate control ($p>0.05$). A broadly similar result was seen with a mixture of catechin plus copper (II) sulphate and vitamin C stored for one day under refrigerated conditions, showing no significant difference in viability to the result with the room stored mixture ($p>0.05$).

These trends were broadly similar to those seen with *S. aureus* NCTC 06751 with mixtures after 1 to 2 days showing reductions in antimicrobial activity to levels shown by copper (II) sulphate controls ($p>0.05$). As seen with *S. aureus* NCTC 06751, vitamin C in general reduced the antimicrobial activity of the catechin plus copper (II) sulphate mixture against *Ps. aeruginosa* NCTC 950. However, addition of vitamin C to a catechin plus copper (II) sulphate mixture tested against *Ps. aeruginosa* NCTC 950 did not result in a viability similar to that seen for *S. aureus* NCTC 06751 in buffer under the same conditions. With *Ps. aeruginosa* NCTC 950 these findings suggest that vitamin C lost some – but not all - activity within this catechin plus copper (II) sulphate and vitamin C combination. Apparently, *Ps. aeruginosa* NCTC 950 had a greater sensitivity to these mixtures than *S. aureus* NCTC 06751 suggesting a particular susceptibility to catechin as found in assays with fresh catechin-copper (II) combinations tested in the same conditions (Figure 4.2).



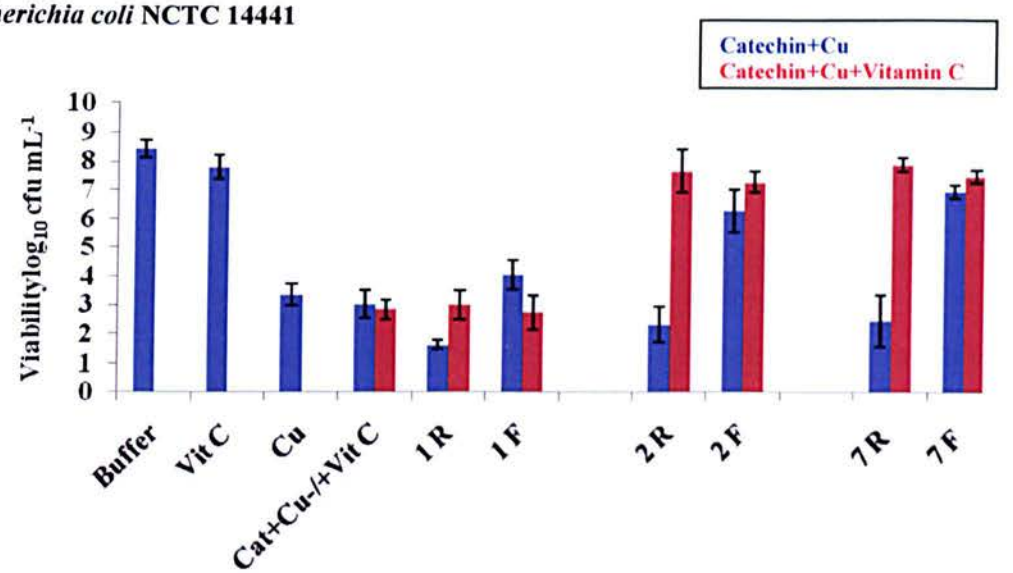
Copper (II) sulphate alone or plus catechin and with, or without, vitamin C all tested freshly made, or following storage for increasing periods either at room temperature or refrigerated

Figure 5.4 Effects of storage at two different temperatures on the antimicrobial activity of a freshly-made solution of catechin combined with copper (II) sulphate as well as a similar solution with added vitamin C against *Proteus mirabilis* NCTC 7827. Vit C = 856 μM (final concentration) vitamin C, Cu = 214 μM (f.c.) copper (II) sulphate, Cat = 214 μM (f.c.) catechin, '+' denotes combinations, '-/+' denotes absence or addition of 856 μM (f.c.) vitamin C (Vit C), blue bars denote mixtures of solutions without vitamin C stored at room temperature in the dark *ca.* 20 °C, red bars denote similar mixtures with added vitamin C stored in the dark in refrigerated conditions *ca.* 5 °C, R = room temperature, F = fridge temperature, 1,2,7 denote length of storage period in days prior to suspension assay. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Sample volumes for fresh mix catechin+Cu+vitamin C were increased to 500 μL to maintain detection of colony forming units. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results with *P. mirabilis* NCTC 7827 showed that the buffer control showed no significant difference in antimicrobial activity to that of the 856 μM (final concentration) vitamin C control ($p>0.05$). However, the results showed that there were significant differences for the buffer control compared to the 214 μM (f.c.) copper (II) sulphate control as well as all the equimolar 214 μM (f.c.) catechin plus copper (II) sulphate samples tested with and without without the presence of added vitamin C (all $p<0.05$). Noteworthy are the results that showed that room stored equimolar 214 μM (f.c.) catechin plus copper (II) sulphate samples appeared more antimicrobially active than similar catechin plus copper (II) sulphate samples stored under refrigerated conditions. However, statistically significant differences were only seen between the 1 day old room stored catechin plus copper (II) sulphate sample, the freshly made sample, and those similar samples stored in refrigerated conditions at 1, 2 and 7 days old ($p<0.05$) (Figure 5.4).

The increased antimicrobial activity of the 1 day old room stored mixture of catechin plus copper (II) sulphate sample compared to the freshly made sample suggested that a new substance may have been formed, which was particularly active against *P. mirabilis* NCTC 7827. The addition of vitamin C reduced the activity of the above mixture against *P. mirabilis* NCTC 7827 possibly due to oxidation processes as described for *S. aureus* NCTC 06751 above.

***Escherichia coli* NCTC 14441**



Copper (II) sulphate alone or plus catechin and with, or without, vitamin C all tested freshly made, or following storage for increasing periods either at room temperature or refrigerated

Figure 5.5 Effects of storage at two different temperatures on the antimicrobial activity of a freshly-made solution of catechin combined with copper (II) sulphate as well as a similar solution with added vitamin C against *Escherichia coli* NCTC 14441. Vit C = 856 μM (final concentration) vitamin C, Cu = 214 μM (f.c.) copper (II) sulphate, Cat = 214 μM (f.c.) catechin, '+' denotes combinations, '-/+' denotes absence or addition of 856 μM (f.c.) vitamin C (Vit C), blue bars denote mixtures of solutions without vitamin C stored at room temperature in the dark *ca.* 20 °C, red bars denote similar mixtures with added vitamin C stored in the dark in refrigerated conditions *ca.* 5 °C, R = room temperature, F = fridge temperature, 1,2,7 denote length of storage period in days prior to suspension assay. Mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Sample volumes for fresh mix catechin+Cu+vitamin C were increased to 500 μL to maintain detection of colony forming units. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

The results with *Escherichia coli* NCTC 14441 showed that the buffer control showed no significant difference in antimicrobial activity when compared to that of the 856 μM (final concentration) vitamin C control (p>0.05). However, the results showed that the buffer control showed significant differences with the 214 μM (f.c.) copper (II) sulphate control as well as all the

equimolar 214 μM (f.c.) catechin plus copper (II) sulphate samples tested without the presence of added vitamin C (all $p<0.05$). Noteworthy are the results that showed that room stored equimolar 214 μM (f.c.) catechin plus copper (II) sulphate samples after 1, 2, and 7 days of storage appeared significantly more antimicrobially active than similar catechin plus copper (II) sulphate samples stored under refrigerated conditions ($p<0.05$) The addition of vitamin C to samples of catechin plus copper (II) sulphate did not enhance the antimicrobial activity against *Escherichia coli* NCTC 14441. On the contrary, vitamin C significantly reduced the activity of catechin plus copper (II) sulphate in 1, 2, and 7 day old room stored samples ($p<0.05$) although under refrigerated conditions no significant differences were seen between catechin plus copper (II) sulphate samples with those without added vitamin C ($p>0.05$) against *Escherichia coli* NCTC 14441 (Figure 5.5).

The above results show *Escherichia coli* NCTC 14441 exhibiting similar results to those seen with *Pr. mirabilis* NCTC 7827 (Figure 5.4) with two exceptions. Firstly, that the antimicrobial activity of the 7 day old room stored mixture without added vitamin C was similar to the fresh mixture with or without the presence of vitamin C (both $p>0.05$). This result suggested, besides the possibility of anomalous viable cell counts, that at room temperature in the absence of vitamin C, antimicrobial activity was preserved against *E. coli* NCTC 14441. The presence of vitamin C did not preserve or increase the antimicrobial activity of the mixture at any stage of the storage period but brought a loss of antimicrobial activity in both room and fridge temperatures against *E. coli* NCTC 14441. Noteworthy is the 1 day old room stored mixture of catechin plus copper (II) sulphate without added vitamin C, which was the only sample of catechin plus copper (II) sulphate whose antimicrobial activity was significantly different from the copper (II) control ($p<0.05$) and the combinations stored at room temperature for 1, 2, and 7 days against *E. coli* NCTC 14441 (all $p<0.05$).

Results from the above figures (5.2, 5.3, 5.4, and 5.5) shown in Figure 5.6 summarise the effects of storage for different periods on the antimicrobial activity of a freshly made solution of equimolar 214 μM (final concentration) catechin combined with copper (II) sulphate against *Staphylococcus aureus* NCTC 06571, *Pseudomonas aeruginosa* NCTC 950, *Proteus mirabilis* NCTC 7827, and *Escherichia coli* NCTC 14441. These results depicted in Figure 5.6 show that *S. aureus* NCTC 06571 was the least susceptible of the four strains of bacterial species tested to the freshly made as well as to the 1, 2, and 7 days old stored mixtures of catechin and copper (II) sulphate. Considering the results from the exposures to freshly made catechin and copper (II) sulphate mixture, those of *S. aureus* NCTC 06571 were significantly different from those of *E. coli* NCTC 14441 ($p<0.05$) but the *S. aureus* NCTC 06571 results were not significantly different from the results for the other two

species, *Ps. aeruginosa* NCTC 950, and *P. mirabilis* NCTC 7827 ($p>0.05$). However, with the stored solutions of catechin and copper (II) sulphate mixture at 1, 2, and 7 days of age, *S. aureus* NCTC 06571 susceptibility was not significantly different from that of *Ps. aeruginosa* NCTC 950 ($p>0.05$). In contrast, the susceptibility of *S. aureus* NCTC 06571 to aged catechin and copper (II) sulphate mixture was significantly different to that of the other two Gram negative species, *P. mirabilis* NCTC 7827, and *E. coli* NCTC 14441 ($p<0.05$).

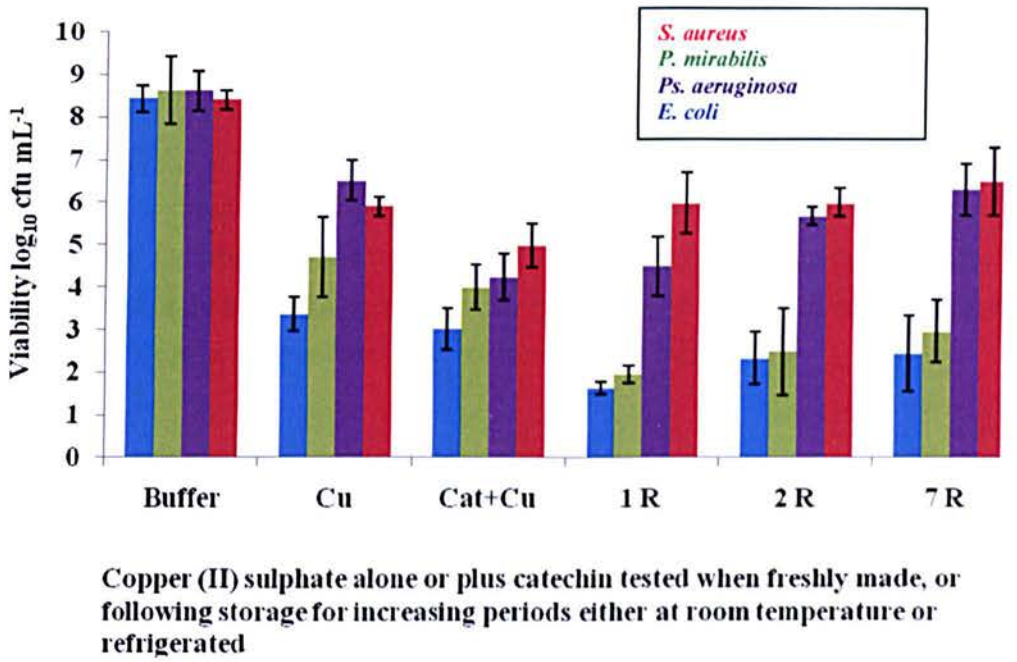


Figure 5.6 Comparison of effects of storage for different periods on the antimicrobial activity of a freshlymade solution of catechin combined with copper (II) sulphate against *Staphylococcus aureus* NCTC 06571, *Pseudomonas aeruginosa* NCTC 950, *Proteus mirabilis* NCTC 7827, and *Escherichia coli* NCTC 14441. Red = *S. aureus*, green = *P. mirabilis*, purple = *Ps. aeruginosa*, blue = *E. coli*; Cu = 214 μM (final concentration) copper (II) sulphate control, Cat+Cu = equimolar 214 μM (f.c.) catechin combined with copper (II) sulphate, R = room temperature (*ca.* 20 °C) stored solution of equimolar 214 μM (f.c.) catechin plus copper (II) mixture kept in the dark, F = fridge temperature (*ca.* 5 °C) stored solution of equimolar 214 μM (f.c.) catechin plus copper (II) mixture kept in the dark, 1,2,7 = length of storage period in days prior to suspension assay. Samples were incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Sample volumes for fresh mix catechin/copper (II) plus vitamin C were increased to 500 μL to maintain detection of colony forming units. Catechin controls were similar to those previously shown (Figure 4.2): *S. aureus* = 7.50 (±0.56), *P. mirabilis* = 7.82 (±0.51), *Ps. aeruginosa* = 7.12 (±0.42), *E. coli* = 8.00 (± 0.56) $\log_{10}$ cfu mL^{-1}. Results were based on triplicate tests from 3 separate experiments. Bars show SEMs.

In summary some general conclusions can be drawn. The activity of the freshly made catechin plus copper (II) sulphate solution, which was stored at room temperature, declined slightly over 7 days against *S. aureus* NCTC 06751 and *Ps. aeruginosa* NCTC 950 (Figure 5.6). The same freshly prepared catechin and copper (II) sulphate mixture, which had been stored at room temperature, increased in antimicrobial activity after 1 day when tested against *P. mirabilis* NCTC 7827 and *E.*

coli NCTC 14441. However, catechin and copper (II) sulphate mixture stored for 2 and for 7 days showed antimicrobial activity similar to that of the freshly made mixture of catechin and copper (II) sulphate ($p>0.05$). This suggests the possibility of the production of a new substance with more antimicrobial activity than the original freshly-made mixture. It is possible that a new substance may have been formed which increased the antimicrobial activity against *P. mirabilis* NCTC 7827 and *E. coli* NCTC 14441, but not against *S. aureus* NCTC 06751 or *Ps. aeruginosa* NCTC 950.

When vitamin C was added to the catechin-copper (II) sulphate mixture similar trends were seen whether solutions were stored at room or fridge temperatures over a 7 day period against all species of bacteria. However, for *E. coli* NCTC 14441 vitamin C preserved, but did not increase the antimicrobial activity of the freshly-made mixture for 1 day. In general, activity was lost within the first or second day of storage. It is likely that vitamin C was oxidised in solution. The presence of vitamin C in these conditions apparently reduced the formation of any hypothetical activated product. It is likely that the colder fridge storage temperature may not have generated this activated product, which could explain why antimicrobial activity did not increase.

5.4 Effects of freeze drying on the activity of a freshly-made solution of catechin-copper (II) sulphate with added vitamin C solutions against four bacterial species

As shown above, a mixture of 214 μM (f.c.) catechin and equimolar copper (II) sulphate with added 856 μM (f.c.) vitamin C, which had been stored in water, showed showed a fall in antimicrobial activity over a 7 day period. Freeze drying removes water and thus reduces the risk of oxidation from atmospheric oxygen. Freeze dried samples were therefore assayed following reconstitution with water to the above concentrations against *Staphylococcus aureus* NCTC 06571, *Pseudomonas aeruginosa* NCTC 950, *Proteus mirabilis* NCTC 7827 and *Escherichia coli* NCTC 14441. The remaining powder was stored at 5 °C in the dark. Subsequently, samples were assayed at intervals of 1 and 2 weeks, and 1, 3 and 12 months against the same species of bacteria (Figure 5.7). Individual mixture components (catechin, copper (II) sulphate, vitamin C) were also freeze dried and tested for comparison.

The results from the above procedures showed that the freeze-dried samples of 214 μM (f.c.) catechin, 856 μM (f.c.) vitamin C, and 214 μM (f.c.) copper (II) sulphate solutions tested periodically during a storage period of one year when reconstituted with water and tested against *Staphylococcus aureus* NCTC 06571, *Pseudomonas aeruginosa* NCTC 950, *Proteus mirabilis* NCTC 7827 and *Escherichia coli* NCTC 14441 showed no significant differences to fresh solutions tested on other occasions (*e.g.*, see Figures, 4.2, 5.2, 5.3, 5.4, and 5.6) (all $p>0.05$). When a freeze-dried equimolar 214 μM (f.c.) catechin and copper (II) sulphate mixture was reconstituted with

water and subsequently tested against the same four species of bacteria similar results were obtained to a similar freshly made mixture tested previously (Figure 4.2) with no significant differences seen (all $p>0.05$). Reconstituted freeze-dried mixture of 214 μM (f.c.) catechin, 856 μM (f.c.) vitamin C, and 214 μM (f.c.) copper (II) sulphate was also periodically tested against *Staphylococcus aureus* NCTC 06571, *Pseudomonas aeruginosa* NCTC 950, *Proteus mirabilis* NCTC 7827 and *Escherichia coli* NCTC 14441 and compared to a freshly made solution during each experiment. No significant differences were seen between the reconstituted freeze-dried and the freshly made solutions in their antimicrobial activity against the four species of bacteria (all $p>0.05$) (Figure 5.7).

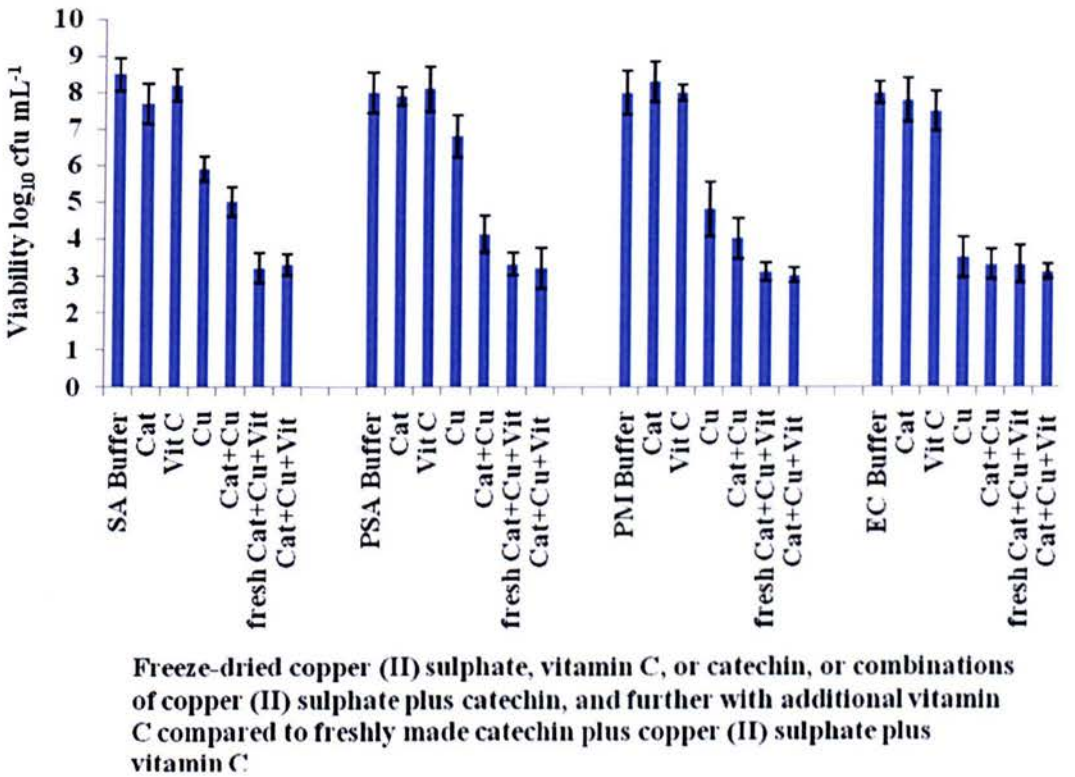


Figure 5.7 Effects of freeze drying a catechin-copper (II) sulphate-vitamin C solution on the antimicrobial activity of *Staphylococcus aureus* NCTC 06571, *Pseudomonas aeruginosa* NCTC 950, *Proteus mirabilis* NCTC 7827, and *Escherichia coli* NCTC 14441. SA = *S. aureus*, PSA = *Ps. aeruginosa*, PM = *P. mirabilis*, EC = *E. coli*, Cat = catechin reconstituted to 214 μM solution (final concentration), Vit C = vitamin reconstituted to 856 μM solution (f.c.), Cu = copper (II) sulphate reconstituted to 214 μM solution (f.c.), Cat+Cu = equimolar catechin combined with copper (II) sulphate reconstituted to 214 μM solution, fresh Cat+Cu+Vit C = freshly made solution of equimolar 214 μM (f.c.) catchin plus copper (II) sulphate plus 856 μM (f.c.) vitamin C, Cat+Cu+Vit = similar combination using freeze-dried components to result in similar concentrations following reconstitution with water. Individual freeze-dried components and mixtures were stored at 5 °C in dry, dark conditions for up to 3 months. Reconstituted mixtures were allowed to stand in the dark for 10 minutes prior to the addition of an inoculum. Samples were incubated in the dark at room temperature for 30 minutes. The plates were incubated at 37 °C for 24 hours. Samples were increased up to 500 μL to maintain detection of colony forming units. Results were based on triplicate tests from 12 separate experiments conducted ovet the course of one year. Bars show SEMs.

These results show that the tested freeze-dried materials tested individually and when combined following reconstitution with water show similar antimicrobial activities to freshly-made solutions of individual components and combinations. Freeze drying combined mixtures of catechin, copper (II) sulphate and vitamin C in the proportions indicated appears to be an optimal way to preserve the mixture for convenience of use.

5.5 Problems caused by stability of reagents in attempts to crystallise catechin with copper (II) sulphate

Aside from the possibility of loss of antimicrobial activity of substances stored in solution which were described and investigated above (Figures, 5.2, 5.3, 5.4, and 5.5) the storage of substances in solution also posed a potential problem for the crystallisation experiments (section 4.8) since periods of up to several days were required on occasion to allow for the complete evaporation of test solvents from crystallisation troughs. Changes in appearance such as colour, presence of precipitation and flocculation, colour of precipitate, were noted during the solvent evaporation process (section 4.8). Typically this changed from a very pale, clear yellow solution to a progressively darker brown colour which could have been due to the formation of high molecular weight polyphenols (Chou *et al.*, 1999). This is possibly due to the oxidation of catechin.

5.6 Conclusions

The storage of WT and copper (II) sulphate for 172 weeks at -20 °C had no effect on subsequent antimicrobial activities when tested at room temperature against *S. aureus* NCTC 06751 and other species of bacteria. However, the activities of an antimicrobial mixture of equimolar catechin plus copper (II) sulphate which had been stored at 5 °C fell over a 7 day period. Addition of vitamin C did not increase or preserve antimicrobial activity against any of the tested species of bacteria. Vitamin C generally reduced the antimicrobial activity of the catechin-copper (II) sulphate solutions after 1 to 2 days at both room and fridge storage conditions.

The catechin plus copper (II) sulphate solution, when stored at room temperature and in the dark, became more active against *E. coli* NCTC 14441 and *P. mirabilis* NCTC 7827, but not against *Ps. aeruginosa* NCTC 950 or *S. aureus* NCTC 06751. It is possible that a catechin oxidation product was produced and this may have higher and more selective activity than catechin itself. The presence of added vitamin C appeared to prevent the formation of this product.

The freeze-dried mixture produced a consistent activity against each test species of bacteria just as the freshly made solution. This suggests that freeze-drying is suitable for preserving the antibacterial activity of this mixture. Other water free preservatives such as in an ointment base may be also be suitable for this mixture (McCarrell *et al.*, 2008).

6.1 General discussion

6.1.1 Introduction

Studies of the antimicrobial activities of different substances follow a variety of investigative methods to determine their effectiveness against bacteria, *e.g.*, using different exposure times and controlling conditions such as temperature or pH. Methods can include qualitative assessments of antimicrobial activity using diffusion assays, *e.g.* such as zones of inhibition and epsilometer tests. If activity is found measurements of viable cell numbers can be subsequently carried out using other methods. Addition of other substances or adjuncts can lead to the development of improved, more effective antimicrobial formulations.

6.1.2 Studies into the methodology of antimicrobial assays

(i) Comparing diffusion assays and viable cell counts

Diffusion assay experiments found varied results when different species of bacteria were exposed to white, green and black tea (WT, GT, and BT) extracts with effects greater against *Staphylococcus aureus* NCTC 06571 than against *Pseudomonas. aeruginosa* NCTC 950 and *Proteus mirabilis* NCTC 7827 (Table 3.1). *P. mirabilis* and *Ps. aeruginosa* showed similar susceptibilities to WT and BT. *Ps. aeruginosa* was less susceptible to GT than *P. mirabilis.* However, when the susceptibilities of *S. aureus* NCTC 06571 and *Ps. aeruginosa* NCTC 950, were compared using suspension assays and viable cell counts, there were no differences between WT, GT, and BT activities (Figure 3.1).

The different results from the two methods possibly can be explained as follows: Suspension assays and viable cell counts provide information on the numbers of surviving cells that are capable of reproduction and thereby potentially extending an infection or developing the likelihood of its occurrence. However, the inhibition zone experiments results of the teas on the bacterial lawn growth depended as much on diffusion of the tea types through the agar gel as any differential effect of the teas on bacteria survivability (Wheat, 2001; Pujol *et al.*, 2008). For example, conceivably GT and BT components diffused faster through the medium than WT and thus, produced 'larger' effects. In contrast, suspension assays incorporating few barriers to diffusion allowed rapid contact between test compounds and cells.

(ii) Limitations on the use of spread plates for viable cell counts

Viable cell counts have an advantage over other methods of investigating the rise or fall of viable cell numbers such as optical density measurements, which do not differentiate directly between live and dead cells. However, the viable count method used in this study has the disadvantage that if multiple viable cells are aggregated or clumped together, which can occur in nature or following different experimental or therapeutic treatments, then following spreading on to plates such a clump of cells, containing more than one viable cell, will only grow into one colony. In appropriate conditions a single non-confluent colony will grow from a single viable cell and indeed is assumed to do so for practical purposes. Practically speaking planktonic cells and those suspended in liquid suspension following agitation *via* vortexing or gentle shaking viable cells are separated from one another and will accordingly grow into separate colonies. However, with clumped viable cells a similar single colony will grow so it is not possible to know whether any colony grew from a single or clump of viable cells. In this study in order to reduce the possible effect of clumped viable cells and increase the likelihood that colonies grew from single viable cells the possible clumping of viable cells was minimised by careful vortexing and mixing of all samples containing cells prior to the removal of any suspension.

Another disadvantage of this method of estimating numbers of viable cells is that some cells may not have recovered sufficiently during the overnight incubation to develop into a colony: so whilst being alive, albeit injured or inhibited in some way, the cells did not grow into visible colonies and were not counted. Preliminary tests that investigated the recovery of non-viable injured cells over a longer period where plates were recounted after 48, and 72 hours showed no further increases in cfus. This indicated that a conventional overnight incubation was sufficient for growth of viable cells (results not shown).

It was also noticed in preliminary experiments that plates which were over three days old showed somewhat lower growth with copper (II) sulphate exposed cells than freshly made plates by over 1 $\log_{10}$ cfu mL^{-1} (results not shown). For this reason freshly made plates were only used for up to three days. It is possible that the older, slightly drier plates challenged the cells in addition to the copper (II) sulphate, resulting in a lower viability measurement for each tested bacterial species.

(iii) Viable counts after only 30 minutes exposure to test substances

Measurements of the activity of plant and other products tested against bacteria are often done over a period of 24 hours or more (Barcia-Macay *et al.*, 2006; Mohamed *et al.*, 2012). Whilst this is of interest to investigate a varying and developing pattern of response over time this approach was not pertinent to this investigation which was to find and develop rapidly acting bactericidal agents for use as disinfective and antiseptic agents for use in food preparation areas and in agricultural

settings. For this reason bacteria in this project were exposed to test substances for 30 minutes only in the majority of experiments. This length of exposure proved entirely adequate as the kinetic studies, described previously (Figure 4.13), showed that the majority of the antimicrobial effect occurred within the first few minutes of exposure.

6.1.3 Effects of copper (II) sulphate and vitamin C without catechin on antimicrobial activity and the effects of catalase

The antimicrobial effects of copper (II) sulphate alone and when combined with vitamin C increased with concentration of either copper (II) sulphate or vitamin C in the combinations (Table 3.3, Figure 4.3). Various suggestions have been made as to the possible mechanisms of action of the injurious effects of copper (II) ions on bacterial cells and include effects on cell metabolism and cell membrane physiology (Kumamoto *et al.*, 2001; Lebedev *et al.*, 2005). The antimicrobial activity of copper (II) sulphate on *S. aureus* NCTC 06571 and *Escherichia coli* NCTC 14441 was completely removed by the added catalase irrespective of whether the antimicrobial activity had been increased by the addition of vitamin C or catechin. This suggested that H_2O_2 generation was the principal cause for the effect against the bacteria (Figures 4.15, 4.17).

Several investigations, including that of Murata and Yano (1990) reported that vitamin C was bactericidal in the presence of trace quantities of copper (II) ions against Gram-negative species, but less so against Gram-positive species. The present study found that vitamin C tested alone at 1712 μM did not affect *S. aureus* NCTC 6571 or *E. coli* NCTC 14441 and this agrees with McCarrell *et al.* (2008) who also found no effect on *S. aureus* NCTC 06571 at concentrations of 43.48 mM. These authors found, however, that vitamin C did affect *Ps. aeruginosa* NCTC 950 and *E. coli* NCTC 12241, at concentrations above 10.87 mM. In this study, *E. coli* was not affected by 1712 μM vitamin C in the 30 minute exposures (results not shown).

Likely explanations for vitamin C effects on bacteria previously suggested include interference with aerobic cell respiration, cell membrane physiology and the generation of reactive oxygen species (Miller, 1969; Lho *et al.*, 1991; Murata and Yano, 1990). It is of note that *E. coli* NCTC 14441 synthesises more catalase in response to vitamin C exposure, which suggests that vitamin C generates ROS (Richter *et al.*, 1988; Smith *et al.*, 2003). The results from this study agree with previous investigations, which suggested that H_2O_2 production from copper (II) ions increased in the presence of vitamin C and following exposure to test species a reduced bacterial viability, as in this case with *S. aureus* NCTC 6571 and *E. coli* NCTC 14441 (Figure 4.3) (Ericsson, 1954; Miller, 1969; Lho *et al.*, 1991; Murata and Yano, 1990). The effect of copper (II) ions plus vitamin C on *E. coli* NCTC 14441 in this study resulted in no detectable colony forming units, which may have been due to an inadequate sampling volume rather than low undetectable numbers of viable cells.

Catalase removed any enhancement of activity caused by vitamin C when added to copper (II) ions (Appendix II: Table A, main treatment (MT) 9, control). Using UV-vis spectroscopy, it could be shown that catalase did not act by complexing copper (II) ion (Figure 4.16). The copper (II) ions appeared to act by generating harmful levels of H_2O_2, probably via Fenton chemistry, and this was increased by vitamin C.

6.1.4 The formation of active and inactive complexes between tea extracts and metal ions against *S. aureus* NCTC 06571

The results in Chapter 3 (Figure 3.4) indicated that white, green and black tea extracts had different activities when tested with 4.8 mM copper (II) sulphate against *S. aureus* NCTC 06571. The WT-copper (II) ion combination was more active than the GT-copper (II) ion or BT-copper (II) ion combinations, which had similar effects. The WT-copper (II) ion and GT-copper (II) ion combinations showed different activities after standing times of 0, 10 or 30 minutes prior to inoculation. It is possible that WT contained compounds which interfered with the availability/activity of free copper (II) ions, possibly by forming inactive complexes. In contrast to the whole WT extract, a WT fraction of < 1 kDa (WTF) when combined with copper (II) sulphate was more active than copper (II) controls (Figure 3.11). These collective findings suggested that whole WT contained substances that deactivated copper (II) ions and these had been removed from the WTF. It is possible that the whole WT extract included large molecular weight polyphenols that formed complexes with copper (II) ions and thus removed the antimicrobial copper (II) ion effects.

In an attempt to explain the different antimicrobial activities of the whole WT and WTF (< 1 kDa) in the presence of copper (II) sulphate, UV-vis spectroscopy was used to investigate whether complexes were formed which could account for these differences. The WTF tested alone showed a lower UV-vis absorbance, than the whole WT although in each case the peak absorbance spectrum was similar and typical of polyphenols (Figure 3.12) (Andjelkovic *et al.*, 2006; Torreggiani *et al.*, 2008; Liu *et al.*, 2010). Tea and other plant polyphenols show increases in absorbance when transition metal ions are added and complexes were formed (Andjelkovic *et al.*, 2006; Torreggiani *et al.*, 2008; Liu *et al.*, 2010). In the present study, WT and WTF showed absorbance peaks at 290 nm and 285 nm respectively, with WTF showing lower absorbance. No change in either WT or WTF absorbance was seen following addition of copper (II) between 190 and 900 nm (Figures 3.12, 3.13).

UV-vis experiments with freshly prepared catechin and heat-treated catechin solutions provided evidence of complex formation with copper (II) ions (Figure 4.11). However, in other experiments

both heat-treated or fresh catechin plus copper (II) sulphate lost some antimicrobial activity when autoclaved together (Figure 4.9). Autoclaving these combinations generated a brown precipitate which probably accounts for the partial loss in activity of the catechin-copper (II) ion mixtures.

6.1.5 Structure-activity relationships of tea catechins against *S. aureus* NCTC 06571 and the selection of catechin as a weak antimicrobial agent for possible enhancement with additives

A literature survey revealed that the tea flavan-3-ol minimum inhibitory concentrations (MIC) against different strains of *S. aureus* were in the following order: *ent*-catechin = *ent*-epicatechin > catechin > epicatechin > epicatechin gallate > epigallocatechin > epigallocatechin gallate (Figure 1.2) (Takahashi *et al.*, 1995; Akiyama *et al.*, 2001; Stapleton *et al.*, 2004a, 2004b; Gibbons *et al.*, 2004; Taguri *et al.*, 2004; Roccaro *et al.*, 2004). The present study initially tested five of these flavanols, (excluding the two less abundant enantiomers) in combination with copper (II) sulphate against *S. aureus* NCTC 06571 (Chen *et al.*, 2008; Lin *et al.*, 2008). The amounts of each catechin required to produce similar levels of antimicrobial activity with the same concentration of copper (II) sulphate followed a similar order except for EGC and EGCG which both required the same concentration.

Catechin (Figure 6.1.1) was selected to investigate enhancement effects that can possibly be achieved by heat treatment and combinations with various adjuncts because of its relatively low activity compared to other flavan-3-ols when combined with copper (II) sulphate against *S. aureus* NCTC 06571.

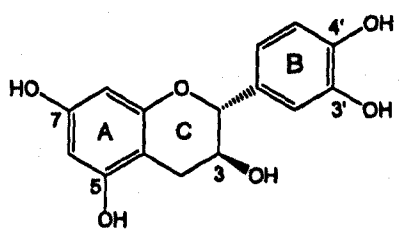


Figure 6.1.1 General structure of tea flavan-3-ols. Isometry occurs at positions 2 and 3 in the C ring where the 3',4'- dihydroxyphenyl and hydroxyl group respectively can each be found in two configurations giving rise to catechin and *ent*-catechin as well as epicatechin and *ent*-epicatechin. Substitutions of OH for H at 5' in the B ring and gallic acid for OH at 3 in the C ring give rise to other tea flavan-3-ols.

In investigations of the activities of tea catechin isomers with equimolar copper (II) sulphate, the stereoisomers *ent*-epicatechin (axial groups at C2 and C3) and *ent*-catechin (axial group at C2 and planar group at C3) showed similar activities and catechin was less active than epicatechin (Figure 4.18). This trend follows the abundance of these substances in nature: enantiomers are relatively

rare, but catechin and epicatechin are much more abundant (Chen *et al.*, 2008; Lin *et al.*, 2008). However, in the H_2O_2 assays catechin generated less H_2O_2 than the two enantiomers and this suggested that the capacity to generate H_2O_2 was not the only factor to consider in relating molecular structure to antimicrobial activity (Figure 4.19). The ability of the different isomers to attach to the cell wall and to complex copper (II) ions is probably also important for ensuring that released H_2O_2 can act at the cell surface, especially the cell membrane. Several investigations previously described the relationship between various flavan-3-ol structures and their binding to and intercalation with cell membranes as predictors of biological activity in general (Caturia *et al.*, 2003; Sirk *et al.*, 2009; Cushnie and Lamb, 2011).

6.1.6 Antimicrobial activity of mixtures of catechin, iron (II) sulphate, iron (III) chloride and copper (II) sulphate against *S. aureus* NCTC 06751 and *E. coli* NCTC 14441

This set of experiments explored whether iron (II/III) salts on their own or in combination with catechin could generate sufficient reactive oxygen species (ROS) concentrations to produce antimicrobial affects. Heat treating iron (II) solutions may reduce antimicrobial activity due to oxidation to iron (III), which is more stable. The present study found that fresh or heat-treated 214 μM (f.c.) iron (II) and (III) solutions resulted in the same viabilities as buffer controls following exposures to *S. aureus* NCTC 06571 and *E. coli* NCTC 14441 in 30 minute exposures (Table 4.5a). However, iron (II) enhanced the activity of catechin against *S. aureus* NCTC 06571 but not against *E. coli* NCTC 14441 (Figure 4.7). These results agree with those of McCarrell *et al.* (2008) who also found that iron (II) sulphate enhanced the activity of pomegranate rind extract (PRE) against *S. aureus* NCTC 06571 but not against *E. coli* NCTC 12241, where PRE reduced the antimicrobial activity of iron (II) sulphate against *E. coli* NCTC 12241 by *ca.* 50%. Such differences between *S. aureus* NCTC 06571 and *E. coli* NCTC 14441 may be due to differences in cell wall structure, cell membrane pump activity, protective chemicals such as enzymes, ion chelating agents such as siderophores, or different types of metabolism (Lebedev *et al.*, 2005; Sirk *et al.*, 2009). It would appear that adding iron (II) to catechin or PRE improved activity against the Gram-positive species but not against the Gram-negative species.

When copper (II) and iron (II) ion solutions were combined antimicrobial activity increased by *ca.* 1 $\log_{10}$ cfu mL^{-1} against *S. aureus* NCTC 06571, but there was no additional effect against *E. coli* NCTC 14441 (Figure 4.7). The effect on *S. aureus* was possibly due to its susceptibility to a possible increased level of extracellular H_2O_2 brought about by oxido-reduction reactions between the copper (II) ions and iron (II) ions through Fenton chemistry. In the case of *E. coli* NCTC 14441, it is possible that siderophores removed iron (II) ions from the extracellular fluid into the cytoplasm removing them from any interactions with copper (II) ions within the vicinity of the cell envelope and thus preventing any increased H_2O_2 generation and further bactericidal effect.

Adding EDTA to the metal (II) salt solutions eliminated their effects on *S. aureus* NCTC 06571 and *E. coli* NCTC 14441 which indicated that the bioavailability of the metal ions may be related to their antibacterial effects.

6.1.7 Antimicrobial activity of catechin, iron (II) and copper (II) sulphate in the presence of vitamin C against *S. aureus* NCTC 06751 and *E. coli* NCTC 14441

Addition of vitamin C to catechin plus iron (II) or plus copper (II) ion solutions or both types of ion together did not improve antimicrobial activity (Figures 4.3, 4.7; Appendix II: Table A, MT's 3-6). This may be due to a greater reactivity of iron (II) ions and catechin with copper (II) ions than with vitamin C and as such may have prevented vitamin C from any further reduction effect on the copper (II) or iron (II) ions.

Previous investigations have shown that copper (II) ions combined with flavanol containing tea extracts can generate bactericidal ROS including H_2O_2 and that the activity of such flavanols may be preserved by vitamin C in aqueous solution (Clifford and Repine, 1982; Hoshino *et al.*, 2000, Hatano *et al.*, 2008). In the present study catechin may have increased its activity with different additions against *S. aureus* NCTC 06751 by generating more ROS/ H_2O_2 by the mechanism outlined above and shown in more detail in Figure 6.1.2.

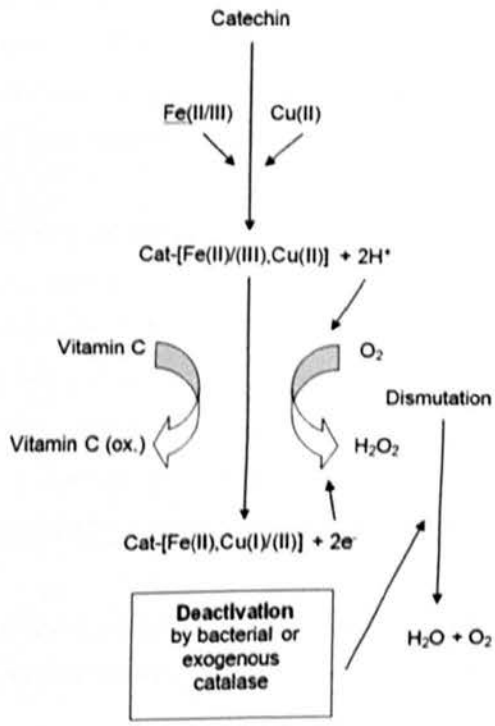


Figure 6.1.2 Suggested reactions of catechin with iron (II) and copper (II) ions and vitamin C. Scheme shows catechin combining with iron (II) and copper (II) ions forming an aqueous complex and releasing protons. A complex or vitamin C could reduce O_2 and with the released protons possibly form H_2O_2 in a dismutation reaction. Iron (II) and copper (II) ions can also form H_2O_2 without catechin via Fenton chemistry. Deactivation of inhibitory H_2O_2 can occur by bacterial or exogenous catalase.

The results obtained with catalase results are consistent with those of Smith *et al.* (2003) who showed that H_2O_2 generated by black wattle polyphenols (*Acacia mearnsii*) could be removed by exogenous catalase, and that *E. coli* mutants lacking catalase were especially susceptible to H_2O_2 whereas oxyR mutants that constitutively overexpress antioxidant enzymes were resistant.

6.1.8 Antimicrobial effects of zinc (II) and manganese (II) ions alone and in various combinations against *S. aureus* NCTC 06751

In experiments with zinc (II) ions, 214 μM (f.c.) zinc (II) sulphate tested against *S. aureus* NCTC 06751 alone; and with added vitamin C; and with further added catechin had no antimicrobial effect in the conditions tested. Zinc (II) ions added to a mixture of copper (II) sulphate combined with catechin and vitamin C showed that it did not affect the activity of the mixture against *S. aureus* NCTC 06751 in similar test conditions. This suggested that in these test conditions, zinc (II) ions had no interactive or interference effects with either the copper (II) ions, or the vitamin C, or the catechin (Figure 4.5).

The addition of 214 μM (f.c.) manganese (II) sulphate to catechin did not enhance activity against *S. aureus* NCTC 06571 (Figure 4.6). A similar finding by McCarrell *et al.* (2008) found that manganese (II) also did not enhance PRE against the same species. In this study the addition of zinc (II) sulphate to a combination of catechin combined with copper (II) sulphate showed no effect on the activity of the catechin-copper (II) combination against *S. aureus* NCTC 06751. Zinc (II) ions also had no effect on activity with the further addition of vitamin C (Figure 4.5).

In experiments with 214 μM (f.c.) manganese (II) ions tested alone or with further 6 fold addition of vitamin C (1284 μM, f.c.) against *S. aureus* NCTC 06751, little antimicrobial activity was seen (Figure 4.6). However, in another study by Inoue *et al.* (1999), 18.2 μM (f.c.) manganese was found to be active against different strains of *S. aureus* in water sampled from hot springs in 30 minute exposures, only when additional iodide ions (7.8 μM) were present and in acidified conditions (pH 2.0 – 3.0). It appears that manganese (II) ions may require specific adjuncts and conditions to show antimicrobial effects against *S. aureus* not reproduced in this study.

6.1.9 Comparison of the antimicrobial activities of catechin with pomegranate rind extract when combined with copper (II) sulphate against *S. aureus* NCTC 06751 and other species of bacteria

Aqueous extracts of the fruit rind of the pomegranate tree (*Punica granatum*) have been shown to be active against *S. aureus* and other species of bacteria (McCarrell *et al.*, 2008, Gould *et al.*, 2009a, 2009b). The activity of pomegranate rind extract (PRE) combined with added 4.8 mM

copper (II) sulphate in the above studies was found to produce a similar level of activity against *S. aureus* to that found in this study with a white tea sub-fraction of < 1 kDa (WTF) within a similar combination against *S. aureus* (Figure 3.26). Both PRE and WTF contain catechin and other polyphenols and possibly may exert their effects in similar ways (de Pascual-Teresa *et al.*, 2000; Chen *et al.*, 2008).

In another species in this study *Ps. aeruginosa* NCTC 950 was shown to be the least susceptible strain to copper (II) sulphate tested alone (Table 3.3). However, following exposure to a combination of catechin and equimolar copper (II) sulphate, *Ps. aeruginosa* showed the greatest reduction in viability when compared to the falls shown by *S. aureus* NCTC 06751, *P. mirabilis* NCTC 7827 and *E. coli* NCTC 14441 tested under the same conditions (Figure 4.2). McCarrell *et al.* (2008) and Gould *et al.* (2009a, 2009b) also reported a similar finding with PRE plus copper (II) sulphate showing that a PRE-copper (II) ion combination had greater effects on Gram-negative species in general than on Gram-positive species including *S. aureus* NCTC 06751. This greater susceptibility of Gram-negative species such as *Ps. aeruginosa* NCTC 950 may result from its specific cell wall features such as type or number of periplasmic proteins or porins (Braud *et al.*, 2010; Elsen *et al.*, 2011). In the present investigation, *S. aureus* NCTC 06571 was the least sensitive to combined catechin plus equimolar copper (II) ions, which may be due in part to a greater defence offered by the different chemical composition and greater width of the Gram-positive cell wall (Figure 4.2) (Taguri *et al.*, 2006; Sirk *et al.*, 2009; Cushnie and Lamb, 2011).

The above findings raised the question as to the possible interactive mechanisms of action of the combined test substances since catechin itself when tested alone was not antimicrobial in these conditions (Figure 3.18). Similar findings were reported by McCarrell *et al.* (2008) and Gould *et al.* (2009a, 2009b) for PRE. It is likely, as has been suggested in other investigations on other tea catechins, that in this study catechin complexed with copper (II) ions led to increased H_2O_2 production compared to catechin or copper (II) ions controls tested on their own (Figure 4.19) (Hoshino *et al.*, 1999). It is possible that catechin increased the reactivity of copper (II) ions within the complex making it more likely that the copper (II) ions would become reduced by catechin (in solution) from copper (II) ions to copper (I) ions, which then spontaneously oxidised to copper (II) ions generating H_2O_2 via Fenton chemistry (Figure 6.1.2). In addition catechin may have negatively affected the stability of bacterial cell walls and membranes, which increased the injurious effects of H_2O_2 (Sirk *et al.*, 2009). It is possible that catechin and other flavanols partly exert their effect against cells by complexing with copper (II) ions and other substances and binding them at the cell membrane producing more localised and intense effects as mentioned above (Hoshino *et al.*, 1999). In this study support for the formation of complexes also comes from the work on solubility of catechin which was increased twofold at room temperature in the presence of equimolar copper (II)

sulphate (Table 4.4). Observed antimicrobial effects of catechin plus copper (II) sulphate combinations are unlikely to be due to a pH effect arising from the addition of acidic copper (II) sulphate to the catechin since the pH of 214 μM (f.c.) catechin and 214 μM (f.c.) copper (II) sulphate is similar (*ca.* pH 6.6) (Figure 4.12) and at this concentration a complex is postulated.

6.1.10 Investigations into possible antimicrobial mechanisms of action using different molar ratios of catechin and adjuncts

(i) Combination ratio effects on antimicrobial activity

Investigations by Esparza *et al.* (2005) and Ghosh *et al.* (2006) showed that individual catechin molecules in aqueous solution bind to specific numbers of metal ions such as copper (II) and zinc (II) ions. This posed the question whether in this study such catechin-metal (II) ion complexed with fixed ratios of components were responsible for antimicrobial effects. If so, then varying the ratio of catechin to adjunct(s) within assays might have resulted in a stoichiometric pattern of antimicrobial activity. Such ratio effects on pH were initially investigated in this study against *S. aureus* NCTC 06751 (see Figure 4.10). The results of these assays showed that a fixed concentration of catechin with increasing amounts of added copper (II) sulphate in the molar ratio of catechin 1: copper (II) sulphate between 0 and 16.0 (where 1 = 214 μM final concentration) following exposure to *S. aureus* NCTC 06751 resulted in the greatest fall in viability between ratios of 1: 0.25 and 1: 0.75 (Figure 4.1). This finding may reflect the binding characteristics of the catechin with copper (II) ions as suggested by Esparza *et al.* (2005) and Ghosh *et al.* (2006). Copper (II) sulphate controls tested at concentrations between 0.25 and 1.00 showed no effect on the activity of the cells suggesting copper (II) tolerance at these concentrations tested in these conditions. In the controls with higher concentrations of copper (II) sulphate between ratios of 1.0 and 16.0 : 1 Ringer's solution the profile of *S. aureus* NCTC 06751 viability following exposure was similar to that if catechin was also present (Figure 4.1). It is possible that at higher copper (II) ions: catechin ratios some of the copper (II) ions will be free and thus able to affect the cells.

Equimolar catechin and copper (II) sulphate mixtures in the presence of increasing vitamin C concentrations progressively increased activity against *S. aureus* NCTC 06751 (Figure 4.3). Similar observations were made in the controls where catechin was absent. This can be explained by the effect of vitamin C on copper (II) ions.

(ii) Effect of different ratios of catechin and copper (II) sulphate pH and antimicrobial activity

A 1:1 molar ratio of copper (II) sulphate and catechin had the greatest effect on pH with higher ratios of copper (II) ions resulting in no further effect on pH (Figure 4.10). Copper (II) ions displaced protons from the catechin OH groups and caused pH to fall. A similar explanation was

suggested for the pH reducing effects of WT and WTF (< 1 kDa) mixed with copper (II) sulphate (Figure 3.15).

(iii) Effect of different ratios of catechin and and copper (II) sulphate on UV-vis absorbance

The presence of copper (II) ions increased the absorbance of catechin at 280 nm at all ratios of copper (II) sulphate tested (Figure 4.11). This increase in absorbance could be due to attached copper (II) ions or perhaps a catechin solvation effect brought about by the added copper (II) sulphate. It should be noted that free copper (II) ions have an absorption maximum at 800 nm where free aqueous copper (II) ions would normally show absorbance.

(iv) Combination ratio effects of catechin and and copper (II) sulphate on crystallisation of mixtures

In further investigations crystallisation studies showed that certain ratios of combined catechin with copper (II) sulphate were more likely to result in crystal formation than others. A ratio of catechin 1: 0.66 copper (II) sulphate generally produced crystals, albeit of a small size of *ca.* 0.25 – 0.50 mm in length (see section 4.8). Whilst strict molar ratios of combined catechin with copper (II) ions may exist in aqueous solution this may not apply to crystals.

Recent work on polyphenol-copper (II) ion complexes by Pirker *et al.* (2012) investigated the effect of pH on the speciation of copper (II) ion in reactions with green tea polyphenols, EGCG and gallic acid. They found that di- or polymeric complexes dominated the copper (II) speciation in the pH 4-8 range – a similar pH range to that measured in the samples containing copper (II) ions in this study. They showed 'that the molecular masses of complexes increase with increasing pH, indicating either coordination of increasing numbers of polyphenol molecules as ligands to the copper or the increasing involvement of polyphenol dimers as ligands in the copper coordination sphere'. If variable stoichiometry occurs during the crystallisation process these findings may help explain why regular crystals were difficult to produce. In addition a conclusion of Pirker *et al.* (2012) may possibly explain the observations seen in this study where increasing UV-vis absorbance was seen with catechin : Cu ratios of up to 1: 16 in the UV-vis results discussed above (Figure 4.11).

6.1.11 Antimicrobial activity of caffeine in the presence of catechin, with copper (II) sulphate and vitamin C against *S. aureus* NCTC 06751

Fungi, lower and higher plants have evolved to manufacture and secrete antimicrobials such as allelopathic chemicals and antibiotics to kill competitive bacteria *e.g.*, in the soil substratum (Nakai *et al.*, 2001; Celenza *et al.*, 2012). In response, bacteria have evolved cell membrane pumps (MDR pumps) to actively remove such harmful substances from their cells. In response to this certain

plants have co-evolved to produce compounds such as alkaloids and certain flavanoids to inhibit MDR pumps (Gibbons *et al.*, 2004). These MDR inhibitors work in synergy with other antimicrobial compounds to defend the plant from bacteria (Raj and Dhala, 1965; Junio *et al.*, 2011). The alkaloid caffeine, found in various plant species, and found in high concentrations in tea and coffee bushes, is believed to act as an inhibitor of MDR pumps and is a proven antimicrobial against food spoilage bacteria (Lewis and Ausubel, 2006; Maletta *et al.*, 2012). In this study, caffeine was therefore tested as a putative enhancer of catechin with and without adjuncts against test bacteria. Caffeine had no effect on the activity of catechin-copper (II) sulphate mixtures, on copper (II) sulphate alone or on a catechin-copper (II) sulphate-vitamin C mixture against *S. aureus* NCTC 06751 (Figure 4.4). This can be explained by the catechin and adjunct mixtures not affecting their antimicrobial actions on bacterial MDR pumps and consequently are probably working in another way. This reasoning supports the suggestion that catechin with adjuncts generate H_2O_2 extracellularly which damages cell envelope structures and does not enter the cell.

6.1.12 Effects on antimicrobial activity of heat treatment of catechin alone and in combination with copper (II) sulphate against *S. aureus* NCTC 06751

Previous studies have shown that heat treatment of aqueous tea leaf extracts as well as well as other polyphenols such as tannic acid solution can raise their antimicrobial activities (Takahashi *et al.*, 1995; Asaka *et al.*, 2000; Kim *et al.*, 2011; Isaacs *et al.*, 2011). In this investigation freshly made catechin solutions heated at 100 °C for 10 or 30 minutes, or autoclaved for 15 minutes resulted in clear, yellowish brown solutions which showed similar antimicrobial activities to freshly made catechin and buffer controls against *S. aureus* NCTC 06751 (Figures 4.8, and 4.9). These colour changes suggested the formation of auto-oxidation products described in other investigations and which occur during the 'fermentation' process in black tea production (Hathway and Seakin, 1957; Bors *et al.*, 1999; Es-Safi *et al.*, 2003; Kim *et al.*, 2011).

Preliminary studies with nuclear magnetic resonance (NMR) to attempt to identify newly formed chemical products within heat-treated catechin solution for 10 minutes at 100 °C initially suggested that less than 20% of the original starter catechin molecules in solution had been converted into new products following the heat treatment process. If this is estimation is correct, this amount was sufficient to produce a significant difference in antimicrobial activity against *S. aureus* NCTC 06751 when it was subsequently combined with copper (II) sulphate and compared to fresh catechin with copper (II) sulphate ($p<0.05$). NMR peak signals for the new products required in-depth processing in order to separate them from background noise as well as from unconverted catechin molecule signals. The progression of this work is suggested in the 'Future work' section below (see Section 6.4). A scheme is proprosed below as to synthesis and the identity of the newly formed substance.

When all three types of heat-treated catechin solutions and freshly made catechin were combined with equimolar copper (II) sulphate and tested against *S. aureus* NCTC 06751, the heat-treated solutions resulted in a greater reduction in *S. aureus* NCTC 06751 viability than did exposure to the non heat-treated solution (Figure 4.8). It seems likely that heating the catechin solution caused an oxidation and produced new products such as quinone methides along with their subsequent reaction products, which may be responsible for the increased activity against *S. aureus* NCTC 06751 (Figure 6.1.3) (Azam *et al.*, 2004; Ferreira, 2012). Findings from other investigations on polyphenols have led to suggestions that increased chemical and antimicrobial activities following heat treatment can be explained in terms of the chemical features of newly formed reaction products (Bors *et al.*, 1999; Es-Safi *et al.*, 2003; Drynan *et al.*, 2010).

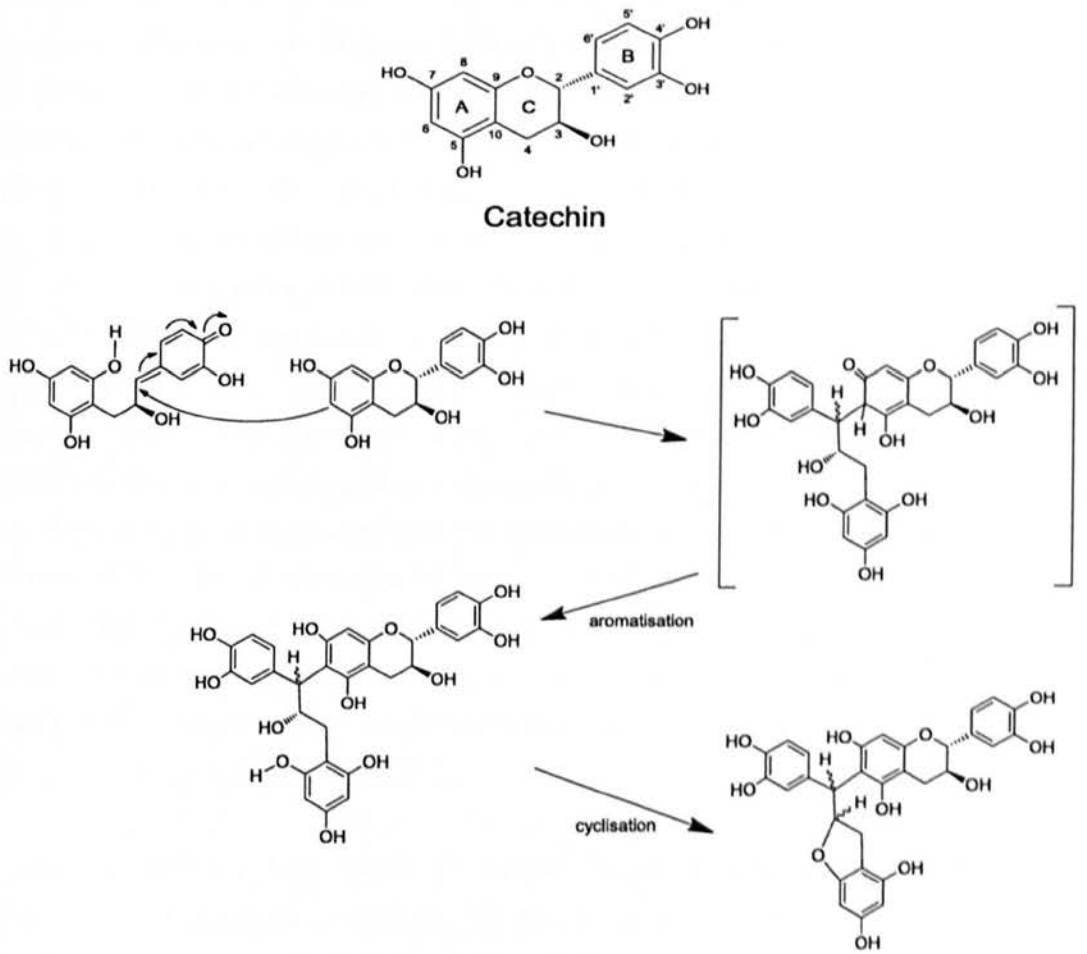


Figure 6.1.3 Suggested possible reactions of catechin in aqueous conditions at natural pH during heat treatment at 100 °C for 10 minutes. The top figure shows the general structure of catechin molecule with the A, B, and C ring positions numbered for subsequent reference. The underlying scheme shows the opening of the catechin C-ring to generate a B-ring quinone methide that acts as an electrophile for coupling with a C-8 or a C-6 of a second catechin moiety. Secondary modifications are then shown that involve cyclization between an aromatic OH-group and the secondary alcohol function at the equivalent of C-3 (Mechanism and text based on Ferreira, 2012).

The scheme shown above (Figure 6.1.3) outlines a putative reaction showing the formation of new chemical products derived from heating catechin solution at 100 °C for 10 minutes. This scheme proposed by Professor Daneel Ferreira of the University of Mississippi (2012) has met with general agreement and is similar to those proposed by other investigators following similar investigations on the chemical effects of the heat treatment of catechin solution (Mochizuki *et al.*, 2002; Drynan *et al.*, 2010).

The final structure shown at the end of the scheme in the above figure possesses certain features of particular note. Firstly, the structure possesses only 9 OH groups although it was formed from 2 component catechin molecules each with 5 OH groups, one being lost through condensation. Assuming that these OH groups can dissociate in aqueous conditions releasing free protons, this reduction of the number of OH groups could have lead to a reduction in the number of protons in the solution which in turn could account for the observed rise in the pH from pH 6.5 to 6.7 following heat treatment (Figure 4.12). The difference in chemical structure, and particularly the number of OH groups, between the fresh catechin and the quinone methide dimer shown above may, on account of the difference in pH, account for the subsequent changes in pH seen when the substances were added to equimolar and to 4.8 mM copper (II) sulphate. In each case addition to both equimolar and 4.8 mM copper (II) sulphate resulted in a pH reduction of *ca.* 0.75 units which suggests a similar degree of proton loss from the fresh and heat-treated catechin solution. However, the greater degree of acidity of the 4.8 mM copper (II) sulphate (*ca.* pH 5.9) compared to the equimolar copper (II) sulphate (pH *ca.* 6.6) appears to have promoted a slightly higher degree of copper (II) ion induced proton loss from the heat-treated catechin than from the freshly prepared catechin (Figure 4.12). This is despite the possibility that heat-treated catechin may have fewer OH groups than the freshly prepared catechin as suggested above and may be related to the types and degree of complex formation shown at different values of pH as demonstrated in other investigations on copper (II) ion complex formation with green and black teas as well as catechins (Goodman *et al.*, 2012; Pirker e*t al.*, 2012).

In equimolar conditions with copper (II) sulphate freshly prepared and heat-treated catechin showed different antimicrobial activities (Figure 4.8). In the pH conditions shown by the two mixtures with values around 5.80 and 5.85 respectively it is possible that the heat-treated catechin is bound with a different number of copper (II) ions than the freshly prepared catechin (Figure 4.12). Such a difference could be related to possible differences in H_2O_2 generation and consequent greater antimicrobial effect when compared to the freshly prepared catechin against *S. aureus* NCTC 06751. However, the degree of copper (II) ion complexation by a catechin, or a derivative, may not solely account for the results seen in Figures 4.8, and 4.18 since the degree of H_2O_2

generation by a complex is not the only factor involved in determining antimicrobial activity (Lebedev *et al.*, 2005; Gradisar *et al.*, 2007; Cushnie & Lamb, 2011; Sharma *et al.*, 2012). In addition the presence of and type of branching in the molecular structure of the catechins has been shown to be important with branched structures with gallic acid substituted at C3 such as in ECG and EGCG which show greater activity by intercalation in the cell membrane (Figure 3.17) (Stapleton *et al.*, 2007; Shah *et al.*, 2008; Bernal *et al.*, 2009; Park and Cho, 2010). It is known that galloylated catechins can generate more H_2O_2 in the presence of copper (II) ions than catechins lacking this feature (Nakayama *et al.*, 2002; Sharma *et al.*, 2012). In addition the gallic acid group is known to be attracted strongly into the cell membrane penetrating deeply by intercalation into its palisade structure and this considerably alters its fluidity and physiological properties (Caturia *et al.*, 2003; Stapleton *et al.*, 2007; Maestre *et al.*, 2010). In this case the dimer product shown in Figure 6.1.3 is branched and this feature may raise its activity in the same way as the branched catechins *i.e.* via intercalation effects. In addition such intercalation could bring the dimer-copper (II) ion complex closer to the cell membrane and so exert a more localised effect on the cell membrane from H_2O_2 generation. The difference in antimicrobial activity between the fresh catechin and the heat-treated catechin derivative combined with equimolar copper (II) sulphate may also be partly determined by differences in ability to destabilise the cell membrane making it more or less susceptible to any free or complexed copper (II) ion generated H_2O_2.

In kinetic studies the rate of effect of 214 μM (f.c.) catechin with combined 4.8 mM copper (II) sulphate on antimicrobial activity within the first 10 seconds of exposure was approximately twice that seen with 4.8 mM copper (II) sulphate alone and half that seen with the heat-treated catechin (Figure 4.14). In comparison 1 μM (f.c.) of the EGCG tested in the same conditions during the first minute of exposure produced an even greater rate than the heat-treated catechin combination (Figure 4.13). The antimicrobial activities of these mixtures largely occurred within the first 10 seconds of exposure suggesting a rapid effect from a highly active substance such as free radicals or ROS like H_2O_2.

Following autoclaving, combinations of freshly made or previously heat-treated catechin with excess copper (II) sulphate both contained insoluble dark brown matter. The antimicrobial activities of these combinations were less than non-autoclaved combinations suggesting the possibility of a reduction in the amount of active substances when the insoluble matter was formed (Figure 4.9).

6.1.13 Effect of storage on the antimicrobial activity of combined catechin with copper (II) sulphate against *S. aureus* NCTC 06751 and other target species of bacteria

The catechin combined with equimolar copper (II) sulphate and stored at room temperature showed a small decline in antimicrobial activity over 7 days against *S. aureus* NCTC 06751 and *Ps. aeruginosa* NCTC 950. However, the same room stored mixture tested against *P. mirabilis* NCTC 7827 and *E. coli* NCTC 14441 showed an increase in activity *ca.* 0.5 $\log_{10}$ unit after one day (Figure 5.6). This additional activity was lost after a further day of storage with cell viabilities following exposure being similar to copper (II) ion controls within 7 days. It appears that a transient substance or substances may have been formed in the mixture which increased the antimicrobial activity of the mixture against *P. mirabilis* NCTC 7827 and *E. coli* NCTC 14441. These may have come about from oxidation as the solution was exposed to the atmosphere and more so in the presence of the copper (II) ions (Es-Safi *et al.*, 2003). It is possible that such substances with increased antimicrobial activity may be similar to those produced by heat-treated catechin solution and discussed above (see section 6.1.12).

When catechin was combined with copper (II) sulphate and vitamin C similar or less antimicrobial activity was seen with both room and fridge stored mixtures tested throughout the 7 day period against all four species of bacteria (Figures, 5.2-5.5). Vitamin C does not appear to preserve the antimicrobial activity of catechin-copper (II) sulphate mixture – apparently the converse being true with vitamin C reducing antimicrobial activity in stored solutions. This may have been due to oxidation of vitamin C in solution or to reducing copper (II) ion availability by forming inactive complexes with copper (II) ions. This overall would result in a reduced capacity to generate hydrogen peroxide, either alone or interactively with catechin molecules. Freeze-drying of fresh solutions of catechin combined with copper (II) sulphate, and vitamin C showed that such mixtures, at least when stored at 5 °C in dry, dark conditions, retained the same level of antimicrobial activity against test species of bacteria as seen with freshly prepared solutions (Figure 5.7). This finding suggests that freeze drying may be a suitable means of preserving the antimicrobial activity of this freshly-made mixture. It is possible that removal of the water during the process removes the medium in which atmospheric oxygen would otherwise oxidise catechin and vitamin C.

6.1.14 The use of combinations in existing and emerging contexts in the control of human, animal, and plant disease

Combinations of antimicrobial agents such as fresh catechin or heat-treated catechin combined with adjuncts may provide a means to reduce the amount of copper (II) salt used in certain classical agricultural formulations such as Bordeaux powder or Cheshunt compound, thus reducing incidental harm to the environment as well as the cost of copper-based products in pest control (Jacob *et al.*, 1997; Emeran *et al.*, 2011; El Hadri *et al.*, 2012). In aquaculture catechin may be used

to enhance the antimicrobial activity of copper (II) ions to control algal and fungal growth on plant and fish products (Guardiola *et al.*, 2012: Yin *et al.*, 2010). Catechin may also find a use in antifouling products where it could activate a lower content of copper (II) ions within antifouling treatments (Perez *et al.*, 2006). Catechin may be used to activate the use of iron (II) against *S. aureus* and perhaps other species of bacteria. Catechin feasibly could be incorporated with other substances and used in new settings such as with iron (II) in nanoparticles in cloth and food wrappings and incorporated into foods such as meat, fish and wine products as a preservative against *S. aureus* and yeasts (Kim *et al.*, 2006; Borkow *et al.*, 2010).

6.2 General conclusions

(i) Viability effects and mechanisms of action of whole tea extracts and adjuncts

GT and BT, but not WT, adjusted to pH 7 tested alone against *S. aureus* NCTC 06571 and *Ps. aeruginosa* NCTC 950 showed similar level of viabilities as buffer controls (Figure 3.1). When combined with of copper (II) sulphate against *S. aureus* NCTC 06571 both GT and BT, but not WT, showed a lower antimicrobial activity than that observed with copper (II) ion controls (Figure 3.4). WT combined with copper (II) ions showed the same antimicrobial activity as copper (II) ions alone.

The above findings parallel those of McCarrell *et al.* (2008) who found that another plant antimicrobial substance, pomegranate rind extract (PRE), rich in polyphenols, had no effect on similar species of target bacteria when tested alone. However, when copper (II) sulphate was added to PRE, the phenol antimicrobial activity was enhanced. In this study, in contrast, copper (II) ions added to the teas did not enhance antimicrobial activity (Figure 3.4). Both GT and BT reduced the activity of copper (II) ions against *S. aureus* NCTC 06571 when compared to the activity of copper (II) controls, whilst WT had no such reduction effect. The reduction effect is attributed to the possible formation of inactive complexes which remove labile copper (II) ions by the GT and BT but not in the case of WT – or at least not as quickly (Figure 3.4).

Considering tea-copper (II) complex formation further, previous studies have shown that flavonoids and other polyphenols which are found in plants, including teas, can form inactive complexes with metal ions such as copper (II) and iron (II) removing them from cell metabolism (Yoshino and Murikami, 1998; Goto and Suyama, 2000; Fernandez *et al.*, 2002). In this study, WT, GT and BT all appeared more soluble and less turbid following addition of copper (II) ions which suggested an increase in solubility of the suspended tea solids, probably via interactions between the copper (II) ions and the tea components. Similar effects of metals on the solubility of tannins have been previously reported (Okuda *et al.*, 1982).

Investigations into interactions between tea components and copper (II) ions using pH measurements showed that WT mixed with copper (II) ions resulted in a fall in pH (Figure 3.15). This fall was greater than would be expected for mixing the two acidic solutions together. This finding suggested a chemical interaction had taken place between the tea components and the copper (II) sulphate which released protons with the simultaneous formation of a complex. Other investigations have shown that manipulating pH causes speciation of different complexes between teas and copper (II) ions (Andjelkovic *et al.*, 2006; Goodman *et al.*, 2012; Pirker e*t al.*, 2012). In this study, the possibility of complex formation between tea components and copper (II) ions was supported by UV-vis spectroscopy which showed absorbance decreased with the addition of copper (II) ions to WT, GT, and BT (Figure 3.13). Yashuda *et al.* (2012) showed that UV-vis absorption of tea components within HPLC studies were affected by the presence of copper (II) ions, supporting the possibility of tea component-copper (II) complex formation. In this study, the absorbance values of the combinations was in the order WT < GT < BT suggesting the possibility of different amounts of component substances interacting with copper (II) ions (Figure 3.13). Independent studies have shown that different types of tea contain different amounts of polyphenols dependent on cultivar types, cultivation and post-harvesting processing methods (Chou *et al.* 1999; Du *et al.* 2006; Lin *et al.* 2008). In this study, the formation of hypothetical inactive complexes between tea components and copper (II) sulphate occurred with the whole WT during a test period of pre-treatment up to 30 minutes as shown when antimicrobial activity was gradually lost during this length of time prior to exposure to inoculums (Figure 3.4).

In other experiments WT plus copper (II) ion combinations as well as copper (II) ion controls required similar levels of catalase to remove antimicrobial effects (Figure 3.14). This suggested that copper (II) ions generated harmful hydrogen peroxide to which the WT did not contribute. However, the amount of hydrogen peroxide generated by the 4.8 mM copper (II) ion controls may be considerable (Goncharuk, 1995). The amount of copper (II) generated hydrogen peroxide possibly exceeded any amount produced by the tea components, and thus additional catalase was not required to remove any small amounts, of hydrogen peroxide, generated by the tea (Figure 3.14). Other investigations, however, found that tea components generate hydrogen peroxide (Arakawa *et al.* 2004; Schuck *et al.*, 2008).

In further investigations against *S. aureus* NCTC 06571 the addition of vitamin C to a combination of WT plus copper (II) ions showed the same antimicrobial activity as copper (II) ions plus vitamin C without the presence of WT (Figure 3.6). It is possible that vitamin C together with copper (II) ions could enhance the antimicrobial activity of active WT components against *S. aureus* but the putative presence of inhibitory compounds within WT prevented this.

Experiments indicated the antimicrobial activity of combinations of tea agents was independent of acidity levels following 30 minute exposures (Figures 3.1, 3.7; Tables 3.5, 4.1). Assays of the effect of pH against *S. aureus* NCTC 06571 were only seen at *ca.* pH 9 when copper (II) ions precipitated and antimicrobial activity was lost. It is known that copper (II) ions are likely to precipitate out of aqueous solutions containing high levels of hydroxide ions such as are found in aqueous alkali (Moghal and Sivapullaiah, 2012).

(ii) Viability effects and mechanisms of action of WT sub-fractions and adjuncts

McCarrell *et al.* (2008) investigating the antimicrobial activity of PRE using diffusion assays found that PRE fractions > 5 kDa had similar effects to those of < 5 kDa against target organisms including *S. aureus* NCTC 06751. In order to test the hypothesis that WT contained different substances that could either enhance or inhibit the antimicrobial activity of copper (II) ions, WT was fractionated to investigate the antimicrobial activities of the individual fractions alone and with copper (II) ions. UV-vis spectroscopy showed that WTF (< 1 kDa) had a lower absorbance than all the other fractions and the whole WT (Figure 3.12). This observation of lower UV-vis absorption of lower molecular weight plant leaf fractions has also been reported in other species (Yen *et al.*, 1996). When combined with copper (II) ions only the fraction containing the smallest molecular weight components of < 1 kDa showed antimicrobial activity, which was greater than whole WT (Figure 3.9).

In this study the white tea fraction (WTF < 1 kDa) showed no antimicrobial activity against *S. aureus* NCTC 06571 when tested alone (Figure 3.9). McCarrell *et al.* (2008) found a similar result with the PRE < 5 kDa fraction tested alone against *S. aureus* NCTC 06571. They also found that a PRE-copper(II) ion combination showed little or no increase in the antimicrobial activity against *S. aureus* NCTC 06571 – a similar result as found in this study when vitamin C was added to the WTF (< 1 kDa) plus copper (II) combination (Figures 3.10, 3.11). In this study, neither WTF (< 1 kDa) nor vitamin C showed any antimicrobial activity when tested in the absence of copper (II) ions (Figures 3.10, 3.11). Similar results were found with PRE < 5 kDa (McCarrell *et al.*, 2008).

WTF (< 1 kDa) or vitamin C combined with copper (II) ions showed a similar antimicrobial activity and this was removed in both cases with catalase (Figure 3.14). Catalase is an enzyme known to breakdown hydrogen peroxide removing its harmful effects against many bacteria (Das and Bishayi, 2009). The amounts of added catalase in each case suggested that both WTF (< 1 kDa) and vitamin C had a similar enhancement with copper (II) ions suggesting a production of similar quantities of H_2O_2, and possibly acting in a similar manner to reduce copper (II) ions in Fenton chemistry (Jomova *et al.*, 2012: Ozyurek *et al.*, 2012). Therefore, the antimicrobial activity

of copper (II) ions found naturally occurring in the whole WT was tested separately and found to show no antimicrobial activity. Following a survey of published reports no studies of the antimicrobial activity of *in vivo* levels of metal ions within plants could be found. Other studies show that metal ions found at higher levels in nature, *e.g.* in spa waters can have antimicrobial effects (Inoue *et al.*, 1999). UV-vis spectroscopy showed that WTF (< 1 kDa) had a lower absorbance than all the other fractions and the whole WT (Figure 3.12). This observation of lower UV-vis absorption of lower molecular weight plant leaf fractions has also been reported in other species (Yen *et al.*, 1996).

Measurements of pH showed that WTF (< 1 kDa) was more acidic than whole WT (Figure 3.15). Previous investigations have shown that following certain types of filtration, tea fractions contain different types of substances and have different pH values (Yoshida *et al.*, 1999; Zimmermann and Gleichenhagen, 2011). In this study WTF (< 1 kDa) also had a greater effect on reducing the pH of added copper (II) sulphate than whole WT (Figure 3.15). This fall in pH was greater than would be expected for a mix of two acidic solutions and suggested a chemical interaction had taken place between the tea components and the copper (II) sulphate with the formation of a complex with the simultaneous release of protons (Figure 3.15). This suggested the formation of acidic complexes between WTF components and copper (II) ions. Similar observations on the interactions of green and black teas with copper (II) ions affecting pH have also been reported by others (Goodman *et al.*, 2012). The activity of the WTF (< 1 kDa) compared to whole WT and WT fractions containing larger MW substances is attributed to the removal of inhibitory substances from the larger MW fractions of the tea which interfere with copper (II) ion availability.

(iii) Bacterial viabilities in the presence of main tea catechins and copper (II) sulphate

The antimicrobial activities of commonly investigated tea catechins combined with 4.8 mM copper (II) ions and tested against *S. aureus* NCTC 06751 in 30 minute exposures showed activities in the order: catechin < epicatechin (EC) < epicatechin gallate (ECG) < epigallocatechin (EGC) = epigallocatechin gallate (EGCG) (Table 3.6). The catechins tested on their own against *S. aureus* NCTC 06751 showed the same viabilities as the buffer controls (Figure 3.17). Previous studies on EC, ECG and ECG showed they produced bactericidal effects when added to non-lethal levels of copper (II) against *E. coli* (Kimura *et al.*, 1998; Hoshino *et al.*, 1999, 2000). In this study catechin was shown for the first time to be enhanced by the addition of copper (II) ions.

(iv) Viability effects and mechanisms of action of the weak antimicrobial catechin compared to its tea flavanol isomers and heat-treated catechin with putative adjuncts

The antimicrobial activity of the weak antimicrobial catechin with equimolar copper (II) sulphate against test species of bacteria were in the order *S. aureus* NCTC 06751 = *Ps. aeruginosa* NCTC

950 = *P. mirabilis* NCTC 7827 < *E. coli* NCTC 14441 (Figure 4.2). Previously, Hoshino *et al.*, 2000 showed that Gram-positive bacteria were less susceptible to tea catechin-copper (II) ion combinations than Gram-negative species. In this study, the order of catechin plus copper (II) ions compared to copper (II) sulphate controls was as follows: *E. coli* NCTC 14441 < *P. mirabilis* NCTC 7827 < *S. aureus* NCTC 06751 < *Ps. aeruginosa* NCTC 950, which suggested that catechin was most effective at raising the antimicrobial activity of copper (II) against *Ps. aeruginosa* NCTC 950 (Figure 4.2). Since this species can be relatively tolerant to copper, this finding was unexpected (Braud *et al.*, 2010; Elsen *et al.*, 2011). McCarrell *et al.* (2008) and Gould *et al.* (2009b) reported similar results, where polyphenol-rich pomegranate rind extract plus copper (II) salt had the greatest effects on *Ps. aeruginosa.* This greater susceptibility of *Ps. aeruginosa* to mixtures may result from its specific cell wall features such as type or number of porins or periplasmc proteins (Braud *et al.*, 2010).

The antimicrobial activity of catechin was enhanced by freshly prepared iron (II) sulphate solutions against *S. aureus* NCTC 06571 but not against *E. coli* NCTC 14441 (Figure 4.7). These results agree with those of McCarrell *et al.* (2008) who also found that iron (II) ions enhanced the activity of the polyphenolic rich pomegranate rind extract against *S. aureus* but not against *E. coli.* It would, therefore, appear that iron (II) ions improved the antimicrobial activity of phenolic catechin or PRE against *S. aureus* but not against tested Gram-negative species. In this study addition of catalase removed antimicrobial activity and this suggests that the catechin-iron (II) ion mixtures were generating harmful hydrogen peroxide (Table 4.5). Heat-treated and autoclaved iron (II) sulphate as well as freshly-made, boiled and autoclaved samples of iron (III) chloride did not enhance catechin against either species of bacteria (Table 4.5). This suggests that in these conditions iron (II) sulphate, presumed to have oxidised to iron (III) sulphate in the heating process, as well as iron (III) ions added directly, as iron (III) chloride, failed to generate noticeable levels of antibacterial hydrogen peroxide. A solution of an iron (III) salt without other Fenton reagents is not likely to generate hydrogen peroxide and consequently showed no antimicrobial activity.

A mixture of freshly made iron (II) and copper (II) sulphates were antimicrobial against *S. aureus* NCTC 06751 and *E. coli* NCTC 14441 (Figure 4.6). Catechin was not enhanced by this mixture. This suggested that the antimicrobial effects were due to the copper (II) ions alone or the metal (II) ion combination and likely to be due to redox cycling between the iron (II) and copper (II) ions generating reactive oxygen species (ROS) (Garcia *et al.*, 2012). The level of the antimicrobial effect in this case was similar to that seen with the catechin-copper (II) ion combination (Figure 4.2). This in turn suggested that the effect on antimicrobial activity of adding either freshly made

iron (II) sulphate or catechin to copper (II) sulphate solutions was similar against *S. aureus* NCTC 06751 and *E. coli* NCTC 14441. The mechanism of action in each case was probably via the generation of H_2O_2 which was removed by either the addition of exogenous catalase, or by EDTA to remove the availability of H_2O_2 generating copper (II) and iron (II) ions (Brown *et al.*, 1998) (Figures 4.15, 4.17) inhibitory substances from the larger MW fractions of the tea which interfere with copper (II) ion availability.

The antimicrobial activity of catechin against *S. aureus* NCTC 06571 was not enhanced by either manganese (II) sulphate or zinc (II) sulphate (Figures 4.4, 4.5). This finding was similar to that found with PRE combined with the same salts against the same strain (McCarrell *et al*, 2008). Addition of equimolar zinc (II) to a catechin-copper (II) combination with excess vitamin C did not affect the antimicrobial activity of the combination. This finding suggested that zinc (II) ions did not interfere with or add to the generation of H_2O_2 (Figure 4.4).

Heat-treated catechin solutions, autoclaved or heated at 100 °C for 10 or 30 minutes respectively showed a similar change in appearance changing from a clear, colourless solution to a clear, similarly intense yellow solution. Similar colour changes in aqueous solutions of catechin have been previously reported (Hathway and Seakins, 1957; Es-Safi *et al.*, 2003). Heat-treated catechin solutions showed no antimicrobial activity when tested alone (Figures 4.8, 4.9). In comparison, in another investigation by Kim *et al.* (2011) tannic acid, another plant polyphenol, antimicrobial activity was enhanced by heat treatment. In the production of black tea by the 'fermentation' process carried out optimally at 24 - 29 °C, antimicrobial activity against bacteria is increased but this is not due to conversion of catechin (Chou *et al.*, 1999; Kim *et al.*, 2011). In this study, heat-treated catechin solution combined with copper (II) ions showed greater antimicrobial activity than fresh untreated catechin solution with copper (II) ions against *S. aureus* NCTC 06751 (Figure 4.8). This suggested that the heat treatment had brought about the conversion of some of the catechin molecules to form new antimicrobial substances that possibly include dimeric quinone methides (see Figure 6.1.3) (Ferreira, 2012). In contrast, autoclaving catechin previously combined with copper (II) sulphate resulted in a solution containing a dark brown residual filamentous precipitate. This mixture produced a lower antimicrobial activity than a non-autoclaved combination against *S. aureus* NCTC 06571 suggesting that the precipitate represented a reduced availability of both catechin and copper (II) ions probably being an insoluble copper (II) ion-phenolic substance (Figure 4.9). Other investigations have shown that polyphenols subjected to heat treatment can form insoluble complexes with copper (II) ions (Goto and Suyama, 2000).

In this study, the formation of any antimicrobial complexes from freshly made catechin, and heat-treated catechin (10 minutes at 100 °C) and copper (II) ions was investigated. One investigation

with pH changes showed a greater decrease in pH of the mixed catechin and copper (II) ion solutions than was expected from combination of the components – a similar observation previously made with the whole WT (Figure 4.12). In this case as with the tea this result was interpreted similarly to the result with the WT combination with copper (II) ions, that a chemical interaction had taken place between the catchins and copper (II) ions forming a complex with the release of protons. In UV-vis experiments combinations of increased concentrations of copper (II) sulphate added to both fresh and heat-treated catechin solution showed progressively increased peak heights in UV-vis spectra suggesting increased binding of copper (II) ions with fresh and heat-treated catechin (Figure 4.11). Other investigations have shown appearances of peaks within UV-vis absorption spectra of flavonoids with the addition of transition metal ions (Andjelkovic *et al.*, 2006; Torreggiani, 2008). In this study, these peaks were removed with the chelation agent EDTA, suggesting that added copper (II) ions bound to the catechin were responsible for the peaks (Figure 4.11). Similar observations were made using EDTA by Andjelkovic *et al.* (2006) investigating polyphenol-iron (II) complex formation. In this study, complexation was also investigated by measuring the solubility of fresh catechin which showed that the addition of copper (II) ions increased the solubility of the catechin suggesting that complexation with copper (II) ions (Table 4.4). Increases in the solubility of polyphenols with the addition of transition metal ions has been previously reported (Miniati and Montanari, 1998; Kostyuk *et al.*, 2007).

Investigations of the effect of combining fresh catechin and copper (II) sulphate in different ratios on crystal formation, on the viability of *S. aureus* NCTC 6571, and on resultant pH of mixtures was investigated (see section 4.8). The results indicated that a complex was formed in a ratio of around 1 part of catechin to approximately around 0.66 to 1.0 molar copper (II) sulphate (*i.e.*, catechin 3 or 2 : copper (II) ion) which is within the range of polyphenol : transition metal ion binding ratios reported in other investigations (Weber, 1988; Kuo *et al.*, 1998; Mira *et al.*, 2002; Fernandez *et al.*, 2002; Esparza *et al.*, 2005; Ghosh *et al.*, 2006) (Figure 4.10).

In consideration of all of the above findings in this study, a saturated catechin-copper (II) ion complex is believed to damage the cell membrane to such an extent that any free uncomplexed labile copper (II) ions have a greater effect on the bacterial cell membrane damaged by the complex than free copper (II) ions might have acting on an intact, undamaged membrane in the absence of a complex. The enhanced antimicrobial action of free copper (II) ions acting in parallel alongside catechin-copper (II) complexes is comparable to disinfective and antiseptic mixtures of copper (II) ions whose action against cells is enhanced in the presence of other additions such as H_2O_2, mineral acid, and vitamin C (Ragab-Depre, 1982).

The antimicrobial activity of catechin and its flavan-3-ol isomers combined with copper (II) sulphate against *S. aureus* NCTC 06751 followed the same trend as averaged values of published minimum inhibitory concentrations (MICs) of the catechins against *S. aureus* when tested in the absence of copper (II) ions (Takahashi *et al.*, 1995; Akiyama *et al.*, 2001; Stapleton *et al.*, 2004a, 2004b; Gibbons *et al.*, 2004; Taguri *et al.*, 2004; Roccaro *et al.*, 2004) (Figures 1.2, 4.18) . This suggests that the copper (II) ions in this study enhanced the flavanol isomers in a manner related to their different molecular structures. The pattern of response in each case against *S. aureus* NCTC 06751 showed a structure-activity relationship dependent on enantiomeric form, epimerisation and the degree of galloylation. This is comparable in part to other studies which investigated the effect of galloylation on catechin activity with respect to membrane binding, bacterial adhesion to mammalian cells as well as bactericidal activity (Sakanaka *et al.*, 1996; Hoshino *et al.*, 2000; Kajiya *et al.*, 2002; Hayakawa *et al.*, 2004). There are relatively few studies comparing the effects of non-galloylated tea catechin isomers on cells (Stapleton *et al.*, 2004a, 2006). In this study with the isomers of catechin, the effect of the enantiomer and the two epimerised isomers was small indicating the relatively small importance of these variations in structure when combined with copper (II) ions against *S. aureus* NCTC 06751 within the conditions tested (Figure 4.18). It was possible to link the observed antimicrobial activities of the isomer-copper(II) ion combinations with their capacity to generate hydrogen peroxide (Figure 4.19).

Comparing the rates of antimicrobial action of copper (II) ions alone with fresh catechin, heat-treated catechin and EGCG combined with copper (II) sulphate against *S. aureus* NCTC 06571 showed all four substances produced their main effect on cell viability within the first minute of exposure (Figure 4.13). This new finding supported the hypothesis that the antimicrobial effect was as a result of a rapidly acting harmful ROS burst of activity (Figure 4.19). The greater activity of the EGCG is believed to be due to the galloyl side chain intercalating with the cell membrane producing a more intense effect against *S. aureus* NCTC 06751 compared to more superficial binding from other catechins (Arakawa *et al.*, 2004; Hayakawa *et al.*, 2004; Singh *et al.*, 2011; Cui *et al.*, 2012). If the heat-treated catechin solution contains branched molecular structures as suggested (Figure 6.1.3) then it is possible that such branched species may work in a similar manner against the cells providing a possible explanation as to the mechanism of the heat-treated catechin-copper (II) ion complex.

Examination of cells previously exposed to antimicrobial mixtures using oil-immersion light microscopy showed no interference with the normal Gram reaction for each cell type suggesting that the mixtures did not affect cell wall components or their reaction to Gram stain reagents sufficiently to make any visible difference compared to cells not exposed to antimicrobial mixtures

(Beveridge, 2001) (see section 4.12). Using electron microscopy to examine cells that have been exposed to harmful mixtures containing catechin, caution must be exercised in interpreting results which suggest agglutination effects from catechin mixtures since agglutinated cells may be an artefact resulting from the preparation process (Figure 4.20). However, with both *S. aureus* NCTC 06751 and *E. coli* NCTC 14441 agglutinated cells were seen in each case. Similar findings have been reported by others investigating the effect of polyphenols on the morphology and structure of cells (Bikels-Goshen *et al.*, 2010; Nakayama *et al.*, 2012). In this study, it is possible that catechin-copper (II) complexes attach to the cell envelope forming a complex which can then join to adjacent cells forming a glue-like bond between the cells causing adhesion and clumping of cells as seen in the SEM images (Figure 4.20).

v) Bacterial viabilities and mechanisms of action of stored solutions of catechin and putative adjuncts at different temperatures

Following storage for 7 days, solutions of combined catechin plus copper (II) sulphate at room temperature and at *ca.* 5 °C lost antimicrobial activity against *S. aureus* NCTC 06751 and *Ps. aeruginosa* NCTC 950 with *P. mirabilis* NCTC 7827 only showing antimicrobial activity levels similar to copper (II) sulphate controls (Figures, 5.2, 5.3, 5.4).

With *E. coli* NCTC 14441, the antimicrobial activity of room stored solution after 7 days was similar to the copper (II) sulphate controls. However, after the same period of storage at 5 °C the antimicrobial activity of the mixture was similar to buffer values which suggested a loss of antimicrobial copper (II) sulphate activity (Figure 5.5). Such a loss of antimicrobial activity with an ageing polyphenol solution, in this case catechin, may parallel the previously observed effects of WT, GT, and BT which reduced the antimicrobial activity of copper (II) sulphate against *S. aureus* NCTC 06571 (Figure 3.4). With both the ageing catechin solution and the tea extracts the observed reduction of the antimicrobial activity of copper (II) ions could be due to similar compound/s being formed in each case. In the first case with the catechin solution this could be by oxidation during the storage period and in the second case with the tea extracts by the accelerated oxidation processes that features as part of the tea 'fermentation' process (Yam *et al.*, 1997, 1998; Chou *et al.*, 1999; Ferrara *et al.*, 2001; Karori *et al.*, 2007; Kim *et al.*, 2011).

With a room stored solution of combined catechin with copper (II) ions the antimicrobial activity against *P. mirabilis* NCTC 7827 and *E. coli* NCTC 14441 increased during the first day of storage (Figures 5.4, 5.5, 5.6). This increase, hypothetically, may be due to a substance which was not formed in the 5 °C stored solution and which may be similar to a more active form – possibly similar to the heat-treated catechin which also showed higher activity than the fresh catechin. The

presence of copper (II) ions could have been important in the production of this hypothetical active compound as shown by Es-Safi *et al.* (2003) in other studies. In this study however, by the second day of storage at room temperature, the solution showed a similar antimicrobial activity as copper (II) ion controls, suggesting the loss of the active substance (Figures 5.4, 5.5, 5.6).

Overall, the changes in colour and antimicrobial activity of aqueous catechin during storage are probably due to progressive changes brought about by oxidative processes. Such processes have been studied to prevent browning of food substances (Segovia-Bravo *et al.*, 2012). Whilst vitamin C added to foods can act as a preserving agent of foods by virtues of its antioxidant properties, vitamin C added to stored catechin-copper (II) ion solutions had no effect on preserving antimicrobial activity (Banon *et al.*, 2007; Hatano *et al.*, 2008). Conversely, added vitamin C lowered antimicrobial activity of stored solutions against all test species of bacteria (Figure 5.6).

Freeze-drying is an established method of preserving the integrity of foodstuffs (Dehkarghanian *et al.*, 2009). Freeze-dried mixtures of catechin combined with copper (II) sulphate and vitamin C and stored in dry conditions at 5 °C produced the same antimicrobial activities as freshly made solutions against *S. aureus* NCTC 06751, *Ps. aeruginosa* NCTC 950, *P. mirabilis* NCTC 7827 and *E. coli* NCTC 14441 (Figure 5.7). Freeze-drying appears to be a suitable method of storing the mixture whilst preserving its antimicrobial activity. Both fresh and freeze-dried catechin-copper (II) sulphate-vitamin C mixtures produced similar antimicrobial activities against the four species suggesting a maximal effect within the conditions tested (Figure 5.7).

(vi) Possible applications of tested and investigated combinations

On account of their demonstated ability to enhance the antimicrobial activity of copper (II) and iron (II) ions in this study, combinations of agents such as fresh and/or heat-treated catechin could theoretically provide a means to reduce the amount of copper (II) ions and iron (II) ions used in certain industrial formulations (see Section 6.1.14 above). Using the enhancing effect of catechin and perhaps its derivatives the amount of these ions used in agriculture, aquaculture and other applications, could be reduced bringing down the cost of transition metal (II) ions in pest control agents. This in turn would reduce the introduction of agricultural run-off and industrial outfall into the environment which can accumulate in food chains showing progressively toxic effects on organisms at each stage in the food chain (Jacob *et al.*, 1997; Emeran *et al.*, 2011; El Hadri *et al.*, 2012).

6.3 Limitations of the experiments in the study

Investigating a minor constituent tea flavanol with relatively low antimicrobial activity such as catechin could be regarded as a less valuable aim than investigating other flavanol compounds which are in higher abundance and with higher known antimicrobial activities such as epigallocatechin gallate (EGCG) (Si *et al.*, 2006; Lin *et al.*, 2008). However, this view is contrary to a main aim of the study which is to enhance the antimicrobial activity of weak, less active, components. A proof of concept in this regard is informative if novel methods of combatting rising levels of antimicrobial resistance are to be found – it is not necessarily possible to predict which compounds when combined with each other or following specific pre-treatments will turn out to be better than others in this regard.

Extracts are a mixture of substances some of which are antimicrobials and are enhanced by adjuncts whilst other components are not antimicrobial and can inhibit the action of adjuncts. Consequently whole extracts such as tea may show little or no antimicrobial activity due to the presence of inhibitory substances as was shown in this study with the whole WT which showed less activity than the WTF.

Using bactericidal suspension assays followed by viable counts of cells whilst better than optical density measurements to establish numbers of living cells, does present a problem of degree of experimental error. This is principally due to the accumulated experimental error from each stage of the suspension assay as well as viable count procedures, principally pipetting and to a lesser extent inadequate mixing, as well as poor spreading leading to confluent colonies and damaged agar concealing colonies. Further to this, cells are likely to sustain varying degrees of injury from antimicrobial samples with a consequence that some injured cells will recover sufficiently to grow and reproduce whereas others will not. In the case of strongly antimicrobial test samples with few remaining viable cells, representative sampling could be a problem if insufficient volume was spread to represent the complete variation in surviving cells.

The use of spread plates to investigate viable cell counts presents a potential difficulty in interpretation if the viable cells are, through inadequate vortexing, clumped together since any such clump would be likely to grow into only one colony giving an underestimation of the actual number of viable cells in the sample. This is likely to be a particular problem if the cells are naturally clumped together in nature such as *S. aureus* let alone caused by exposure to antimicrobial substances.

Using electron and light microscopy to examine the effects of harmful substances on cells presented a potential problem in using centrifugation within the methods used to prepare the sample for examination. Centrifugation can cause agglutination of the cells by compacting them under centrifugal force. However, the results indicated that only samples exposed to antimicrobial mixtures containing catechin showed cell agglutination whereas controls did not (Figure 4.20).

6.4 Future work

(i) Investigating the antimicrobial activity of catechin on susceptible target microbes

Since *Ps. aeruginosa* NCTC 950 was the most susceptible species of those tested with the equimolar catechin–copper (II) ion mixture, further investigation of this species and related species of *Pseudomonas* that cause numerous problems of infection in medical, veterinary, agricultural, food storage, and other settings is warranted (Hingst *et al.*, 1987; Wagner *et al.*, 2008; Elsen *et al.*, 2011). Such investigations could include testing similar flavanols combined with a range of different adjuncts. Species of bacteria and other microbes, such as certain strains of *Ps. aeruginosa*, yeasts, rusts and blights, that show some copper tolerance, may also be more susceptible to copper (II) ions if catechin is present, and more so if other adjuncts are present.

(ii) Enhancing the activity of antimicrobial agents such as catechin by multiple additions

Alongside the catechins present in tea sub-fractions, other studies have shown WT contains other sub-components such as glycosides and polysaccharides some of which are highly active against *S. aureus* and other species of bacteria (Engelhardt *et al.*, 1993; Lin *et al.*, 2008). Junio *et al.* (2011) showed that three different flavonoid sideroxylins isolated from the flowering plant golden seal (*Hydrastis canadensis*) had no antimicrobial activity when tested alone, whereas each acted synergistically with the alkaloid berberine when tested against a range of microbes. In the quest for novel treatments to combat antimicrobial resistance, it would be useful to know what levels of antimicrobial activity could be reached by combining different active novel subcomponents, and in what proportions these would optimally act against target species of bacteria (Inoue *et al.*, 1999). Optimal combination ratios could then perhaps be further enhanced with other additives such as transition metal (II) ions to overcome the various defence mechanisms present in bacteria and other microbes. It would be pertinent to this study to investigate the effect of heat-treated catechin combined with iron (II) against *S. aureus* 06751 since fresh catechin was active against this species with iron (II) ions.

(iii) Enhancing the antimicrobial activity of agents by heat treatment

Whilst the application of heat to substances in normal atmospheric conditions is assumed to lead to oxidation with a commensurate loss in antimicrobial activity, this study along with others has

shown that this is not necessarily the case (Asaka *et al.*, 2000; Landry *et al.*, 2009; Kim *et al.*, 2011). It would be advantageous if heat treatment studies particularly with polyphenols might feature more often in experiments involving natural products, at least as controls.

As an extension to the work in this investigation and others (Asaka *et al.*, 2000; Es-Safi *et al.*, 2003; Kim *et al.*, 2011) catechin derivatives maybe further studied to investigate which derivatives following heat treatment show the highest biological activities against target species. The antimicrobial activity of catechin solution following heat treatment seen against *S. aureus* NCTC 06571 could be investigated further against other target species to establish whether the effect of heat-treated catechin is limited to certain species or is a general enhancement effect against different types, species, and strains of bacteria. Since a similar antimicrobial activity was seen following heating catechin solution after 10 and 30 minutes against *S. aureus* NCTC 06571 it would be informative to investigate the antimicrobial activity of heating catechin solution following specific periods of heat treatment *e.g.*, 0.5, 1, 2, or 5 minutes in order to ascertain what length of heating results in optimal antimicrobial activity. Assuming that such a length of heating time can be established, chemical analysis can then be performed to identify any new substances present in the solution which may be causing the rise in antimicrobial activity. Further to this, similar experiments with varied heating temperatures from 20 °C by 10 °C steps to 100 °C would answer the question whether active compounds were produced in optimal amounts at a specific temperature. Interestingly, after losing antioxidant activity following heat treatment, some polyphenol antioxidants apparently show greater antimicrobial activity *e.g.*, those within polyphenol-rich bark extract of the cinnamon tree (*Cinnamomum verum*) (Hsieh 2000; Baker, 2012).

In the investigations on autoclaved catechin solutions it was observed that catechin solutions combined with copper (II) sulphate prior to autoclaving lost antimicrobial activity following the process when subsequently tested against *S. aureus* NCTC 06751. It would be informative to know the identity of the chemical constituents of the brown precipitate as well as what remained in solution. This would provide further information about the location of the added copper (II) ions.

(iv) Enhancing the activity of antimicrobial agents such as catechin by storage

The putative transitory substance or substances which appeared to be present in one day old catechin-copper (II) ion solution and which enhance the antimicrobial activity of the solution against the Gram-negative species, *P. mirabilis* NCTC 7827 and *E. coli* NCTC 14441 but not *S. aureus* NCTC 06571 or *Ps. aeruginosa* NCTC 950 warrant further investigation. Such transient substances may be similar to the derivative substances present in heat-treated catechin solution. Since the antimicrobial activity of these transients seem to disappear after *ca.* 24 hours it raises the

question of how long it takes for such compounds to form? Time trials could be carried out to investigate the effect of time and of temperature on the process. If simply allowing antimicrobial solutions to stand for a certain period of time at a certain temperature produces rises in antimicrobial activity this would be a benefit in developing effective antimicrobial mixtures. At an optimal point in development when the solution shows the maximal antimicrobial activity it could then be freeze-dried to preserve this activity.

(v) Preserving the activity of solutions of antimicrobial mixtures

Aside from the case of freeze-dried mixtures, a loss in antimicrobial activity was observed during storage of catechin solutions (aside from the exception described above). Such a loss in activity may be due to a catechin autoxidation/ polymerisation. Investigating the exclusion of oxygen, other than by adding vitamin C from the solution may show a reduction in the loss of antimicrobial activity of the solution and would suggest a means by which such solutions may be preserved.

(vi) Mechanism of action of catechin and related flavan-3-ols with adjuncts

The examination of cells using electron microscopy at increased magnifications following exposure to antimicrobial mixtures may assist in elucidating the mode of action of catechin-copper (II) ion mixtures. This could show changes in morphology and cell structure if they occur following exposure (Hamilton-Miller and Shah, 1999; Shah *et al.*, 2008; Bikels-Goshen *et al.*, 2010).

If agglutination of cells by catechin-copper (II) ion complexes occur this would help to explain why numbers of cfus fall following exposure to such mixtures. The extent of any agglutination of viable cells may possibly be measured by the use of enzymes to separate cells. Agglutinated viable cells perhaps might be separated using extended vortexing or an anti-coagulating enzyme such as trypsin? Following such a procedure, a more precise number of remaining viable cells remaining could be investigated. Optical density methods could then also be used for a more accurate estimation of total cell numbers.

In structure-activity relationship investigations of the antimicrobial activity of the main tea flavanols epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) were often found to be the most active (Figure 1.2). Occasionally, epigallocatechin (EGC) shows equal if not greater activity against test species of bacteria which warrants a reconsideration of the role of the B ring featuring 3 OH groups compared to the galloyl group substitution of the C ring, found in ECG and EGCG, against different species and in different conditions (Hayakawa *et al.*, 2004).

This study found that tea flavanol isomers of catechin showed varying antimicrobial activities against *S. aureus* NCTC 06571 (Table 3.6). Further investigations showed that the different

isomers also when combined with copper (II) ions generated different amounts of H_2O_2 (Figure 4.19). This suggests that specific molecular features could enhance the generation of H_2O_2 when combined with copper (II) ions. The roles of each of the catechin three rings, the presence of galloyl moieties, the effect of epimerisation, the activities of the different enantiomers, and specific position of important functional groups within an antimicrobial agent all could be differentiated further.This approach could also be used to investigate the antimicrobial mechanisms of other diverse flavanols. This information would be useful in the discovery and development of novel therapeutic agents.

In this study the addition of zinc (II) ions did not affect the action of catechin when tested alone or with added adjuncts (Aarestrup and Hasman, 2004; Cavaco *et al.*, 2011; Iinuma and Tsuboi, 2012). Zinc (II) ions appeared not to block the binding of catechin to copper (II) ions (Figure 4.5). However, blocking may be investigated using the addition of borate ions which may in turn prevent the binding of catechin to other substrates (Mochizuki *et al.*, 2002). This finding may be a useful tool in investigating the mechanisms of action of flavanols with transition metal ion adjuncts.

Investigation of the solubilities of tea extracts and heat-treated catechin combined with adjuncts such as transition metal (II) ions could provide additional information on the formation of catechin complexes as well as the numbers and proportions of sub-components involved (Table 4.4). Such investigations could be matched with precise pH and UV-vis measurements of a range of putative sub-component ratios. Additionally to assist further, attempts at crystallisation of catechin and other flavanols with copper (II) ions and other substances such as caffeine using a checkerboard type experiment to investigate the effects of pH against ratios of combinations, together with an atmosphere free from oxygen (see section 4.8) may prove fruitful.

(vii) Further investigations of kinetics of catechin with different adjuncts against target microbes

Findings in this study showed that fresh and heat-treated catechin solutions when combined with copper (II) ion produced rapid antimicrobial effects, reducing the viability of *S. aureus* NCTC 06571 within seconds of contact (Figure 4.13). Such studies could be repeated with other species of microbe such as *Ps. aeruginosa* NCTC 950 where a 30 minute exposure of catechin plus copper (II) ion combination provided the greatest effect, and also with *E. coli* NCTC 14441 where the effect of catechin was minimal (Figure 4.2). Other microbes such as yeasts, and mycoplasmas would also be good potential targets to examine the defensive capabilities conferred by the cell wall or lack of one, respectively.

In addition findings from such investigations would assist in identifying types of bacteria as suitable targets for specific types of antimicrobial mixtures when particularly rapid action is required such as brief contact disinfective and antiseptic agents. Such experiments to compare the initial rate of reaction of all the catechins tested as well as a range of catechin derivatives may also give further clues as to molecular features favourable towards rapid antimicrobial activity.

(viii) Medicinal and industrial applications of catechin formulations

Plant products can often show high levels of binding to textiles, skin and leather products, metals, ceramic substances, plastics and other artificial surfaces, and transition metal ions. On account of these characteristics flavanol products and certain derivatives may find application in enhancing the effects of copper and other substances such as antiseptic fabrics and glazes, and within antifouling paints as well as surface coatings of coppered hospital surfaces. The possibility of a partial replacement of copper (II) ions within such contexts such as by catechin or by iron (II) salts, or both, within formulations may also prove useful for practical and environmental applications, where it is advantageous to minimise the use of harmful copper (II) salts. Investigations to apply antimicrobial combinations of substances in non-liquid contexts warrant further attention.

6.5 Final comments

EGCG when combined with copper (II) sulphate acted faster than catechin or the catechin derivatives, which were produced by heat treatment against *S. aureus* NCTC 06571. The ability of flavan-3-ols to form complexes with ROS generating metal ions which can bind to cell walls is probably as important as providing a source of reducing power on to Fenton agents to generate antimicrobial H_2O_2.

Since the different flavanols tested with copper (II) ions produced varying antimicrobial activities it is probable that all the flavanol-copper (II) ion mixtures produced different amounts of H_2O_2 as well as having different levels of attraction, binding and intercalation with the cell membrane. The size of the antimicrobial effect of these factors is also dependent on the conditions and the species and strain of bacterium. Generally speaking, hydrogen peroxide, produced by complexes on the outer surface of the cell and causing injury there, would not be exposed to any protective intracellular protective enzymes. With species of bacteria that are more tolerant to copper (II) ions it may be that an assembly of cell wall substances serves to reduce the effect of H_2O_2 and other ROS. Such substances normally forming part of the membrane structure may in turn be destabilised by agents such as catechin or adjuncts enabling more injurious effects from H_2O_2.

Since vitamin C failed to preserve the antimicrobial activity of catechin-copper (II) ion solution it is cautionary to note that vitamin C does not always necessarily act as an effective preservation agent and indeed may even reduce the antimicrobial activity of treatments as shown in this study, at least those stored in aqueous solution.

Tea compounds and stored catechin with adjuncts may find application for adsorbing transition metal ions possibly with a view to using such by-products, *e.g.* from the tea industry as well as spent domestic and catering tea products in adsorbing harmful metal ions such as copper (II) ion from waste water and run-off which may be present in unsafe, environmentally harmful concentrations.

References

Aarestrup, F.M. and Hasman, H. 2004. Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride and antimicrobial substances used for disinfection. *Veterinary Microbiology,* 100 (1-2), 83-89.

Abib, R.T., Quincozes-Santos, A., Nardin, P., Wofchuk, S.T., Perry, M.L., Goncalves, C.A., and Gottfried, C. 2008. Epicatechin gallate increases glutamate uptake and S100B secretion in C6 cell lineage. *Molecular & Cellular Biochemistry,* 310, 153-158.

Addy, M., Greenman., Renton-Harper, P., Newcombe, R. and Doherty, F. 1997. Studies of stannous fluoride toothpaste and gel. 2. Effects on salivary bacterial counts and plaque regrowth in vivo. *Journal of Clinical Periodontology*, 24, (2), 86-91.

Akhavan, O., Kalaee, M., Alavi, Z.S., Ghiasi, S.M.A. and Esfandiar, A. 2012. Increasing the antioxidant activity of green tea polyphenols in the presence of iron for the reduction of graphene oxide. *Carbon,* 50 (8), 3015-3025.

Akiyama, H., Fujii K., Yamasaki, O., Oono T, and Iwatsuki, K. 2001. Antibacterial action of several tannins against *Staphylococcus aureus. Journal of Antimicrobial Chemotherapy,* 48, 487-491.

Aksuner, N., Henden, E., Aker, Z., Engin, E. and Satik, S. 2012. Determination of essential and non-essential elements in various tea leaves and tea infusions consumed in Turkey. *Food Additives & Contaminants Part B – Surveillance,* 5 (2), 126-132.

Alamuri, P., Lower, M., Hiss, J.A., Himpsl, S.D., Schneider, G., and Mobley, H.L.T., 2010 Adhesion, Invasion and agglutination mediated by two trimeric autotransporters in human uropathogen *Proteus mirabilis. Infection & Immunity,* 78, 4882-4894.

Almajano, M.P., Carbo, R., Jimenez, J.A.L., and Gordon, M.H. 2008. Antioxidant and antimicrobial activities of tea infusions *Food Chemistry,* 108, 55–63.

Alvarez, L.G., Webb, C.R., and Holmes, M.A. 2011. A novel field-based approach to validate the use of network models for disease spread between dairy herds. *Epidemiology & Infection,* 139, 1863-1874.

An, B-J., Kwak, J-H., Son, J-H., Park, J-M., Lee, J-Y., Jo, C., and Byun, M-W. 2004. Biological and anti-microbial activity of irradiated green tea polyphenols. *Food Chemistry,* 88, 549-555.

Anderson, J.C., McCarthy, R.A., Paulin, S. and Taylor, P.W. 2011. Anti-staphylococcal activity and beta-lactam resistance attenuating capacity of structural analogues of (-)-epicatechin gallate. *Bioorganic & Medicinal Chemistry Letters,* 21 (23), 6996-7000.

Andjelkovic, M, Van Camp, J., De Meulenaer, B., Depaemelaere, G., Socaciu, C., Verloo, M., and Verhe, R. 2006. Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry,* 98, 23-31.

Ankolekar, C., Johnson, D., Pinto, M.D., Johnson, K., Labbe, R. and Shetty, K. 2011. Inhibitory potential of tea polyphenolics and influence of extraction time against *Helicobacter pylori* and lack of inhibition of beneficial lactic acid bacteria. *Journal of Medicinal Food,* 14 (11), 1321-1329.

Ansart, S., Perez, L., Jaureguiberry, S., Danis, M., Bricaire, F., and Caumes, E. 2007. Spectrum of dermatoses in 165 travelers returning from the tropics with skin diseases. *American Journal of Tropical Medicine & Hygiene,* 76, 184-186.

Antimicrobial resistance surveillance in Europe 2010, *Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net),* Revised December, 2011.
http://www.ecdc.europa.eu/en/publications/Publications/Forms/ECDC_DispForm.aspx?ID=774

Arakawa, H., Maeda, M., Okubu, S., and Shimamura, T. 2004. Role of hydrogen peroxide in bactericidal action of catechin. *Biological & Pharmaceutical Bulletin,* 27 (3), 277-281

Arora, D.S., and Kaur, J. 1999. Antimicrobial activity of spices. *International Journal of Antimicrobial Agents,* 12, 257-262.

Asaka, M., Murai, K., Nakanishi, R., and Aoyama, Y. 2000. An increase in antibacterial activity of green tea infusion by heat-treatment. *Journal of the Japanese Society of Food Science,* 47,708-715.

Auger, C., Al-Awwadi, N., Bornet, A., Rouanet, J.M., Gasc, F., Cros, G., and Teissedre, P.L. 2004. Catechins and procyanidins in Mediterranean diets. *Food Research International,* 37 (3), 233-245.

Aween, M.M., Hassan, Z., Muhialdin, B.J., Eljamel, Y.A., Al-Mabrok, A.S.W., and Lani, M.N. 2012. Antibacterial activity of *Lactobacilus acidophilus* strains isolated from honey marketed in Malaysia against selected multiple antibiotic resistant (MAR) Gram-postive bacteria. *Journal of Food Science*, 77, 364-371.

Azam, S., Hadi, N., Khan, N.U. and Hadi, S.M. 2004. Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicology in Vitro*, 18 (5), 555-561.

Baker, J., Sitthisak, S., Sengupta, M., Johnson, M., Jauaswai, R.K. and Morrissey, J.A. 2010. Copper stress induces a global response in *Staphylococcus aureus* and represses sae and agr expression and biofilm formation. *Applied and Environmental Microbiology*, 76 (1), 150-160.

Baker, T.I. 2012. *Personal Communication.*

Ball. A.R., Casadei, G., Samosorn, S., Bremner, J.B., Ausubel, F.M., Moy, T.I. and Lewis K. 2006. Conjugating berberine to a multidrug resistance pump inhibitor creates an effective antimicrobial. *ACS [American Chemical Society] Chemical Biology*, 1 (9), 594-600.

Bandara, B.P. 2012. *Personal communication.*

Banon, S., Diaz, P., Rodriguez, M., Garrido, M.D. and Price, A. 2007. Ascorbate, green tea and grape seed extract increase the shelf life of low sulphite beef patties. *Meat Science*, 77 (4), 626-633.

Barcia-Macay, M., Seral, C., Mingeot-Leclercq, M.P., Tulkens, P.M. and van Bambeke, F. 2006. Pharmacodynamic evaluation of the intracellular activities of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. *Antimicrobial Agents and Chemotherapy*, 50 (3), 841-851.

Basu, P., Katterle, B., Andersoson, K.K. and Dalton, H. 2003. The membrane associated form of methane mono-oxygenase from *Methylococcus capsulatus* (Bath) is a copper/iron protein. *Biochemical Journal*, 369, 417-427.

Battikh, H., Bakhrouf, A. and Ammar, E. 2012. Antimicrobial effect of Kombucha analogues. *Lebensmittel-Wissenschaft & Technologie (LWT-Food Science & Technology)*, 47, (1), 71-77.

Bekele, B., Tetemke, M. and Mogessie, A. 2006. Yeasts and lactic acid flora of tej, an indigenous Ethiopian honey wine. *Food Microbiology.* 23, 277-282.

Bellotti, N., Deya, C.,.del Amo, B. and Romagnoli, R. 2010. Antifouling paints with zinc "tannate". *Indian Engineering Chemical Research*, 49, 3386-3390.

Bernal, P., Zloh, M. and Taylor, P.W. 2009. Disruption of d-alanyl esterification of *Staphlococcus aureus* cell wall teichoic acid by the beta-lactam resistance modifier (-)-epigallocatechin gallate. *Journal of Antimicrobial Chemotherapy*, 63 (6), 1156-1162.

Berry, A.M., Davidson, P.M., Masters, J., Rolls, K. and Ollerton, R. 2011. Effects of three approaches to standardized oral hygine to reduce bacterial colonization and ventilator associated pneumonia in mechanically ventilated patients: A randomised control trial. *International Journal of Nursing Studies*, 48 (6), 681-688.

Betts, J.W., Kelly, S.M., and Haswell, S.J. 2011. Antibacterial effects of theaflavin and synergy with epicatechin against clinical isolates of *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*. *International Journal of Antimicrobial Agents*, 38 (5), 421-425.

Beveridge, T.J. 2001. Use of the Gram stain in microbiology. *Biotechnic & Histochemistry*, 76 (3), 111–118.

Bikels-Goshen, T., Landau, E., Saguy, S. and Shapira, R. 2010. Staphylococcal strains adapted to epigallocatechin gallate (EGCG) show reduced susceptibility to vancomycin, oxacillin and ampicillin, increased heat tolerance, and altered cell morphology. *International Journal of Food Microbiology*, 138 (1-2), 26-31.

Bondarenko, O., Ivask, A., Kakinen, A. and Kahru, A. 2012. Sub-toxic effects of CuO nanoparticles on bacteria: Kinetics, role of Cu ions and possible mechanisms of action. *Environmental Pollution*, 169, 89-91.

Borkow, G., Okon-Levy, N. and Gabbay, J. 2010. Copper oxide impregnanted wound dressing: biocidal and safety studies. *Wounds – a compendium of clinical research and practice*, 22, 301-310.

Borochov-Neori, H., Judeinstein, S., Harari, M., Bar-Ya'akov, I., Patil, B.S., Lurie, S. and Holland, D. 2011. Climate effect on anthocyanin accumulation and composition in the pomegranate (*Punica granatum* L.) fruit arils. *Journal of Agricultural & Food Chemistry*, 59, 5325-5334.

Bors, W., Michel, C., and Stettmaier, K. 2000. Electron Paramagnetic Resopnance Studies of Radical Species of Proanthocyanidins and Gallate Esters. *Archives of Biochemistry and Biophysics*, 374 (2), 347-355.

Bosch, E.H., Van Dorne, H. and De Vries, S. 2000. The lactoperoxidase system: the influence of iodide and the chemical and antimicrobial stability over the period of about 18 months. *Journal of Applied Microbiology*, 89 (2), 215-224.

Braud, A., Geoffroy, V., Hoegy, F., Mislin, G.L.A. and Schalk, I.J. 2010. Presence of the siderophores pyoverdine and pyochelin in the extracellular medium reduces toxic metal accumulation in *Pseudomonas aeruginosa* and increases bacterial metal tolerance. *Environmental Microbiology Reports*, 2, 419-425.

Brown, J.E., Khodr, H., Hider, R.C. and Rice-Evans, A. 1998. Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochemical Journal*, 330, 1173-1178.

Butler, L.M. and Wu, A.H. 2011. Green and black tea in relation to gynaecologic cancers. *Molecular Nutrition & Food Research*, *55* (6), 931-940.

Carlsson, S., Wiklund, N.P., Engstrand, L., Weitzberg, E. and Lundberg, J.O.N. 2001. Effects of pH, nitrite, and ascorbic acid on nonenzymatic nitric oxide generation and bacterial growth in urine. *Nitric Oxide – Biology & Chemistry*, 5 (6), 580-586.

Caturia, N., Vera–Sampler, E., Villalain, J., Mateo, C.R. and Micol, V. 2003. The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes. *Free Radical Biology & Medicine*, 34 (6), 648-662.

Cavaco, L.M., Hasman, H. and Aarestrup, F.M. 2011. Zinc resistance of *Staphylococcus aureus* of animal origin is strongly associated with methicillin resistance. *Veterinary Microbiology*, 150, 344-348.

Cavet, M.E., Harrington, K.L., Vollmer, T.R., Ward, K.W. and Zhang, J.Z. 2011. Anti-inflammatory and anti-oxidative effects of the green tea polyphenol epigallocatechin gallate in human corneal epithelial cells. *Molecular Vision*, 17, 60-61.

Celenza, G., Segatore, B., Setacci, D., Bellio, P., Brisdelli, F., Piovano, M., Garbarino, J.A., Nicoletti, M., Perilli, M. and Amicosante, G. 2012. *In vitro* antimicrobial activity of pannarin alone and in combination with antibiotics against methicillin resisitant *Staphylococcus aureus* clinical isolates. *Phytomedicine*, 19, 596-602.

Cevallos-Casals, B.A., Byrne, D., Okie, W.R. and Cisneros-Zevallos, L. 2006. Selecting new peach and plum genotypes rich in phenolic compounds and enhanced functional properties. *Food Chemistry*, 96 (2), 273-280.

Chan-Rodriguez, D., Ramon-Sierra, J., Lope-Ayoya, J., Sauri-Duch, E., Cuevas-Glory, L. and Ortiz-Vazquez, E. 2012. Antibacterial properties of honey produced by *Melipona beechii* and *Apis mellifera* against foodborne microorganisms. *Food Science & Biotechnology*, 21, 905-909.

Challice, J.S. 1973. Phenolic compounds of Genus *Pyrus* .6. Distribution of phenols amongst various tissues of *Pyrus* stem. *Journal of the Science of Food & Agriculture*, 24, 285-293.

Chen, D., Milacic, V., Chen, M.S., Wan, S.B., Lam, W.H., Huo, C., Landis-Piwowar, K.R., Cui, Q.C., Wali, A., Chan, T.H. and Dou, Q.P. 2008. Tea polyphenols, their biological effects and potential molecular targets. *Histology & Histopathology*, 23 (4), 487-496.

Chen, Q., Zhao, J., Cahitep, S. and Guo, Z. 2008. Simultaneous analysis of main catechin contents in green tea (*Camellia sinensis* L.) by Fourier transform near infrared reflectance (FT-NIR) spectroscopy. *Food Chemistry*, 113 (4), 1272-1277.

Cheruiyot, E.K., Mumera, L.M., Ngetlich, W.K., Hassanali, A., Wachira, F. and Wanyoko, J.K. 2008. Shoot epicatechin and epigallocatechin contents respond to water stress in tea [*Camellia sinensis* (L.) O. Kuntze]. *Bioscience Biotechnology & Biochemistry*, 72 (5), 1219-1226.

Chiancone, E., Ceci, P., Ilari, A., Ribacchi, F. and Stefani, S. 2004. Iron and proteins for iron storage and detoxification. *Biometals*, 17 (3), 197-202.

Cho, Y.S., Oh, J.J. and Oh, K.H. 2010. Antimicrobial activity and biofilm formation inhibition of green tea polyphenols on human teeth. *Biotechnology and Bioprocess Engineering*, 15 (2), 359-364.

Chosa, H., Toda, M., Okubo, S., Hara, Y. and Shimamura, T. 1992. [Antimicrobial and microbicidal activities of tea and catechins against *Mycoplasma*]. *Kansenshogaku zasshi.* [*The Journal of the Japanese Society for Infectious Diseases*], 66 (5), 606-611.

Chou, C.C., Lin, L.L. and Chung, K.T. 1999. Antimicrobial activity of tea as affected by the degree of fermentation and manufacturing season. *International Journal of Food Microbiology*, 48, 125-130.

Choudary, D.K., Johri, B.N. and Prakash, A. 2008. Volatiles as priming agents that initiate plant growth and defence responses. *Current Science*, 94 (5), 595-604.

Chowdary, N.B., Salama, V., Sharman, D.D., Mala, V.R. and Qadri, S.M.H. 2011. Efficacy of nutrients and fungicides against alternaria leaf blight of mulberry. *Indian Journal of Sericulture*, 50 (1), 70-74.

Chung, K.T., Wong T.Y., Wei, C.I., Huang, Y.W. and Lin, Y. 1998. Tannins and human health : A review. *Critical Reviews in Food Science & Nutrition*, 38 (6), 421-464.

Cinco, M., Banfi, E., Tubaro, A. and Della Loggia, R. 1983. A microbiological survey on the activity of a hydro alcoholic extract of chamomile. *International Journal of Crude Drug Research*, 21 (4), 145-151.

Clark, S., Porter, R., McComb, B., Lippert, R., Olson, S., Nohner, S. and Shivaprasad, H.L. 2010. Clostridial dermatitis and cellulitis: an emerging disease of turkeys. *Avian Diseases*, 54, 788-794.

Clements, A., Young, J.C., Constantinou, N., Frankel, G. 2012. Infection strategies of enteric pathogenic *Escherichia coli. Gut microbes*, 3, 71-87.

Clifford, D.P. and Repine, J.E. 1982. Hydrogen peroxide mediated killing of bacteria, *Molecular and Cellular Biochemistry*, 49, 143-149.

Cooper, J.E. and Needham, J.R. 1981. The starling (*Sturnus vulgaris*) as an experimental-model for staphylococcal infection of the avian foot. *Avian Pathology*, 10, pp. 273+

Costabile, A., Kolida, S., Klinder, A., Gietl, E., Bauerlein, M., Frohberg, C., Landschütze, V. and Gibson, G.R. 2010. A double-blind, placebo-controlled, cross-over study to establish the bifidogenic effect of a very-long-chain-inulin extracted from the globe artichoke (*Cynara scolymus*) in healthy human subjects. *British Journal of Nutrition*, 104 (7), 1007-1017.

Costabile, A., Snaghi, S., Martin-Pelaez, S., Mueller-Harvey, I., Gibson, G.R., Rastall, R.A. and Klinder, A. 2011. Inhibition of *Salmonella Typhimurium* by tannins *in vitro*. *Journal of Food Agriculture & Environment*, 9 (1), 119-124.

Couzinet-Mossion, A., Balayssac, S., Gilard, V., Malet-Martino, M., Potin-Gautier, M. and Behra, P. 2010. Interaction mechanisms between caffeine and polyphenols in infusions of *Camellia sinensis* leaves. *Food Chemistry*, 119 (1), 173-181.

Cowan, M.M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews*. 12, 564-582.

Cui, Y., Oh, Y.J., Lim, J., Youn, M., Lee, I., Pak, H.K., Park, W., Jo, W. and Park, S. 2012. AFM [Atomic force microscopy] study of the differential inhibitory effecst of the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) against Gram-positive and Gram-negative bacteria. *Food Microbiology*, 29 (1), 80-87.

Cushnie, T.P.T., Taylor, P.W., Nagaoka, Y., Uesato, S., Hara, Y. and Lamb, A.J. 2008. Investigation of the antibacterial activity of 3-*O*-octanoyl-(-)-epicatechin. *Journal of Applied Microbiology*, 105 (5), 1461-1469.

Cushnie, T.P.T. and Lamb, A.J. 2011. Recent advances in understanding the antibacterial properties of flavonoids. *International Journal of Antimicrobial Agents*, 38, 99-107.

Dagostin, S., Scharer, H.J., Pertot, I., Tamm, L. 2011. Are there alternatives to copper for controlling grapevine downy mildew in organic viticulture? *Crop Protection*, 30, 776-778.

Darwish, A.M., Mitchell, A. and Strauss, D.L. 2012. Evaluation of a 4-h static copper sulphate treatment against experimental infection of *Flavobacterium columnare* in channel catfish (*Ictalurus punctatus*). *Aquaculture research*, 43, 688-695.

Das, D. and Bishayi, B. 2009. Staphylococcal catalase protects intracellularly survived bacteria by destroying H(2)O(2) produced by the murine peritoneal macrophages. *Microbial Pathogenesis*, 47, 57-67.

Delbes-Paus, C., Dorchies, G., Chaabna, Z., Callon, C., Montel, M.C. 2010. Contribution of hydrogen peroxide to the inhibition of *Staphylococcus aureus* by *Lactococcus garvieae* in interaction with raw milk microbial community. *Food Microbiology*, 27, 924-932.

Dehkarghanian, M., Lacroix, M., and Vijayalakshmi, M.A. 2009. Antioxidant properties of green tea polyphenols encapsulated in caseinate beads. *Dairy Science & Technology*, 89 (5), 485-499.

de Moraes, C.M.B., Melo, D.A.D., Santos, R.C.V., Bitencourt, S., Mesquita, F.C., de Oliveira, F.D., Rodriguez-Caballo, E., Bartrons, R., Rosa, J.L., Ventura, F.P., de Oliveira, J.R. (2012). Antiproliferative effect of catechin in GRX cells. *Biochemsirty & Cell Biology-Biochemie et Biologie Cellulaire* , 90, 575-584.

De Pascual-Teresa, S., Santos-Buelga, C. and Rivas-Gonzales, J.C. 2000. Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *Journal of Agricultural and Food Chemistry*, 48 (11). 5331-5337.

Dignum, M.J.W., Kerlera, J. and Verpoorte, R. 2001. Vanilla production: Technological, chemical, and biosynthetic aspects. *Food Reviews International*, 17, 119–120.

Diker, K.S., Akan, M., Hascelik, G. and Yurdakok, M. 1991. The bactericidal activity of tea against *Campylobacter jejuni* and *Campylobacter coli*. *Letters in Applied Microbiology*, 12 (2), 34-35.

Diker, K.S. and Hascelik, G. 1994. The bactericidal activity of tea against *Helicobacter pylori*. *Letters in Applied Microbiology*, 19 (5), 299-300.

Donkor, E.S., Newman, M.J., Tay, S.C.K., Dayie, N.T.K.D., Bannerman, E., Olu-Taiwo, M. 2011. Investigation into the risk of exposure to antibiotic residues contaminating meat and egg in Ghana. *Food Control*. 22, 303-313.

Dowling, D.P., Betts, A.J., Pope, C., McConnell, M.L., Eloy, R. and Arnaud, M.N. 2003. Anti-bacterial silver coatings exhibiting enhanced activity through the addition of platinum. *Surface & Coatings Technology*, 163, 637-640.

Drynan, J.W., Clifford, M.N., Obuchowicz, J. and Kuhnert, N. 2010. The chemistry of low molecular weight black tea polyphenols. *Natural Products Reports*, 27 (3), 417-462.

Du, Y.Y., Liang, Y.R., Wang, H., Wang, K.R., Lu, J.L., Zhang, G.H., Lin, W.P., Li, M. and Fang, Q.Y. 2006. A study on the chemical composition of albino tea cultivars. *Journal of Horticultural Science & Biotechnology*, 81 (5), 809-812.

Dufresne, C.J. and Farnworth, E.R. 2001. A review of latest research findings on the health promotion properties of tea. *Journal of Nutritional Biochemistry*, 12, 404-421.

Dziri, S., Hassen, I., Fatnassi, S., Mrabet, Y., Casabianca, H., Hanchi, B., and Hosni, K. 2012. Phenolic constituents, antioxidant and antimicrobial activities of rosy garlic (*Allium roseum* var. *odoratissimum*) *Journal of Functional Foods*, 4, 423-432.

Ebi, G.C., Ifenacho, C.J. and Kamalu, T.N. 1999. Antimicrobial properties of *Uvaria chamae* bark. *Fitoterapia*, 70, 621-624.

El Hadri, H., Chery, P., Jalabert, S., Lee, A., Potin-Gautier, M. and Lespes, G. 2012. Assessment of diffuse contamination of agricultural soil by copper in Aquitaine region by using French national databases. *Science of the Total Environment*, 441, 239-247.

El Modafar, C., Clerivet, A. and Macheix, J.J. 1996. Flavan accumulation in stems of *Platanus* x *acerifolia* seedlings inoculated with *Ceratocystis fimbriata* f. sp. *platani*, the canker stain disease agent. *Canadian Journal of Botany - Revue Canadienne de Botanique*. 74, 1982-1987.

Elsen, S., Ragno, M. and Attree, I. 2011. PtrA is a periplasmic protein involved in Cu tolerance in *Pseudomonas aeruginosa*, *Journal of Bacteriology*, 193 (13), 3376-3378.

Engelhardt, U.H., Finger, A. and Kuhr, S. 1993. Determination of flavone C-glycosides in tea. *Zeitung fur Lebensmittel Unters Forschung*, 197 (3), 239-44.

Emeran, A.A., Sillero, J.C., Fernandez-Aparico, M. and Rubiales, D. 2011. Chemical control of fava bean rust (*Uromyces viciae-fabae*). *Crop Protection*, 30 (7), 907-912.

Ericsson,Y.1954. Effect of ascorbic acid oxidation on mucoids and bacteria in body secretions. *Acta Pathologica Microbiologica Scandinavica*, 35, 573-583.

Esparza, I., Salinas, I., Santamaria, C., Garcia-Mina, J.M. and Fernandez, J.M. 2005. Electrochemical and theoretical complexation studies of Zn and Cu with individual polyphenols. *Analytica Chimica Acta*, 543 (1-2), 267-274.

Es-Safi, N-E., Le Guernevé C., Fulcrand H., Cheynier, V. and Moutournet, M. 2000. Xanthylium salts formation involved in wine colour-changes. *International Journal of Food Science Technology*, 35, (1), 63-74.

Es-Safi, N-E., Cheynier, V. and Moutournet, M. 2003. Effect of copper oxidation of (+)-catechin in a model solution system. *International Journal of Food Science Technology*, 38, 153-163.

Espirito Santo, C., Lam, E.W., Elowsky, C.G., Quarenta, D., Domaille, D.W., Chang, C.J. and Grass, G. 2011. Bacterial killing by dry metallic copper surfaces. *Applied Environmental Microbiology*, 77, 794-802.

Fardiaz, S. 1995. Antimicrobial activity of coffee (*Coffea robusta*) extract. *ASEAN Food Journal*, 10 (3), 103-106.

Feng, J.F., Wang, Y.L., Zhan, X.C., Luo, L., Li, L.L. and Li, C.R. 2009. Theoretical studies on the shelf life of 10% ascorbic acid solution. *Acta Chimica Sinica*, 67 (6), 459-465.

Fernandez, M.T., Mira, M.L., Florêncio, M.H. and Jennings, K.R. 2002. Iron and copper chelation by flavonoids: an electrospray mass spectrometry study. *Journal of Inorganic Biochemistry*, 92 (2), 105-111.

Ferrara, L., Montesano, D. and Senatore, A. 2001. The distribution of minerals and flavonoids in the tea plant *(Camellia sinensis)*. *Farmaco*, 56 (5-7), 397-401.

Ferrazzano, G.F., Amato, I., Ingenito, A., De Natale, A. and Pollio, A. 2009. Anti-cariogenic effects of polyphenols from plant stimulant beverages (coca, coffee, tea). *Fitoterapia*, 80 (5), 255-262.

Ferreira, D. 2012. *Personal communication.* Dr. Daneel Ferreira, Chair, Department of Pharmacognosy, Research Professor in Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi, University, MS 38677, USA.

Feucht, W., Treutter, D. and Christ, E. 1997. Role of flavanols in yellowing beech trees of the Black Forest. *Tree Physiology*, 17 (5), 335-340.

Fisher, A.E.O. and Naughton, D.P. 2004. Metal ion chelating peptoids with potential as anti-oxidants: complexation studies with cupric ions. *Journal of Inorganic Biochemistry*, 98, 343-346.

Fitzgerald, D.J., Stratford, M., Gasson, M.J., Uekert, J., Bos, A. and Narbad, A. 2004. Mode of antimicrobial action of vanillin against *Escherichia coli, Lactobacillus plantarum* and *Listeria innocua. Journal of Applied Microbiology*, 97 (1), 104-113.

Fleurat-Lessard, P., Dedaldechamp, F., Thibault, F., Bere, E. and Roblin, G. 2011. Antifungal effects of iron sulphate on grapevine fungal pathogens. *Sciencia Horticulturae*, 130, 517-523.

Floret, D. 2001. Clinical aspects of staphylococcal and streptococcal toxinic diseases. *Archives de Pediatrie*, 8 (4), 762S-768S.

Friedman, M., Henika, P.R., Levin, C.E., Mandrell, R.E. and Kozukue, N. 2006. Antimicrobial activities of tea catechins and theaflavins and tea extracts against *Bacillus cereus. Journal of Food Protection.* 69 (2), 354-361.

Franco, W., Hsu, W.Y. and Simmone, A.H. 2010. Survival of *Salmonella* and *Staphylococcus aureus* in mexican red salsa in a food service setting. *Journal of Food Protection*, 73, 1116-1120.

Garcia, C.R., Angele-Martinez, C., Wilkes, J.A., Wang, H.C., Battin, E.E. and Brumaghim, J.L. 2012. Prevention of iron- and copper-mediated DNA damage by catecholamine and amino acid neurotransmitters, L-DOPA, and curcumin: Metal binding as a general antioxidant mechanism. *Dalton Transactions*, 41 (21), 6458-6467.

Garratt, L. 1996. The return of infectious disease. *Global Issues*, 1, 20-26.

Ghassempour, A., Mollayi, S., Farzaneh, M., Sharif-Tehrani, A. and Aboul-Enein, H.Y. 2011. Variation in catechin, epicatechin and their enantiomers concentrations before and after wheat cultivar-*Puccinia triticina* infection. *Food Chemistry*, 125 (4), 1287-1290.

Ghosh, K.S., Maiti, T.K., Mandal, A. and Daspupta, S. 2006. Copper complexes of (-)-epicatechin gallate and (-)-epigallocatechin gallate act as inhibitors of Ribonuclease A *FEMS Letters*, 580 (19), 4703-4708.

Gibbons, S., Moser, E. and Kaatz, G.W. 2004. Catechin gallates inhibit multidrug resistance (MDR) in *Staphylococcus aureus. Planta Medica*, 70, 1240-42.

Goncharuk, V.V., Savluk, O.S., Potapchenko, N.G. and Ilyashenko, V.V. 1995. Disinfection by hydrogen peroxide in the presence of metal ions as catalysts of its decomposition. *Dopovidi Natsional'noyi Akademiyi Nauk Ukrayiny*, 0 (6), 123-127.

Goodman, B.A., Severino, J.F., and Pirker, K.F. 2012. Reactions of green and black teas with Cu(II). *Food & Function*, 3, 399-409.

Gordon, N, and Wareham, DW. 2010. Antimicrobial activity of the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) against clinical isolates of *Stentrophomonas maltophilia. International Journal of Antimicrobial Agents*, 36, 129-131.

Goto, M. and Suyama, K. 2000. Occlusion of transition metal ions by new adsorbents synthesised from plant polyphenols and animal fibrous proteins. *Applied Biochemistry and Biotechnology*, 84-86, 1021-1038.

Gould, S.W., Fielder, M.D., Kelly, A.F. and Naughton, D. 2009a. Anti-microbial activities of pomegranate rind extracts: enhancement by cupric sulphate against clinical isolates of *S. aureus*, MRSA and PVL positive CA-MSSA. *BMC Complementary & Alternative Medicine*, 9, 23.

Gould, S.W.J., Fielder, M.D., Kelly, A.F., El Sankary, W. and Naughton, D.P. 2009b. Antimicrobial pomegranate rind extracts: enhancement by Cu(II) and vitamin C against clinical isolates of *Pseudomonas aeruginosa. British Journal of Biomedical Science*, 66, 129-132.

Graça, A., Abadias, M., Salazar, M, and Nunes C. 2011. The use of electrolyzed water as a disinfectant for minimally processed apples. *Postharvest Biology & Technology*, 61,172-177.

Gradisar, H., Pristovsek P., Plaper, A. and Jerala, A. 2007. Green tea catechins inhibit bacterial DNA gyrase by interaction with its ATP binding site. *Journal of Medicinal Chemistry*, 50 (2), 264-71.

Greger, M. 2007. The human/animal interface: emergence and resurgence of zoonotic infectious diseases. *Critical Reviews in Microbiology*, 33, 243-299.

Grube, M., Furnkranz, M., Zitzenbacher, S., Huss, H. and Berg, G. 2011. Emerging multi-pathogen disease caused by *Didymella bryoniae* and pathogenic bacteria on Styrian oil pumpkin. *European Journal of Plant Pathology*, 131, 539-548.

Guardiola, F.A., Cuesta, A., Meseguer, J. and Esteban, M.A. 2012. Risks of using antifouling biocides in aquaculture. *International Journal of Molecular Science*, 13, 1541-60.

Hagerman, A.E., Dean, R.T. and Davies, M.J. 2003. Radical chemistry of epigallocatechin gallate and its relevance to protein damage. *Archive of Biochemistry & Biophysics*, 414 (1), 115-120.

Hamilton-Miller, J.M.T., and Shah, S. 1999. Disorganisation of cell division of methicillin-resistant *Staphylococcus aureus* by a component of tea (*Camellia sinensis*): a study by electron microscopy. *FEMS Microbiology Letters*, 176, 463-469.

Hara-Kudo, Y., Yamasaki, A., Sasaki, M., Okubo, T., Minai, Y., Haha, M., Kondo, K. and Sugita-Konishi, Y. 2005. Antibacterial action on pathogenic bacterial spores by green tea catechins. *Journal of the Science of Food & Agriculture*, 85 (14), 2354-2361.

Hassan, M.A., El-Naggar, M.Y., and Said, W.Y. 2001. Physiological factors affecting the production of an antimicrobial substance by *Streptomyces violates* in batch cultures. *Egyptian Journal of Biology*, 3, 1-10.

Hassan, M.S., Amna, T., Pandeya, D.R., Hamza, A.M., Bing, Y.Y., Kim, H.C. and Khil, M.S. 2012. Controlled synthesis of Mn_2O_3 nanowires by hydrothermal method and their bactericidal and cytotoxic impact: a promising future material. *Applied Microbiology and Biotechnology*, 95 (1), 213-222.

Hatano, T., Kusada, M., Hori, M., Shiota, S., Tsuchiya, T. and Yoshida, T. 2003. Theasinensin A, a tea polyphenol formed from (-)-epigallocatechin gallate, suppresses antibiotic resistance of methicillin-resistant *Staphylococcus aureus. Planta Medica*, 69 (11), 984-989.

Hatano,T., Kusada, M., Inada, K., Ogawa, T.O., Shiota, S., Tsuchiya, T. and Yoshida, T. 2005. Effects of tannins and related polyphenols on methicillin resistant *Staphylococcus aureus. Phytochemistry*, 66 (17), 2047-2055.

Hatano, T., Tsugawa, M., Kusuda, M., Taniguchi, S., Yoshida, T., Shiota, S. and Tsuchiya, T. 2008. Enhancement of antibacterial effects of epigallocatechin gallate using ascorbic acid, *Phytochemistry*, 69, 3111-3116.

Hathway, D.E. and Seakins, J.W.T. 1957. Enzymic oxidation of catechin to a polymer structurally related to some phlobatannins. *Biochemical Journal*, 67, 239-245.

Hayakawa, F., Kimura, T., Hoshino, N. and Ando T. 1999. DNA cleavage activities of (-)-epigallocatechin, (-)-epicatechin, (+)-catechin, and (-)-epigallocatechin gallate with various kinds of metal ions: *Bioscience Biotechnology & Biochemistry*, 63, 1654-1656.

Hayakawa, F., Ishizu, Y., Hoshino, N., Yamaji, A., Ando, T. and Kimura, T. 2004. Prooxidative Activities of Tea Catechins in the Presence of Cu^{2+}. *Bioscience Biotechnology & Biochemistry*, 68 (9), 1825-1830.

Hingst, V., Maiwald, M. and Sonntag, H.G. 1987. Investigations on the enzymatic degradation of formaldehyde by isolates of the species *Pseudomonas aeruginosa*. *Zentralblatt fur Bacteriologie Mikrobiologie und Hygiene Serie B- Umwelthygiene Krankenhaushygiene Arbeitshygiene Präventative Medizin*, 184 (20), 167-181.

Hoellein, L. and Holzgrabe, U. 2012. Ficts and facts of epinephrine and norepinephrine stability in injectable solutions. *International Journal of Pharmaceutics*, 434 (1-2), 468-480.

Hoshino, N., Kimura, T., Yamaji, A. and Ando, T. 1999. Damage to the cytoplasmic membrane of *Escherichia coli* by catechin-copper (II) complexes, *Free Radical Biology and Medicine*, 27 (11-12) 1245-1250.

Hoshino, N., Kimura, T., Hayakawa, F., Yamaji, A. and Ando, T. 2000. Bactericidal activity of catechin-copper (II) complexes against *Staphylococcus aureus* compared with *Escherichia coli*. *Letters in Applied Microbiology*, 31, 213-217.

Hseuh, P., Chen, W., Luh, K. 2005. Relationships between antimicrobial use and antimicrobial resistance in Gram-negative bacteria causing nosocomial infections from 1991-2003 at a university hospital in Taiwan. *International Journal of Antimicrobial Agents*, 26, 463-472.

Hsieh, P-C. 2000. Antimicrobial effect of cinnamon extract. *Taiwanese Journal of Agricultural & Chemical Food Science*, 38, 184-193.

Hsieh, P-C., Mau, J-L. and Huang, S-H. 2001. Antimicrobial effect of various combinations of plant extracts. *Food Microbiology*, 18, 35-43.

Hung, C.Y., and Yen, G.C. 2002. Antioxidant activity of phenolic compounds isolated from *Mesona precumbens* Hemsl. *Journal of Agricultural and Food Chemistry*, 50 (10), 2993-2997.

Imade, G., Sagay, A., Egah, D., Onwuliri, D., Grigg, M., Egbodo, C., Thacher, T., Potts, M. and Short, R. 2008. Prevalence of HIV and other sexually transmissible infections in relation to lemon or lime juice douching among female sex workers in Jos, Nigeria. *Sexual Health*, 5, 55-60.

Ikigai, H., Nakae, T., Hara, Y. and Shimamura, T. 1993. Bactericidal catechins damage the lipid bilayer. *Biochimica et Biophysica Acta*, 1147 (1), 132-136.

Isaacs, C.E., Wen, G.Y., Xu, W., Jia, J.H., Rohan, L., Corbo, C., Di Maggio, V., Jenkins, E.C. and Hillier, S. 2008. Epigallocatechin gallate inactivates clinical isolates of *Herpes simplex* virus. *Antimicrobial Agents & Chemotherapy*, 52, 962-970.

Iinuma, K., and Tsuboi, I. 2012. Zinc ascorbate has superoxide dismutase-like activity and *in vitro* antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*. *Clinical, cosmetic and investigational dermatology*, 5, 135-140.

Ikeda, I., Kobayashi, M., Hamada, T., Tsuda, K., Goto, H., Imaizumi, K., Nozawa, A., Suigimoto, A. and Kuda, T. 2003. Heat-epimerized tea catechins rich in gallocatechin gallate and catechin gallate are more effective to inhibit cholesterol absorption than tea catechins rich in epigallocatechin gallate and epicatechin gallate. *Journal of Agricultural and Food Chemistry*, 51 (25), 7303-7307.

Inoue, T., Inoue, S. and Kubota, K. 1999. Bactericidal activity of manganese and iodide ions against *Staphylococcus aureus*: A possible treatmnent for acute atopic dermatitis. *Acta Dermato-Venerologica*, 19 (5), 360-362.

Isogai, E., Isogai, H., Hirose, K., Hayashi, S. and Oguma, K. 2001. *In vivo* synergy between green tea extract and levofloxacin against enterohemorrhagic *Escherichia coli* O157 infection. *Current Microbiology*, 42, 248-251.

Isaacs, C.E., Xu, W.M., Merz, G., Hillier, S., Rohan, L., and Wen, G.Y. 2011. Digallate dimers of (-)-epigallocatechin gallate inactivate *Herpes simplex* virus. *Antimicrobial Agents and Chemotherapy*, 55 (12), 5646-5653.

Jackson, E.L. and Hudson, C.S. 1937, A new method of determining ring structures and alpha and beta configurations of glycosides. *Journal of the American Chemical Society*, 59, 994-1003.

Jacob, C. K. and Idicula, S.P. 1997. Effect of fungicide spraying on pink disease incidence in *Hevea brasiliensis*. *Indian Journal of Natural Rubber Resreach*, 10 (1-2), 48-52.

Jansen, R., Gerth, K., Steinmetz, H., Reinecke, S., Kessler, W., Kirschning, A. and Müller, R. 2011. Elansolid A3, a unique *p*-quinone methide antibiotic from *Chitinophaga sancti*. *Chemistry*, 17, (28), 7739-7744.

Johnson, B.J., Delehanty, J.B., Lin, B. and Ligler, F.S. 2008. Immobilized proanthocyanidins for the capture of bacterial lipopolysaccharides. *Analytical Chemistry*, 80 (6), 2113-2117.

Jomova, K., Baros, S. and Valko, M. 2012. Redox active metal-induced oxidative stress in biological systems. *Transition Metal Chemistry*, 37 (2), 127-134.

Jungeblut, C.W. 1939. A further contribution to vitamin C theory in experimental poliomyelitis. *Journal of Experimental Medicine*, 70, 315.

Junio, H.A., Sy-Cordero, A.A., Arlene, A., Ettefagh, K.A., Burns, J.T., Micko, K.T., Graf, T.N., Richter, S.J., Cannon, R.E., Oberlies, N.H. and Cech, N.B. 2011. Synergy-directed fractionation of botanical medicines: a case study with goldenseal (*Hydrastis canadensis*). *Journal of Natural Products*. 74, 1621-1629.

Joubert, E., Manley, M. and Botha, M. 2008. Evaluation of spectrophotometric methods for screening of green rooibos (*Aspalathus linearis*) and green honey bush (*Cyclopia gensitoides*) extracts for high levels of bio-active compounds. *Phytochemical Analysis*, 19 (2), 169-178.

Kajiya, K., Kumazawa, S. and Nakayama, T. 2002. Effects of external factors on the interaction of tea catechins with lipid bilayers. *Bioscience Biotechnology and Biochemistry*, 66 (11), 2330-2335.

Karak, T. and Bhagat, R.M. 2010. Trace elements in tea leaves, made tea and tea infusion: A review. *Food Research International*, 43, 2234-2252.

Karori, S.M., Wachira, F.N., Wanyoko, J.K. and Ngure, R.M. 2007. Antioxidant capacity of different types of tea products. *African Journal of Biotechnology*, 6 (19), 2287-2296.

Kaur, R.P., Dikshit, R.P. and Hoondal, G.S. 2005. Synergistic antimicrobial activity of tea & antibiotics. *Indian Journal Medical Research*, 122, 80-84.

Keefe, G. 2012. Update on control of *Staphylococcus aureus* and *Streptococcus agalactiae* for management of mastitis. *Veterinary Clinics of North America-Food Animal Practice*, 28, 203+

Khalil, R.K.S. 2010. Influence of gallic acid and catechin polyphenols on probiotic properties of *Streptococcus thermophilus* CHCC 3534 strain. *World Journal of Microbiology & Biotechnology*, 26, 2069-2079.

Khalil, O.A.K., Oliveira, O.M.M.F., Vellosa, J.C.R, de Quadros, A.U., Dalposso, L.M., Karam, T.K. and Mainardes, R.M. 2012. Curcumin antifungal and antioxidant activities are increased in the presence of ascorbic acid. *Food Chemistry*, 133 (3), 1001-1005.

Kim, M.S., Lee, W.K., Kim, H.Y., Kim, C., Ryu, Y.W. 1998. Effect of environmental factors on flavanol glycoside production and phenylalanine ammonia-lyase activity in cell suspension cultures of *Gingko biloba*. *Journal of Microbiology & Biotechnology*, 8, 237-244.

Kim, T.N., Feng, Q.L., Kim, J.O., Wu, J., Wang, H., Chen, G.C. and Cul, F.Z. 1998. Antimicrobial effecst of metal ions (Ag^{+}, Cu^{2+}, Zn^{2+}) in hydroxyapatite. *Journal of Materials Science-Materials in Medicine*, 9 (3), 129-134.

Kim, K.M., Lee, B.Y., Kim Y.T., Choi, S.G., Lee, J., Cho, S.Y. and Choi, W.S. 2006. Development of antimicrobial edible film incorporated with green tea extract. *Food Science & Biotechnology*, 15 (3), 478-481.

Kim, T.J., Silva, J.L., and Jung, Y.S. 2011. Enhanced functional properties of tannic acid after thermal hydrolysis. *Food Chemistry*, 126, 116-120.

Kim, Y., Goodner, K.L., Park, J.D., Choi, J. and Talcott, S.T. 2011. Changes in antioxidant phytochemicals and volatile composition of *Camellia sinensis* by oxidation during tea fermentation. *Food Chemistry*, 129, 1331-1342.

Kimura, T., Hoshino, N., Yamaji, A., Hayakawa, F. and Ando, T. 1998. Bactericidal activity of catechin-copper (II) complexes on *Escherichia coli* ATCC11775 in the absence of hydrogen peroxide. *Letters in Applied Microbiology*, 27, 328-330.

Kofink, M., Papagiannopoulos, M. and Galensa, R. 2007. (-)-Catechin in cocoa and chocolate: Occurence and analysis of an atypical flavan-3-ol enantiomer. *Molecules*, 12 (7), 1274-1288.

Kuhnert, N., Clifford, M.N. and Müller, A. 2010. Oxidative cascade reactions yielding polyhydroxy-theaflavins and theacitrins in the formation of black tea thearubigins: Evidence by tandem LC-MS. *Food Function*, 1, 180-199.

van Koningsveld, G.A., Gruppen, H., de Jonge, H.H.J., Wijngaards, G., van Boekel, M.A.J.S., Walstral, P. and Voragen, A.G.J. 2002. The solubility of potato proteins from industrial potato juice as influenced by pH and various additives. *Journal of the Science of Food and Agriculture*, 82 (1), 134-142.

Kopteva, Z.H.P., Zanina, V.V., Kopteva A.E., Serednitskii, Y.A.A., Suprun, V.V., Bodak V.S. 1988. Bactericidal properties of oil bitumen-based and coal-based anticorrosion materials. *Mikrobiologicheskii Zhurnal*, 50, 87-91.

Kostyuk, V.A., Potapovich, A.I., Kostyuk, T.V. and Cherian, M.G. 2007. Metal complexes of dietary flavonoids:Evaluation of radical scavenger properties and protective activity against oxidative stress *in vivo*. *Cellular and Molecular Biology*, 53 (1), 62-69.

Kumamoto, M., Sonda, T., Nagayama, K. and Tabata, M. 2001. Effects of pH and metal ions on antioxidative activities of catechins. *Bioscience Biotechnology & Biochemistry*, 65 (1), 126-132.

Kuo, S.M., Leavitt, P.S. and Lin, C.P. 1998. Dietary flavonoids interact with trace metals and affect metallothionein level in human intestinal cells. *Biological Trace Element Research*, 62 (3), 135-153.

Lambers, F.A.E., Prins, M, Thomas, X., Molenkamp, R., Kwa, D., Brinkman, K., van der Meer, J.T.M., and Schinkel, J. 2011. Alarming incidence of hepatitis C virus re-infection after treatment of sexually acquired acute hepatitis C virus infection in HIV-infected MSM. *Aids*, 25, F21-F27.

Landry, B.K., Nadworny, P.L., Omotoso, O.E., Maham, Y., Burrell, J.C. and Burrell, R.E. 2009, The kinetics of thermal instability in nanocrystalline silver and the effect of heat treatment on the antibacterial activity of nanocrystalline silver dressings. *Biomaterials*, 30, (36), 62929-62939.

Lebedev, V.S., Volodina, L.A., Deinega, E.Y. and Fedorov, Y.I. 2005. Structural modifications of the surface of *Escherichia coli* bacteria and copper-induced permeability of plasma membrane. *Biofizika*, 50 (1), 107-113.

Leber, C.K. 2006. Topical wound dressing and making same. *US Patent* 2006094996.

Lehrke, G., Hernaez, L., Mugliaroli, S.L., von Staszewski, M. and Jagus, R.J. 2011. Sensitisation of *Listeria* innocua to inorganic acids by natural antimicrobials. *LWT-Food Sience & Technology*, 44 (4), 984-991.

Lespinas, F., Dupuy, G., Revol, F. and Aubry, C. 1989. Enzymic urea assay: a new colorimetric method based on hydrogen peroxide measurement. *Clinical Chemistry*, 35 (4), 658-662.

Levitt. S, Steyn, K., Dave, J. and Bradshaw, D. 2011. Chronic noncommunicable diseases and HIV-AIDS on a collision course: relevance for health care delivery, particularly in low resource settings-insights from South Africa. *American Journal of Clinical Nutrition*, 94, 1690-1696.

Lewis, K. and Ausubel, F.M. 2006. Prospects for plant-derived antibacterials. *Nature Biotechnology*, 24 (12), 1504-1507.

Lho, I.H., Kishikawa, S., Kamesaki, Y., Kato, F. and Murata, A. 1991. Effects of iron (II)-ascorbate complex on various bacteria. *Nippon Nogeikagaku Kaishi - Journal of the Japan Society for Bioscience Biotechnology and Agrochemistry*, 65 (12), 1761-1768.

Li, Y., Du, Y.M. and Zou, C. 2009. Effects of pH on antioxidant and antimicrobial properties of tea saponins. *European Food Research & Technology*, 228 (6), 1023-1028.

Lim, Y.C., and Cha, Y.Y. 2011. Epigallocatechin-3-gallate induces growth inhibition and apoptosis of human anaplastic thyroid carcinoma cells through suppression of EGFR/ERK pathway and cyclin B1/CDK1 complex. *Journal of Surgical Oncology*, 104, 776-780.

Lin, L-Z, Chen, P. and Harnley, J. 2008. New phenolic components and chromatographic profiles of green and fermented teas. *Journal of Agricultural & Food Chemistry*, 56, 8130-8140.

Liu, E.H., Qi, L.W., and Li, P. 2010. Structural relationship and binding mechanisms of five flavonoids with bovine serum albumin. *Molecules*, 15, 9092-9103.

Liu, H.M., Guo, J.H., Cheng, Y.J., Liu, P., Long, C.A., Deng, B.X. 2010. Inhibitory activity of tea polyphenol and *Hanseniospora uvarum* against *Botrytis cinera* infections. *Letters in Applied Microbiology*, 51, 258-263.

Lo Vecchio, A. and Zacur, G.M. 2012. *Clostridium difficile* infection: an update on epidemiology, risk factors, and therapeutic options. *Current Opinion in Gastroenterology*, 28, 1-9.

Luna, M.C., Tudela, J.A., Martinez-Sanchez, A., Allende, A., Marin, A. and Gil, M.I. 2012. Long-term deficit and excess of irrigation influences quality and browning related enzymes and phenolic metabolism of fresh-cut iceberg lettuce (*Lactuca sativa* L.). *Postharvest Biology and Technology*, 73, 37-45.

Mabe, K., Yamada, M., Oguni, I. and Takahashi, T. 1999. *In vitro* and *in vivo* activities of tea catechins against *Helicobacter pylori*. *Antimicrobial Agents and Chemotherapy*, 43 (7), 1788-1791.

McCarrell, E.M., Gould, S.W. Fielder, M.D., Kelly, A.F., El Sankary, W. and Naughton, D. 2008. Antimicrobial activities of pomegranate rind extracts: enhancement by addition of metal salts and vitamin C. *BMC Complementary & Alternative Medicine*, 8, 64.

Maestre, R., Micol V., Funes, L. and Medina, I. 2010. Incorporation of grape seed extract in membranes and relation with efficacy in muscle foods. *Journal of Agriculture and Food Chemistry*, 58 (14), 8365-8374.

Maletta, A.B. and Were, L.M. 2012. Effect of coffee filtrate, methylglyoxal, glyoxal, and caffeine on *Salmonella Typhimurium* and *S. enteritidis* survival in ground chicken breasts. *Journal of Food Science*, 77 (2), M135-M141.

Mandal, S., DebMandal, M., Pal, N.K. and Saha, K. 2010. Antibacterial activity of honey against clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhi. *Asian Pacific Journal of Tropical Medicine*, 3, 961-964.

Maurizio, B., Saverio, B., Giancarlo, P., Federica, R. and Arnaldo, C. 2008. Inhibition of human prostate cancer progression by administration of green tea catechins: A two years later follow-up update. *Journal of Urology*, 179 (4), 224.

Mavroidi, N. 2008. Transmission of zoonoses through immigration and tourism. *Veterinaria italiana*, 44 (4), 651-656.

Mehrotra, S., Ashwani, K.S. and Shoma, P.N. 2010. Comparative antimicrobial activities of neem, amla, aloe, Assam tea and clove extracts against *Vibrio cholera*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Journal of Medicinal Plants Research*, 4 (18), 2473-2478.

Meltzer, S.M., Monk, B.J., and Tewari, K.S. 2008. Green tea catechins for treatment of external genital warts. *American Journal of Obstetrics & Gynecology*, 200, 233.

Miller, T.E. 1969. Killing and lysis of Gram-negative bacteria through the synergistic effects of hydrogen peroxide, ascorbic acid, and lysozyme. *Journal of Bacteriology*, 98 (3), 949-955.

Mimica-Dukic, N., and Bozin, B. 2007. Essential oils from Lamiaceae species as promising antioxidant and antimicrobial agents. *Natural Product Communications*, 2 (4), 445-452.

Ming, L.J. and Epperson, J.D. 2002. Metal binding and structure-activity relationship of the metalloantibiotic peptide bacitracin. *Journal of Inorganic Biochemistry*, 91 (1), 46-58.

Miniati, M. and Montanari, L. 1998. Extraction and purification of rutin from tobacco leaf protein technology wastes. *Italian Journal of Food Science*, 10 (4), 339-349.

Mira. L., Fernandez, M.T., Santos, M., Rocha, R., Florencio, M.H. and Jennings, K.R. 2002. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radical Research*, 36 (11), 1199-1208.

Mitchell, C., Manhart, L.E., Thomas, K.K., Agnew, K. and Marrazo, J.M. 2011. Effect of sexual activity on vaginal colonization with hydrogen peroxide-producing lactobacilli and *Gardnerella vaginalis*. *Sexually Transmitted Diseases*, 38, 1137-1144.

Mitsumoto, M., O'Grad, M.N., Kerry, J.P. and Buckley, D.J. 2005. Addition of tea catechins and vitamin C on the sensory evaluation, colour and lipid stability during chilled storage in cooked or raw beef and chicken patties. *Meat Science*, 69 (4), 773-779.

Mochizuki, M., Yamazaki, S., Kano, K. and Ikeda. T. 2002. Kinetic analysis and mechanical aspects of autoxidation of catechins. *Biochimica et Biophysica Acta – General Subjects*, 1569 (1-3), 35-44.

Moghal, A.A.B. and Sivapullaiah, P.V. 2012. Retention characteristics of Cu^{2+}, Pb^{2+}, and Zn^{2+} from aqueous solutions by two types of low lime fly ashes. *Toxilogical and Environmental Chemistry*, 94 (10), 1941-1953.

Mohamed, A.F., Nielsen, E.I., Cars, O. and Friberg, L.E. 2012. Pharmacokinetic-pharmacodynamic model for gentamicin and its adaptive resistance with predictions of dosing schedules in neonates. *Antimicrobial Agents and Chemotherapy*, 56 (1), 179r-188.

Montalban-Lopez, M., Sanchez-Hidalgo, M, Valdivia, E., Martinez-Bueno, M and Maqueda, M. 2011. Are bacteriocins underexploited? NOVEL applications for OLD antimicrobials. *Current Pharmaceutical Biotechnology*, 12 (8), 1205-1220.

Montuelle, B., Dorigo, U., Berard, A., Volat, B., Bouchez, A., Tlili, A., Gouy, V., and Pesce, S. 2010. The periphyton as a multimetric bioindicator for assessing the impact of land use on rivers: an overview of the ArdiSres-Morcille experimental watershed (France). *Hydrobiologia*, 657 (1), 123-141.

Moonen, B., Cohen, J.M., Snow, R.W., Slutsker, L., Drakeley, C., Smith, D.L., Abeyasinghe, A.A., Rodriguez, M.H., Maharaj, R., Tanner, M. and Targett, G. 2010. Malarial elimination 3 Operational strategies to achieve and maintain malarial elimination. *Lancet*, 376, 1592-1603.

Mueller-Harvey, I. 2011. *Personal communication.*

Munro, L.A. and Wallace, I.S. 2011. Analysis of farmed fish movements between catchments identifies a simple compartmentalised management strategy for bacterial kidney disease in Scottish aquaculture. *Aquaculture*, 338, 300-303.

Murata, A., Yano, N. 1990. Killing effect of vitamin C on bacteria and yeasts. *Vitamins (Japan)*, 64 (12), 709-713.

Murata, A., Kinoshita, R., Inque, Y., and Kato F. 1991. Mechanism of bactericidal action of ascorbic acid on *Escherichia coli*. *Vitamins (Japan)*, 65 (9), 439-445.

Nakai, S., Inoue, Y., Hosomi, M. and Murikami, A. 2000. *Myriophyllum spicatum* released allelopathic polyphenols inhibiting growth of blue-green algae *Microcystis aeruginosa*. *Water Research*, 34 (11), 3026-3032.

Nakai, S., Inoue, Y. and Hosomi, M. 2001. Algal growth inhibition effects and inducement modes by plant-producing phenols. *Water Research*, 35 (7), 1855.1859.

Nakayama, T., Ichiba, M., Kuwubara, M., Kajiya, K and Kumazawa, S. 2002. Mechanisms and structural specificity of hydrogen peroxide formation during oxidation of catechins. *Food Science and Technology Research*, 8 (3), 261-267.

Nakayama, M., Shigemune, N., Tsugukuni, T., Jun, H., Matsushita, T., Mekada, Y., Kurahachi, M. and Miyamoto, T. 2012. Mechanism of the combined anti-bacterial effect of green tea extract and NaCl against *Staphylococcus aureus* and an *Escherichia coli* 0157:H7. *Food Control*, 25 (1), 225-232.

Nas, M.N. 2004. *In vitro* studies on some natural beverages as botanical pesticides against *Erwinia amylovora* and *Curtobacterium flaccumfaciensis* subsp. *Poinsettiae. Turkish Journal of Agriculture and Forestry*, 28 (1), 57-61.

Navas, E. 2002. Problems associated with potential massive use of antimicrobial agents as prophylaxis or therapy of a bioterrorist attack. *Clinical Microbiology and Infection*, 8 (8), 534-539.

Ndounla, J., Spuhler, D., Kenfack, S., Wethe J. and Pulgarin, C. 2013. Inactivation by solar-Fenton in pet bottles of wild enteric bacteria of natural well water: Absence of regrowth after one week of subsequent storage. *Applied catalysis B – Environmental*, 129, 309-317.

Neves, A.L.D., Komescu, M.C. and Di Matteo, M.A.S. 2010. Effects of Green Tea Use on Wound Healing. *International Journal of Morphology*. 28, 905-910.

Niaounakis, M. 2011. Olive-mill wastewater in antiquity, environmental effects and applications. *Oxford Journal of Archeology*, 30, 411-425.

Nirmal, N.P. and Benjakul, S. 2012. Inhibition kinetics of catechin and ferulic acid on polyphenoloxidase from cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*). *Food Chemistry*, 131, 569-573.

Nishiyama, M.F., Coasta, M.A.F., da Costa, A.M., de Souza, C.G.M., Boer, C.G., Bracht, C.K. and Peralta, R.M. 2010. Brazilian green tea (*Camellia sinensis* var *assamica*): effect of infusion time, mode of packaging and preparation on the extraction efficiency of bioactive compounds on the stability of the beverage. *Ciência e Tecnologia de Alimentos*, 30, 191-196.

Nuri, M. 2004 *In vitro* studies on some natural beverages as botanical pesticides against *Erwinia amylovora* and *Curtobacterium flaccumfaciensis* subsp. *Poinsettiae. Turkish Journal of Agriculture & Forestry*, 28, 57-61.

Okuda, T., Mori, K., Shiota, M. and Ida, K. 1982. Effect on interaction of tannins with co-existing susbstances. 2. Reduction of heavy-metal ions and solubilisation of precipitates. *Yakugaku Zasshi – Journal of the Pharmaceutical Sociey of Japan*, 102 (8), 735-742.

Oliveira, D.L., Costabile, A., Wilbey, R.A., Grandison, A.S., Duarte, L.C. and Roseiro, L.B. 2012. *In vitro* evaluation of the fermentation properties and potential prebiotic activity of caprine cheese whey oligosaccharides in batch culture systems. *Biofactors*, 38 (6), 440-449.

Oms-Oliu, G., Odriozola-Serrano, I., Soliva-Fortuny, R., Eliz-Martinez, P. and Martin-Bellosos, O. 2012, Stability of health-related compounds in plant foods through the application of non thermal processes. *Trends in Food Science & Technology*, 23 (2), 111-123.

Ozcelik, B., Orhan, I. and Toker, G. 2006. Antiviral and antimicrobial assessment of some selected flavonoids. *Zeitschrift für Naturforschung*, 61, 632-638.

Ozyurek, M., Gungor, N., Baki, S., Guclu, K. and Apak, R. 2012. Development of a silver nanoparticle-based method for the antioxidant capacity measurement of polyphenols. *Analytical Chemistry*, 84 (18), 8052-8059.

Papagianni, M., Avramadis, N., Filioussis, G., Dasiou, D. and Ambrosiadis, I. 2006. Determination of bacteriocin activity within bioassys carried out on a solid and liquid substrates ; assessing the factor 'indicator microorganism'. *Microbial Cell Factories*, 5, Article Number: 30 DOI: 10.1186/1475-2859-5-30.

Paramasivam, S., Thangaradjou, T. and Kannan, L. 2007. Effect of natural preservatives on the growth of histamine producing bacteria. *Journal of Environmental Biology*, 28 (2), 271-274.

Park, K.D. and Cho, S.J. 2010. Synthesis and antimicrobial activities of 3-*O*-alkyl analogues of (+)-catechin: Improvement of stability and proposed action mechanism. *European Journal of Medicinal Chemistry*, 45, 1028-1033.

Parsonnet, J., Hansmann, N.A., Seymour, J.L., Delaney, M.L., DuBois, A.M., Modern, P.A., Jones, M.B., Wild, J.E. and Onkerdonk, A.B. 2010. Persistence survey of toxic shock syndrome toxin-1 producing *Staphylococcus aureus* and serum antibodies to this antigen in five groups of menstruating women. *BMC Infectious Diseases*, 10, 249.

Perez, M., Guillermo, B., Garcia, M., del Amo, B. and Stupak, M. 2006. Cupric Tannate: A low copper content antifouling pigment. *Progress in Organic Coatings*, 55, 311-315.

Perez-Llamas, F., Gonzalez, D., Cabrera, L., Espinosa, C., Lopez, J.A., Larque, E., Almajano, M.P. and Zamora, S. 2011. White tea consumption slightly reduces iron absorption but not growth, food efficiency, protein utilization, or calcium, phosphorus, magnesium, and zinc absorbtion in rats. *Journal of Physiology & Biochemistry*, 67, (3), 331-337.

Perumalla, A.V.S. and Hettiarachchy, N.S. 2011. Green tea and grape seed extracts – potential applications in food safety and quality. *Food Research International*, 44, 827-839.

Peyton, B.M., Wilson, T. and Yonge, D.R. 2002. Kinetics of phenol biodegradation in high salt solutions. *Water Research*, 36 (19), 4811-4820.

Pirker, Katharina F; Baratto, Maria Camilla; Basosi, Riccardo; Goodman and Bernard A. 2012. The influence of pH on the speciation of copper (II) in reactions with the green tea polyphenols, epigallocatechin gallate and gallic acid. *Journal of Inorganic Chemistry*, 112, 10-16.

Podlesek, Z. and Comino, A. 1994. Antagonists of bacitracin. *Letters in Applied Microbiology*. 19 (2), 102-104.

Pretorius, J.C., Magama, S. and Zietsman, P.C. 2003. Purification of antibacterial compounds from *Euclea crispa* subsp. *crispa* (Ebenaceae) leaves. *South African Journal of Botany*. 69, 589-586.

Punyasiri, P.A.N., Abeysinghe, I.S.B. and Kumar, V. 2005. Preformed and induced chemical resistance of tea leaf against *Exobasidium vexans* infection. *Journal of Chemical Ecology*. 31 (6), 1315-1324.

Pujol, I., Pastor, F.J., Lazera, M.D. and Guarro, J. 2008. Evaluation of the neo-sensitabs (R) diffusion method for determining the antifungal susceptibilities of *Cryptococcus gattii* isolates, using three different agar media. *Revista Iberoamericana de Micologia*. 25 (4), 215-220.

Purcaro, R., Schrader, K.K., Burandt, C., Della Grecca, M. And Meepagala, K.M. 2009. Algicide constituents from *Swinglea glutinosa*. *Journal of Agricultrural & Food Chemistry*. 57, 10632-10635.

Ragab-Depre, N.J. 1982. Water disinfection with the hydrogen peroxide-ascorbic-copper(II) system. *Applied and Environmental Microbiology*. 44 (3), 555-560.

Raj, C.V. and Dhala, S. 1965. Effect of naturally occurring xanthines on bacteria. I. Antimicrobial action and potentiating effects on antibiotic spectra. *Applied Microbiology*. 13, 432-6.

Relja, B., Tottel, E., Breig, L., Henrich, D., Schneider, H., Marzi, I. and Lehnert, M. 2011. Effects of green tea catechins on the pro-inflammatory response after haemmorrhage/resuscitation in rats. *British Journal of Nutrition*, 105, 1791-1797.

Richter, H.E., Switala J. and Loewen, P.C. 1988. Effect of ascorbate on oxygen-uptake and growth of *Escherichia-coli-B*. *Canadian Journal of Microbiology*, 34 (6), 822-825.

Roccaro, A.S., Blanco, A.R., Giuliano, F., Rusciano, D. and Enea, V. 2004. Epigallocatechin-gallate enhances the activity of tetracycline in staphylococci by inhibiting its efflux from bacterial cells. *Antimicrobial Agents & Chemotherapy*, 48, 1968-1973.

Roekel, M. and Fernandez, K. 2012. Use of biomass for the removal of heavy metals at low concentrations from freshwater Chilean Atlantic salmon farms. *Aquacultural Engineering*, 49, 1-9.

Sakanaka, S., Aizawa, M., Kim, M. and Yamamoto, T. 1996. Inhibitory effects of green tea polyphenols on growth and cellular adherence of an oral bacterium, *Porphyromonas gingivalis*. *Bioscience Biotechnology and Biochemistry*, 60 (5), 745-749.

Salaman, M.K. 2004. Resistant bacterial infections treated with vitamin C. *Health Freedom News*. http://www.thenhf.com/health_freedom_news_14.htm accessed 29/01/08

Sannella, A., Messori, L., Casini, A., Francesco Vincieri, F., Bilia, A.R., Maiori, G. and Severini, C. 2006. Antimalarial properties of Green Tea. *Biochemistry & Biophysics Research Communication*, 353 (1),171-181.

Sannasiddappa, H., Costabile, A., Gibson, G.R. and Clarke, S.R. 2011. The influence of *Staphylococcus aureus* on gut microbial ecology in an *in vitro* continuous culture human colonic model system. *PloS (Public Library of Science) one*, 6 (8), 23227

Schiffenbauer, M., Ghandi, J., George, G., Goldshteyn, A., Livshits, E. and Welsh, N. 2004. The anti-bacterial, anti-fungal, and anti-viral effect of white tea. *American Society Microbiology*, 104, 538.

Schmidt, J., Joost, I., Skaar, E.P., Hermann, M. and Bischoff, M. 2012. Haemin represses the haemolytic acivity of *Staphylococcus aureus* in an sae-dependent manner. *Microbiology - Society of General Microbiology*, 158, 2619-2631.

Schrader, K.K., Nanayakkara, N.P.D., Tucker, C.S., Rimando, A.M., Ganzera, M. and Schaneberg, B.T. 2003. Novel derivatives of 9,10-anthroquinone are selective algicides against the musty-odor cyanobacterium *Oscillatoria peronata*. *Applied & Environmental Microbiology*, 69, 5319-5327.

Schuck, A.G., Ausubel, M.B., Zuckerbaum, H.I. and Babich, H. 2008. Theaflavin-3,3'-digallate, a component of black tea: An inducer of oxidative stress and apoptosis. *Toxicology in Vitro*, 22 (3), 598-609.

Schuier, M., Sies, H., Illek, B. and Fischer, H. 2005. Cocoa-related flavonoids inhibit CFTR-mediated chloride transport across T84 human colon epithelia. *Journal of Nutrition*, 135 (10), 2320-2325.

Scoparo, C.T., de Souza, L.M., Dartora, N., Sassaki, G.L. Gorin, P.A.J. and Iacomini, M. 2012. Analysis of *Camellia sinensis* green and black teas via ultra high performance liquid chromatography assisted by liquid-liquid partition and two-dimensional liquid chromatography (size exclusion x reversed phase). *Journal of Chromatography*, 1222, 29-37.

Segovia-Bravo, K.A., Garcia-Garcia, P., Lopez-Lopez, A., and Garrido-Fernandez, A. 2012. Effect of inert atmosphere on the postharvest browning of manzanilla olives and optimization by response surface methodology of the aqueous treatments. *Journal of Food Science*, 77 (5), S194-S201.

Sharma, A., Gupta, S., Sarethi, I.P., Dang, S. and Gabrani R. 2012. Green tea extract: Possible mechanism and antibacterial activity on skin pathogens. *Food Chemistry*, 135, 672-675.

Shah, S., Stapleton, P.D. and Taylor, P.W. 2008. The polyphenol (-)-epicatechin gallate disrupts the secretion of virulence-related proteins by *Staphylococcus aureus*. *Letters in Microbiology*, 46, 181-185.

Sharangi, A.B. 2009. Medicinal and therapeutic properties of tea (*Camellia sinensis* L.) – a review. *Food Research International*, 42 (5-6), 529-535.

Shigemune. N., Nakayama, M., Tsugukuni, T., Hitomi, J., Yoshizawa, C., Mekada, Y., Kurahachi, M and Miyamoto, T. 2012. The mechanisms and effect of epigallocatechin gallate (EGCg) on the germination and proliferation of bacterial spores. *Food Control*, 27, 269-274.

Shimamura, T., Zhao, W-H. and Hu, Z-Q. 2007 Mechanism of action and potential for use of tea catechin as an anti-infective agent. *Anti-infective Agents in Medicinal Chemistry*, 6, 57-62.

Shiono, M., Matsugaki, N., and Takeda, K. 2008. Structure of commelinin, a blue complex pigment from the blue flowers of *Commelina communis*. *Proceedings of the Japan Academy Series B – Physical and Biological Sciences*, 84 (10), 452-456.

Shorr, A.F., Haque, N., Taneja, C., Zervos, M., Lamerato, L., Kothari, S., Zilber, S., Donabedian, S., Perri, M.B., Spalding, J. and Oster, G. 2010. Clinical and economic outcomes for patients with health-care associated *Staphylococcus aureus* pneumonia. *Journal of Clinical Microbiology*, 48 (9), 3258-3262.

Si, W.D., Gong, J., Tsao, R., Kalab, M., Yang, R. and Yin, T.L. 2006. Bioassay-guided purification and identification of antimicrobial components in Chinese green tea extract. *Journal of Chromatography*, A 1125 (2), 204-210.

Simões, M., Bennett, R.N., and Rosa, E.A.S. 2009. Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. *Natural Products Report*. 26, 746-757.

Singh, B.N., Shankar, S. and Srivastava, R.K. 2011. Green tea catechin, epigallocatechin-3-gallate (EGCG): Mechanisms, perspectives and clinical applications. *Biochemical Pharmacology*, 82, 1807-1821.

Singh, S., Pandey, A., Kumar, B. and Palni, L.M.S. 2010. Enhancement in growth and quality parameters of tea [*Camellia sinensis* (L.) O. Kuntze] through inoculation with arbuscular mychorizal fungi in an acid soil. *Biology & Fertility of Soils*, 46 (5), 427-433.

Sipos, P., Gyory, H., Hagymasi, K., Ondrejka, P. and Blasovics, A. 2004. Special wound healing methods used in ancient eygpt and the mythological background. *World Journal of Surgery*, 28, 211-216.

Sirk, T.W., Brown, E.F., Sum, A.K. and Friedman, M. 2008. Molecular dynamics study on the biophysical interactions of seven green tea catechins with lipid bilayers of cell membranes. *Journal of Agricultural and Food Chemistry*, 56 (17), 7750-7758.

Sirk, T.W., Brown, E.F., Friedman, M. and Sum, A.K. 2009. Molecular binding of catechins to biomembranes: Relationship to biological activity. *Journal of Agriculture*, 57, 6720-6728.

Sivarooban, T., Hettikrachiky, N.S. and Johnson, M.G. 2008. Transmission electron microscopy study of *Listeria monocytogenes* treated with nisin in combination with either grape seed or green tea extract. *Journal of Food Protection*, 71 (10), 2105-2109.

Smith, A.H., Imlay, J.A. and Mackie, R.I. 2003. Increasing the oxidative stress response allows *Escherichia coli* to overcome the inhibitory effects of condensed tannins. *Applied and Environmental Microbiology*, 69 (6), 3406-3411.

Sokmen, A., Vardar-Unlu, G., Polissiou, M., Daferera, D., Sokmen, M. and Donmez, E. 2003. Antimicrobial activity of essential oil and methanol extracts of *Achillea sintenisii* Hub. Mor. (Asteraceae), *Phytotherapy Research*, 17, 1005-1010.

Song, J.M. and Seong, B.L. 2007. Tea catechins as a potential alternative anti-infectious agent. *Expert Review of Anti-Infective Therapy*, 5, 497-506.

Sreeramulu, G., Zhu, Y. and Knol, W. 2000. Kombucha fermentation and its antimicrobial activity. *Journal of Agricultural Food Chemistry*, 48, 2589-2594.

Stapleton, P.D., Shah, S., Anderson, J.C., Hara, Y., Hamilton-Miller, J.M.T. and Taylor, P.W. 2004. Modulation of beta-lactam resistance in *Staphylococcus aureus* by catechins and gallates. *International Journal of Antimicrobial Agents*, 23, 462-467.

Stapleton, P.D., Shah, S., Hamilton-Miller, J.M.T., Hara, Y., Nagaoka, Y., Kumagai, A., Uesato, S. and Taylor, P.W. 2004. Anti-*Staphylococcus aureus* activity and oxacillin resistance modulating capacity of 3-O-acyl-catechins. *International Journal of Antimicrobial Agents*, 24, 374-380.

Stapleton, P.D., Shah, S., Hara, Y. and Taylor, P.W. 2006. Potentiation of catechin gallate-mediated sensitisation of *Staphylococcus aureus* to oxacillin by nongalloylated catechins. *Antimicrobial Agents & Chemotherapy*, 50, 752-755.

Stapleton, P.D., Shah, S., Ehlert, K., Hara, Y. and Taylor, P.W. 2007. The beta-lactam-resistance modifier (-)-epicatechin gallate alters the architecture of the cell wall of *Staphylococcus aureus*. *Microbiology-SGM*, 153, 2093-2103.

Stewart, G.S.A.B., Jassim, S.A.A., Denyer, S.P., Newby, P., Linley, K. and Dhir, V.K. 1998. The specific and senstitive detection of bacterial pathogens within 4 h using bacteriophage amplification. *Journal of Applied Microbiology*, 84, 777-783.

Stockfith, E., Beti, H., Orasan, R., Grigorian, F., Mescheder, A., Tawfik, H. and Thielert, C. 2008. Topical polyphenon (R) E in the treatment of external genital and perianal warts: a randomized controlled trial. *British Journal of Dermatology*, 158, 1329-1338.

Su, P., Henriksson, A., Nilsson, C., and Mitchell H. 2008. Synergistic effect of green tea extract and probiotics on the pathogenic bacteria, *Staphylococcus aureus* and *Streptococcus pyogenes. World Journal of Microbiology & Biotecnology*, 24 (9), 1837-1842.

Sultana, N. Arayne, M.S. and Sabri, R. 2005. Eryhromycin synergism with essential and trace elements. *Pakistan Journal of Pharmaceutical Science*, 18 (2), 35-39.

Sultana, N. and Arayne, M.S. 2007. *In vitro* activity of cefadroxil, cephalexin, cefatrizine and cefpirome in presence of essential and trace elements. *Pakistan Journal of Pharmaceutical Science*, 20 (4), 305-310.

Sun, L.M., Zhang, C.L. and Li, P. 2011. Characterization, antimicrobial activity, and mechanism of a high-performance (-)-epigallocatechin-3-gallate (EGCG)-Cu-II/polyvinyl alchohol (PVA) nanofibrous membrane. *Journal of Agricultural and Food Chemistry*, 59 (9), 5087-5092.

Sun, Y., Hung, W.C., Chen, F.Y., Lee, C.C. and Huang, H.W. 2009. Interaction of tea catechin (-)-epigallocatechin gallate with lipid bilayers. *Biophysical Journal*, 96 (3), 1026-1035.

Suzuki, Y., Miyoshi, N. and Isemura, M. 2012. Health-promoting effects of green tea. *Proceedings of the the Japan Academy Series B-Physical & Biological Sciences*, 88, (3), 88-101.

Szabo, G. and Nemeth, Z. 2010. Use of functional toothpaste made with nanotechnology, in the treatment of oral mucosa diseases. *Fogorvosi szemle*, 103 (2), 39-41.

Tabasco, R., Sanchez-Patan, F., Monagas, M., Bartolome, M., Moreno-Arribas, M.V., Pelaez, C. and Requena, T. 2011. Effect of grape polyphenols on lactic acid bacteria and bifidobacteria growth: Resistance and metabolism. *Food Microbiology*, 28 (7), 1345-1352.

Tabib, Z., Hagler, W.M. and Hamilton, P.B. 1982. Comparison of physiological-parameters useful for assessment of activity of antifungal agents in feed and ingredients. *Journal of Food Protection*, 45 (11), 1030-1037.

Taguri ,T., Tanaka, T. and Kouno, I. 2004. Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. *Biolological & Pharmaceutical Bulletin*, 27, 1965-1969.

Taguri, T., Tanaka, T. and Kouno, I. 2006. Antibacterial spectrum of plant polyphenols and extracts depending on hydroxyphenyl structure. *Biological & Pharmaceutical Bulletin*, 29 (11), 2226-2235.

Takabayashi, F., Harada, N., Yamada, M., Muroshisa, B. and Oguni, I. 2004. Inhibitory effect of green tea catechins in combination with sucralflate on *Helicobacter pylori* infection in Mongolian gerbils *Journal of Gastroenterology*, 39 (1), 61-63.

Takahashi, O., Cai, Z., Toda, M., Hara, Y. and Shimamura,T. 1995. Appearance of antibacterial activity of oxacillin against methicillin resistant *Staphylococcus aureus* (MRSA) in the presence of catechin. *Journal of the Japanese Association of Infectious Diseases*, 69, 1126-1134.

Takahashi., T., Aso, Y., Kasi, W. and Kondo, T. 2010. Improving the antimicrobial acivity against *Staphylococcus aureus* of composite sheets containing wasted tea leaves by roasting. *Journal of Wood Science*, 56, 403-410.

Tamba, Y, Yoshitani, T., and Yamazaki, M. 2006. Single giant unilamellar vesicle method reveals effect of antimicrobial peptide, magainin 2, and antibacterial substance, catechin, on membrane permeability and membrane structure. *IEEE International Symposium on Micro-NanoMechatronics and Huamn Science*, 225-230

Tejero-Sarinena, S., Barlow, J., Costabile, A., Gibson, G.R. and Rowland, I. 2012. *In vitro* evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. *Anaerobe*, 18 (8), 530-538.

Theobald, S., Pfeiffer, P., Zuber, U. and Konig, H. 2008. Influence of epigallocatechin gallate and phenolic compounds from green tea on the growth of *Oenococcus oeni*. *Journal of Applied Microbiology*, 104, 566-572.

Tiwari, R.P., Bharti, H.D., Kaur, R.P., Dikshit, R.P., and Hoondal, G.S. 2005. Synergistic antimicrobial activity of tea & antibiotics. *Indian Journal of Medical Research*, 122, 80-84.

Tkalec, M., Car, D., Gospocic, J., Kriziac, I., Duz, K. and Vidakovic-Cifrek, Z. 2012. Response of *Kalanchoe daigremontiana* to wounding and infection with *Agrobacrterium tumefaciens*. *Periodicum Biologorum*, 114 (1), 83-90.

Torreggiani, A., Jurasekova, Z., Sanchez-Cortes, S. and Tamba, M. 2008. Spectroscopic and pulse radiolysis studies of the antioxidant properties of (+)-catechin: metal chelation and oxidising radical scavenging. *Journal of Raman Spectroscopy*, 39, 265-275.

Tran, T.T.T., Quan, T.D., Nguyen, T.H.A., and Sung, T.V. 2012. Cytotoxic and antimicrobial alkaloids from *Fissistigma poilanei* (Annonaceae) collected in Vietnam. *Natural Product Research*, 26 (14), 1296-1302.

Tsan M.F., Douglass, K.H. and McIntyre, P.A. 1977. Hydrogen-peroxide production and killing of *Staphylococcus aureus* by human polymorphonuclear leukocytes. *Blood*, 49 (3), 437-444.

Uekusa, Y., Takeshita, Y., Ishii, T., Kamihara, M., Kumasawa, S., Nakamura, K., Naito, A. and Nakayama, T. 2008. Interaction between tea catechins and biological membranes. *Yakugaku Zasshi – Journal of the Pharmaceutical Society of Japan*, 128, 137-138.

Unno, K., Yamamoto, H., Maeda, K., Takabayashi, F., Yoshida, H., Kikunaga, N., Takamori, N., Asahina, S., Iguchi, K., Sayama, K. and Hoshino, M. 2009. Protection of brain and pancreas from high-fat diet: Effects of catechin and caffeine. *Physiology & Behaviour*, 96, 262-269.

Van Asbeck, B.S., Marcelis, J.H., Marx, J.J.M, Struyvenberg, A., van Kats, J.H. and Verhoef, J. 2005. Inhibition of bacterial multiplication by the iron chelator deferoxamine: Potentiating effect of ascorbic acid. *European Journal of Clinical Microbiology & Infectious Diseases*, 2 (5), 426-431.

Vandeputte, O., Kienrebeogo, M., Rajaonson, S., Diallo, B., Mol, A., El Jaziri, M. and Baucher, M. 2010. Identification of catechin as one of the flavonoids from *Combretum albiflorum* bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. *Applied Environmental Microbiology*, 76, 243-253.

Vargas-Reus, M.A., Memarzadeh, K., Huang, J., Ren, G.G.G. and Allaker, R.P. 2012. Antimicrobial activity of nanoparticulate metal oxides against peri-implantitis pathogens. *International Journal of Antimicrobial Agents*, 40 (2), 135-139.

Volcheck, G.W. 2004. Clinical evaluation and management of drug hypersensitivity. *Immunology and Allergy Clinics of North America*, 24 (3), 357-371.

von Staszewski, M., Pilosof, A.M.R. and Jagus, R.J. 2011. Antioxidant and antimicrobial performance of different Argentinian green tea varieties as affected by whey proteins. *Food Chemistry*, 125, 186-192.

Voravuthikunchai, S.P., and Mitchell, H. 2008. The inhibitory and killing activities of medicinal plants against multiple antibiotic-resisitant *Helicobacter pylori*. *Journal of Health Science*, 54, 81-88.

Ververidis, F., Trantas, E., Douglas, C., Vollmer, G., Kretschmar, G. and Panopoulos, N. 2007. Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part 1: Chemical diversity, impacts on plant biology and human health *Biotechnology Journal*, (2) 1214-1234.

Wagner, V.E., Filiatrault, M.J., Picardo, K.F., and Iglewski, B.H. 2008. *Pseudomonas aeruginosa* virulence and pathogenesis issues. *Pseudomonas: Genomics & Molecular Biology*, 129-158.

Wang, K.B., Liu, F., Liu, Z.H., Huang, J.A., Xu, Z.X., Li, Y.H., Chen, J.H., Gong, Y.S. and Yang, X.H. 2011. Comparison of catechins and volatile compounds among different types of tea using high perfomanace chromatograph and gas chromatograph mass spectrometer. *International Journal of Food Science and Technology*, 46 (7), 1406-1412.

Wang, L.Y, Wei, K., Jiang, Y.W., Cheng, H., Zhou, J., He, W., Zhang, C.C. 2011. Seasonal climate affects on flavanols and purine alkaloids of tea (*Camellia sinensis* L.). *European Food Research & Technology*, 233, (6), 1049-1056.

Weber, G. 1988. HPLC with electrochemical detection of metal-flavonoid-complexes isolated from food. *Chromatographia*, 26, 133-8.

Weisburger, J.H. 1997. Tea and health: A historical perspective. *Cancer Letters*, 114 (1-2), 315-317.

Weisburger, J.H. and Chung, F.L. 2002. Mechanisms of chronic disease causation by nutritional factors and tobacco products and their prevention by tea polyphenols. *Food & Chemical Toxicology*, 40 (8), 1145-1154.

Wheat, P.F. 2001. History and development of antimicrobial susceptibility testing methodology. *Journal of Antimicrobial Chemotherapy*, 48 (1), 1-4.

Williams, S.N., Pickwell, G.V. and Quattrochi, L.C. 2003. A combination of tea (*Camellia sinensis*) catechins is required for optimal inhibition of induced *CYP1A* expression by green tea extract. *Journal of Agriculture & Food Chemistry*, 51 (22), 6627-6634.

Winward, G.P., Avery, L.M., Stephenson, T. and Jefferson, B. 2008. Essential oils for the disinfection of grey water. *Water Research*, 42 (8-9), 2260-2268.

Wingfield, M.J., Slippers, B. and Wingfield, B. 2010. Novel associations between pathogens, insects and tree species threaten world forests. *New Zealand Journal of Forestry Science*, 40 (Suppl. S), S95-S104.

Wolinsky, L.E., Cuomo, J., Quesada, K., Bato, T. and Camargo, P.M. 2000. A comparative pilot study of the effects of a dentrifice containing green tea bioflavonoids, sanguinarine or triclosan on oral bacterial biofilm formation. *Journal of Clinical Dentistry*, 11, 53-58.

Wolf, R., Tufano, M.A., Ruocco, V., Grimaldi, E., Donnarumma, G. and Baroni, A. 2006. Quinine sulphate inhibits invasion of some bacterial skin pathogens. *International Journal of Dermatology*, 45 (6), 661-663.

Wolfram, S. 2007. Effects of green tea and EGCG on cardiovascular and metabolic health. *Journal of the American College of Nutrition*, 26 (4), 373S-388S.

Wolska, K.I., Grzes, K. and Kurek, A. 2012. Synergy between novel antimicrobials and conventional antibiotics or bacteriocins. *Polish Journal of Microbiology*, 61 (2), 95-104.

Wood, J.P., Calfee, M.W., Clayton, M., Griffin-Gatchalian, N. and Touati, A. 2011. Optimizing acidifed bleach solutions to improve sporicidal efficacy on building materials. *Letters in Applied Microbiology*, 53 (6), 668-672.

Wu, H.Q., Li, Q., Zhou, X.D., Kolosov, V.P., and Perelman, J.M. 2012. Theaflavins extracted from black tea inhibit mucous hypersecretion induced by cigarette smoke in rats. *Inflammation*, 35, 271-279.

Wu, S-C., Yen, G-C., Wang, B-S., Chiu, C-K., Yen, W-J., Chang, L-W. And Duh, P-D. 2007. Antimutagenic and antimicrobial activities of Pu-erh tea. *LWT (Lebensmittel-Wissenschaft & Technologie)-Food Science Technology*, 40, 506-512.

Xie, B., Shi, H., Chen, Q. and Ho, C-T. 1993. Antioxidant properties of fractions and polyphenol constituents from green, oolong, and black teas. *Proceedings of the National Science Council Republic of China Part B Life Sciences*, 17 (2), 77-84.

Yam, T.S., Shah, S. and Hamilton-Miller, J.M.T. 1997. Microbiological activity of whole and fractionated crude extracts of tea (*Camellia sinensis*), and of tea components. *FEMS Microbiology Letters*, 152, 169-174.

Yam, T.S., Hamilton-Miller, J.M.T., and Shah, S. 1998. The effect of a component of tea (*Camellia sinensis*) on methicillin resisitance. *PB2'* synthesis, and β-lactamase production in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, 42, 211-216.

Yang, Y., Mandal, A.K., Bredeston, L.M., Gonzalez-Flecha, F.L. and Arguello, J.M. 2007. Activation of *Archaeoglobus fulgidus* Cu+-ATPase CopA by cysteine. *Biochimica et Biophysica Acta-Biomembranes*. 1768 (3), 495-501.

Yashuda, M., Matsuda, C., Ohshiro, A., Nouye, K., and Tabata M. 2012. Effects of metal ions (Cu^{2+}, Fe^{2+}, and Fe^{3+}) on HPLC analysis of catechins. *Food Chemistry*, 133 (2), 518-525.

Yen, G.C., Wu, S.C. and Duh, P.D. 1996. Extraction and identification of antioxidant components from the leaves of mulberry (*Morus alba* L*). Journal of Agricultural and Food Chemistry*, 44 (7), 1687-1690.

Yi, S.M., Zhu, J.L., Fu, L.L. and Li, J.R. 2010.Tea polyphenols inhibit *Pseudomonas aeruginosa* through damage to the cell membrane. *International Journal of Food Microbiology*, 144 (1), 111-117.

Yin, P., Zhao, L., Li, K., Qi, Y., Feng, D. and Bai, Y. 2010. Removal of red tide algae by a slow releasing copper ions algicide. *Huanjing Kexue*, 21 (5), 12-16.

Yoshida, Y., Kiso, M. and Goto, T. 1999. Efficiency of the extraction of catechins from green tea. *Food Chemistry*, 67 (4), 429-433.

Yoshino, M. and Murikami, K. 1998. Interaction of iron with polyphenolic compounds: Applications to antioxidant characterisation. *Analytical Biochemistry*, 257 (1), 40-44.

Zamora, L.L. and Perez-Gracia, M.T. 2012. Using digital photography to implement the McFarland method, *Journal of the Royal Society Interface*, 9 (73), 1892-1897.

Zevenhuizen, L.P.T.M., Dolfing, J., Eshuis, E.J., and Scholtenkoerselman, I.J. 1979. Inhibitory effects of copper on bacteria related to the free ion concentration. *Microbial Ecology*, 5, 139-146.

Zimmermann, B.F. and Gleichenhagen M. 2011. The effect of ascorbic acid, citric acid and low pH on the extraction of green tea: How to get the most out of it. *Food Chemistry*, 124 (4), 1543-1548.

Zago, M.P., Verstraeten, S.V. and Oteiza, P.I. 2000. Zinc in the prevention of Fe^{2+} initiated lipid and protein oxidation. *Biological Research*, 33 (2) 143-150.

Zhang, Y., Hu, Y.B., Yang, B.Y., Ma, F., Lu, P., Li, L.M., Wan, C.S., Rayner, S. and Chen, S.Y. 2010. Duckweed (*Lemna minor*) as a model plant system for the study of human microbial pathogenesis. *Plos One*, 5, (10), e13527.

Zhao, Y., Chen, P., Lin, L.Z., Harnly, J.M., Yu, L.L. and Li, Z.W. 2011. Tentative identification, quantitation, and principal component analysis of green pu-erh, green, and white teas using UPLC/DAD/MS. *Food Chemistry*, 126 (3), 1269-1277.

Zhu, S., Moreno, K.X., Jenkins, R.M. and Walmsley, J.A. 2008. Interactions of 1,5-naphthrydine with $Pd(en)Cl_2$ or $[Pd(en)(H_2O)_2](NO_3)_2$ in aqueous solution. *Dalton Transactions*, 45, 6401-6408.

Appendix I

Assays of putative adjuncts and tea extracts

Figure or Table numbers in brackets that follow section titles refer to those in the main text

A. The antimicrobial activity of copper (II) sulphate tested at different exposure times against three different species of bacteria (Table 3.3)

Inoculum	330 µL of test or control substance	700 µL of putative adjunct with concentrations used	Change to protocol
S. aureus *Ps. aeruginosa* *P. mirabilis*	330 µL Ringer's solution	4.8, 9.6, 19.2 mM copper (II) sulphate	10 minute as well as 30 min. exposures

Key: *S. aureus* = *Staphylococcus aureus* NCTC 06751, *Ps. aeruginosa* = *Pseudomonas aeruginosa* NCTC 950, *P. mirabilis* = *Proteus mirabilis* NCTC 7827

B. The antimicrobial activity of different concentrations of vitamin C against *Staphylococcus aureus* NCTC 06751

Inoculum	330 µL of test or control substance	700 µL of putative adjunct with concentrations used
Staphylococcus aureus NCTC 06751	Ringer's solution	1 , 5, 10, 100 µM; 1, 10, 15, 20 mM vitamin C

C. The antimicrobial activity of copper (II) sulphate at different concentrations with the further addition of vitamin C against *Staphylococcus aureus* NCTC 06751 (Figures 3.2, 3.3)

Inoculum	330 µL of test or control substance	Volumes of putative adjunct(s) and concentration(s) used plus any additional controls
Staphylococcus aureus NCTC 06751	Ringer's solution	700 µL 1, 10, 100 µM; 1, 5, 10 mM copper (II) sulphate
	Ringer's solution	350 µL 9.6 mM copper (II) sulphate mixed with 350 µL 9.6 mM and with 19.2 mM vitamin C
	Ringer's solution	700 µL 4.8 mM vitamin C

D. Antimicrobial activity of WT combined with pH adjusted Ringer's solution on the viability of *Staphylococcus aureus* NCTC 06571

Inoculum	330 µL test or control substance	700 µL Ringer's solution adjusted to specific pH value
Staphylococcus aureus NCTC 06751	WT	Ringer's solution adjusted to pH 3
	WT	Ringer's solution adjusted to pH 5
	WT	Ringer's solution adjusted to pH 7
	WT	Ringer's solution adjusted to pH 9

E. Antimicrobial activity of WT, GT, and BT tested alone against *Staphylococcus aureus* NCTC 06751 and *Pseudomonas aeruginosa* NCTC 950 as well as the same teas combined with 4.8 mM copper (II) sulphate for different standing times prior to addition of inoculum against *Staphylococcus aureus* NCTC 06751* (Figures 3.1, 3.4, Table 3.3)

Inocula	**330 µL of test or control substance**	**700 µL of putative adjunct(s) and concentration(s) used plus any additional controls**	**Modifications to protocol:** Standing time of mixed reagents prior to addition of inoculum
Staphylococcus aureus NCTC 06751, *Pseudomonas aeruginosa* NCTC 950	Ringer's solution White tea (WT) Green tea (GT Black tea (BT)	4.8 mM, copper (II) sulphate Ringer's solution Ringer's solution Ringer's solution	
Staphylococcus aureus NCTC 06751 (only)	WT GT BT	4.8 mM copper (II) sulphate 4.8 mM copper (II) sulphate 4.8 mM copper (II) sulphate	10 & 30 minutes 10 & 30 minutes 10 & 30 minutes

*Following the results of the above experiment a standing time of 10 minutes was adopted for all subsequent suspension assays since this period of time did not affect subsequent antimicrobial activity in WT, whereas the 30 minutes standing time reduced activity to buffer control values. No antimicrobial activity was seen with 10, or 30 minutes standing time with both the GT and BT.

F. Antimicrobial activity of WT combined with 4.8 mM copper (II) sulphate and with copper (II) sulphate alone during an extended incubation of 80 minutes on the viability of *Staphylococcus aureus* NCTC 06571 (Figure 3.5)

Inoculum	**330 µL test or control substance**	**Volumes of putative adjuncts used plus additional controls**	**Modifications to protocol**
Staphylococcus aureus NCTC 06751	Ringer's solution WT	700 µL 4.8 mM, copper (II) sulphate 700 µL Ringer's solution	Samples withdrawn to stop solutions at 0, 10, 30, 60, 65, 70, 75, and 80 minutes after the introduction of inoculum

G. Antimicrobial activity of WT combined with equimolar 4.8 mM copper (II) sulphate and vitamin C on the viability of *Staphylococcus aureus* NCTC 06571 (Figure 3.6)

Inoculum	**330 µL test or control substance**	**Volumes of putative adjuncts used plus any additional controls**
Staphylococcus aureus NCTC 06751	Ringer's solution	700 µL 4.8 mM, copper (II) sulphate
	WT	700 µL Ringer's solution
	WT	700 µL 4.8 mM, copper (II) sulphate
	Ringer's solution	700 µL 4.8 mM vitamin C
	WT	700 µL 4.8 mM vitamin C
	Ringer's solution	350 µL 9.6 mM copper (II) sulphate plus 350 µL 9.6 mM vitamin C
	WT	350 µL 9.6 mM copper (II) sulphate plus 350 µL 9.6 mM vitamin C

H. Antimicrobial activity of WT combined with pH adjusted 4.8 mM copper (II) sulphate on the viability of *Staphylococcus aureus* NCTC 06571 (Figure 3.7 and text p. 57)

Inoculum	330 µL test or control substance	700 µL putative adjunct used plus controls
Staphylococcus aureus NCTC 06751	Ringer's solution	4.8 mM copper (II) sulphate adjusted to pH 5.5
	Ringer's solution	4.8 mM copper (II) sulphate adjusted to pH 9.0
	WT	Ringer's solution
	WT	4.8 mM copper (II) sulphate adjusted to pH 5.5
	WT	4.8 mM copper (II) sulphate adjusted to pH 9.0
	Ringer's solution	Ringer's solution adjusted to pH 3.0
	Ringer's solution	Ringer's solution adjusted to pH 5.5
	Ringer's solution	Ringer's solution adjusted to pH 9.0

I. Antimicrobial activity of whole WT and a white tea sub-fraction (WTSN) tested alone and when combined with endogenous levels of copper (II) and with 4.8 mM copper (II) sulphate on the viability of *Staphylococcus aureus* NCTC 06571 (Table 3.5, Figure 3.8)

Inoculum	330 µL test or control substance	700 µL putative adjunct used plus any additional control
Staphylococcus aureus NCTC 06751	WT	Ringer's solution
	WTSN	Ringer's solution
	WT	0.56 µM copper (II) sulphate
	WTSN	0.48 µM copper (II) sulphate
	Ringer's solution	4.8 mM copper (II) sulphate
	WT	4.8 mM copper (II) sulphate
	WTSN	4.8 mM copper (II) sulphate

Note: 0.56 and 0.48 µM added copper (II) sulphate corresponds to levels of copper (II) of 0.14 and 0.12 mg L^{-1}, found WT and WTSN.

J. Antimicrobial activity of WT sub-fractions (< 5 kDa, < 1 kDa) alone and with combinations of 4.8 mM copper (II) sulphate and equimolar vitamin C on the viability of *Staphylococcus aureus* NCTC 06571 (Figures 3.9, 3.10)

Inoculum	330 µL test or control substance	Volumes of putative adjuncts used plus any additional control
Staphylococcus aureus NCTC 06751	Ringer's solution	700 µL 4.8 mM vitamin C
	Ringer's solution	700 µL 4.8 mM copper (II) sulphate
	Ringer's solution	350 µL 9.6 mM copper (II) sulphate plus 350 µL 9.6 mM vitamin C
	WT	700 µL Ringer's solution
	WTF (< 5 kDa)	700 µL Ringer's solution
	WTF (< 5 kDa)	700 µL 4.8 mM copper (II) sulphate
	WTF (< 5 kDa)	700 µL 4.8 mM vitamin C
	WTF (< 5 kDa)	350 µL 9.6 mM copper (II) sulphate plus 350 µL 9.6 mM vitamin C
	WTF (< 1 kDa)	700 µL Ringer's solution
	WTF (< 1 kDa)	700 µL 4.8 mM copper (II) sulphate
	WTF (< 1 kDa)	700 µL 4.8 mM vitamin C
	WTF (< 1 kDa)	350 µL 9.6 mM copper (II) sulphate plus 350 µL 9.6 mM vitamin C

K. Effects of adding catalase on antimicrobial activity of whole WT and WT sub-fraction (< 1 kDa) with adjuncts against *S. aureus* NCTC 06571 (Figure 3.13)

Inoculum	330 µL test or control substance	Volumes of putative adjuncts used plus any additional control
Staphylococcus aureus NCTC 06751	Ringer's solution	700 µL 4.8 mM copper (II) sulphate
	Ringer's solution	350 µL 9.6 mM copper (II) sulphate plus 350 µL 9.6 mM vitamin C
	WT	700 µL 4.8 mM copper (II) sulphate
	WTF (< 1 kDa)	700 µL 4.8 mM copper (II) sulphate
	WTF (< 1 kDa)	350 µL 9.6 mM copper (II) sulphate plus 350 µL 9.6 mM vitamin C
	Ringer's solution	700 µL 4.8 mM copper (II) sulphate plus 300 µg mL^{-1} bovine catalase*
	WT	700 µL 4.8 mM copper (II) sulphate plus 300 µg mL^{-1} bovine catalase
	WTF (< 1 kDa)	700 µL 4.8 mM copper (II) sulphate plus 600 µg mL^{-1} bovine catalase

	Ringer's solution	700 µL 4.8 mM copper (II) sulphate plus 600 µg mL^{-1} bovine catalase
	WTF (< 1 kDa)	350 µL 9.6 mM copper (II) sulphate plus 350 µL 9.6 mM vitamin C plus 600 µg mL^{-1} bovine catalase

*Catalase added immediately prior to inoculums in each case

L. Investigation of pH changes following the addition of different concentrations of copper (II) sulphate to WT, WTF < 1 kDa, and freshly made and heat treated catechin (Figures 3.14, 4.10, 4.12)

330 µL of test or control substance with concentrations used	**Volumes of test or control substance with concentrations used**
330 µL WT	700 µL Ringer's solution
330 µL Ringer's solution	700 µL 4.8mM copper (II) sulphate
330 µL WT	700 µL 4.8mM copper (II) sulphate
330 µL WTF (< 1000 Da)	700 µL Ringer's solution
330 µL WTF (< 1000 Da)	700 µL 4.8mM, copper (II) sulphate
330 µL 1000 µM catechin	700 µL Ringer's solution
330 µL 1000 µM catechin	700 µL 472 µM copper (II) sulphate
330 µL 1000 µM catechin	700 µL 4.8mM, copper (II) sulphate Ringer's solution
330 µL 1000 µM catechin, heat treated at 100°C for 10 minutes	700 µL Ringer's solution
330 µL 1000 µM catechin, heat treated at 100°C for 10 minutes	700 µL 472 µM copper (II) sulphate
330 µL 1000 µM catechin, heat treated at 100°C for 10 minutes	700 µL 4.8 mM, copper (II) sulphate
330 µL 1000 µM catechin	700 µL aliquots of each of the following: 118, 236, 331.5, 472, 944, 1888, 3776 µM copper (II) sulphate (which gave precise molar ratios of x8.0, x4.0, x2.0, x1.0, x0.75,x0.5, x0.25 to 1 of catechin
330 µL Ringer's solution	700 µL aliquots of each of the following: 118, 236, 331.5, 472, 944, 1888, 3776 µM copper (II) sulphate
330 µL 1000 µM catechin	Deionised water (simple dilution of catechin) 700 µL 525 µL 350 µL 175 µL
330 µL 1000 µM catechin	Acidified water (pH 6.6) Deionised water 700 µL (x1) 0 µL 525 µL (x0.75) 125 µL 350 µL (x0.5) 350 µL 175 µL (x0.25) 525 µL

Appendix II

Assays of putative adjuncts and catechin

A. Details of main catechin treatments and accompanying controls with profiles of additives used in the catechin plus copper (II) and iron (II) assays along with investigations of their reaction mechanisms against test bacteria using catalase.

Main treatments (MT + number, in bold) plus controls (Ref. to fig/table no. in main text in brackets)	Additives (final concentrations shown)						
	Catechin (μM)	Iron (II) or Iron(III) (μM)	Copper (II) (μM)	Vitamin C (μM)	EDTA (μM)	Catalase (μg mL^{-1})	Inocula (0.5 McFarland standard)
Buffer (Fig. 4.2)	Lambda[b]						*Sa, Ec, Psa, Pm,*
Catechin alone (Figs. 4.1, 4.2, 4.13, 4.14, 4.15)	214						"
Ringer's pH: 3.5	Ringer's						"
pH: 5.5	Ringer's						"
pH: 7.2 (Table 4.1)	Ringer's						"
Cu(II): fresh (Fig. 4.2)	Ringer's		214				"
MT1. Catechin + copper(II) (Fig. 4. 2)	214		214				"
Fe(II): fresh, (Fig 4.7)	Ringer's	214 Fe(II): fresh					*Sa, Ec*
heated,	Ringer's	heated					"
autoclaved (Table 4.5a)	Ringer's	autoclvd.					"
Fe(III): fresh,	Ringer's	214 Fe(III): fresh					"
heated,	Ringer's	heated					"
autoclaved (Table 4.5a)	Ringer's	autoclvd.					"
Cu(II): heated	Ringer's		214				"
MT2. Catechin + fresh, or heated or autoclaved Fe(II) or Fe(III) (Table 4.5a)	214	214 Fe(II): fr. (fg. 4.7)					"
	214	heated					"
	214	autoclvd.					"
	214	214 Fe(III): fresh					"
	214	heated					"
	214	autoclvd.					"
Fe(II) + Cu(II) (Fig. 4.7)	Ringer's	214 Fe(II) fresh	214				"

MT3. Catechin + Fe(II)+Cu(II) (Fig. 4.7)	214	214 Fe(II) fresh	214				"
Vit. C alone (Fig. 4.3)	Ringer's			1712			"
Catechin + vit. C	214			1712			"
Cu(II) + vit. C alone (Fig. 4.3)	Ringer's		214 214 214	428 856 1712			" " "
MT4. Catechin + Cu(II) + vit. C (Fig. 4.3)	214 214 214		214 214 214	428 856 1712			" " "
Fe(II) + vit. C	Ringer's	214 Fe(II) fresh		856			"
MT5. Catechin + Fe(II) + vit. C	214	214 Fe(II) fresh		856			"
Fe(II) + Cu(II) + vit.C	Ringer's	214 Fe(II) fresh	214	856			"
MT6. Catechin + Fe(II) + Cu(II) + vit. C (Fig. 4.7)	214	214 Fe(II) fresh	214	856			"
EDTA alone (Table 4.5b)	Ringer's				2140		"
Fe(II) + Cu(II) + EDTA (Table 4.5b)	Ringer's	214 Fe(II) fresh	214		535		"
MT7. Catechin + Fe(II) + Cu(II) + EDTA (Table 4.5b)	214	214 Fe(II) fresh	214		1070		"
MT8. Cu(II) + catalase (Fig. 4.15)	Ringer's		214			0 - 200 0 - 600	" "
Catalase alone	Ringer's					600	"
Catechin + catalase	214					600	" "
Fe(II) + Cu(II) +catalase (Table 4.5c)	Ringer's	214 Fe(II) fresh	214			600	
Vit. C + catalase	Ringer's			856		600	"
MT9. Catechin +	214		214	856		0 - 200	" "

Cu(II) + vit. C + catalase (Fig. 4.17)						0 - 600	
Cu(II) + Vit.C + catalase	214		214			0 - 200 0 - 600	"
MT10.Catechin + Fe(II) + Cu(II) + catalase	Ringer's	214 Fe(II) fresh	214			600	"
catechin + Fe(II) + catalase	214	214 Fe(II) fresh				600	"
catechin + Cu(II) + catalase	214		214			600	

Key : Fe(II) = aqueous iron(II) sulphate heptahydrate; **Fe(III)** = aq. iron(III) chloride solution; **Cu(II)** = aq. copper(II) sulphate pentahydrate; **EDTA** = aq. Ethylenediamine acetic acid disodium salt; **Catalase** = bovine liver catalase solution 50 mg mL^{-1} with an activity of *ca.* 47 kU mg^{-1} protein. **Inocula:** *Sa* = *S. aureus* = *Staphylococcus aureus* NCTC 06571, *Psa* = *Ps.aeruginosa* = *Pseudomonas aeruginosa* NCTC 950, *Pm* = *P.mirabilis* = *Proteus mirabilis* NCTC 7827, *Ec* = *E. coli* = *Escherichia coli* NCTC 14441. Results are shown as viabilities with SEM's in brackets based on at least 3 replicates; ranges are shown where appropriate. Figure and Table numbers refer to illustrations in the data chapters. Main treatements are numbered as MT and shown in bold text with accompanying controls shown in non-bold text.

Notes on Methodology

The assay mixtures made up to produce the final concentrations shown above used following volumes and concentrations of solutions shown below:

Main treatments (MT + number, shown in bold) and controls:
Buffer: [b]All assays featured 1000μL control samples of lambda buffer adjusted to pH 7.2 as a control.
Ringer's solution adjusted to different pH values: 1000 μL Ringer's solution adjusted to pH 3.5, or 5.5, or 7.2
(+)-Catechin alone: 330 μL 1000 μM (+)-catechin + 700 μL Ringer's solution
Cu(II) alone, fresh, heated: 330 μL Ringer's solution + 700 μL 472 μM $Cu(II)SO_4$

MT1: 330 μL 1000 μM (+)-catechin + 700 μL 472 μM $Cu(II)SO_4$
Fe(II) alone, fresh, heated, autoclaved: 330 μL Ringer's solution + 700 μL 472 μM $Fe(II)SO_4$ either freshly made, or freshly made and then heated ,or freshly made and then autoclaved
Fe(III) alone, fresh, heated, autoclaved: 330 μL Ringer's solution + 700 μL 472 μM $Fe(III)Cl_3$ prepared as Fe(II) above
Fe(II) + Cu(II): 330 μL Ringer's solution + 350 μL 944 μM $Fe(II)SO_4$ + 350 μL 944 μM $Cu(II)SO_4$

MT2: 330 μL 1000 μM (+)-catechin + 700 μL 472 μM [f]$Fe(II)SO_4$ or $Fe(III)Cl_3$, in each case either freshly made, or freshly made and then heated ,or freshly made and then autoclaved

MT3: 330 μL 1000 μM (+)-catechin + 350 μL 944 μM $Fe(II)SO_4$ + 350 μL 944 μM $Cu(II)SO_4$
Vitamin C alone: 330 μL Ringer's solution + 700 μL 1510 μM vit. C
(+)-Catechin + vit. C: 330 μL 1000 μM (+)-catechin + 700 μL 1510 μM vit. C
Cu(II) + vit. C alone: 330 μL Ringer's solution + 350 μL 944 μM $Cu(II)SO_4$ + 350 μL 1888 μM, or 3776 μM ,or 7552 μM vit. C respectively

MT4: 330 μL 1000 μM (+)-catechin + 350 μL 944 μM $Cu(II)SO_4$ + 350 μL 1888 μM, or 3776 μM, or 7552 μM vit. C respectively
Fe(II) + vit. C: 330 μL Ringer's solution + 350 μL 944 μM $Fe(II)SO_4$ + 350 μL 3776 μM vit. C

MT5: 330 μL 1000 μM (+)-catechin + 350 μL 944 μM $Fe(II)SO_4$ + 350 μL 3776 μM vit. C
Fe(II) + Cu(II) + vit. C: 330 μL Ringer's solution + 175 μL 1888 μM $Fe(II)SO_4$ + 175 μL 1888 μM $Cu(II)SO_4$ + 175 μL 3776 μM vit. C

MT6: 330 μL 1000 μM (+)-catechin + 175 μL 1888 μM $Fe(II)SO_4$ + 175 μL 1888 μM $Cu(II)SO_4$ + 175 μL 3776 μM vit. C
EDTA alone: 330 μL Ringer's solution + 700 μL 2140 μM EDTA solution
Fe(II) + Cu(II) + EDTA: 330 μL Ringer's solution + 175 μL 1888 μM $Fe(II)SO_4$ + 175 μL 1888 μM $Cu(II)SO_4$ + 175 μL 2140 μM EDTA

MT7: 330 µL 1000 µM (+)-catechin + 175 µL 1888 µM $Fe(II)SO_4$ + 175 µL 1888 µM $Cu(II)SO_4$ + 175 µL 2140 µM EDTA

MT8: 330 µL Ringer's solution + 700 µL 472 µM $Cu(II)SO_4$ + [c]catalase at concentrations specified
[c]Catalase alone: 1000 µL Ringer's solution + catalase at concentration specified
(+)-Catechin + catalase: 330 µL 1000 µM (+)-catechin + 700 µL Ringer's solution + catalase at concentration specified
Fe(II) + Cu(II) + catalase: 330 µL Ringer's solution + 350 µL 944 µM $Fe(II)SO_4$ + 350 µL 944 µM $Cu(II)SO_4$ + catalase at concentration specified
Vit. C + catalase: 650 µL Ringer's solution + 350 µL 3776 µM vit. C + catalase at concentration specified

MT9: 330 µL 1000 µM (+)-catechin + 350 µL 944 µM $Cu(II)SO_4$ + 350 µL 3776 µM vit. C + catalase at concentrations specified
Cu(II) + vit. C + catalase: 650 µL Ringer's solution + 350 µL 3776 µM vit. C + catalase at concentrations specified

MT10: 330 µL 1000 µM (+)-catechin + 350 µL 944 µM $Fe(II)SO_4$ + 350 µL 944 µM $Cu(II)SO_4$ + catalase at concentration specified
(+)-Catechin + Fe(II) + catalase: 330 µL 1000 µM (+)-catechin + 700 µL 472 µM $Fe(II)SO_4$ + catalase at concentration specified
(+)-Catechin + Cu(II) + catalase: 330 µL 1000 µM (+)-catechin + 700 µL 472 µM $Cu(II)SO_4$ + catalase at concentration specified
[c]Catalase was added immediately prior to addition of inoculum

Further details of catechin suspension assays: fresh catechin with other putative adjuncts (zinc (II) and manganese (II) sulphate, vitamin C, caffeine); fresh catechin and other flavanols with molar ratios of copper (II) sulphate; treated catechin with copper (II) sulphate. Figure or Table numbers in brackets that follow section titles refer to those in the main text.

B. Effects of adding catechin and vitamin C to manganese (II) sulphate against *Staphylococcus aureus* NCTC 06751 (Figure 4.6)

Inoculum	330 µL 1000 µM catechin or control	Volumes of putative adjuncts used plus any additional control
Staphylococcus aureus NCTC 06751	Catechin	700 µL 472 µM manganese (II) sulphate
	Ringer's solution	700 µL 472 µM manganese (II) sulphate
	Ringer's solution	350 µL 944 µM manganese (II) sulphate plus 350 µL 1888 µM vitamin C
	Ringer's solution	350 µL 944 µM manganese (II) sulphate plus 350 µL 5664 µM vitamin C

C. Effects of adding caffeine to fixed ratios of copper (II) sulphate and vitamin C against *Staphylococcus aureus* NCTC 06751 (Figures 4.3, 4.4)

Inoculum	Volume of test substance or control	Volumes of putative adjuncts used plus additional controls
Staphylococcus aureus NCTC 06751	330 µL 0.01, 0.1, 0.5, 1% caffeine	700 µL Ringer's solution
	330 µL Ringer's solution	700 µL 472 µM copper (II) sulphate
	330 µL Ringer's solution	350 µL 944 µM copper (II) sulphate plus 350 µL 1888 µM vitamin C
	330 µL 1000 µM catechin	472 µM copper (II) sulphate
	330 µL 1000 µM catechin	350 µL 944 µM copper (II) sulphate plus 350 µL 1888 µM vitamin C
	330 µL *2.34 % caffeine	472 µM copper (II) sulphate
	330 µL *2.34 % caffeine	350 µL 944 µM copper (II) sulphate plus 350 µL 1888 µM vitamin C
	175 µL 1000 µM catechin plus 175 µL *4.68% caffeine	472 µM copper (II) sulphate
	175 µL 1000 µM catechin plus 175 µL *4.68% caffeine	350 µL 944 µM copper (II) sulphate plus 350 µL 1888 µM vitamin C

*final concentration in assay of 0.5%

D. Effects of adding zinc (II) sulphate to combinations of catechin plus copper (II) sulphate plus vitamin C against *Staphylococcus aureus* NCTC 06751 (Figure 4.5)

Inoculum	Volume of test substance or control	Volumes of putative adjuncts used plus additional controls
Staphylococcus aureus NCTC 06751	330 µL Ringer's solution	700 µL 472 µM zinc (II) sulphate
	330 µL Ringer's solution	350 µL 944 µM zinc (II) sulphate plus 350 µL 1888 µM vitamin
	330 µL 1000 µM catechin	700 µL 472 µM zinc (II) sulphate
	330 µL 1000 µM catechin	350 µL 944 µM zinc (II) sulphate plus 350 µL 1888 µM vitamin
	330 µL Ringer's solution	350 µL 944 µM zinc (II) sulphate plus 350 µL 944 µM copper (II) sulphate
	330 µL 1000 µM catechin	333.3 µL 1416 µM zinc (II) sulphate plus 333.3 µL 1416 µM copper (II) sulphate plus 333.3 µL 2832 µM vitamin C
	330 µL 1000 µM catechin	333.3 µL 1416 µM zinc (II) sulphate plus 333.3 µL 1416 µM copper (II) sulphate plus 333.3 µL 5664 µM vitamin C
	330 µL 1000 µM catechin	333.3 µL 1416 µM zinc (II) sulphate plus 333.3 µL 1416 µM copper (II) sulphate plus 333.3 µL 8496 µM vitamin C

E. The effect of heating catechin solution at 100 °C for 10 and for 30 minutes on subsequent antimicrobial activity, and when combined with equimolar copper (II) sulphate against *Staphylococcus aureus* NCTC 06751 (Figure 4.8)

Inoculum	330 µL catechin or control	700 µL of putative adjunct
Staphylococcus aureus NCTC 06751	Ringer's solution	472 µM copper (II) sulphate
	1000 µM catechin heated for 10 minutes at 100 °C	Ringer's solution
	1000 µM catechin heated for 30 minutes at 100 °C	Ringer's solution
	1000 µM catechin freshly made	Ringer's solution
	1000 µM catechin heated for 10 minutes at 100 °C	472 µM copper (II) sulphate
	1000 µM catechin heated for 30 minutes at 100 °C	472 µM copper (II) sulphate

F. The effect of autoclaving freshly made and previously heat treated catechin and copper (II) sulphate solution in different combinations on subsequent antimicrobial activity against *Staphylococcus aureus* NCTC 06751 (Figure 4.9)

Inoculum	330 µL catechin or control	700 µL of putative adjunct
Staphylococcus aureus NCTC 06751	Ringer's solution	472 µM copper (II) sulphate
	Ringer's solution	472 µM copper (II) sulphate autoclaved
	500 µM catechin freshly made	Ringer's solution
	500 µM catechin freshly made and autoclaved	Ringer's solution
	500 µM catechin heated for 10 minutes at 100°C	Ringer's solution
	500 µM catechin heated for 10 minutes at 100°C and autoclaved	Ringer's solution
	500 µM catechin freshly made	472 µM copper (II) sulphate
	500 µM catechin freshly made and autoclaved	472 µM copper (II) sulphate
	500 µM catechin freshly made	472 µM copper (II) sulphate autoclaved
	500 µM catechin freshly made and autoclaved	472 µM copper (II) sulphate autoclaved
	500 µM catechin freshly made and autoclaved with the adjunct prior to mixing with inoculum	472 µM copper (II) sulphate
	500 µM catechin heated for 10 minutes	472 µM copper (II) sulphate

	at 100°C	
	500 μM catechin heated for 10 minutes at 100°C and autoclaved	472 μM copper (II) sulphate
	500 μM Catechin heated for 10 minutes at 100°C	472 μM copper (II) sulphate autoclaved
	500 μM catechin heated for 10 minutes at 100°C and autoclaved	500 μM Catechin freshly made and autoclaved
	500 μM catechin heated for 10 minutes at 100°C and autoclaved with the adjunct prior to mixing with inoculum	472 μM copper (II) sulphate

G. Investigation of reaction mechanisms using kinetic studies (Figures 4.13, 4.14)

The rate of antimicrobial activity of test agents with putative adjuncts (measured as a reduction in cell viability over time) was investigated to find out if certain antimicrobial combinations would produce their inhibitory effects on cells faster than other test combinations. If this proved to be true then the rate experiments could also provide information on whether certain chemical functional groups within the molecular structure of the tea catechins were more active than others. In Experiment I, samples were made up as per suspension assays with concentrations of 1000 μM catechin and 1μM EGCG which previously gave similar levels of activity in independent assays with added 4.8 mM copper (II) sulphate against *S. aureus* NCTC 06751 (Table 3.4), to reach a point where resulting viability was *ca.* 3 $\log_{10}$ cfu mL^{-1} after 30 minutes exposure. Rates of action of test agents against *S. aureus* NCTC 06751 were compared in excess copper(II) sulphate conditions to avoid any rate limitations caused by low availabilty of copper (II) ions. In a second experiment fresh and heat treated catechin were tested in a similar way but with samples withdrawn at 0, 10, and 40 seconds. Rate of antimicrobial activity was expressed in both experiments as a fraction of the numbers of cfu's seen at a particular time interval divided by the original number of buffer control cfu's at time zero.

Test or control substance with concentrations used	700 μL of test or control substance with concentrations used	Time samples withdrawn
330 μL Ringer's solution 330 μL 1000 μM freshly made catechin 330 μL 1000 μM catechin, heat treated at 100°C for 10 minutes 330 μL 1 μM epigallocatechin gallate (EGCG)	700 μL 4.8 mM, copper (II) sulphate	Experiment I: 0, 12, 3, 5, 10 ,15 minutes In Expt. II: 0, 10, 40 seconds. EGCG not tested.

H. Investigation of structure-activity relationship of catechin and its flavanol isomers (Figure 4.18)

In order to find out whether the functional groups of the molecular structure of catechin had any influence on its antimicrobial activity when combined with copper (II) sulphate against *S. aureus* NCTC 06571 flavanol isomers of catechin were investigated using the suspension assay methods described above. The antimicrobial activity of catechin was compared to *ent*-catechin, epicatechin and *ent*-epicatechin, heat treated catechin (100°C for 10 minutes), as well as catechin solution stored at room temperature for 14 days at room temperature and exposed to the atmosphere, all against *S. aureus* NCTC 06571. The catechin solutions and its isomers were tested alone as well as combined with equimolar copper (II) sulphate.

330 µL test or control substance with concentrations used	**700 µL of test or control substance with concentrations used**
330 µL 1000 µM freshly made catechin	Ringer's solution
330 µL 1000 µM ent-catechin	Ringer's solution
330 µL 1000 µM ent-epicatechin	Ringer's solution
330 µL 1000 µM catechin, heat treated at 100°C for 10 minutes	Ringer's solution
330 µL 1000 µM catechin solution, stored at room temperature for 14 days, exposed to the atmosphere	Ringer's solution
330 µL Ringer's solution	700 µL 472 µM, copper (II) sulphate
330 µL 1000 µM freshly made catechin	700 µL 472 µM, copper (II) sulphate
330 µL 1000 µM ent-catechin	700 µL 472 µM, copper (II) sulphate
330 µL 1000 µM ent-epicatechin	700 µL 472 µM, copper (II) sulphate
330 µL 1000 µM catechin, heat treated at 100°C for 10 minutes	700 µL 472 µM, copper (II) sulphate
330 µL 1000 µM catechin solution, stored at room temperature for 14 days, exposed to the atmosphere	700 µL 472 µM, copper (II) sulphate

Appendix III

Assays of stored mixtures

A. The effect of storage at for periods up to 3 years at -20 °C on solutions of catechin and copper (II) sulphate solution on subsequent antimicrobial activity against *Staphylococcus aureus* NCTC 06751 (Figure 5.1)

Inoculum	330 µL test substance	700 µL of putative adjunct
Staphylococcus aureus NCTC 06751	Ringer's solution	4.8 mM copper (II) sulphate (freshly made)
	Ringer's solution	4.8 mM copper (II) sulphate (stored)
	WT (fresh)	Ringer's solution
	WT (stored)	Ringer's solution
	WT (fresh)	4.8 mM copper (II) sulphate (stored)
	WT (stored)	4.8 mM copper (II) sulphate (stored)

Aliquotted frozen samples were thawed from time to time and compared with freshly made solutions

B. The effect of storage at room temperature on catechin solution on subsequent antimicrobial activity when added to copper (II) sulphate activity against *Staphylococcus aureus* NCTC 06751 (Figure 4.8)

Inoculum	330 µL catechin or control	700 µL of putative adjunct
Staphylococcus aureus NCTC 06751	Ringer's solution	472 µM copper (II) sulphate
	1000 µM catechin stored for 14 days	Ringer's solution
	1000 µM catechin stored for 14 days	472 µM copper (II) sulphate

C. The effect of storage at different temperatures on solutions of catechin and copper (II) sulphate solution and with the further addition of vitamin C on subsequent antimicrobial activity against a panel of four different species of bacteria (Figures 5.2 – 5.6)

Inocula	1000 µL of test mixtures stored at room temperature and at 5°C	Length of storage prior to assay
Staphylococcus aureus NCTC 06751	*321 µM combined equimolar catechin plus copper (II) sulphate (room temperature)	0, 1, 2, and 7 days
Pseudomonas aeruginosa NCTC 950	321 µM combined equimolar catechin plus copper (II) sulphate (5 °C)	0, 1, 2, 7 and days
Proteus mirabilis NCTC 7827	321 µM combined equimolar catechin plus copper (II) sulphate plus 1284 µM vitamin C (room temperature)	0, 1, 2, 7 and days
Escherichia coli NCTC 14441	321 µM combined equimolar catechin plus copper (II) sulphate plus 1284 µM vitamin C (5 °C)	0, 1, 2, 7 and days

*Final concentrations in assays following addition of inoculum: catechin 214 µM; copper (II) sulphate, 214 µM; vitamin C. 856 µM.

F. The effect of freeze drying and storage of combined catechin plus copper (II) sulphate plus vitamin C solution on subsequent antimicrobial activity against a panel of four different species of bacteria (Figure 5.7)

Inocula	**1000 µL solution of combined fresh components**	**1000 µL solution of freeze dried mixture**
Staphylococcus aureus NCTC 06751 *Pseudomonas aeruginosa* NCTC 950 *Proteus mirabilis* NCTC 7827 *Escherichia coli* NCTC 14441	321 µM catechin plus 321 µM copper (II) sulphate	321 µM combined equimolar catechin plus copper (II) sulphate

Freeze dried substances were stored at 5°C. Final concentrations in assays following addition of inoculum: catechin 214 µM; copper (II) sulphate, 214 µM; vitamin C. 856 µM. Amounts of freeze dried full mixture required to make up 5 mL solutions for suspension assays = 0.0062 g $5mL^{-1}$

Appendix IV Publications

Holloway, A., Gould, S.W.J., Fielder, M.D., Naughton, D.P., and Kelly, A.K. 2011. Enhancement of antimicrobial activities of whole and sub-fractionated white tea by addition of copper (II) sulphate and vitamin C against *Staphylococcus aureus*; a mechanistic approach. *BMC Complementary and Alternative Medicine*. 11, 115-124.

Holloway, A.C., Mueller-Harvey, I., Gould, S.W.J., Fielder, M.D., Naughton, D.P., and Kelly, A.K. 2012.The effect of copper(II), iron(II) sulphate, and vitamin C combinations on the weak antimicrobial activity of (+)-catechin against *Staphylococcus aureus* and other microbes. *Metallomics*. 4 (12), 1280-1286.