THE UNIVERSITY OF HULL

# Microreactor synthesis of labelled polyphenols: a route to antibacterial modes of action in important hospital pathogens

being a thesis submitted for the Degree of Doctor of Philosophy

in the University of Hull

by

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**Declaration**

I would like to declare that all the work in this thesis is from my own work, unless stated otherwise. The work has nether been accepted or being submitted for any other degrees.

**Acknowledgements**

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## Dedication

I would like to dedicate this thesis to my family, future wife, pepper and my old friend Mr Bernard Betts snr

**Abstract**

The aims of the PhD were to synthesize deuterium labelled epicatechin in a microreactor, discover if theaflavin and epicatechin possess antibacterial action against important hospital pathogens and to determine if synergy exists between polyphenols and between polyphenol and current antibiotics. Other aims were to determine the minimum inhibitory concentrations of theaflavin and a theaflavin:epicatechin combination against the bacteria *Stenotrophomonas maltophilia*, to synthesize theaflavin and deuterium labelled theaflavin in a microreactor and to link the continuous flow process to antimicrobial testing for future mode of action analysis.

A 2-step microreactor synthesis using in a T-shaped microreactor successfully produced deuterium labelled epicatechin in position 8. Mass spectrometry and H-NMR confirmed deuterium labelled epicatechin had been produced. The 2-step synthesis produced a yield in excess of 90%. A 4-step micro reactor synthesis of deuterium labelled epicatechin, was found to be unsuccessful after step 2 of the synthesis. A 1-step method for the microreactor production of planar epicatechin was also shown to be unsuccessful in a microreactor.

Antimicrobial testing of theaflavin, epicatechin and a 2:1 combination of theaflavin and epicatechin was performed against 4 clinical isolates of MRSA, 6 clinical isolates of *Acinetobacter baumannii* and 6 clinical isolates of *Stenotrophomonas maltophilia*. Results from the disc diffusion assay confirmed that epicatechin produced no antibacterial action and theaflavin produced strong antibacterial action. The combination of theaflavin and epicatechin produced higher antibacterial activity than theaflavin alone indicating synergy between the two polyphenols. Minimum inhibitory concentrations (MICs) of theaflavin and the theaflavin:epicatechin combination (2:1) were determined against 9 clinical isolates and one control isolate of *Stenotrophomonas maltophilia* using a microtiter assay. Results from the microtiter assay used indicated that the MIC for theaflavin was between 400 and 800 µg/mL. The MIC for the theaflavin:epicatechin combination (2:1) was between 200 and 400 µg/mL.

A 1-step microreactor synthesis of theaflavin from epicatechin and epigallocatechin using extracted polyphenol oxidase was shown to be successful, producing high yields. Using the same methodology, the synthesis of deuterium labelled theaflavin was undertaken using epigallocatechin and the deuterium labelled epicatechin. However, this reaction was shown to be unsuccessful. The antibacterial action of microreactor synthesized theaflavin against *Acinetobacter baumannii* in a continuous flow process was investigated. Bacterial viability was tested using the resazurin indicator method. No viable cells were observed from bacterial samples exposed to ≥ 4 hours of the continuous flow of theaflavin products. This indicated that the theaflavin produced antibacterial action after this time of exposure. At exposure times less than 4 hours, viable cells were detected.

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## Publications

*Published papers*

J. W. Betts., S. P. Kitney., Y. Fu., W. Peng., S. M. Kelly and S. J. Haswell (2010) Production of deuterium labelled (-)-epicatechin in a microreactor. *Chemical Engineering Journal.* **167**(2-3). 545-547.

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

*Papers in preparation*

Enzymatic synthesis of theaflavin in a continuous flow microreactor.

Minimum inhibitory concentrations of theaflavin and combinations with epicatechin and quercetin against clinical isolates of *Stenotrophomonas maltophilia*.

Antagonistic and synergistic antibacterial effects of polyphenols combined with clinical antibiotics against hospital isolates of *Stenotrophomonas maltophilia* and *Acinetobacter baumannii.*

*Conference presentations*

J. W. Betts, S. P. Kitney, S. M. Kelly and S. J. Haswell (2010) Production of deuterium labelled (-)-epicatechin in a microreactor – a route to toxicological studies. Poster presented at IMRET 2010 conference in Kyoto, Japan.

J. W. Betts, W. Peng, Y. Fu, S. M. Kelly and S. J. Haswell (2008) Chemical synthesis of theaflavin in a microreactor. Poster presented at SES conference (2008) at Lancaster University.

J. W. Betts, W. Peng, Y. Fu, S. M. Kelly and S. J. Haswell (2008) Chemical synthesis and biological activity of theaflavin in a microreactor. Poster presented at ARF student conference (2008) at Hull University.

**Abbreviations**

| | | |
|---|---|---|
| EC | - | Epicatechin |
| TF | - | Theaflavin |
| EGC | - | Epigallocatechin |
| EGCG | - | Epigallocatechin gallate |
| MS | - | Mass spectrometry |
| $^{1}$H-NMR | - | Proton nuclear magnetic resonance |
| $^{13}$C-NMR | - | Carbon 13 nuclear magnetic resonance |
| PBS | - | Phosphate buffered saline |
| DMSO | - | Dimethyl sulfoxide |
| MIC | - | Minimum inhibitory concentration |
| LDL | - | Low-density lipoprotein |
| DNA | - | Deoxyribonucleic acid |
| RNA | - | Ribonucleic acid |
| SCLC | - | Small cell lung carcinoma |
| FDA | - | Food and drug administration (US) |
| CDC | - | Centre for disease control |
| WHO | - | World heath organisation |
| ICU | - | Intensive care unit |
| MRSA | - | Methicillin-resistant *Staphylococcus aureus* |
| VRE | - | Vancomycin-resistant enterococci |
| PDMS | - | Polydimethyl siloxane |
| PMMA | - | Polymethyl methacrylate |
| HF | - | Hydrogen fluoride |
| D-labelling | - | Deuterium labelling |
| THF | - | Trifluoromethanesulfonate |
| HPLC | - | High performance liquid chromatography |
| UV | - | Ultraviolet |
| HCL | - | Hydrochloric acid |
| NBS | - | N-bromosuccinimide |
| $CCl_4$ | - | Carbon tetrachloride |
| DCM | - | Dichloromethane |
| $NaBD_4$ | - | Sodium borodeuteride |
| NIS | - | N-iodosuccinimide |
| TFA | - | Trifluoroacetic acid |
| TBDMS | - | Tert-butyldimethylsilyl |
| DMF | - | Dimethylformamide |
| $MgSO_4$ | - | Magnesium sulphate |
| TLC | - | Thin layer chromatography |
| $NaSO_4$ | - | Sodium sulphate |
| MW | - | Molecular weight |
| *spp* | - | Species |
| HIV | - | Human immunodeficiency virus |
| CBA | - | Columbia blood agar |
| ISO | - | Iso-sensitive |
| BSAC | - | British society for antimicrobial chemotherapy |
| NCTC | - | National collection of type cultures |
| AMP | - | Ampicillin |
| CN | - | Gentamycin |
| IPM | - | Imipenem |
| TE | - | Tetracycline |

| | | |
|---|---|---|
| FOX | - | Cefoxitin |
| CIP | - | Ciprofloxacin |
| CLM | - | Clarithromycin |
| P | - | Penicillin G |
| PPO | - | Polyphenol oxidase |
| NADPH | - | Nicotinamide adenine dinucleotide phosphate |
| rpm | - | Rotations per minute |

# Chapter 1

Introduction to polyphenols, synthesis and microfluidics

## 1.1 Introduction

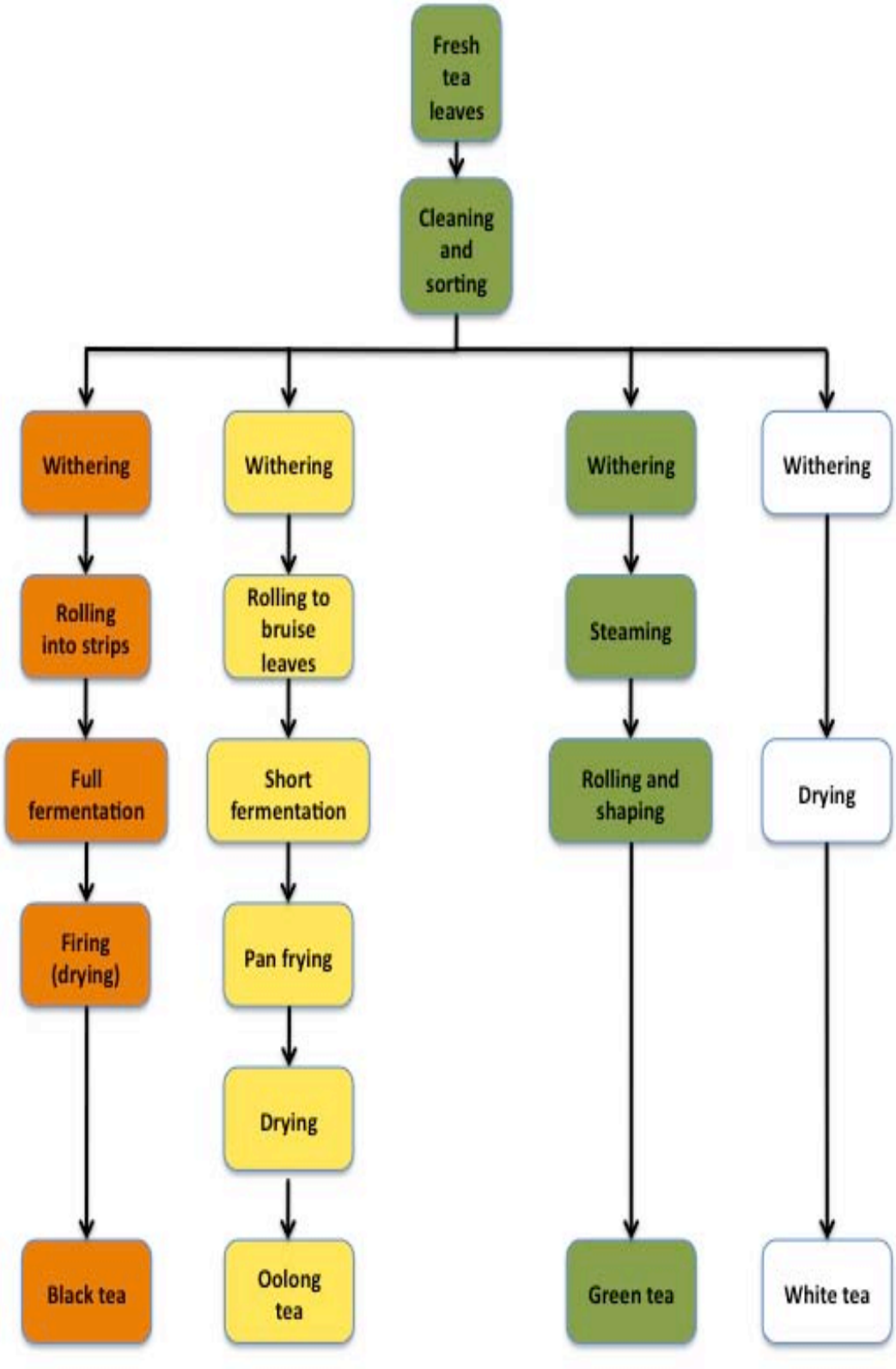
### 1.1.1 Tea and its contents

Green tea like black tea is produced from the plant *Camellia sinensis* (Zaveri, 2006). *Camellia sinensis* is an evergreen shrub with leaves between 3 and 10 inches long (Taylor, 1998), which grows in over 30 countries (Gupta *et al.,* 2002). These tea trees often produce fragrant white flowers (figure 1) and are typically grown in conditions such as in acidic soils, high or low altitude and high humidity (Dufresne and Farnworth, 2001) and therefore can be cultivated in many regions of the world including China, Japan, Kenya, India, Brazil and Argentina (Mitscher and Dolby Toews, 2008).

Figure 1. The leaves and white flowers of *Camellia sinensis*.

Although there is only one tree/shrub from which tea is produced, there are many of different varieties of tea including black tea, green tea, oolong tea and white tea Sharangi (2009). The main difference between them is their oxidation state, with the most oxidation occurring in black tea (Jhoo *et al*., 2004). Accordingly teas can be processed in a variety of ways depending on the product required (See figure 2).

Figure 2. Methods of tea processing (teazonline, 2011)



Tea is the second most consumed beverage in the world, of this 72% is black tea and 26% is green tea and 2 % other varieties (Katiyar and Mukhtar, 1996). The consumption of tea has occurred for over five thousand years and was thought of as a highly prized tonic to keep the body in a healthy condition (Mitscher and Dolby Toews, 2008). Archaeological data suggests that infusions from tea and other wild pants, was practiced by our ancestors up to five hundred thousand years ago (Jelinek, 1978). Although it was thought to have originated as a beverage in Japan, the earliest evidence of tea comes from China where traditional healers such as Sheg Nung lived in 2730 BC (Kuroda and Hara, 2004). In Japan for hundreds of years a tradition called the tea ceremony has existed. Tea ceremonies where originally conducted by warriors and were thought of as spiritual events.

Although for many years green tea has been consumed more in the eastern world it has in more recent years become very popular in the west due to its link with health benefits (Marion *et al.,* 2003). However, black tea is still the most popular choice as it makes up 72% of the worlds total tea production (Sharangi, 2009).

The health benefits of tea are linked to their active ingredients called polyphenols (Marion *et al.,* 2003). These polyphenolic compounds are part of the flavonoid group, which are also found in red wine, black grapes, cocoa, beans and Chinese rhubarb (Rein *et al.,* 2000). It is estimated that in the US 500-1000mg of flavonoids including those found in tea are consumed everyday (Cushnie and Lamb, 2005). Catechins are the most common polyphenolic compounds in green tea whereas theaflavins are more common in black tea (Matsuo, Tanaka and Kouno, 2009) and inpart the reddish brown colour to the tea from the theaflavins. This colour difference between black and green tea can be seen in figure 3.

Figure 3. The change in colour of tea leaves which relates to flavonoid content.


Theaflavins represent 0.4-1.8% of the dry weight of black tea (Sharma, bari and Singh, 2009). Catechins form 80% of the polyphenolic content of green tea and represent 20-30% of the dry weight of the tea leafs. Catechins in green tea leafs include epicatechin, catechin, epigallocatechin and epigallocatechin gallate (Lorenz *et al.,* 2004). Examples of the structures of catechins and theaflavins can be seen in figures 4 and 5. The leaves contain other compounds including caffeine, amino acids, minerals (fluoride), aromatic oils, vitamins, proteins and carbohydrates (Khan and Mukhtar, 2007). The quantities of these compounds vary between types of tea and can affect its smell, taste and colour (Mitscher and Dolby Toews, 2008).

Figure 4. The structure of the black tea flavanoids, theaflavin, theaflavin-3'-monogallate and theaflavin-3-3'-digallate and their molecular weights

Figure 5. The structures of the green tea catechins, epigallocatechin gallate, epicatechin, epigallocatechin, epicatechin gallate, catechin and their molecular weights

### 1.1.2 Bioavailability and stability of polyphenols

In humans, polyphenol concentrations in plasma often do not exceed 1 μM even after the consumption of 10–100 mg of an individual polyphenol (Scalbert and Williamson, 2000). However, due to polyphenol metabolites formed in the body's tissues, the total polyphenol concentration in plasma is likely to be higher. Many studies focus on polyphenol activity in vitro that do not take factors such as metabolism or bioavailability into consideration that could affect their activity in vivo (Williamson and Manach, 2005). However, there have been demonstrations that tea polyphenols have strong in vivo activity in humans (Serafini, Ghiselli and Ferro-Luzzi, 1996). It was suggested that polyphenol absorption took place in the upper gastrointestinal system due to the speed of the in vivo response after any tea was consumed.

Catechins have been shown to have short half-lives in plasma often forming conjugates with sulfate or methyl groups after 2-3 hours (Bell *et al.,* 2000). An exception to this is with epigallocatechin gallate where research has demonstrated that up to 100% of the polyphenol remains unconjugated after 1 hour (Lee *et al.,* 2002).

There are various factors including those biochemical and chemical that can affect polyphenol absorption and polyphenol bioavailability can also be linked to stability.

As many of the benefits of green and black tea are thought to come from their active components there stability is the environment is very important. A number of research groups have investigated the stability of catechins on several occasions, with some having described an effect called "browning" (Lopez-Toledano *et al.,* 2002) whereby phenolic compounds degrade. The "browning" of these phenolics can be caused by enzymatic and non-enzymatic oxidation reactions. These reactions can be seen in a variety of products for example, in damaged fruit where a red/brown "bruise" is produced (Tanaka *et al.,* 2002). The bruise is the result of surface damage to the fruit, which allows

oxygen below the surface of the skin resulting in an oxidation reaction of phenolics contained in the fruit. The stability of catechins has a great impact on their usage especially in the food/beverage market and also in their applications in the medical industry.

Temperature has been shown to have an effect on the stability of catechins. Results from various studies have shown that boiling catechins at 98°C for 7 hours contributes to 15-20% of their degradation (Chen *et al.,* 2001 and Zhu *et al.,* 1997). It was noted by Chen *et al.,* (2001) that autoclaving catechins at 120°C for 20 minutes reduces their stability by 24%, although this was thought to be partially pH dependant.

Research groups have also invested pH and its contribution to the stability/degradation of catechins (Zhu *et al.,* 1997). In this study it was noted that in green tea catechins, the lower the pH the more stable the catechins were. It was also noted that in alkaline solutions the catechins were less stable and therefore more easily absorbed in the intestine. Another issue that has been raised is the effect of mixing flavanols with milk. In 2007 Lorenz *et al*., discovered that when adding milk to a solution containing flavanols the protective health effects are prevented. A possible reason the investigation gave for this was that the protein in the milk led to the formation of complexes with catechins in the tea.

A study by Hatano *et al.,* (2008) discovered that the addition of ascorbic acid to polyphenolic solutions prolonged its usage. This was a very useful discovery in terms of the potential storage lifespan of polyphenols for food or medicinal uses. Synergy has also been seen in other research, where the cholesterol-lowering effects of green tea extracts were increased by the addition of theaflavin (Maron *et al.,* 2003).

### 1.1.3 Benefits of tea polyphenols

#### 1.1.3.1 General benefits of polyphenols

There have been many investigations to determine the health benefits of flavanols, which include anticancer (Cooper, Morre and Morre, 2005; Sadava *et al.,* 2007), anti- inflammatory (Sang *et al.,* 2004), antioxidant (Dobashi *et al.,* 2008), antiviral (Williamson *et al.,* 2006) and antibacterial effects (Navarro-Martinez *et al*, 2005; Vijaya *et al.,* 1995). Other benefits of green and black tea components include stroke prevention (Fraser, Mok and Lee, 2007), a protective effect from liver damage that can be caused by anticancer drugs (El-Beshbishy, 2005) and its use as a food preservative (Yilmaz, 2006). It has been proposed that flavanols, including theaflavin, improve vascular function (Schewe, Steffen and Sies, 2008). Epicatechin has been particularly noted to have positive effects, with its metabolites having a protective effect against oxidized low-density lipoprotein (LDL) that would otherwise cause oxidative stress to endothelial cells. However, there are doubts about the positive effect of flavanols on cardiovascular health, with some researchers stating that there is inconclusive evidence and the need for further controlled studies in this area (Riemersma *et al.,* 2001). Recent research has also proposed that polyphenols could be used in the preservation of mammalian tissue without the need for cryopreservation Han *et al.,* 2006). Previous studies have also demonstrated that tea polyphenols can increase that rate of fat oxidation, leading to weight loss (Dulloo *et al*., 1999). However, another study using green tea found that it had no effect on weight maintenance following a low energy diet in obese subjects (Kovacs *et al.,* 2004).

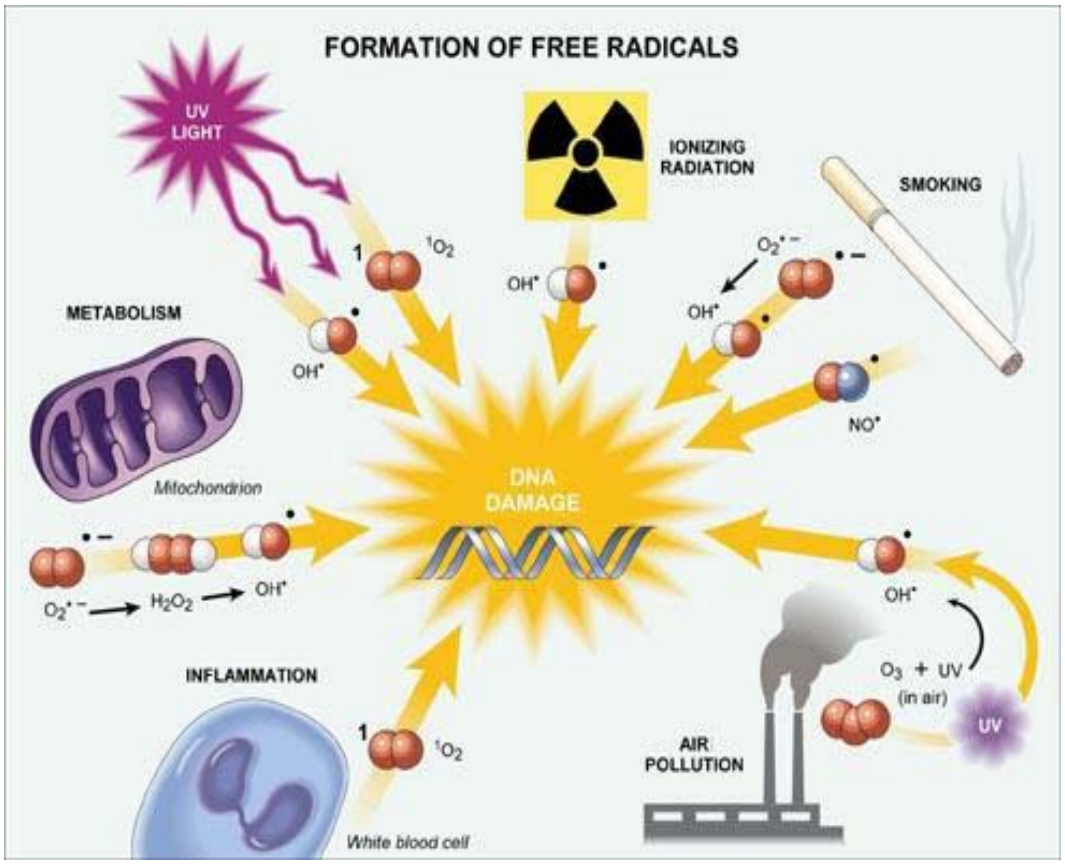
#### 1.1.3.2 Polyphenols as antioxidants

The basis for many of their reported benefits are thought to be the flavanols role as antioxidants (Almajano *et al.,* 2008). Antioxidants are defined as materials that are able to neutalize the effects of free radicals and thus prevent the oxidative damage they cause to biological systems (Azzi *et al*., 2004). Antioxidants are classified into two groups: enzymatic and non-enzymatic antioxidants (Ratnam *et al.,* 2006). Green and black tea polyphenols are classed as non-enzymatic antioxidants. Previous research has shown that

consumption of antioxidants in the human diet has fallen over thousands of years and is now far less than that of our ancestors (Benzie, 2003) possibly due to higher processing of food. Antioxidants including ascorbic acid (vitamin C) and catechins have been proposed to have a protective affect against free radicals and other oxidative agents (Collins *et al.,* 1996; Frei and Higdon, 2003).

Free radicals are molecules with an unpaired electron making them highly reactive (Bettelheim and March, 1998). One very important example of a free radical is the reactive oxygen species (ROS) ·O-H (hydroxyl radical) that can be formed when high energy such as radiation reacts with $H_2O$. Free radicals are produced naturally in mitochondria to aid energy production (Del Prete *et al.,* 2008), but they also can be generated by a variety of environmental factors including pollution, radiation, ingested chemicals (see figure 6) and can contribute to diseases such as heart disease, cancer and diabetes (Ratnam *et al.,* 2006).

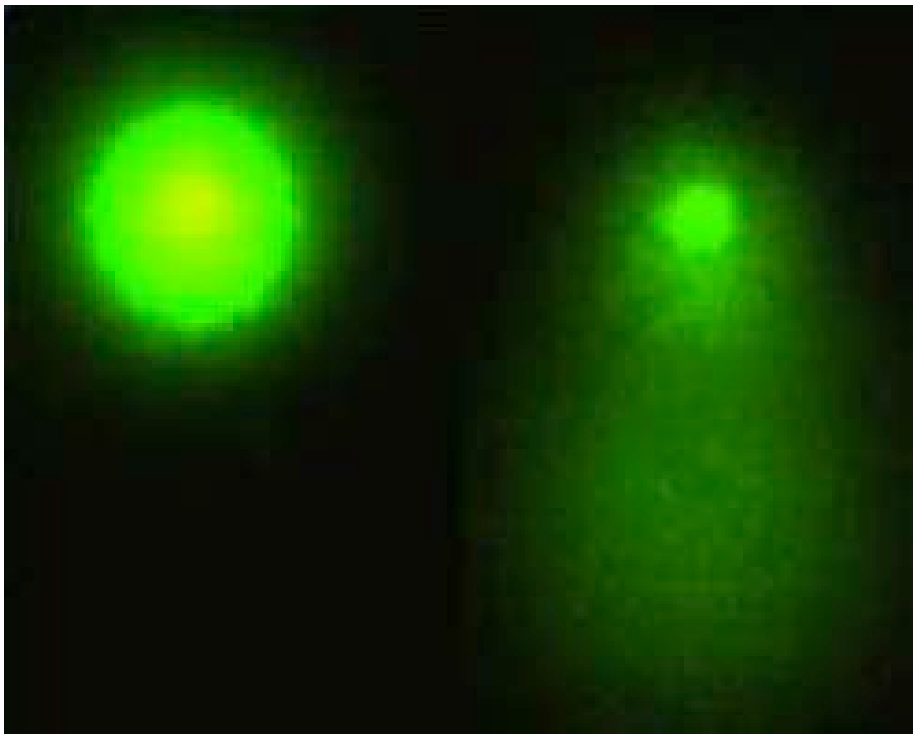
Figure 6. An overview of the environmental and biological causes of free radical formation (Lifeinnovate, 2008).



Reactive oxygen species are also used by neutrophil granulocytes to destroy infectious agents such as bacteria when engulfed (Ligeti and Mocsai, 1999). Although free radicals have benefits, if uncontrolled they can also disrupt DNA replication, transcription and translation (Riso *et al.,* 2002). These disruptions can cause mutations, cancers or even lead to cell death (Labieniec and Gabryelak, 2005). It is therefore crucial that the integrity of DNA in cells remains and replication of healthy DNA can therefore continue (Riso *et al.,* 2002).

DNA damage can be monitored using various methods including the Comet assay (Plazar *et al*., 2007). Using a Comet assay, damage to the DNA caused by any oxidative damage can be detected. A Comet assay involves single cell electrophoresis and produces a fluorescent Comet style image when DNA damage has occurred (Plazar *et al.,* 2008). If no DNA damage has occurred, an intact circle of DNA will be seen. This is due to fragments of DNA travelling further through the electrophoresis gel than intact DNA. An example of a comet assay can be seen in Figure 7 showing undamaged DNA on the left and damaged DNA on the right giving the classic Comet tail.

Figure 7. An example of a comet assay (Rowland, 2008)



The green tea catechins epigallocatcechin gallate and epicatechin have been shown to have a free radical scavenging ability (Li *et al.,* 2007), which can have a great impact on DNA damage and lipid oxidation (Yumiko, Takeshi and Hiroshi, 1998). However, theaflavin from black tea has been shown to have an equal or greater antioxidant ability than epigallocatechin gallate (Leung *et al*., 2001 and Luczaj and Skrzydlewska, 2005). Polyphenolic compounds related to theaflavin found in black tea have also been found to inhibit oxidative DNA damage (Lodovici *et al.,* 2000). In this study DNA damage in colon mucosa in rats was prevented by the ingestion of black tea polyphenols. One study also found that due to its antioxidant properties, epicatechin gave antidegenerative effects in rats with induced Alzheimer's disease (De Ruvo *et al.,* 2000).

Many also agree with the role of antioxidants in the prevention or even treatment for cancers. An example of this was reported by Sadava *et al.,* in 2007 where it was shown that green tea extracts do have anticancer effects and could be used as a treatment of drug resistant Small-cell lung carcinoma (SCLC). However, previously in 2005 the U.S Food and Drug Administration (FDA) dismissed anticancer effects of green tea claiming "it is highly unlikely". Other research groups suggest the catechin epigallocatechin gallate in green tea actually causes oxidative damage, which may lead to carcinogenesis (Furukawa *et al.,* 2003). Other research suggested that taking high doses of antioxidants may cause harm and that the benefits of pro-oxidnants such be investigated (Gutteridge and Halliwell, 2010). However, in 2008 a study found theaflaivin-3,3'-digallate, inhibited the growth of tumour cells due to the compound generating reactive oxygen species (Schuck *et al.,* 2008). It was proven in one study that the antioxidant abilities of epigallocatechin gallate came from the trihydroxyphenyl B ring (Valcic *et al.,* 1999). The study also proposed that the epigallocatechin gallate phenoxyl radical that is initially formed in an antioxidant reaction, could be used as a novel marker for epigallocatechin gallate reactions in living systems.

Alternative or additional research into flavanols and their interaction and ability to inhibit various enzymes and other types of proteins has been reported

(Cushnie and Lamb, 2005). Although very little research has been undertaken to discover the exact structural relationship and proposed activity, some groups (Tsuchiya *et al.,* 1996) have found that 5,7-dihydroxylation of the A ring and 2',4'- or 2',6'-dihydroxylation of the B ring of specific flavanones are very important in there antibacterial activity.

### 1.1.3.3 Antibacterial activity of polyphenols and infection

An area where polyphenols have significant benefits is in microbiological diseases and antimicrobial action. Microbiology is defined as the study of microscopic multicellular and unicellular organisms and covers areas including bacteriology, medical microbiology, virology and mycology (Madigan *et al.,*2009). Microbial disease is a vastly important area and affects human populations on every continent. Figures from the World Health Organisation (WHO) in 2004 showed that over 16.8% of deaths in the world are as a result of an infection (WHO, 2004) and in low income countries it increases to over 34%.

Areas of particular concern are hospital-acquired infections, opportunistic pathogens and drug resistance. Hospital-acquired infections often affect the elderly, individuals with a suppressed or poor immune system and patients who have had surgery and are often very costly to treat (Sheng *et al.,* 2005). Data suggests that patients staying on an intensive care unit (ICU) increases their risk of developing a nosocomial infection by up to 10 times that of general wards (Weber *et al*. 1999). However, hospital-acquired infections can cause additional problems when the infection is caused by a resistant strain of bacteria. For example infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *enterococci* (VRE) can result in patient mortality (Albrich *et al*., 1999).

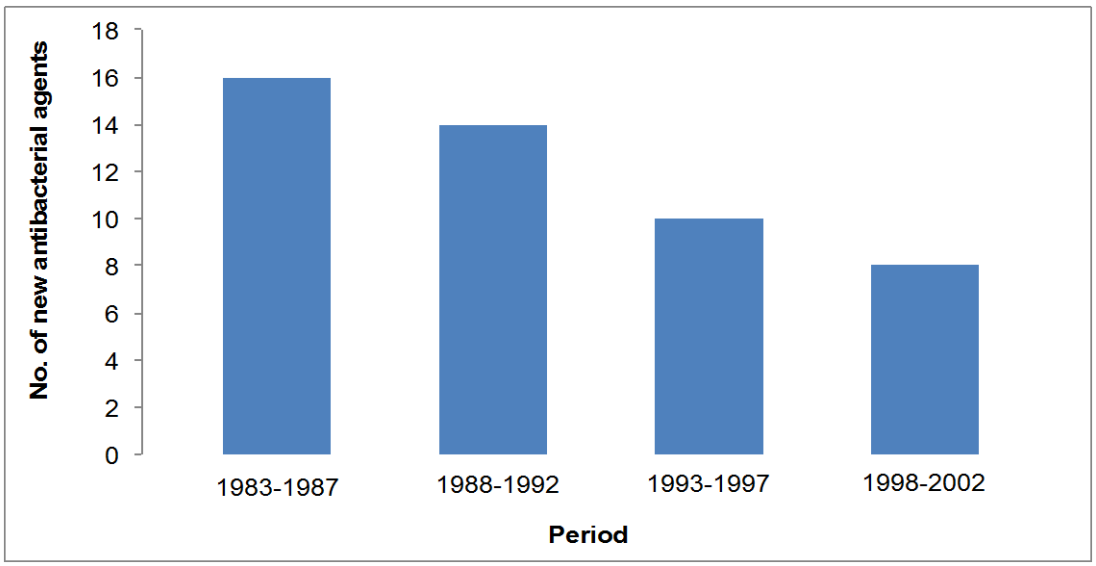
Opportunistic infections are often caused by bacteria, which naturally colonise the human body but do not usually cause harm in healthy individuals. However, they can cause serious infections in immunocompromised individuals (Madigan *et al.,* 2009). An example of a opportunistic pathogen is *Acinetobacter*

*baumannii* which has been shown to cause serious infection in various individuals including wounded soldiers or victims of natural disasters (Joly-Guillou, 2005).

Antibiotic resistance can occur via several routes including over prescription of antibiotics, full courses of antibiotics not being taken by patients and bacterial mutation (Arias and Murray, 2009). Resistance is not a new topic, since the first antibiotics were used resistance has occurred, for example only one year after methicillin was introduced in 1960, resistant strains were seen (Maltezou and Giamarellou, 2006). Since that time many more resistant strains have appeared and despite the reduction in the prescriptions of antibiotics such as fluoroquinolones, numbers of resistant strains to the drug including *Escherichia coli,* have increased (Soulsby, 2005).

With production and approval of new antimicrobial drugs reducing in the last 30 years (see figure 8), and further research indicating a reduction of 56% in 1998-2002 when compared to 1983-1987 (Spellberg *et al*., 2004), the drugs used to treat bacterial infections is rapidly reducing.

Figure 8. New antimicrobial agents between 1983 and 2002 (Spellberg *et al*., 2004)



Due to the increase in resistance and the decrease in the production of new antimicrobial drugs, using natural compounds such as polyphenols could be useful in creating new antimicrobial chemotherapies.

Polyphenols have shown to have significant antibacterial activity against various bacterial species including MRSA and also reduce the resistance to certain antibiotics (Anderson *et al.,* 2005). It has also been shown that green tea polyphenols inhibit *Helicobacter* growth *in vivo* and *in vitro* (Stoicov, Saffari and Houghton, 2009). This is a significant finding as *Helicobacter* has been linked to causing stomach cancer.

Previous research has suggested that tea catechins can suppress the emergence of resistance due to their antimutagenic properties (Pillai *et al.,* 2001). It was discovered by Zhao *et al.,* (2002) that epigallocatechin gallate inhibited penicilinase produced by penicillin resistant *Staphylococcus aureus* resulting in the restoration of the antibiotics effects. This is a major benefit as these synergistic effects could be used to restore the other antibiotics. Tea polyphenols have also been shown to have synergy with other antioxidants such as ascorbic acid where it was found to increase the antibacterial effect and stability of epigallocatechin gallate (Hatano *et al.,* 2008).

Black tea theaflavins have previously been shown to have strong antibacterial effects against *Bacillus cereus* with theaflavin-3, 3'-digallate having the greatest effect (Friedman *et al.,* 2006). However, in a past study it was shown that when comparing the antimicrobial of tea with various degrees of fermentation, black tea showed the least activity (Chou, Lin and Chung, 1999).

The consumption of tea has also been shown to significantly reduce caries in animals and humans (Wu and Wei, 2002). Research studies have shown that tea polyphenols inhibit the glucan produced by bacteria used to adhere to tooth enamel and cause plaque (Kuroda and Hara, 2004). Without this glucon

bacteria are no long able to proliferate and as a result acid induced tooth decay is reduced. It has been suggested that supplements of green tea in a diet would be advantageous in dental management (Sharma *et al.,* 2007).

Tea flavanoids have also been shown to block the harmful effects of bacterial toxins including the neurotoxin produces by *Clostridium botulinum,* which causes the disease botulism (Friedman, 2007). According to the research by Satoh *et al.,* (2002) black tea flavanoids blocked the action of bolulinum neurotoxcins in mice.

The mechanisms of the antibacterial action of polyphenols have been proposed and include inhibiting enzymes including DNA gyrase whereby epigallocatechin gallate has been shown to bind to the ATP binding site of the gyrase B subunit and disrupting activity (Gradisar *et al.,* 2007). The study by Gradisar *et al.,* (2007) also showed that it is the benzopryan ring of epigallocatechin gallate that blocks the active site. In previous research the mechanism was suggested to result from direct binding of the polyphenols the peptide structure of bacterial components (Shimamura, Zhao and Hu, 2007). A further previous study indicated that tea catechins, in particular, epigallocatechin gallate can cause leakage from bacterial cell membranes (Friedman, 2007). The study also stated that due to significant differences in human and bacterial cell membrane structures results in bacterial and not human cell death. Another proposed mode of action is the inhibition of bacterial energy metabolism in a similar to respiratory-inhibiting antibiotics (Cushnie and Lamb, 2005)

Although there are proposed mechanisms of polyphenol antibacterial activity more research is needed to confirm the modes of action. To accurately understand the antimicrobial modes of action of compounds including polyphenols, isotopic labelling can be undertaken. Previous research had used deuterium labelling to identify the modes of action of compounds (Paaren *et al.,* 1981). By labelling a compound it could be possible to either trace where it is

having and effect or monitor metabolites (Kohri *et al.,* 2001) to determine which part of the compound is giving the antimicrobial effects.

### 1.1.4 Stable isotopic Labelling

The use of stable isotope labelled compounds such as epicatechin can be a very useful way of identifying the potential health benefits of flavanols. Labelled compounds can for example be used in metabolic studies (Allen, Shachar-Hill and Ohlrogge, 2007). The synthesis of labelled compounds can be notoriously difficult, expensive and usually requires a number of synthetic steps (Hooper, Watts and Wiles, 2008). Compounds of interest can be labelled with a variety of markers, which include $^{13}C$, $^{14}C$, fluorescence fluophores and deuterium (Lundberg *et al.,* 2007 and Zheng *et al.,* 2008).

Fluorescent fluorophore markers such as a protein, nucleic acid or reactive probe are bonded covalently to another molecule (Matthews, Van Holde and Ahern, 1999). The fluoropore emits part of its excitation energy by non-radiant transfer to surrounding molecules as a result of absorbing ambient energy (Reed, Holmes, Weyers and Jones, 2003) and fluorescence is produced.

Fluorescent markers are very useful in biological studies such as cell sorting and microscopy (Stryer, 1998). However, they are very sensitive to environmental conditions such as temperature and pH (Parak, Pellegrino and Plank, 2005). Another problem with fluorescent labels is that they are often large and can alter the overall structure and potentially the properties of a compound, which in turn could bias results in a metabolic study.

Using a carbon isotope represents an alternative option and a number of carbon isotopes can be used including $^{11}C$, $^{13}C$ and $^{14}C$ (Solomans and Fryhle, 2006). All carbon isotopes stable and unstable (radioactive) have the same atomic number and protons as carbon but they all have variations in their neutrons and therefore a different mass.

The use of a $^{13}C$ marker has great benefits such as its stability and integration into the overall structure of the compound investigated (Flores *et al.,* 2002). Using $^{14}C$ has similar benefits in that it too can be integrated into the overall structure. Another benefit of using $^{14}C$ is that it is detectable due to the radiation it omits (EVS, 2005). Using the carbon isotopes $^{13}C$ or $^{14}C$ in the molecular structure of a compound, effectively allows monitoring of the compounds metabolic pathway (Allen, Shachar-Hill and Ohlrogge, 2007). Carbon isotopes have also been found to be effective in nutrition studies where ingested plant material labelled with $^{13}C$ (Svejcar, Judkins and Boutton, 1993). In this study changes in the isotope ratios were monitored by mass spectrometry. Although this is an effective method of labelling a compound and has been used in flavanoids in previous research, it often involves many reaction steps and more complicated stereochemistry (Nay, Arnaudinaud and Vercauteren, 2002). However, a simple method of producing $^{14}C$ labelled epicatechin, was undertaken in 1974, by Eastmond and Gardner. In their proposed method $^{14}C$ labelled epicatechin and $^{14}C$ procyanidins were extracted from the seeds of *Aesculus hippocastanum* after the plant had been fed with sodium acetate-1-$^{14}C$. This methodology was effective in using the plants natural production system to yield $^{14}C$ epicatechin. Theoretically this could be performed using the tea plant *Camellia sinensis*. Whereas $^{13}C$ is a stable carbon isotope $^{14}C$ is unstable (radioactive) and therefore there are associated risks when it is used including radiation exposure (CNSC, 2003).

Deuterium labelling involves the substitution of hydrogen with deuterium atom. Deuterium is an isotope of hydrogen having the same atomic number but a different mass (Riley, 2000). This is due to the nucleus of hydrogen containing one proton, whereas the nucleus of deuterium contains a proton and a neutron (Solomons, T. W. G. and Fryhle, C. B. 2006).

Deuterium labelling often involves multiple steps and has been used by many research groups to label compounds such as procyanidins (Pierre, Cheze and Vercauteren, 1997) and polyphenolic compounds (Buffnoir *et al.,* 1998). This approach has been the chosen method for this study as similar reactions have

been proven to be successful in various batch reactions (Kohri *et al*., 2001 and Peng and Yao, 2009). Due to the change in mass when substituting hydrogen with deuterium it is an excellent marker and can be used in a wide variety of research including monitoring the deep-water access of plants (Panuelas and Filella, 2003) and in metabolic studies.

### 1.1.5 Microfluidics

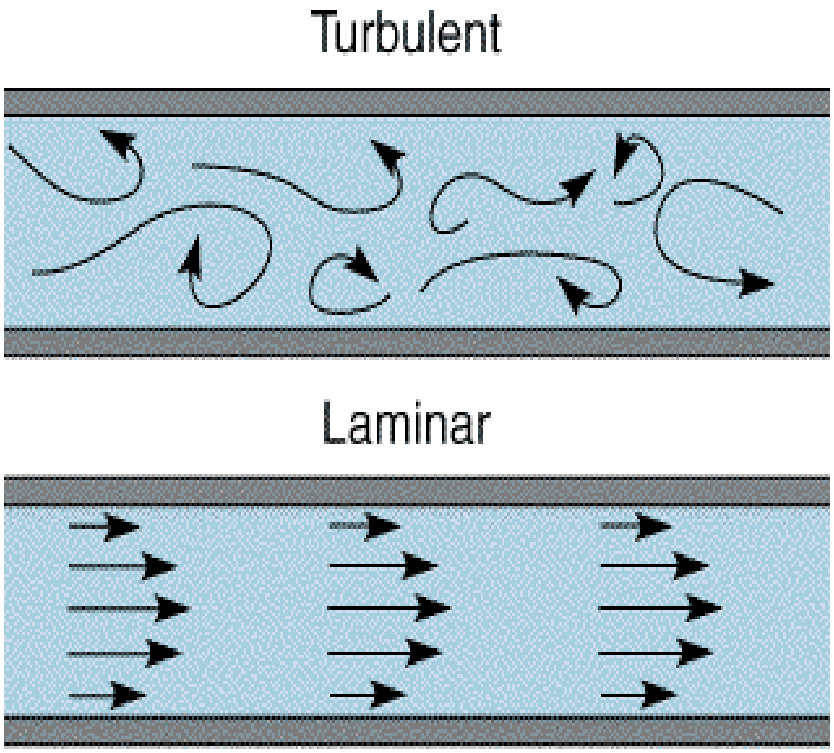
Microfluidics is a subject that deals with the manipulation of small volumes of fluids using channels with dimensions in the range of 10 to many hundreds of micrometres (Whitesides, 2006). Microfluidics encompasses a range of disciplines including chemistry, biology, fluid mechanics and surface science (Gomez, 2008). Other important disciplines are electronics and fabrication. Microfluidics, lab-on-chip or micro total analysis systems (μTAS) can be used to integrate processes including PCR, detection and analysis onto a chip in the micrometer scale (Ong *et al*., 2008). The first microfluidic systems were created in the mid-1970's with the production of miniaturized gas chromatography system (Terry, 1979). Since then microfluidic systems have flourished and are an integral part of modern day research (Haswell, 2006).

#### 1.1.5.1 Flow of fluids in a microfluidic device

The flow of fluids in a microfluidic device, have different characteristics than in larger (macro) devices, which can be altered using a variety of techniques, including the use of obstacles and/or micromixers (deMello, 2006 and Wong *et al*., 2003). A fluid can be defined as a material that can continually deform under any shear stress that is applied (Ong *et al*., 2008). There are three parameters that are important when characterizing a fluid. They are pressure, $P$, viscosity, $\eta$, and density, $p$ (Tabeling, 2005). If the viscosity gradient of a fluid is directly proportional to its shear stress it can be defined as a Newtonian fluid, whereas a fluid is defined as a non-Newtonian if viscosity is changed with shear stress (Nguyen and Wereley, 2002). The behaviour of fluids are dominated by factors such as, fluidic resistance, surface tension and energy dissipation and in microfluidics these factors are exploited (Kirby, 2010). Using obstacles in the flow channels of a microfluidic device can generate a mixture of turbulent and

laminar flows (Tabeling, 2005). These techniques can be used in chemical synthesis to mix reagents. Laminar flow is often seen in microfluidics and is described as streamlined flow, where parallel layers flow along side each other with no disruption (Ong *et al.,* 2008). The difference between Laminar and turbulent flow can been seen in figure 9.

Figure 9. The differences of laminar and turbulent flow in a microfluidic device (Cambridge, 2011)




The type of flow in a system can be determined from the Reynolds number (deMello, 2006). An equation used to determine the Reynolds number can be seen in equation 1.

Equation 1. The equation to determine the Reynolds number (deMello, 2006)

$$\mathbf{R_e = \rho v d/\eta}$$

Where:

**$R_e$ =** Reynolds number

**ρ =** Density of fluid ($Kg/m^3$)

**v** = Velocity of flowing fluid (m/s)

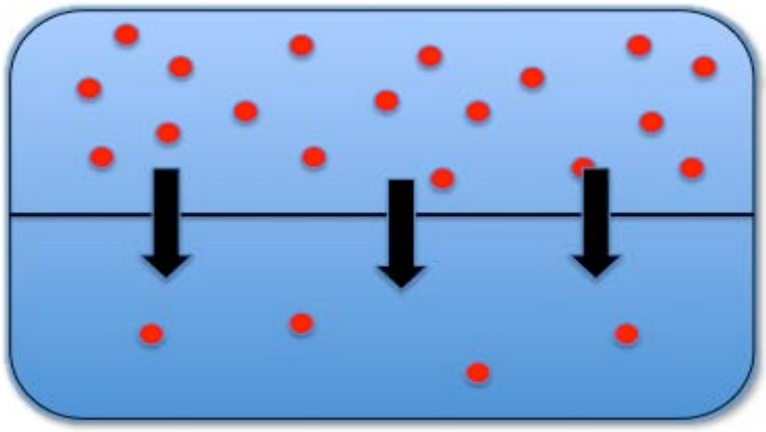
**d** = Diameter of channel (m)

**η** = □Viscosity of fluid ($Ns/m^2$)

If $R_e$ < 10, the flow will be laminar, if $R_e$ > 2000, the flow will be turbulent. In between $R_e$ < 10 and >2000 is classed as an intermediate regime.

In microchannels $R_e$ is very small and as a result reagents mix via diffusion (deMello, 2006). Diffusion is defined as the movement of particles from areas of higher concentration to those of a lower concentration (Isaacs, 1996) and relates to Flick's law. A representation of diffusion can be seen in figure 10.

Figure 10. A representation of diffusion and the movement of particles from a high to a low concentration (Tabeling, 2005)



Flick's law describes this movement of particles from high to low concentrations in his equation (Tabelling, 2005), which can be seen in equation 2, where *J* is the diffusion flux, D is the diffusion coefficient, $\phi$ is the concentration and *x* is the position.

Equation 2. The equation for Flick's first law

$$J = -D\frac{\partial \phi}{\partial x}$$

Flick's second law in concerned with the predicting how diffusion results in the concentration field to change over time (Tabelling, 2005). The equation for Flick's second law can be seen in equation 3, where *t* is the time, D is the diffusion coefficient, $\phi$ is the concentration and *x* is the position.

Equation 3. Showing the equation for Flick's second law

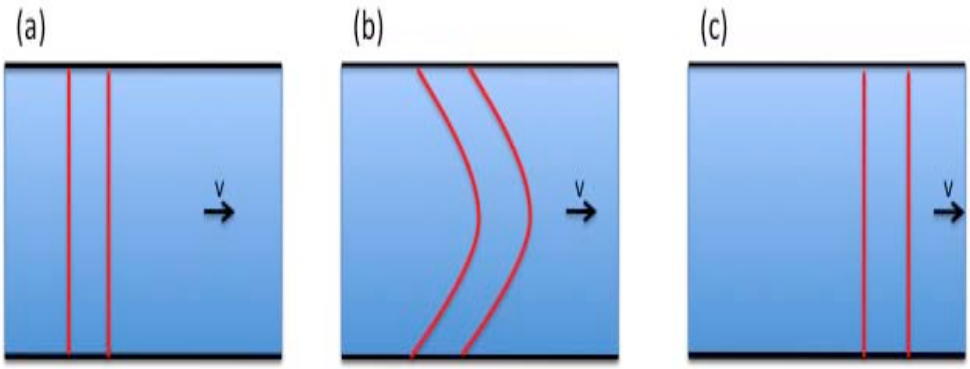
$$\frac{\partial \phi}{\partial t} = D\frac{\partial^2 \phi}{\partial x^2}$$

A very important requirement when diffusion is used for mixing of reagents in a microfluidic device, is the absence of convection (Heeren *et al.,* 2006). As diffusion is not instantaneous, it can be predicted. Therefore due to the short channel length in a microfluidic device it is important that reaction flow rates allow adequate time for total mixing of reagents by diffusion. Other transport mechanisms in microfluidic systems are convection, where heat transfer effects transport and migration, where molecules are transport due to a driving force such as an electric field (Ong *et al*., 2008).

Another key factor when describing flow in microfluidics is Taylor dispersion, which Sir Geoffrey Taylor described in 1953 (Azer, 2005). This is a

phenomenon whereby a solution in a capillary tube or cylindrical microchannel with a Poiseuille flow (Bruus, 2008) has a initial flat concentration, but when diffusion is neglected friction on the sides of the solution results in a stretched paraboloid-shaped plug (see figure 11). However, with diffusion the profile of the solution is evened out. If diffusion did not come into play, the solution would become increasing stretched (Kirby, 2010).

Figure 11. The Taylor dispersion problem (Bruus, 2008). V is poiseuille flow, a showing normal flow with a flat profile solute, b with no diffusion a paraboloid plug forms, c with diffusion profile evens out.



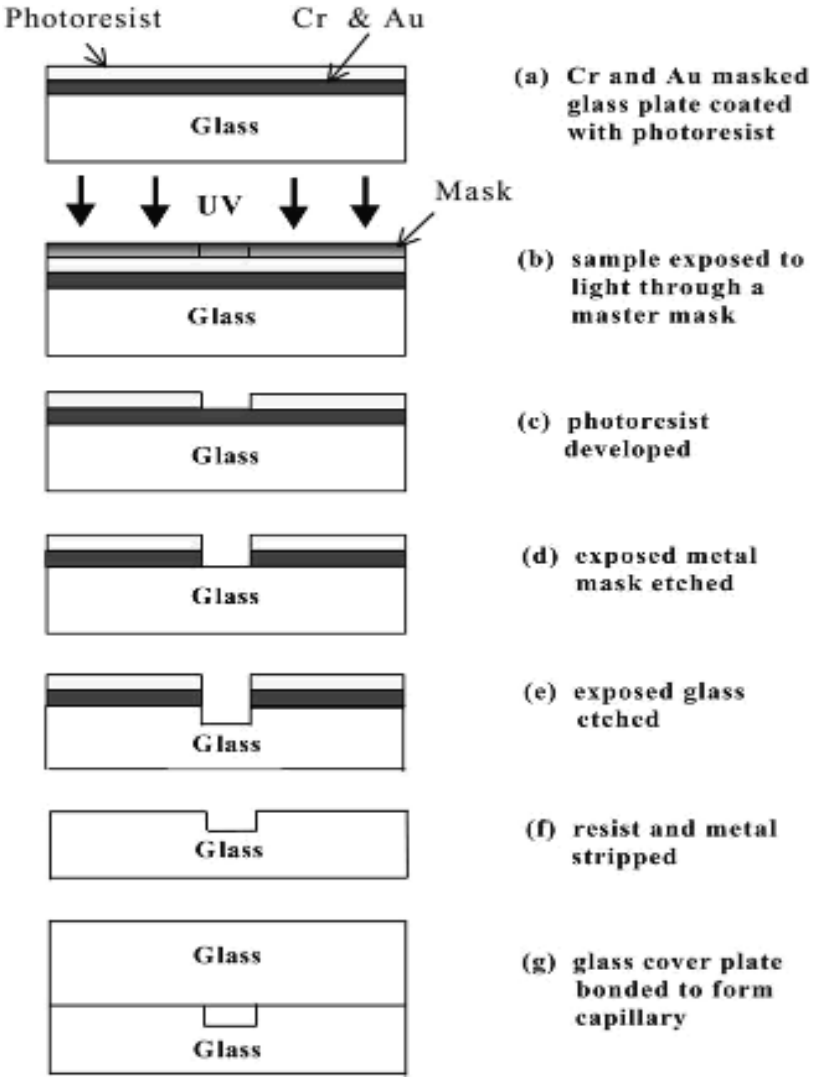

The flow of cells and particles can also be manipulated in a microfluidic device by altering flow rates and by using acoustophoresis where acoustic force is used (Evander *et al.,* 2008).

### 1.1.5.2 Manufacturing microfluidic devices

Microfluidic devices can be produced using different materials including glass, metals, polymers such as polydimethyl siloxane (PDMS) or polymethyl methacrylate (PMMA) and ceramics (Haswell and Skelton, 2000). The type of material used in the fabrication of a microfluidic device is dependant on various factors including reproducibility of the fabrication and the materials chemical compatibility (Fletcher *et al*., 2002). The fabrication methods used today for glass are based on those using photolithography and etching, which were

developed in the 1970s (Gravesen, Branebjerg and Jensen, 1993; McCreedy and Wilson 2001 and Tabeling, 2005). The fabrication of glass microreactors, which are preferable for chemical synthesis, involves using pre-produced mask (McCreedy, 2001) and hydrogen fluoride (HF) to etch channels in glass in series of steps (Fletcher *et al.,* 2002), which can be seen in figure 12.

Figure 12. The steps involved in the fabrication of a glass microreactor taken from Fletcher *et al.,* (2002).




### 1.1.5.3 Advantages and uses of microfluidics

Microreactors have been shown to have many advantages in chemical synthesis (Janasek, Franzke and Manz, 2006). These advantages include a

greater level of reaction control, an increase in product yield and purity and a lower volume of reagents required (Haswell, 2006). Another benefit of microreactors is in the area of health and safety where due to reduced reactants, factors such as managing heat transfer are more easily and safely controlled (Haswell and Skelton, 2000). Reactions in microfluidic reactors can also be relatively easily optimized making them much more efficient than traditional batch reactions (Whitesides, 2006). Microreactors and MEMS (microelectro mechanical systems) have their uses in various fields including chemical analysis and biological screening (Pennemann *et al.,* 2004). Various reactions have been performed in microreactors including multiple step chemical synthesis (Wiles *et al.,* 2003) and labelling (Hooper, Watts and Wiles, 2008).

### 1.2 General aims

The aims in chapter 2 of this project are to synthesis deuterium labelled epicatechin for the use in the synthesis of deuterium labelled theaflavin

In chapter 3 the aim of the research will be to discover if there is any antibacterial effect of theaflavin against hospital isolates of MRSA, *A. baumannii* and *S. maltophilia* and to discover if any antibacterial synergy occurs between theaflavin and epicatechin against those isolates.

The aims in chapter 4 are to enzymatically synthesize theaflavin and deuterium labelled theaflavin in a microreactor as a continuous flow process whereby the microreactor synthesis of theaflavin and the antimicrobial testing are combined in order to discover the antimicrobial modes of action.

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# Chapter 2

Organic synthesis of deuterium labelled (-)-epicatechin in a microreactor

## 2.1 Introduction

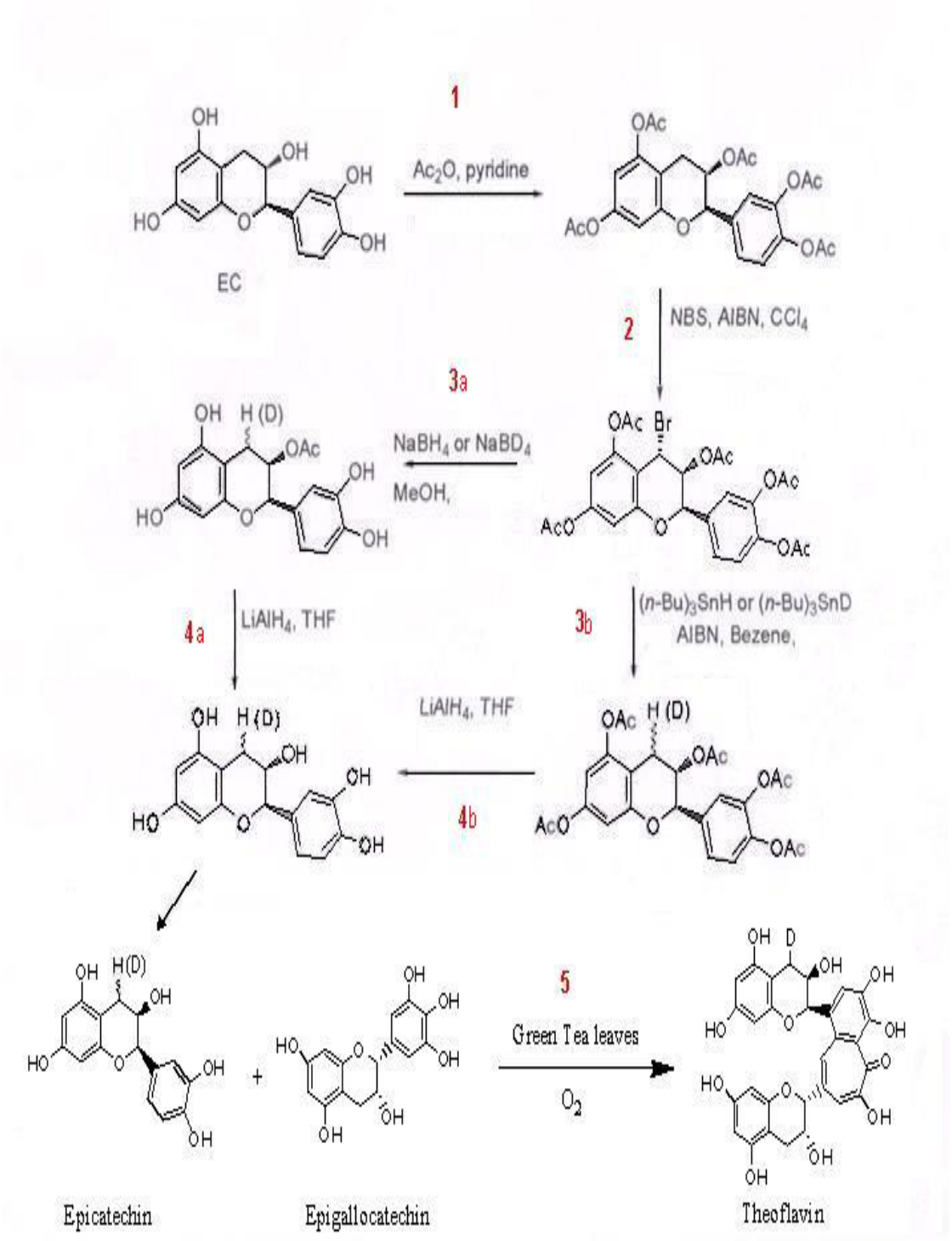
### 2.1.1 Synthesis of deuterium labelled epicatechin and theaflavin

Producing theaflavin involves an oxidation reaction between epicatechin and epigallocatechin with an enzyme catalyst (Jhoo *et al*., 2004). This has been performed on several occasions in batch reactions using oxidative enzymes extracted from various fruits and plants (Tanaka *et al.,* 2002). Producing a D-labelled theaflavin molecule would require the same final reaction with the exception that the EC would have be previously D-labelled. One method for D-labelling theaflavin is based the method reported by Kohri *et al.,* (2001) to D-label epigallocatechin gallate and involves 5 steps, which can be seen in figure 13. This scheme was presented to us by our research partners at Unilever Shanghai who carried out the reactions in batch (Peng and Yao, 2009).

Previous groups have performed a similar reaction to that of step 5 in figure 13 (Jhoo *et al*., 2004). This was performed in a batch reaction with a crude enzyme extract to produce and unlabelled theaflavin. The reaction undertaken by Jhoo *et al.,* (2004) only produced a yield of 15.2%. This is a very poor yield and if a similar reaction was used for the final step of producing labelled theaflavin, it was be very costly. However this was dramatically improved by Sharma, Bari and Singh (2009) who converted 85% of catechins to theaflavins using an immobilized polyphenol oxidase in their batch reaction. This result has never been achieved in a continuous flow system such that it could be used as method to manufacture labelled theaflavin.

It is theoretically possible to perform the series of reactions shown in figure 13 in a microfluidic reactor, with the potential benefit of a greater yield than in previous batch reactions.

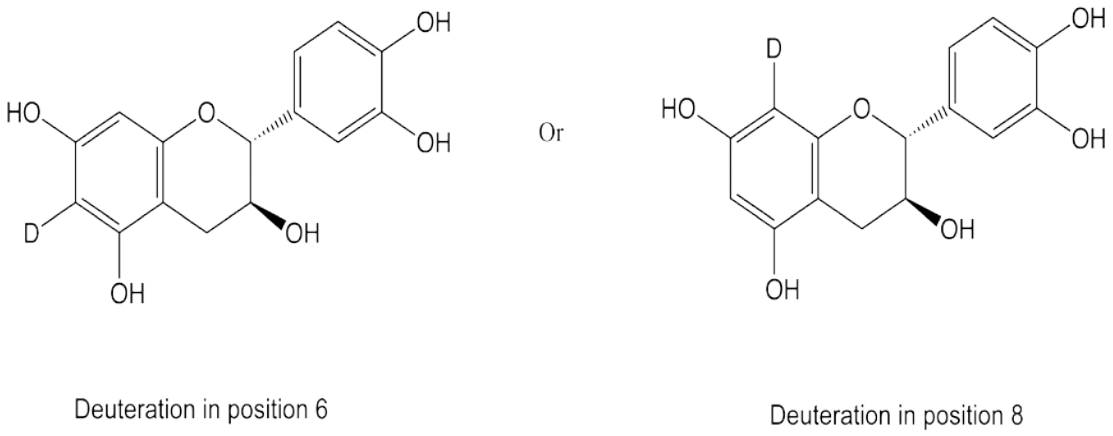
Figure 13. Reaction scheme adapted from Kohri *et al.,* (2001) by Peng and Yao (2009) showing steps for 2 synthetic routes of producing a deuterium labelled epicatechin and theaflavin in batch reactions.



Chemical synthesis has been performed on many occasions in a microfluidics reactor (Haswell, 2006). D-labelling has also been performed using a microreactor, more recently by Hooper, Watts and Wiles (2008) with great success. In their reactions phenol derivatives were labelled with deuterium giving yields in excess of 92%.

When examining the structure of epicatechin another option of deuterium labelling is present. Previous research by Kiehlmann *et al.,* (1988) shows that in epicatechins stereoisomer catechin, a deuterium label can be added in position 6 or 8 (see figure 14).

Figure 14. Positions of deuterium labelling in catechin (Kiehlmann *et al.,* 1988)




Deuteration in position 6

Deuteration in position 8

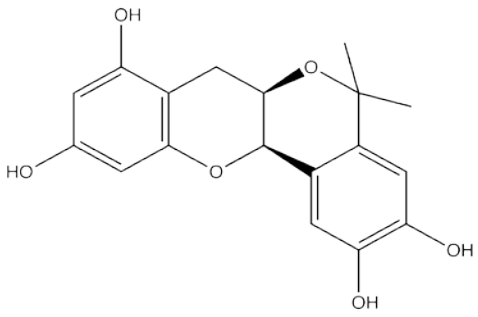
A rapid microreactor synthesis of labelling epicatechin using a scheme based on this work could also be achieved. However in the work by Kiehlmann *et al.,* (1988), deuterium labelled catechin was easily converted back to catechin in acidic conditions

### 2.1.2 Synthesis of modified catechins

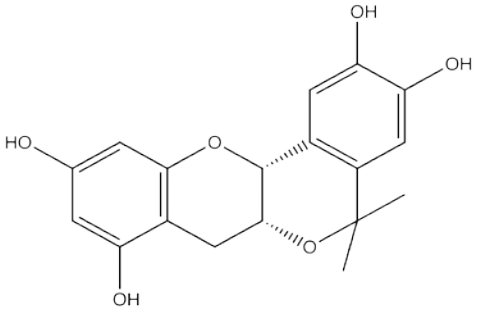
Various groups have attempted to synthesize modified catechins in order to increase their antioxidant capabilities. In 1990, Van der Western, Steenkamp and Ferreira showed what they called “an unusual reaction” where they reacted

various flavan-3-ols, including epicatechin, catechin and epigallocatechin with acetone and tetrabromomethane. The result was a simple acid catalysed reaction, where acetone was incorporated into the carbon framework of the flavan-3-ols, leading to the formation of a tetracyclic ring, which can be seen in figure 15.

Figure 15. Showing planar epicatechin and planar catechin




Planar epicatechin




Planar catechin

Unfortunately the yield of planar epicatechin and catechin in the method by Van der Western, Steenkamp and Ferreira (1990) was very low. The yield for planar epicatechin was 23% and for catechin it was only 24%. However, the yield for planar catechin was increased to 71% by Fukuhara *et al*., (2002) by utilizing a new method. This method involved the reaction of catechin with borontrifluoride diethyletherate and acetone at 0°C for 3 hours. The research group used a new method in 2006, which gave a 76.3% yield of planar catechin (Hakamata *et al.,* 2006). In this new method catechin was reacted with acetone and Trimethylsilyl trifluoromethanesulfonate in THF at -5°C for 12 hours. From the research carried out in 2002 and 2006 planar epicatechin was shown to have increased antioxidant capabilities. In a theoretical study (Wang and Zhang, 2005) it was proposed that the increased radical scavenging activity was due to an electron transfer mechanism and not hydrogen transfer as in the case of unmodified polyphenols. In 2009, Fukuhara *et al.,* practically confirmed that planar catechin had a 5-fold increase in radical-scavenging activity in comparison to catechin. The extra activity was proposed to arise from the “modifications affecting O-H

bond dissociation enthalpy or stabilization of the phenoxyl radical" (Fukuhara *et al*., 2009).

It is noticeable that since Van der Western, steenkamp and Ferreira (1990), there had been no further research conducted on the production of planar epicatechin. It is likely that planar epicatechin could show increased antioxidant capabilites comparable to planar catechin, and therefore would be beneficial to synthesize. Since the yield was so poor in batch a microfluidic approach may give better results due to enhanced mixing conditions.

## 2.2 Aims

The aims of this project are to produce deuterium labelled epicatechin in a microreactor by adapting batch methods by Kohri *et al.,* (2001), Peng and Yao (2009) and Kiehlmann, Leto and Cherniwchan (1988). It is hoped that the stable deuterium labelled epicatechin produced will be be used in future metabolic studies and in the deuterium labelled theaflavin which will be used to determine its antimicrobial modes of action. Further aims of this project will be to synthesize planar epicatechin in a microreactor in order to test if like planar catechin it had increased antioxidant and antibacterial effects.

## 2.3 Materials and methods

**General:** All chemicals were used as supplied and were purchased from Sigma-Aldrich UK, Fisher Scientific UK and Unilever China. All solvents were all stored over molecular sieves.

**Analytical methods**: All NMR experiments were recorded using a JEOL JNM-ECP400 FT NMR spectrometer (400MHz ($^{1}$H), 99.5MHz ($^{13}$C)) All data recorded are given a chemical shifts in δ (ppm). All $^{1}$H HMR multiplicity and integration were downfield from tetramethylsilane and $^{13}$C NMR from $CDCl_3$. All NMR samples were unless stated otherwise run in deuterated chloroform solutions.

All MS experiments were performed using a Thermo-Finnigan LCQ Classic MS system with thermo separations LC including binary gradient pumping system and AS3000 autosampler.

Melting point data were collected using an Olympus BH2 microscope and a Mettler FP52 heat platform and FP5 controller. The heating rate was 10°C/min up to a temperature of 140°C were the rate was lowered to 1°C/min. All elemental analysis was performed using a Fison EA 1108 CHN.

### 2.3.1 Methods for testing the stability of EC

#### 2.3.1.1 Production of EC solution

A simple stability test for epicatechin over time was carried out using 10 mM solutions of EC. To produce a 10 mM solution of EC, 0.0291 g was weighed out and dissolved in 1 mL of 100% methanol in a 15 mL tube. To this 9 mL of water was added and then vortexed to mix. The tube was wrapped in aluminium foil to protect against any possible photo degradation and stored in the fridge until it was required for analysis. New solutions were prepared at 20 day intervals, up to and including 100 days after the first solution had been prepared.

#### 2.3.1.2 Analysis of EC solutions

The analysis of the EC solutions was performed using high performance liquid chromatography (HPLC). 10 µL samples of each solution were in turn injected into the HPLC machine. The elutant was a 20/80 mix of methanol and water, with a flow rate of 1000 mL/min. A Gemini 5 micron C18 (150 x 4.80mm) column was selected to gain the best retention time. The wavelength of the UV lamp was set to 280 as this was found to show catechins more effectively in this HPLC setup.

### 2.3.2 Synthesis of deuterium labelled epicatechin – Method 1

The initial methodology for this series of reactions was based on that reported by Kohri *et al.,* 2001, adapted for the labelling of epicatechin and theaflavinin

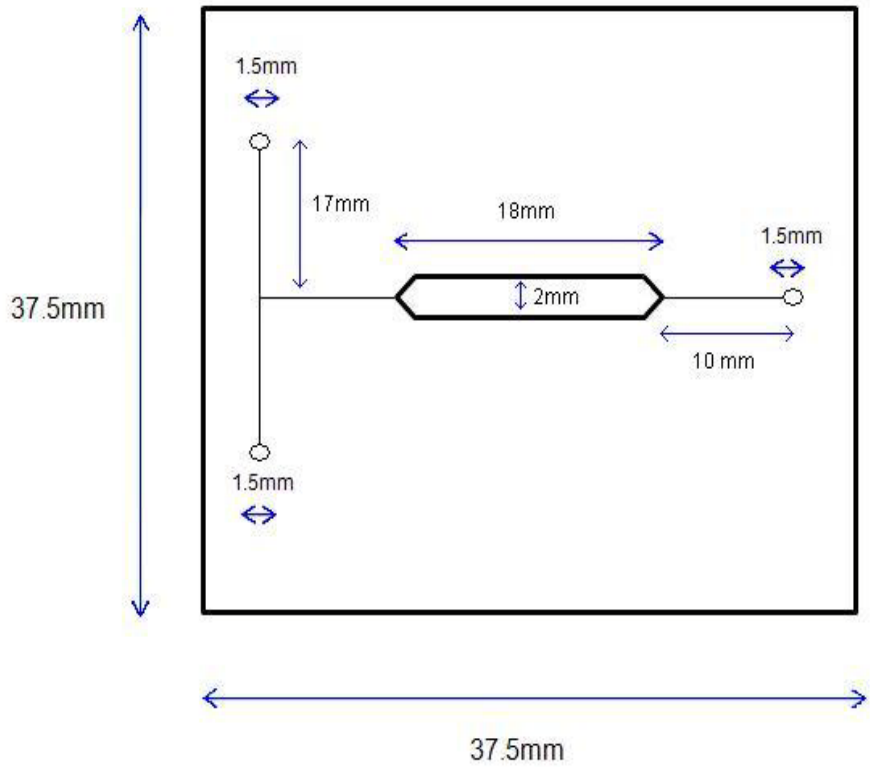
batch by Peng and Yao (2009). The adapted reaction scheme can be seen in figure 13.

#### 2.3.2.1 Microfluidic reactor design and preparation

The microreactor used in the labelling reactions was specifically designed and produced to allow the input of two separate solutions. The microreactor, which can be seen in figure 16 had channels etched using the technique shown in figure 10 to give a depth of 60 µm which would allow mixing to occur over its length. The channel width unless otherwise stated was 100 µm.

Before use, the microfluidic reactor was washed with methanol to clean the channels and then flushed with acetic anhydride to prevent any residue methanol reacting with the reactants in the experiment. Methanol and water were used to flush the microreactor after the reaction had taken place, to avoid any crystal structures forming in the channels of the reactor, which might lead to future blockages.

Figure 16. Microreactor design used in the multi step synthesis

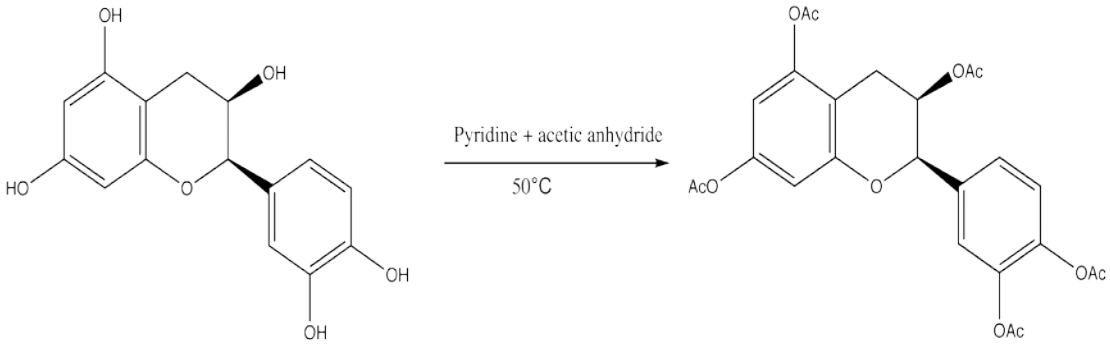



### 2.3.2.2 Production and optimisation of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin in a microreactor

The synthesis of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin was performed in a T-shaped microreactor as seen in figure 15. A reaction scheme for this shown in figure 17.

A typical reaction involved flowing 3.5 mL of 20 mM epicatechin in pyridine alongside 0.5 mL of acetic anhydride in 3 mL of pyridine at 50 °C. The reaction was controlled by a syringe pump using a flow rate of either 20/15/10/5/2.5 µL/min. Products were collected into a vial containing methanol, which acted as a quencher. This was to confirm that the reaction actually occurred in the microreactor and not in the vial.

Figure 17. The reaction scheme for the production of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin




Products collected were dissolved into diethyl ether and washed with 1M HCl several times before then being washed with brine. The organic layer was dried over $MgSO_4$ filtered and concentrated under reduced pressure, which gave a yellow viscous oil. This was recrystilized in ethanol giving a white solid. The product was weighed before $^{1}$H'NMR, $^{13}$C NMR, CHN and melting point analysis was carried out.

### 2.3.2.3 Bromination of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin in a microreactor

In this reaction 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin was brominated in a T-shaped microreactor (see figure 16) using N-bromosuccinimide (NBS) in a 1:1 reaction. The reaction scheme for this reaction can be seen in figure 18.

Figure 18. The reaction scheme for the bromination of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin with NBS

To 5 mL of carbon tetrachloride ($CCl_4$) and 5 mL of acetic acid 0.025 g of NBS was added and dissolved in a round bottomed flask whilst warming in a water bath at 40 °C for five minutes. Once the NBS had dissolved 0.05 g of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin was added and mixed. In this reaction a free radial initiator was used called 1,1'azobis(cyclohexanecarbonitrile). This was used as it was found to be a more efficient initiator (Sigma-Aldrich, 2009) than the 2,2'azobis(2-methylpropionitrile) (AIBN) used by Kohri *et al.,* (2001) and Peng and Yao (2009) in their bromination of EGCG and EC respectively. A 1mM solution was used and prepared by dissolving 0.0024 g of 1,1'azobis(cyclohexanecarbonitrile), in 10 mL of $CCl_4$.

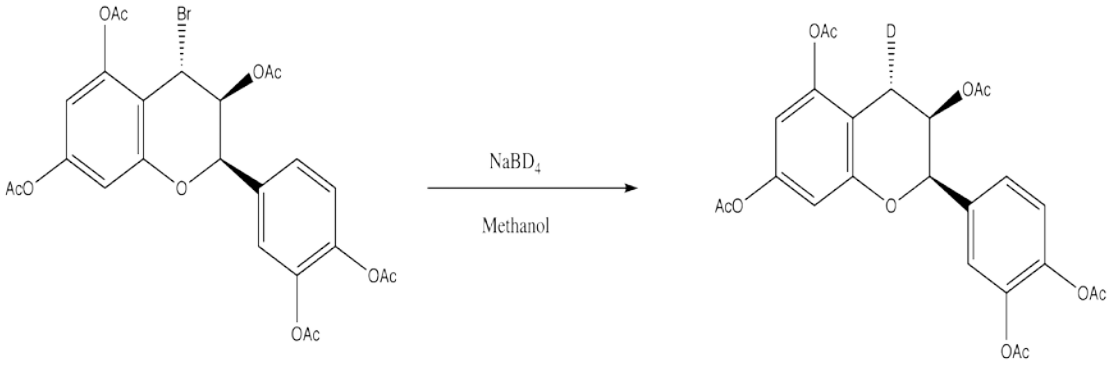
A typical reaction involved flowing 10 mL the 1:1 NBS/3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin in a $CCl_4$/acetic acid solution alongside 10 mL of the 1mM 1,1'azobis(cyclohexanecarbonitrile)/ $CCl_4$ solution at 60 °C through a T-shaped microreactor. The reaction was controlled by a syringe pump using a flow rate of 5 µL/min. Products were collected into a vial containing water, which acted as a quencher. This was to confirm that the reaction actually occurred in the microreactor and not in the collecting vial.

Once the reaction was complete the product solution was washed with dichloromethane (DCM) and distilled water to remove the succinimide. The water was removed into a beaker and the products were washed again with DCM. The product solution was dried using magnesium sulphite, filtered and concentrated under reduced pressure leaving a dry red coloured powder. The product was weighed before $^{1}$H'NMR and LC/MS data were collected.

### 2.3.2.4 Substitution of bromine with deuterium

In this reaction $NaBD_4$ was used in a 1:4 reaction with the brominated 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin to substitute the bromine with a deuterium. The reaction scheme for this can be seen in figure 19.

Figure 19. The reaction scheme for the substitution of bromine using $NaBD_4$



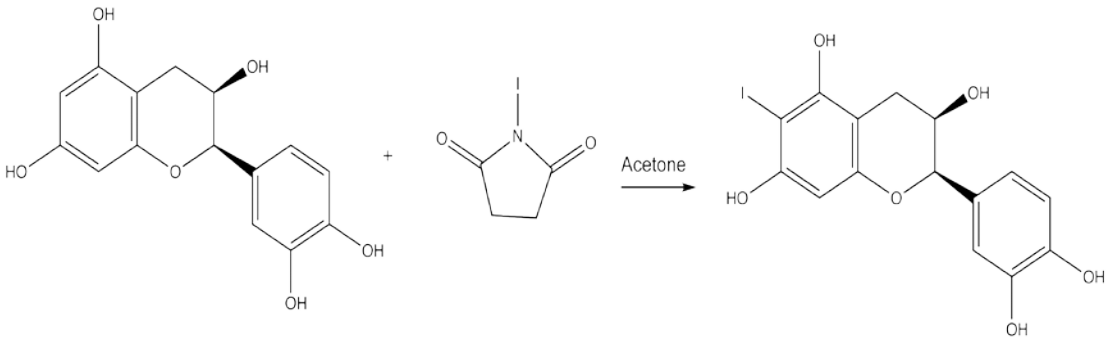

For this reaction 0.06 g of crude bromination product was dissolved in 5 mL of dry methanol and flowed alongside 0.002 g $NaBD_4$ in 5 mL of dry methanol in a T-shaped microreactor (Figure 16). Although the reaction is a 1:4 ($NaBH_4$:bromo-3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin) an excess was used due to the presence of succinimide from the previous step. The reactants were flowed alongside each other at 25 °C. The reaction was controlled by a syringe pump using a flow rate of 5 µL/min. Products were to be collected into a vial containing water, which acted as a quencher.

### 2.3.3 Microreactor synthesis of deuterium labelled epicatechin – Method 2

#### 2.3.3.1 Microreactor synthesis of 6-iodoepicatechin

In this 1:1 reaction based on the batch method by Kiehlmann *et al.,* (1988), N-iodosuccinimide was reacted with epicatechin in a continuous flow microreactor. A reaction scheme of this can be seen in figure 20.

Figure 20. The reaction scheme for the iodination of epicatechin



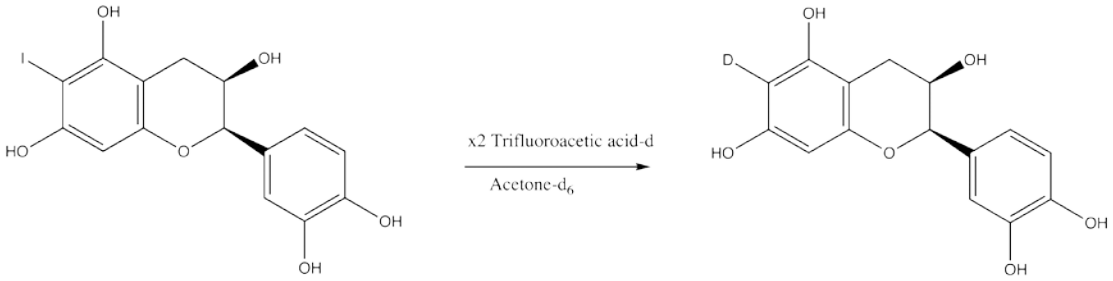

In this reaction 0.058g of epicatechin was dissolved in 10 mL of dry acetone and flowed through a T-shaped microreactor (figure 16) alongside 0.045 g of N-iodosuccinimide in 10 mL of acetone at 25 °C. The reaction was controlled by a syringe pump using a flow rate of 10 µL/min. Products were collected into a vial containing methanol, which acted as a quencher. This was to confirm that the reaction actually occurred in the microreactor and not in the collecting vial.

Products were concentrated under reduced pressure leaving a brown/beige coloured powder. The product was weighed before $^{1}$H'NMR and LC/MS data were collected. Due to the insolubility of 6-iodoepicatechin in D-chloroform, $^{1}$H'NMR analysis was performed in acetone-$d_6$.

#### 2.3.3.2 Microreactor synthesis of deuterated epicatechin

In this 1:2 reaction the deuteration of 6-iodoepicatechin with trifluoroacetic acid-d (deuterated TFA) was attempted in a T-shaped microreactor (see figure 16). A reaction scheme for this reaction can be seen in Figure 21.

Figure 21. Showing the deuteration of 6-iodoepicatechin



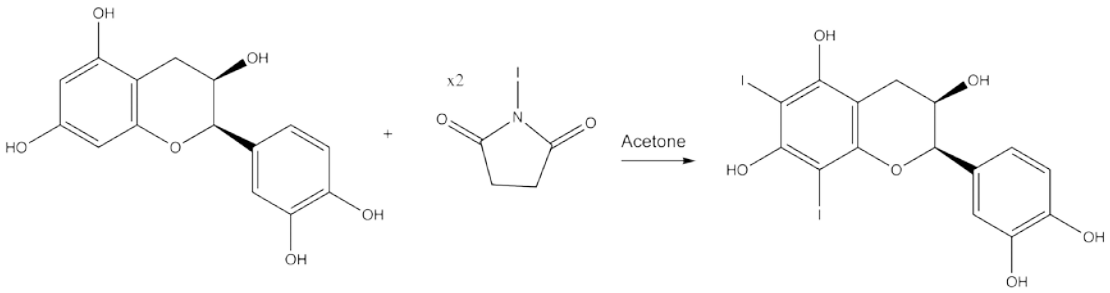

To prepare the reactants 0.10 g of crude 6-iodoepicatechin was dissolved in 5 mL of acetone-$d_6$. For the 1:2 reaction 0.046 g of deuterated TFA was needed. Therefore 31 µL of a 1.493 g/mL solution of deuterated TFA was added to 5 mL of acetone-$d_6$. Both solutions were flowed through the T-shaped microreactor at 25 °C. The reaction was controlled by a syringe pump with a flow rate of 10 µL/min. Products were collected into a vial and then concentrated under reduced pressure leaving a purple/brown oil. The product was weighed before $^1$H'NMR and MS analysis was performed in acetone-$d_6$. The compound was stored in water for 24 h and analysed again to check its stability.

### 2.3.4 Microreactor synthesis of double deuterium labelled epicatechin

#### 2.3.4.1 Microreactor synthesis of double iodinated epicatechin

In this 2:1 reaction based on the batch method by Kiehlmann *et al.,* (1988), N-iodosuccinimide was reacted with epicatechin in a continuous flow microreactor. A reaction scheme of this can be seen in figure 22.

Figure 22. The reaction scheme for the double iodination of epicatechin




In this reaction 0.058 g of epicatechin was dissolved in 10 mL of dry acetone and flowed through a T-shaped microreactor (figure 16) alongside 0.09 g of N-iodosuccinimide in 10 mL of acetone at 25 °C. The reaction was controlled by a syringe pump using a flow rate of 10 µL/min. Products were collected into a vial containing methanol, which acted as a quencher. This was to confirm that the reaction actually occurred in the microreactor and not in the collecting vial.

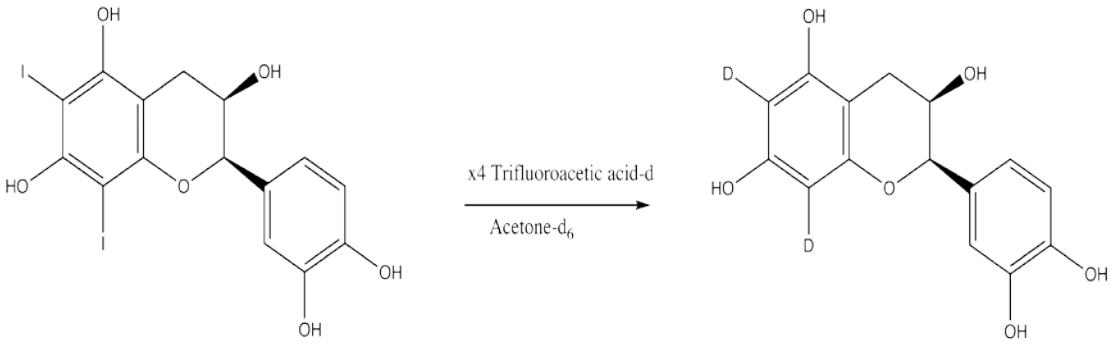
Products were concentrated under reduced pressure leaving a brown/beige coloured powder. The product was weighed before $^{1}$H'NMR and MS data were collected. As with 6-iodoepicatechin, the solubility of 6,8- iodoepicatechin produced was poor in D-chloroform. Therefore $^{1}$H'NMR analysis was performed in acetone-$d_6$.

### 2.3.4.2 Microreactor synthesis of double deuterated epicatechin

In this 1:4 reaction the double deuteration of 6,8-iodoepicatechin with trifluoroacetic acid-d (deuterated TFA) was attempted in a T-shaped microreactor (see figure 16). A reaction scheme for this reaction can be seen in figure 23.

To prepare the reactants 0.15 g of crude 6,8-iodoepicatechin was dissolved in 5 mL of acetone-$d_6$. For the 1:4 reaction 0.092 g of deuterated TFA was needed. Therefore 62 µL of a 1.493 g/mL solution of deuterated TFA was added to 5 mL of acetone-$d_6$. Both solutions were flowed through the T-shaped microreactor at 25 °C.

Figure 23. The reaction scheme for the double deuteration of 6,8-iodoepicatechin




The reaction was controlled by a syringe pump with a flow rate of 10 µL/min. Products were collected into a vial and then concentrated under reduced pressure leaving a purple oil. The product was weighed before $^{1}$H'NMR and MS analysis was performed in acetone-$d_6$. The compound was stored in water for 24 h and analysed again to check its stability.

### 2.3.5 Microreactor synthesis of deuterium labelled epicatechin – Method 3

This methodology is adapted from the batch method by Buffnoir, Rolando and Russell (1998). Unlike Buffnoir, Rolando and Russell (1998), tert butyldimethysilyl groups were added to protect the epicatechin OH groups instead of benzyl groups. It was felt that the removal tert-butyldimethylsilyl groups would be a more straight forward to carry out in a microreactor under acidic conditions. A reaction scheme of the deuteration in method 3 can be seen in figure 24.

Figure 24. Method 3 for deuterating epicatechin

#### 2.3.5.1 Microreactor synthesis of tert-butyldimethylsilyl protected epicatechin

The method to protect epicatechin with tert-butyldimethylsilyl groups was based on that of Runarsson *et al.,* (2008) in their preparation of (TBDMS)-chitosan. A reaction scheme for this reaction can be seen in figure 25.

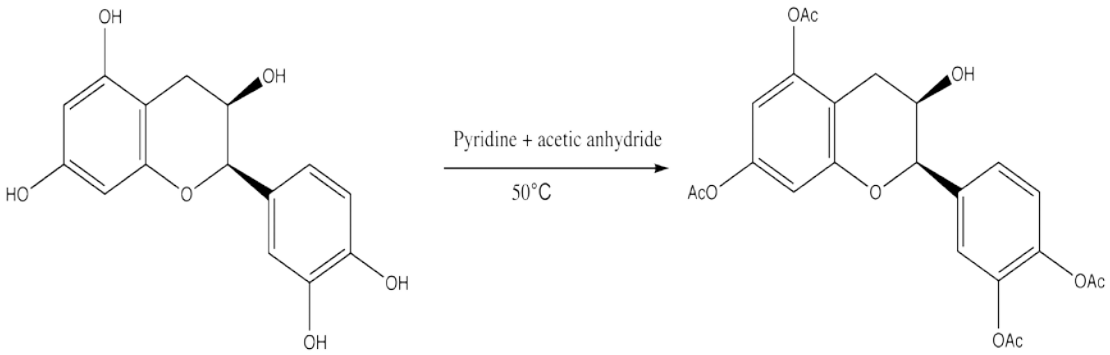
Figure 25. The reaction scheme for the addition of tert-butyldimethylsilyl protecting groups

In preparation of this reaction 0.058 g of epicatechin was dissolved in 10 mL of pre-warmed DMF. Also to 10 mL of prewarmed DMF 0.06 g of tert-butyldimethyl chlorosilane and 0.054 g of imidazole were added and dissolved. Both solutions were flowed through a T-shaped microreactor (Figure 16) at 5/10/15 µL/min. Products were collected into a vile containing water to quench the reaction. The products were extracted into DCM and washed 3 times with brine. The DCM layer was extracted onto $MgSO_4$, filtered and dried under reduce pressure. Products were dissolved in chloroform-d and analysed using $^1$H'NMR.

#### 2.3.5.2 Microreactor synthesis of 3,3',4',7-*O*-tetraacetyl-(-)-epicatechin

The synthesis of 3,3',4',7-*O*-tetraaacetyl-(-)-epicatechin was performed in a T-shaped microreactor as seen in figure 16. A reaction scheme for this seen in figure 26.

Figure 26. The reaction scheme for the production of 3,3',4',7-*O*-tetraacetyl-(-)-epicatechin




In this reaction 3.5 mL of 20 mM (0.02 g) epicatechin in pyridine was flowed alongside 0.25 mL of acetic anhydride in 3.25 mL of pyridine at 50 °C. The reaction was controlled by a syringe pump using a flow rate of 5 µL/min. Products were collected into a vial containing methanol, which acted as a quencher. This was to confirm that the reaction actually occurred in the microreactor and not in the vial.

Products collected were dissolved into diethyl ether and washed with 1M HCl several times before then being washed with brine. The organic layer was dried over $MgSO_4$ filtered and concentrated under reduced pressure, which gave a yellow viscous oil. This was recrystilized in ethanol giving a white solid. Thin layer chromatography (TLC) was performed in ethanol:diethyl ether (1:3) giving 3 spots. The spots were analysed via $^1$H'NMR showing the centre spot to be 3,3',4',7-*O*-tetraacetyl-(-)-epicatechin. The product was weighed before $^1$H'NMR and MS analysis were performed.

### 2.3.5.3 Microreactor synthesis of oxidised 3,3',4',7-*O*-tetraaacetyl-(-)-epicatechin

In this 1:1 Dess-Martin periodinane:3,3',4',7-*O*-tetraaacetyl-(-)-epicatechin oxidation reaction for which a reaction scheme can be seen in figure 27.

Figure 27. Showing the reaction scheme for the Dess-Martin reaction

Dess-Martin periodinane was flowed alongside 3,3',4',7-*O*-tetraaacetyl-(-)-epicatechin in a T-shaped microreactor which can be seen in figure 16. A 10 mM stock solution of Dess-Martin periodinane was made by adding 0.5 mL of 0.3M Dess-Martin (from SigmaAldrich, UK) to 15 mL of DCM. 5 mL of this stock solution was flowed alongside a 5 mL of a 10 mM solution of 3,3',4',7-*O*-tetraaacetyl-(-)-epicatechin in DCM at 10/15/20 μL/Min at room temperature. Products were collected into a vial containing water to quench the reaction. Products were washed with water several times and the DCM layer was concentrated under reduced pressure giving a yellow oil. Products were analysed via $^{1}$H'NMR.

### 2.3.6 Synthesis of planar epicatechin in a microreactor

#### 2.3.6.1 Microreactor synthesis of planar epicatechin – Method 1

Based on the batch methodology reported by Hakamata *et al.,* (2006) trifluoromethanesulfonate and acetone was reacted with epicatechin in a continuous flow microreactor. The reaction scheme of this can be seen in figure 28.

Figure 28. Showing the reaction scheme for method 1 of planar epicatechin production.

For this reaction 0.05 g of epicatechin was dissolved in 5 mL of acetone and flowed through a T-shaped microreactor (figure 16) alongside 5 μL of trifluoromethanesulfonate in 5 mL of THF at flow rates of either 5 or 10 μL/min. To keep the reactants cool, the microreactor was placed on ice. Reactants were collected in a vial containing diethyl ether. Contents of the vial were washed 3 times with brine and the diethyl ether layer was drained and then dried over $Na_2SO_4$. To purify the products column chromatography using toluene-acetone-methanol (7:3:1) was undertaken Products were dried under reduced pressure giving a yellow oil. $^1$H'NMR was then used to analyse the product.

### 2.3.6.2 Microreactor synthesis of planar epicatechin – Method 2

This reaction is based on the batch methodology described by Fukuhara *et al.,* (2002) in which borontrifluoride diethyletherate and acetone was reacted with epicatechin in a continuous flow microreactor. The reaction scheme of this can be seen in figure 29.

Figure 29. Showing the reaction scheme for method 2 of planar epicatechin production.

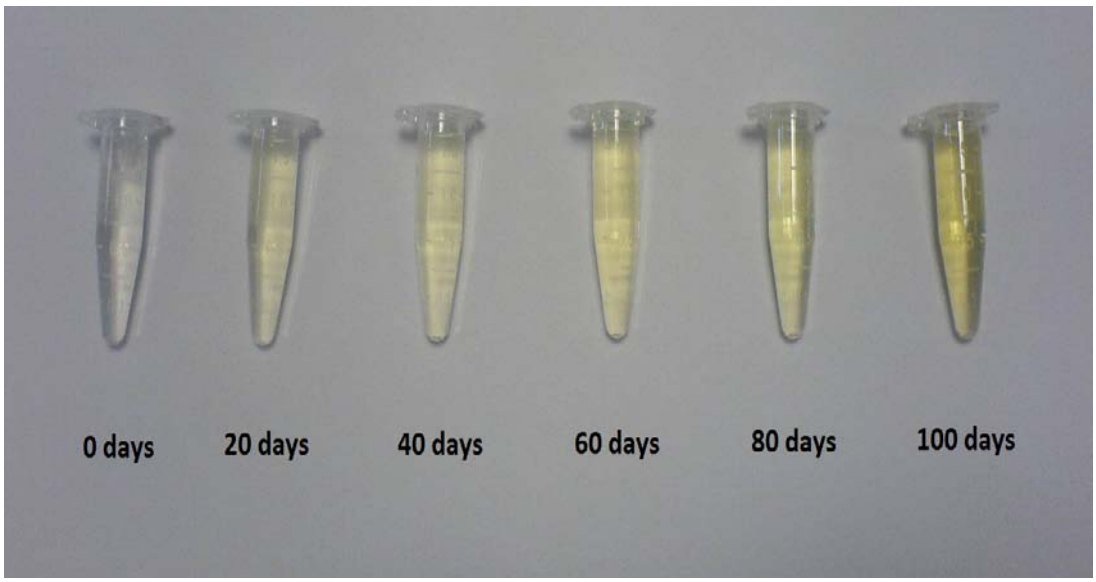
For this reaction 0.05 g of epicatechin was dissolved in 5 mL of acetone and flowed through a T-shaped microreactor (figure 16) alongside 151 μL of borontrifluoride diethyletherate in 4.85 mL of acetone at flow rates of either 5 or 10 μL/min. To keep the reactants cool, the microreactor was placed on ice. Reactants were collected in a vial containing diethyl ether. Contents of the vial were washed 3 times with brine and the diethyl ether layer was drained and then dried over $Na_2SO_4$. To purify the products column chromatography using Dichloromethane:methanol (10:1) was undertaken. Products were dried under reduced pressure giving a yellow oil. $^1$H'NMR was then used to analyse the product.

## 2.4 Results and discussion

### 2.4.1 Results for the stability of EC

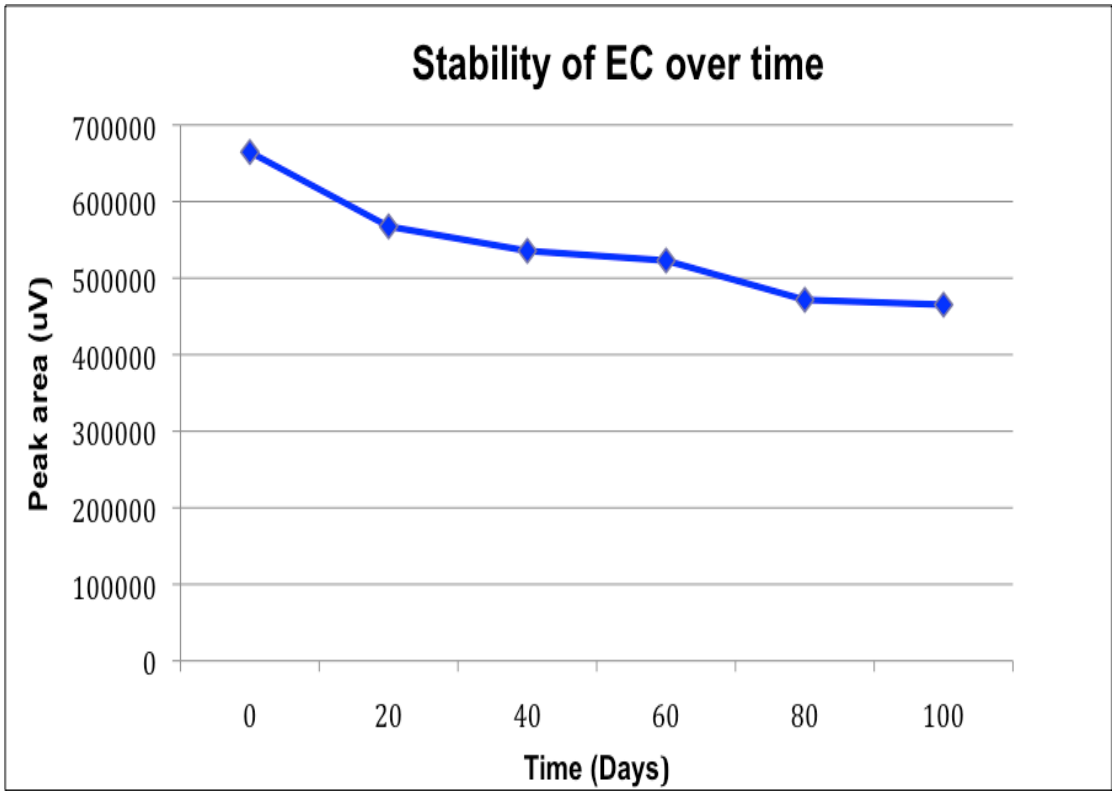
When observing the different EC solutions by eye, it was noticeable that a colour change occurred over time. This colour change, which can be seen in figure 30, increases in intensity with the age of the solution. At 0 days the sample solution of EC is transparent. However, as the age of the sample solution increases so does the yellow colour, with the 100 day old solution having the darkest colour. This indicates some oxidation/polymerization products were being produced.

Figure 30. Colour change of epicatechin solutions over time




When performing the HPLC on the stored samples the retention time of the EC peaks is between 1 minute and 34 seconds and 1 minute and 48 seconds. When analysing the HPLC data the peak area for EC is seen to reduce over time. In figure 31 the peak area can be seen to drop from 664648 to 465260 over the period of 100 days. This equates to a drop in EC content of 30% over this duration and indicates some oxidation/polymerization had occurred. Although the peak for EC reduced over time, no new peaks from oxidation/polymerization products were observed. This would suggest that either the wavelength the detector was set at could not pick up any products or products were carried off the HPLC column in the elutant very quickly and therefore were not detected.

Figure 31. Graph to show the reduction in epicatechin concentration over time



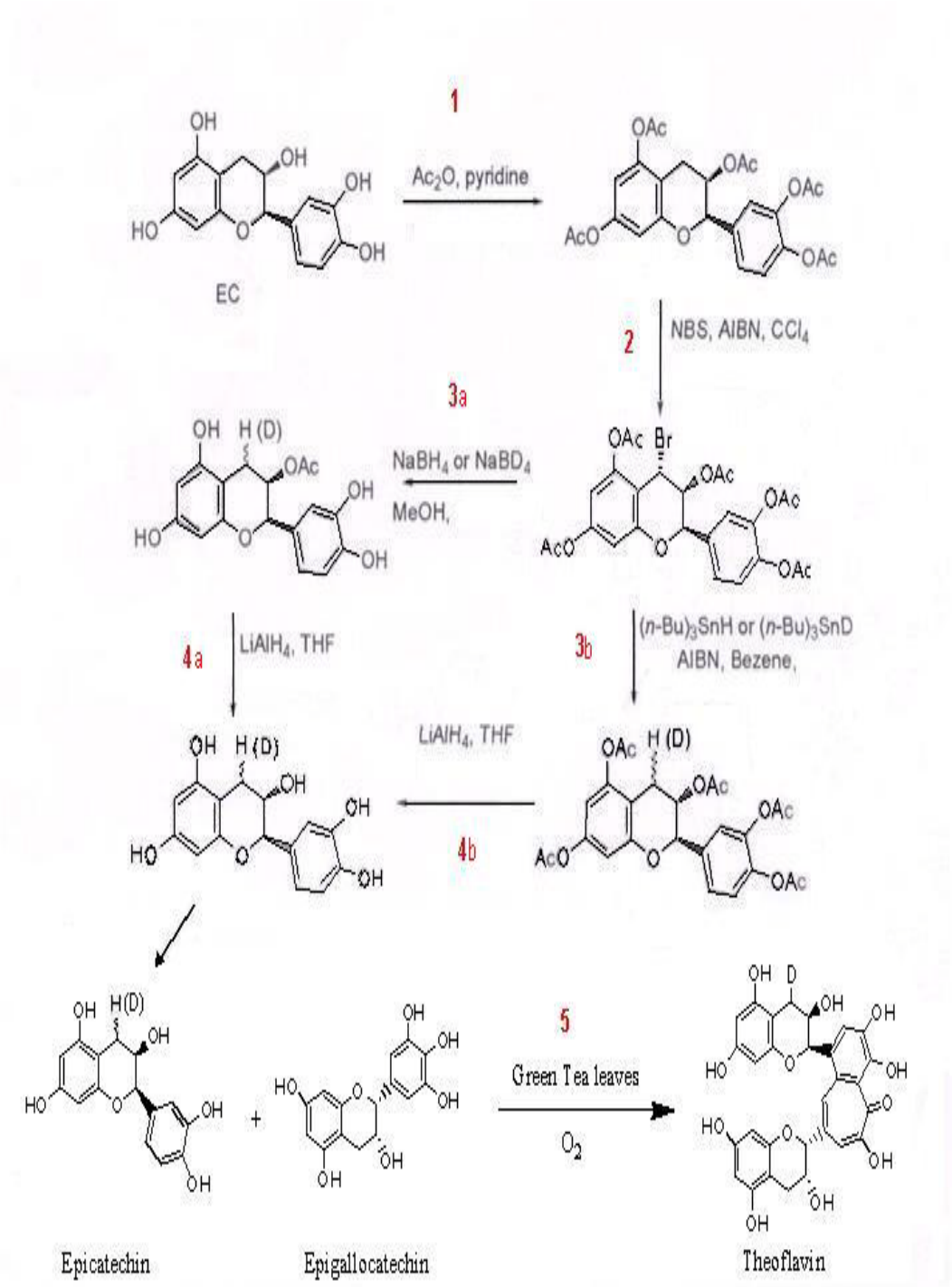

Peak area calculated from HPLC data (280 nm) using a Gemini 5 micron C18 (150 x 4.80mm) column.

Any further investigation would require experimenting with solvent ratios and/or flow rates to detect and analyse and oxidation/polymerisation products.

### 2.4.2 Results for microreactor synthesis of labelled of epicatechin - Method 1

The following results are from method 1 of the microreactor synthesis of deuterium labelled epicatechin. In figure 32 the adapted reaction scheme can be seen showing the steps involved for this synthetic route.

Figure 32. Reaction scheme adapted from Kohri *et al.,* (2001) by Peng and Yao (2009) showing steps for 2 synthetic routes of producing a deuterium labelled epicatechin and theaflavin in batch reactions.



### 2.4.2.1 Optimization and production 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin in a microreactor

The 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin produced at 20 µL/min gave a melting point of 151-153.3 °C which compared to results from past literature results of 152-153 °C (Hergert and Kurth, 1953) was acceptable. The yield produced from this reaction was 57.14% (0.020 g). From the elemental analysis undertaken (See table 1), the results show the compound produced is that expected.

Table 1. Showing elemental analysis from 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin produced from a reaction at 20 µL/min

$C_{25}H_{24}O_{11}$ (MW= 500.45 g/mol)

| Analysis % Expected | | Results % Found |
|---|---|---|
| C | 60.00 | 60.27 |
| H | 4.83 | 5.03 |
| N | 0.00 | 0.00 |

From this reaction the following $^{1}$H'NMR and $^{13}$C-NMR data is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR ($CDCl_3$) 7.37 (1H, d, J=0.72 Hz, H-2'), 7.28 (1H, d, J=2.8Hz, H-6'), 7.21 (1H, d, J=8.4Hz, H-5'), 6.67 (1H, d, J=2.36 Hz, H-6'), 6.57 (1H, d, J=2.2 Hz, H-8'), 5.39 (1H, m, H-3'), 5.11 (1H, bs, H-2'), 2.98 (1H, dd, J=4.4, 22.36Hz, H-4'), 2.89 (1H, dd, J=2.2, 9.0Hz, H-4), 2.31, 2.30, 2.30, 2.28 (4x3H,4xs, 4x$COCH_3$), 1.92 (3H, xs, $COCH_3$).

$^{13}$C-NMR ($CDCl_3$) 170.5, 169.1, 168.5, 168.2, 168.1 (O<u>C</u>O$CH_3$), 154.9 (C-8a), 150.1 (C-7), 149.7 (C-5), 142.1, 141.9 (C-3', 4'), 136.4 (C-1), 124.4 (C-6'), 123.4 (C-5'), 121.9 (C-2'), 110.1 (C-4a). 108.8 (C-6), 108.1 (C-8), 77.9 (C-2), 66.6 (C-3), 22.7 (C-4), 21.1, 20.8, 20.8, 20.7, 20.7 (OCO<u>C</u>$H_3$).

The 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin produced at 15 µL/min gave a melting point of 150.8-153.1 °C which compared to results from past literature results of 152-153 °C (Hergert and Kurth, 1953) was acceptable. The yield produced from this reaction was 65.7% (0.023 g). From the elemental analysis undertaken (See table 2), the results show the compound produced is as expected.

Table 2. Showing elemental analysis from 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin produced from a reaction at 15 µL/min

$C_{25}H_{24}O_{11}$ (MW= 500.45 g/mol)

| Analysis % Expected | | Results % Found |
|---|---|---|
| C | 60.00 | 60.02 |
| H | 4.83 | 4.76 |
| N | 0.00 | 0.00 |

From this reaction the following $^{1}$H'NMR and $^{13}$C-NMR data is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR ($CDCl_3$) 7.36 (1H, d, J=2.6 Hz, H-2'), 7.28 (1H, d, J=2 Hz, H-6'), 7.21 (1H, d, J=8.4 Hz, H-5'), 6.67 (1H, d, J=2.2 Hz, H-6), 6.57 (1H, d, J=2.2 Hz, H-8), 5.39 (1H, m, H-3), 5.11 (1H, bs, H-2), 2.98 (1H, dd, J=4.2, 22 Hz, H-4), 2.89 (1H, dd, J=3.1, 12.8 Hz, H-4), 2.30, 2.30, 2.28, 2.27 (4x3H,4xs, 4x$COCH_3$), 1.92 (3H, xs, $COCH_3$).

$^{13}$C-NMR ($CDCl_3$) 107.4, 169.0, 168.5, 168.2, 168.1 (O<u>C</u>O$CH_3$), 154.9 (C-8a), 150.1 (C-7), 149.7 (C-5), 142.2, 141.9 (C3', 4'), 136.5 (C-1), 124.4 (C-6'), 123.4 (C-5'), 121.9 (C-2'), 110.1 (C-4a), 108.8 (C-6), 108.0 (C-8), 77.9 (C-2), 66.6 (C-3), 22.1, 20.8, 20.8, 20.6, 20.6 (OCO<u>C</u>$H_3$).

The 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin produced at 10 µL/min gave a melting point of 151.8-152.9 °C which compared to results from past literature results of 152-153 °C (Hergert and Kurth, 1953) was acceptable. The yield produced from this reaction was 82.9% (0.029 g). From the elemental analysis undertaken (See table 3), the results show the compound produced is as expected.

Table 3. Showing elemental analysis from 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin produced from a reaction at 10 µL/min

$C_{25}H_{24}O_{11}$ (MW= 500.45 g/mol)

| Analysis % Expected | | Results % Found |
|---|---|---|
| C | 60.00 | 59.81 |
| H | 4.83 | 4.82 |
| N | 0.00 | 0.00 |

From this reaction the following $^{1}$H'NMR and $^{13}$C-NMR data is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR ($CDCl_3$) 7.36 (1H, d, J=2.7 Hz, H-2'), 7.28 (1H, d, J=2.2 Hz, H-6'), 7.20 (1H, d, J=8.2 Hz, H-5'), 6.67 (1H, d, J=2.4 Hz, H-6), 6.57 (1H, d, J=2.2 Hz, H-8), 5.39 (1H, m, H-3), 5.11 (1H, bs, H-2), 2.98 (1H, dd, J=4.4, 22.2 Hz, H-4), 2.89 (1H, dd, J=3.1, 12.6 Hz, H-4), 2.31, 2.30, 2.30, 2.28 (4x3H,4xs, 4x$COCH_3$), 1.92(3H, xs, $COCH_3$)

$^{13}$C-NMR ($CDCl_3$) 169.6, 168.1, 167.5, 167.3, 167.2 (O<u>C</u>O$CH_3$), 154.0 (C-8a), 149.1 (C-7), 148.8 (C-5), 141.3, 140.9 (C3', 4'), 135.5 (C-1), 123.5 (C-6'), 122.5 (C-5'), 121.0 (C-2'), 109.2 (C-4a), 107.9 (C-6), 107.1 (C-8), 77.0 (C-2), 65.7 (C-3), 21.8, 20.1, 19.9, 19.8, 19.7 (OCO<u>C</u>$H_3$)

The 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin produced at 5 µL/min gave a melting point of 151.9-153.2 °C which compared to results from past literature results of 152-153 °C (Hergert and Kurth, 1953) and was acceptable. The yield produced from this reaction was 90.9% (0.0318 g). From the elemental analysis undertaken (See table 4), the results show the compound produced is as expected.

Table 4. Showing elemental analysis from 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin produced from a reaction at 5 µL/min

$C_{25}H_{24}O_{11}$ (MW= 500.45 g/mol)

| Analysis % Expected | | Results % Found |
|---|---|---|
| C | 60.00 | 60.05 |
| H | 4.83 | 4.82 |
| N | 0.00 | 0.00 |

From this reaction the following $^{1}$H'NMR and $^{13}$C-NMR data is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR ($CDCl_3$) 7.36 (1H, d, J=1.8 Hz, H-2'), 7.28 (1H, d, J=2.0 Hz, H-6'), 7.21 (1H, d, J=8.4 Hz, H-5'), 6.67 (1H, d, J=2.2 Hz, H-6), 6.57 (1H, d, J=2.2 Hz, H-8), 5.38 (1H, m, H-3), 5.11 (1H, bs, H-2), 2.98 (1H, dd, J=4.6, 22.4 Hz, H-4), 2.88 (1H, dd, J=1.8, 17.8 Hz, H-4), 2.33, 2.30, 2.30, 2.28 (4x3H,4xs, 4x$COCH_3$), 1.92(3H, xs, $COCH_3$)

$^{13}$C-NMR ($CDCl_3$) 170.5, 169.0, 168.4, 168.1, 168.1 (O<u>C</u>O$CH_3$), 154.9 (C-8a), 149.7 (C-7), 149.7 (C-5), 142.0, 141.9 (C3', 4'), 135.8 (C-1), 123.4 (C-6'), 123.2 (C-5'), 122.0 (C-2'), 109.6 (C-4a), 108.8 (C-6), 108.1 (C-8), 77.0 (C-2), 66.6 (C-3), 26.0, 21.1, 20.8, 20.7, 20.6 (OCO<u>C</u>$H_3$)

The 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin produced at 2.5 µL/min gave a melting point of 152.0-153.3 °C which compared to results from past literature results of 152-153 °C (Hergert and Kurth, 1953) was acceptable. The yield produced from this reaction was 94.3% (0.033 g). From the elemental analysis undertaken (See table 5), the results show the compound produced is as expected. This was a very high yield indicating that microreactors when optimized can equal or supersede traditional batch reactions

Table 5. Showing elemental analysis from 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin produced from a reaction at 2.5 µL/min

$C_{25}H_{24}O_{11}$ (MW= 500.45 g/mol)

| Analysis % Expected | | Results % Found |
|---|---|---|
| C | 60.00 | 60.03 |
| H | 4.83 | 4.82 |
| N | 0.00 | 0.00 |

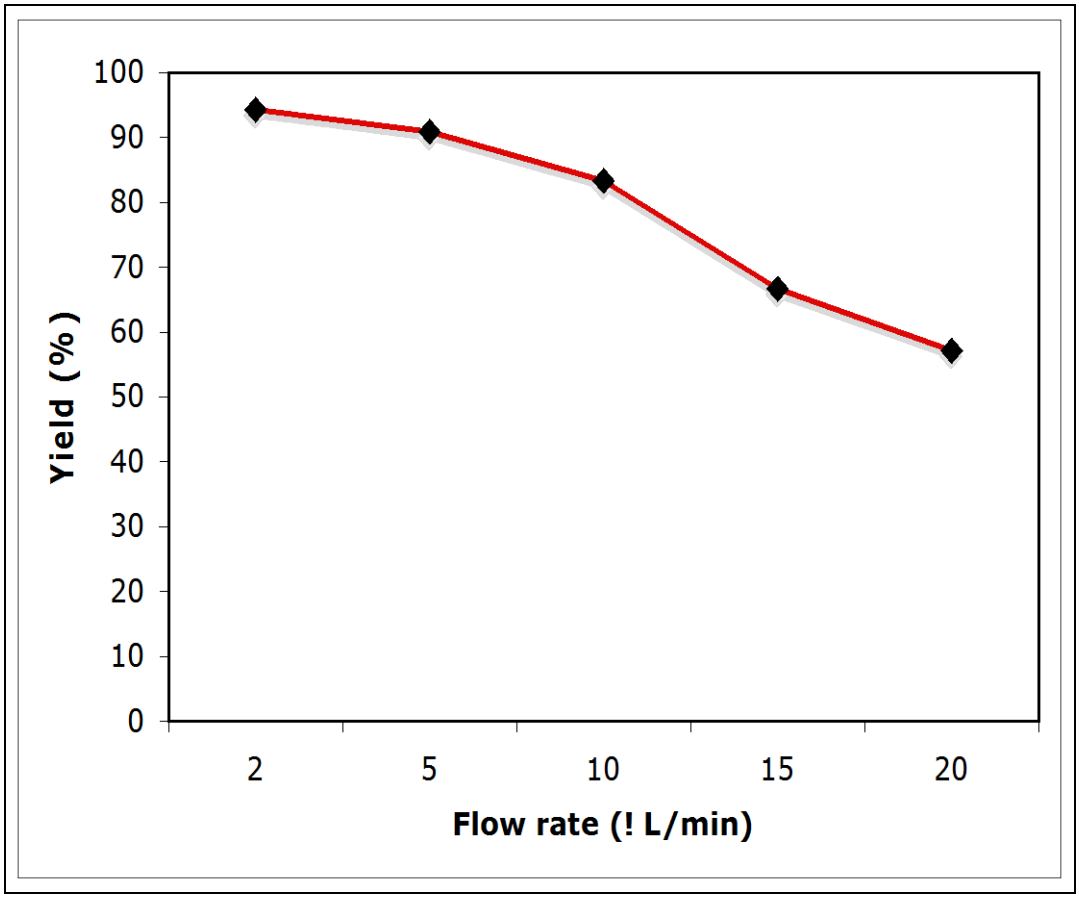
From this reaction the following $^{1}$H'NMR and $^{13}$C-NMR data is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR ($CDCl_3$) 7.36 (1H, d, J=1.8 Hz, H-2'), 7.28 (1H, d, J=2.0 Hz, H-6'), 7.21 (1H, d, J=8.2 Hz, H-5'), 6.67 (1H, d, J=2.2 Hz, H-6), 6.50 (1H, d, J=2.2 Hz, H-8), 5.38 (1H, m, H-3), 5.11 (1H, bs, H-2), 2.99 (1H, dd, J=4.6, 58.1 Hz, H-4), 2.88 (1H, dd, J=1.64, 58.1 Hz, H-4), 2.30, 2.30, 2.29, 2.28 (4x3H,4xs, 4x$COCH_3$), 1.92 (3H, xs, $COCH_3$)

$^{13}$C-NMR ($CDCl_3$) 169.5, 168.0, 167.5, 167.4, 167.2 (O<u>C</u>O$CH_3$), 153.9 (C-8a), 149.2 (C-7), 148.8 (C-5), 141.3, 141.1 (C3', 4'), 135.6 (C-1), 123.4 (C-6'), 122.5 (C-5'), 121.1 (C-2'), 110.2 (C-4a), 108.8 (C-6), 108.5 (C-8), 77.1 (C-2), 65.8 (C-3), 25.1, 21.2, 20.8, 20.7, 20.6 (OCO<u>C</u>$H_3$)

From the optimization data a graph of % yield against flow rate was plotted to show visually how the yield is affected by flow rate in a microreactor (Figure 33).

Figure 33. Graph showing the correlation between flow rate and yield obtained for the synthesis of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin




From the data it can be clearly seen that the slower the flow rate the higher the yield. A flow rate of 2.5 µL/min produced the highest yield of 94.3%. All elemental, melting point, $^{13}$C-NMR and $^{1}$H'NMR data all confirm that in each reaction 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin was produced. Flow rates less than 2.5 µL/min were not tested, as the benefits of a slightly higher yield would be negated by the reduction in the rate of the product synthesised. For example, it would take almost 7 hours to collect 1 mL of a 20mM solution of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin for analysis at 2.5 µL/min, however, it would take over 16 hours to flow the same solution at 1 µL/min.

### 2.4.2.2 Bromination of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin in a microreactor

In this reaction (see figure 32, step 2) 0.06 g of brominated 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin was produced. From this reaction the following H'NMR is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR ($CDCl_3$) 7.36 (1H, d, J=1.84 Hz, H-2'), 7.28 (1H, d, J=1.6 Hz, H-6'), 7.21 (1H, d, J=8.28 Hz, H-5'), 6.67 (1H, d, J=2.36 Hz, H-6), 6.50 (1H, d, J=2.2 Hz, H-8), 5.36 (1H, m, H-3), 5.12 (1H, bs, H-2), 2.98 (1H, dd, J=13.6, 25.2 Hz, H-4), 2.88 (1H, dd, J=19.6, 24.0 Hz, H-4), 2.37, 2.36, 2.30, 2.28 (4x3H,4xs, 4x$COCH_3$), 1.91 (3H, xs, $COCH_3$)

The $^{1}$H'NMR data indicates that the bromination occurred as a mixture in the H-8 and H-6 positions, shown in figure 34. These bromination positions were also found in batch reactions by Kiehlmann and Tracey (1986) and not on the centre tetrahydropyran ring as based on the research by Kohri *et al.,* (2001) and Peng and Yao (2009). Variations in flow rates, initiators and temperatures where tested and none resulted in the 4-bromo 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin.

Figure 34. 6-bromo and 8-bromo 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin

In the acetylation reaction there were peaks in the H'NMR at regions 6.67 ppm and 6.50 ppm where as in the $^{1}$H'NMR for the bromination product the peaks at 6.67 ppm and 6.50 ppm remained but the size of the peaks was significantly reduced. This indicates that bromination was not specific and occurred in either position 6 or 8. The reaction was confirmed with LC/MS where a single mass peak was seen at 580 m/z indicating that single bromination had occurred and a proton had been lost. Although these were not the positions needed for bromination it could still be used to label epicatechin. It also showed that the bromination of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin was possible in a microreactor, although like in batch reactions bromination occurs as a mixture in position 6 or 8.

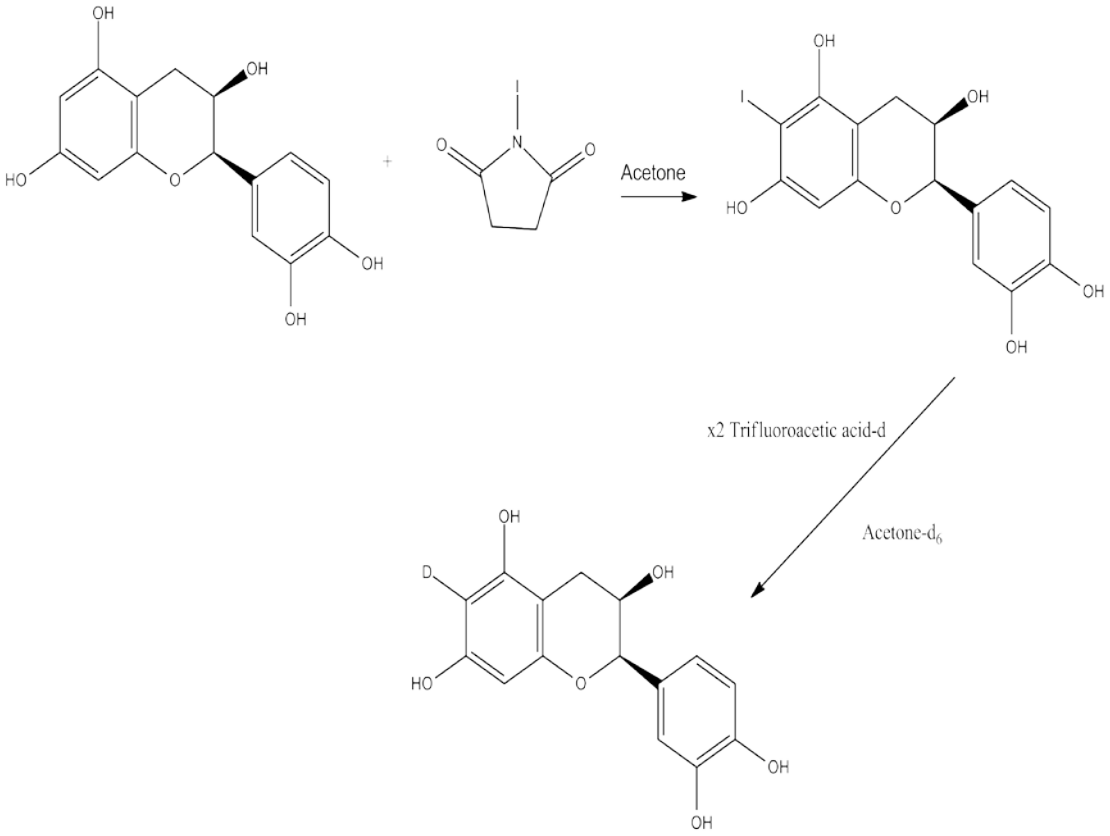
#### 2.4.2.3 Substitution reaction

In this reaction (see figure 32, step 3a) no result could be produced when using a microreactor. This was due to excessive gas production from the $NaBD_4$ resulting in high back pressure and causing the syringe to become disconnected from the microreactor. Unfortunately due to the nature of this reaction it could not be safely repeated again in a microreactor. In the reaction scheme in figure 8 the alternative would be to choose step 3b. Unfortunately this was not an option due to the reactants use in this method contain tin compounds, which are difficult to remove and can cause cell death (Nguyen, Foller and Lang, 2008). Due to the future usage of the labelled compounds with microbial and possibly mammalian cells, this was too high a risk factor and therefore other methods needed to be considered.

### 2.4.3 Results for microreactor synthesis of deuterium labelled epicatechin – Method 2

The following results are from the second synthetic route used to deuterium label epicatechin in a microreactor. In figure 35 a reaction scheme can again be seen for this 2-step synthesis.

Figure 35. Reaction scheme for method 2 of the microreactor synthesis of deuterium labelled epicatechin.




### 2.4.3.1 Iodination of epicatechin in a microreactor

The reaction produced a crude product with a weight of 0.1 g with LC/MS showing a very small mass peak at 290 m/z and much larger mass peak at 416.8 m/z. Although some starting material was still present, H'NMR data from the crude product showed that the reaction was almost 100% complete. From this reaction the following $^{1}$H'NMR is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR (400 MHz, $CD_3COCD_3$) 6.95 (1H, d, J=1.36 Hz, H-2'), 6.81 (1H, d, J=2.2 Hz, H-6'), , 6.14 (1H, d, J=2.76 Hz, H-8), 5.45 (1H, m, H-3), 4.87 (1H, bs, H-2), 2.90 (1H, dd, J=19.2, 4.67 Hz, H-4), 2.70 (1H, dd, J=16.6, 2.2 Hz, H-4)

$^{13}$C'NMR (400 MHz, $CD_3COCD_3$) 157.4 (C-8a), 156.3 (C-7), 156.2 (C-5), 145.2, 141.1 (C-3', C-4'), 131.8 (C-1'), 119.2 (C-6'), 115.7 (C-2'), 115.0 (C-5'), 100.5 (C-4a), 96.2 (C-8), 79.8 (C-6), 67.2 (C-2), 66.4 (C-3), 29.6 (C-4)

A peak was also seen at 2.61 ppm indicating succinimide. As in the research by Kiehlmann *et al.,* (1988) we noticed the disappearance of a peak at 6.25 ppm indicating that the proton in that region had been lost and 6-iodoepicatechin had been produced. However a direct comparison with the work of Kiehlmann *et al.,* (1988) could not be made, due to the variation in peaks produced by catechin and epicatechin. The $^{1}$H'NMR and the LC/MS data confirmed that the iodination had been successful and proved for the first time that a polyphenol could be iodinated in a microreactor. Although this reaction in effect produces a similar result as the bromination reaction, it is position specific and protecting the OH groups was not necessary. This increases speed of production and therefore costs can be reduced.

### 2.4.3.2 Deuteration of 6-iodoepicatechin in a microreactor

In this reaction, a purple/brown oil was produced indicating that there free iodine in the mixture. The product oil weighed 0.105 g. LC/MS spectra of the product showed a main mass peaks at 292 m/z and a smaller peak at 293 m/z indicating the mass of single and double D-labelled epicatechin with the additional deuterium most likely being from hydrogen transfer with one of the phenol groups. From this reaction the following $^{1}$H'NMR is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR (400 MHz, $CD_3COCD_3$) 6.95 (1H, d, J=1.36 Hz, H-2'), 6.81 (1H, d, J=2.2 Hz, H-6'), , 6.14 (1H, d, J=2.76 Hz, H-8), 5.45 (1H, m, H-3), 4.87 (1H, bs, H-2), 2.90 (1H, dd, J=18.1, 4.76 Hz, H-4), 2.70 (1H, dd, J=16.2, 2.2 Hz, H-4)

$^{13}$C'NMR (400 MHz, $CD_3COCD_3$) 157.5 (C-8a), 157.0 (C-7), 156.6 (C-5), 145.2, 145.1 (C-3', C-4'), 132.4 (C-1'), 119.4 (C-6'), 115.7 (C-2'), 115.2 (C-5'), 100.1 (C-4a), 99.8 (C-8), 95.6 (C-6), 79.9 (C-2), 66.6 (C-3), 29.6 (C-4)

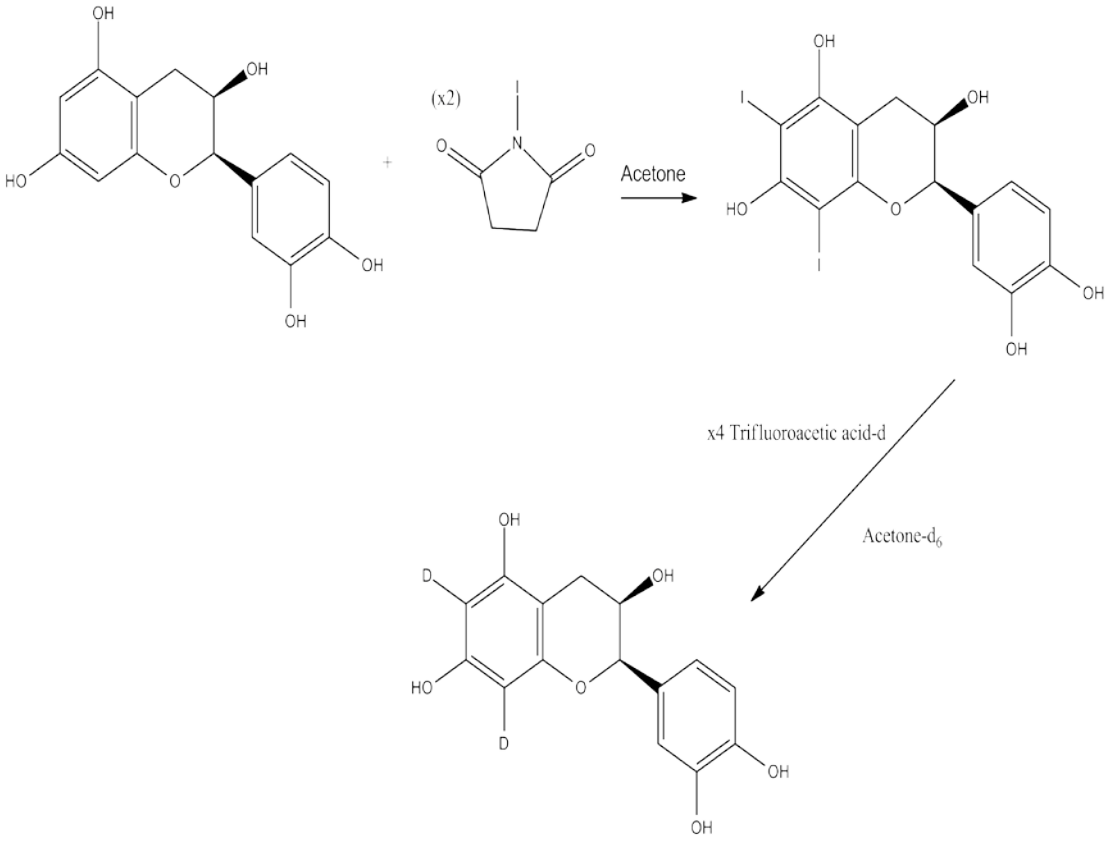
The $^{1}$H'NMR spectra showed that only one peak remained at 6.14 ppm and the peak at 6.25 ppm had not returned. Although it appeared from the colour of the product that there was some free iodine present, LC/MS and $^{1}$H'NMR spectra indicated that the conversion was very high. This represented the first occasion epicatechin had been deuterium labelled in a microreactor

The immediate stability results of deuterium labelled epicatechin showed that in water it was stable for up to 2 hours. LC/MS performed at 0, 1 and 2 hours after storage showed the main mass peak remained at 292 m/z. The mass peak at 293 m/z had been reduced indicating hydrogen transfer had again occurred and the extra deuterium being lost. However this does not represent the stability in serum and this would need to be investigated further if it were to be used in toxicological studies.

### 2.4.4 Results for Double deuteration of epicatechin

The following results are from the second synthetic route used to deuterium label epicatechin in a microreactor. In figure 36 a reaction scheme can again be seen for this 2-step synthesis.

Figure 36. Reaction scheme for the microreactor synthesis of double deuterium labelled epicatechin.




### 2.4.4.1 Double iodination of epicatechin

The reaction produced a crude product with a weight of 0.15 g with LC/MS showing very small mass peaks at 290 m/z and 416.8 m/z and a much larger mass peak at 543.6 m/z. This LC/MS and H'NMR data indicated that the reaction produced both single and double iodinated products, with the greater amount being double deuterated. From this reaction the following $^{1}$H'NMR is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR (400 MHz, $CD_3COCD_3$) 6.95 (1H, d, J=1.36 Hz, H-2'), 6.81 (1H, d, J=2.2 Hz, H-6'), 6.14 (1H, d, J=2.76 Hz, H-8), 5.45 (1H, m, H-3), 4.87 (1H, bs, H-2), 2.90 (1H, dd, J=19.2, 4.67 Hz, H-4), 2.70 (1H, dd, J=16.6, 2.2 Hz, H-4)

$^{13}$C'NMR (400 MHz, $CD_3COCD_3$) 157.4 (C-8a), 156.3 (C-7), 156.2 (C-5), 145.2, 141.1 (C-3', C-4'), 131.8 (C-1'), 119.2 (C-6'), 115.7 (C-2'), 115.0 (C-5'), 100.5 (C-4a), 94.1 (C-8), 76.8 (C-6), 67.2 (C-2), 66.4 (C-3), 29.6 (C-4)

A large peak in the $^{1}$H'NMR spectra was also seen at 2.61 ppm indicating succinimide. As in the single iodination the disappearance of the peak at 6.25 ppm was noticed. The $^{1}$H'NMR spectra revealed that the peak at 6.14 ppm had also been reduced, signifying a second proton had been replaced in some of the epicatechin. The $^{1}$H'NMR and the LC/MS data did confirm that the double iodination had been achieved but the reaction had not been as successful as with the single iodination. However it did prove for the first time that a polyphenol could not only be iodinated, but double iodinated in a continuous flow microreactor.

### 2.4.4.2 Deuteration of 6,8 –iodoepicatechin

In this reaction, a purple oil was produced. This would indicate, that as in the single deuteration some free iodine was present. The product oil weighed 0.111 g. LC/MS spectra of the product showed a main mass peaks at 293 m/z and a smaller peaks at 292 m/z 294 m/z indicating the mass of single and double D-labelled epicatechin with the majority being double deuterated. The mass peak at 294 m/z would have been most likely from proton exchange From this reaction the following $^{1}$H'NMR is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR (400 MHz, $CD_3COCD_3$) 6.95 (1H, d, J=1.36 Hz, H-2'), 6.81 (1H, d, J=2.2 Hz, H-6'), 6.14 (1H, d, J=2.76 Hz, H-8), 5.45 (1H, m, H-3), 4.87 (1H, bs, H-2), 2.90 (1H, dd, J=18.1, 4.76 Hz, H-4), 2.70 (1H, dd, J=16.2, 2.2 Hz, H-4)

$^{13}$C'NMR (400 MHz, $CD_3COCD_3$) 157.5 (C-8a), 157.0 (C-7), 156.6 (C-5), 145.2, 145.1 (C-3', C-4'), 132.4 (C-1'), 119.4 (C-6'), 115.7 (C-2'), 115.2 (C-5'), 100.1 (C-4a), 99.9 (C-8), 96.6 (C-6), 79.9 (C-2), 66.6 (C-3), 29.6 (C-4)

The $^{1}$H'NMR spectra showed that a small peak had remained at 6.14 ppm and the peak at 6.25 ppm had not returned. From the $^{1}$H'NMR and LC/MS spectra it can be concluded that double and single deuteration had occurred. It can also be concluded that the double iodination is not as successful as single deuteration. This could be due to the iodination in the first reaction being less effective in position 8. However, this represents the first occasion epicatechin had been double deuterated labelled in a microreactor.

The immediate stability results of deuterium labelled epicatechin showed that in water it was not stable. LC/MS performed at 0 hours showed a main mass peak at 293. However after 1 and 2 hours of storage in water the main mass peak was 292 m/z. The mass peaks at 293 m/z and 294 m/z had disappeared indicating hydrogen transfer had again occurred and the deuterium in position 8 had also been lost. This shows that's although double iodination of epicatechin is possible in a microreactor only deuteration in position 6 is stable for > 2 hours. However this does not represent the stability in serum and this would need to be investigated further if it were to be used in toxicological studies.

### 2.4.5 Results for the synthesis of deuterium labelled epicatechin – Methodology 3

The following results are from the third synthetic route used to try and deuterium label epicatechin in a microreactor. In figure 37 the scheme can again be seen for this series of reactions.

Figure 37. Method 3 for the microreactor synthesis of deuterated epicatechin

### 2.4.5.1 Results for tert-butyldimethylsilyl protection

The $^{1}$H'NMR spectra obtained from the protection products showed multiple peaks masking any product peaks that would be visible. Even after further purification was undertaken the products could not be seen in the $^{1}$H'NMR spectra. From this it was determined that the reaction was either unsuccessful, or of such low yield that it would not be valuable enough to replicate. It was decided that even though they have reduced stability, acetyl groups would be used for protection.

### 2.4.5.2 Results for the production of 3,3',4',7-*O*-tetraacetyl-(-)-epicatechin

From LC/MS f the purified product one main mass peak was seen at 458.3 m/z indicating acetylation had occurred in four positions. Spectra from $^{1}$H'NMR also confirmed this showing only four acetyl groups. When comparing the $^{1}$H'NMR with that of pentaacetly-(-)-epicatechin it was clear that the acetyl group in position 5 was missing. This confirmed that 3,3',4',7-*O*-tetraacetyl-(-)-

epicatechin had been produced. In this reaction the purified product weighed 0.025 g giving a yield of 78.1%.

From this reaction the following $^{1}$H'NMR is reported where: d = doublets, dd = doublet of doubles, m = multiplet, s = singlet and bs = broad singlet.

$^{1}$H'NMR ($CDCl_3$) 7.36 (1H, d, J=1.8 Hz, H-2'), 7.28 (1H, d, J=2.0 Hz, H-6'), 7.21 (1H, d, J=8.2 Hz, H-5'), 6.67 (1H, d, J=2.2 Hz, H-6), 6.50 (1H, d, J=2.2 Hz, H-8), 5.38 (1H, m, H-3), 5.11 (1H, bs, H-2), 2.99 (1H, dd, J=4.6, 58.1 Hz, H-4), 2.88 (1H, dd, J=1.64, 58.1 Hz, H-4), 2.30, 2.30, 2.29, 2.28 (4x3H,4xs, 4x$COCH_3$)

### 2.4.5.3 Results for Dess-Martin reaction

When reading the $^{1}$H'NMR spectra for this reaction it was clear that it was unsuccessful. Multiple peaks in all regions were observed and therefore masking any product peaks that could possibly have been present. This could have been due to the protecting groups not being sufficient for the reaction, resulting in oxidation at multiple regions on the compound. When analysing products from all flow rates tested the results were the same. A possible solution to this would be to use benzyl groups to protect epicatechin. Unfortunately when consulting the literature, protecting epicatechin with benzyl gives very poor yields in batch (20%, Tckmantel, Kozikowski and Ramanczyk, 1999) making the procedure more costly. Transferring this reaction to microreactor production would be unlikely to increase the yield. A possible alternative would be to follow the work of Hiipakka *et al*., (2002) and produce tert-butldimethulsilyl (TBDMS) protected epicatechin in a 4-step methodology, not a one step process. In this case epicatechin would be protected with acetyl groups prior to the TBDMS reaction. This would give the more stabile TBDMS protecting groups and possibly lead to a more successful Dess-Martin reaction.

### 2.4.6.1 Results for microreactor synthesis of planar epicatechin - Method 1

The following results are from the first synthetic route used to try and synthesize planar epicatechin in a microreactor. In figure 38 a scheme can again be seen for this reaction.

After examining the $^{1}$H'NMR spectra of the products from this reaction it was clear the reaction had not been successful. Even after purification multiple peaks were seen and when comparing to the research of Van der Western, Steenkamp and Ferreira (1990) no product resembling planar epicatechin or catechin could be seen.

Figure 38. The reaction scheme for method 1 of the microreactor synthesis of planar epicatechin.

With the exception of Van der Western, Steenkamp and Ferreira (1990) no other group has attempted to produce planar epicatechin and instead have opted for the production of planar catechin. This could be due to the fact that the either the yield was so poor in their reaction or the stereochemistry for

epicatechin i.e. both the B ring and the OH group attached to the C ring are projecting into the same plane, makes it unlikely for a planar compound to form. However, with catechin the B ring and OH group on the C ring are in different planes and when the reaction to form planar catechin takes places the two are moved into line, straightening/creating the planar molecule.

### 2.4.6.2 Results for the microreactor synthesis of planar epicatechin - Method 2

The following results are from the first synthetic route used to try and synthesize planar epicatechin in a microreactor. In figure 39 a scheme can again be seen for this reaction.

Figure 39. Showing the reaction scheme for method 2 of planar epicatechin production.

As with the methodology 1 the $^{1}$H'NMR spectra did not show the expected product and multiple peaks were shown making interpretation difficult. This did not change even when the flow rate of the reaction was altered.

## 2.5 Conclusions

In conclusion, it has been shown for the first time that the synthesis of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin is possible in a microfluidic reactor and that very high yields can be achieved, equal to or better than those performed in previous batch methods. It has also been shown that in this reaction, flow rate does have a significant impact on both the yields and product purity. In addition, the methodology described can also be applied to various other types of protecting group chemistry, to aid in not only optimisation but also to increase product yields.

It was discovered that the bromination of 3,3',4',5,7-*O*-pentaacetyl-(-)-epicatechin could not occur in the position originally thought and in practice the bromine prefers the electron rich position 6 or 8. It was found that in step 3 shown in figure 13 was not possible in a microreactor due to increased pressure in the resulting in backflow.

A faster two-step method was shown to be effective in producing D-labelling epicatechin. This was the first time 6-iodoepicatecin and 6-deuterium labelled epicatechin had been produced in a continuous flow microreactor. The compound was shown to be stable in water for 2 hours. Future investigations could investigate the stability of deuterated epicatechin over 24 hours in various solvents. If it were to be used in toxicological studies the stability in serum should also be investigated.

It was also shown that double iodination and deuteration of epicatechin could also be performed in a microreactor, although not as successful as single iodination/deuteration. Stabiliy testing also confirmed that the second deuterium was not stable in position 8 most likely due to easier proton transfer in water.

A third methodology for labelling epicatechin was proven to be unsuccessful due to problems with protecting epicatechin. Future investigations should look

into protecting epicatechin using a 4-step methodology with TBDMS. With increased stability the Dess-Martin step in the synthesis should be more successful.

It was also shown that using two different methods, planar epicatechin could not be produced in a microreactor at this time. It was predicted that the procedures appear to be successful only with catechin due to its altered stereochemistry.

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# Chapter 3

## Antimicrobial and synergistic effects of tea polyphenols against problematic hospital pathogens

## 3.1 Introduction

### 3.1.1 Hospital pathogens, antibiotics and resistance

#### 3.1.1.1 Antibiotic groups

There are many different types of antibiotics, which are used to treat bacterial infections. Antibiotics are put in groups that relate to there modes of action (Table 6). Many groups of antibiotics have experienced resistance and manufacturers have modified their structures in order for their use to be continued.

Table 6. Important antibiotics and their modes of action and bacterial targets (Madigan *et al.,* 2009)

| Antibiotic Group | Examples of Antibiotic group | Mode of action | Bacterial targets |
|---|---|---|---|
| Penicillins (β-lactam) | Ampicillin, penicillin | Disrupts peptidoglycan synthesis used in bacterial cell walls | Gram-positive and some gram negative |
| Aminoglycosides | Gentamycin | Binding to bacterial 30S ribosome leading to loss of protein production vital to cell growth | Mostly gram-negative, some gram positive e.g. *Staphylococcus spp.* |
| Carbapenems | Imipenem | Inhibition of cell wall synthesis | Gram-positive and gram-negative |
| Cephalosporins (β-lactam) | Cefoxitin, cefotaxime | Disruption of cell wall synthesis | 5 generations of antibiotic some more effect against gram-positive or gram-negative |
| Glycopeptides | Vancomycin | Disruption of cell wall synthesis | Gram-positive |
| Macrolides | Clarithromycin | Inhibition of bacterial protein synthesis due to binding to ribosome 50S | Gran-positive |
| Quinolones | Ciprofloxacin | Inhibits DNA replication | Gram-positive and gram-negative |
| Sulfonamides | Sulfadiazine | Folate synthesis inhibition, leading to blocking of nucleic acid synthesis | Gram-positive and gram-negative |
| Tetracyclines | Tetracycline | Binds to 30S ribosome subunit preventing mRNA translation | Gram-positive and gram-negative |

There have been 5 previous generations of cephalosporins antibiotics, where modifications have been made to combat continuing resistance (Lynch and Zhanel, 2009). Several bacterial species including MRSA are resistant to many types of antibiotics, especially beta-lactams.

There are 5 mechanisms of acquired bacterial resistance (Greenwood *et al.,* 2008). These include:

- Prevention of antibiotic entry to the cell
- Antibiotic removal
- Antibiotic destruction
- Target site modification
- Metabolic by-pass

Prevention of entry to a bacterial cell is often due to the modification of proteins in the cell wall, which are able to form channels or pores called porins (Vila, Marti and Sanchez-Cespedes, 2007). Antibiotics such as Beta-lactams pass through these porins and are therefore dependant on their size (Greenwood *et al.,* 2008). By reducing the size of the porins the bacterium can prevent the antibiotic from passing through the cell wall and into the cell.

Antibiotic removal is a mechanism of resistance in which a bacterium actively removes an antibiotic from itself. This occurs via the synthesis of a membrane protein, which actively facilitates the efflux of the antibiotic preventing accumulation and reaching the concentration required for inhibition (Kumar and Schweizer, 2005). This can occur with many groups of antibiotics including Beta-lactam, quinolone and aminoglycoside antibiotics and has been shown in bacteria such as *Escherichia coli* and *Heliocobacter pylori* (Falsafi, Ehsani and Niknam, 2009).

Antibiotic destruction is a method of resistance where the bacteria activity destroys or modifies the antibiotic thereby inhibiting it from reaching its target (Davies, 1994). This mechanism was described in 1973 (Benveniste and Davies) against aminoglycoside antibiotics, where enzymes produced by *Actinomycetes spp.* acetylated the 6'-amino group of gentamycin. This modification inactivated the antibiotic. Enzymes called Beta-lactamases are produced by many resistant bacteria such as MRSA and modify/inactivate Beta-lactam antibiotics for example penicillin and ampicillin.

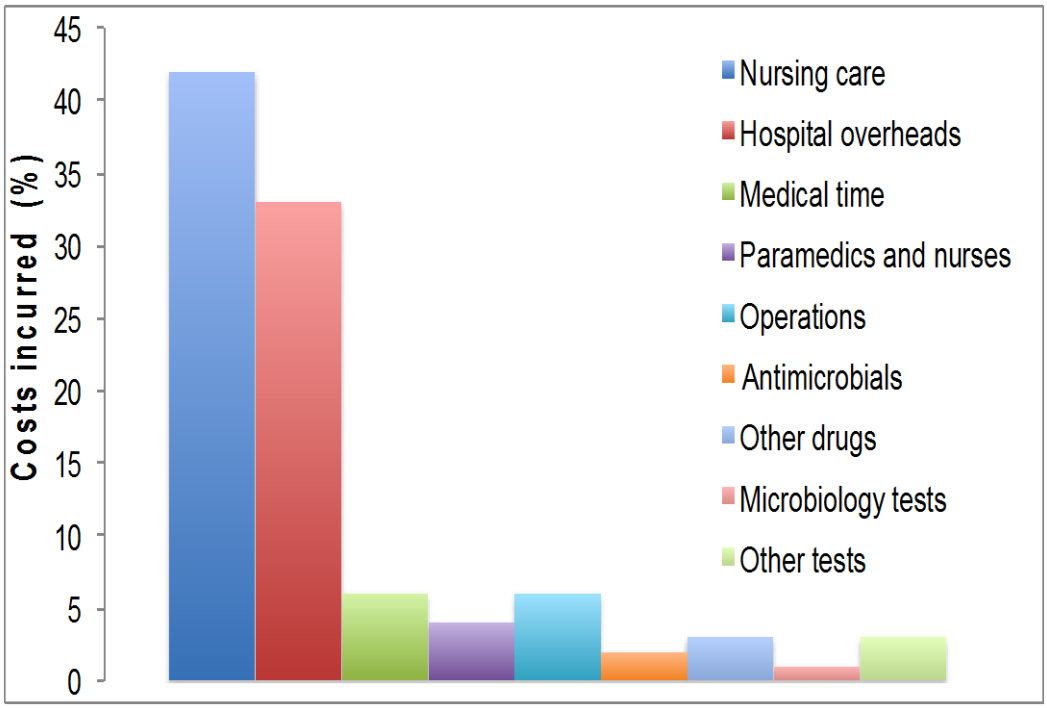
Target site modifications confer resistance and are often from pre-existent mutant strains (Greenwood *et al.,* 2008). These mutations give rise to resistance in a single step and like other mechanisms of resistance, become prevalent due to the failure of an individual to complete a course of antibiotics. Types of modifications include those caused by modified nucleosides in ribosomal RNA (Vester and Long, 2009), which results in the antibiotic being unable to bind to the area of importance. If effective the antibiotic would hinder any ribosomal activity. Use of duel/combination treatments can be effective in treating resistance infections (Rahal, 2006).

Metabolic by-pass is another mechanism that can convey I high level of resistance to antibiotic in bacteria. This mechanism has been shown to give resistance to trimethoprim (Tait and Amyes, 1994), an antibiotic that inhibits dihydrofolate reductase, an enzyme required for the metabolism of folic acid. In this mutation described by Tait and Amyes (1994) plasmid encoded dihydrofolate reductases arise that are not sensitive to trimethroprim.

### 3.1.1.2 Hospital pathogens and resistance

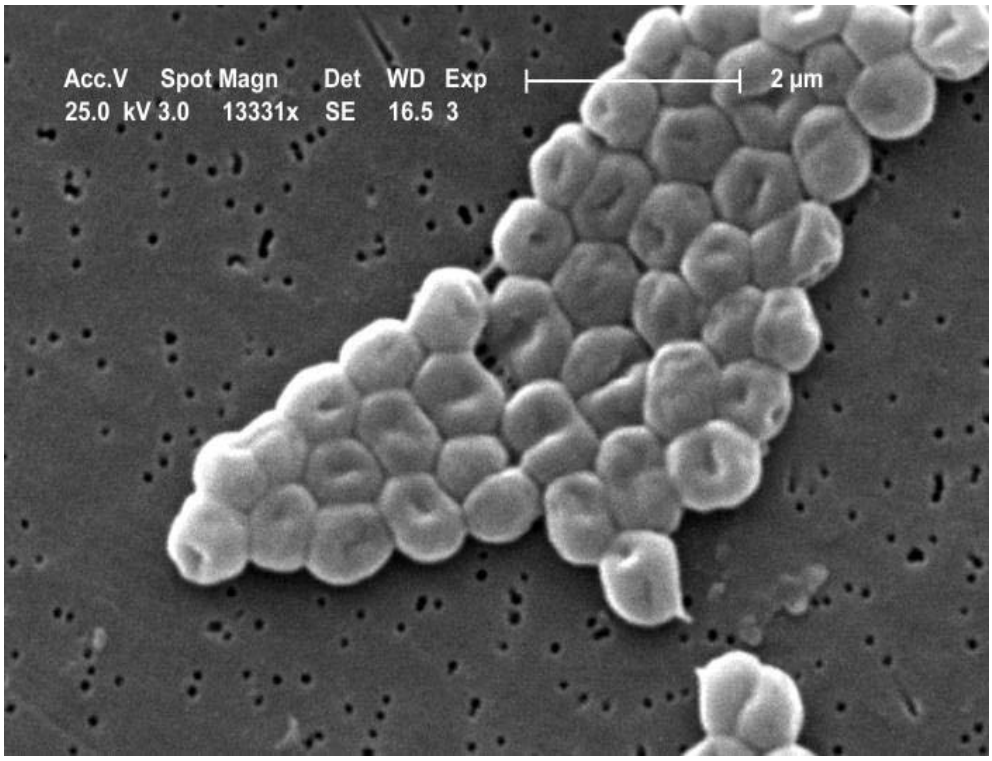
There are many pathogens, which can cause a variety of infections in many different situations. In the US alone there are almost 1.7 million healthcare-associated infections every year with mortality due to these infections affecting 99,000 patients (Guilbeau, 2010). In the UK there in evidence to suggest that the number of hospital-acquired infections are continuing to increase and are responsible for up to 5000 deaths every year (Scott, 2004). Many of these infections are caused by organisms, which are resistant to many clinical antibiotics often causing significant increase in patient stay and the cost of their treatment (Rowland *et al.,* 1999). The distribution of these costs can be seen in figure 40. In Canada, infections caused by MRSA alone, result in an estimated cost of $42-49 million a year (Kim, Oh and Simor, 2001). Four pathogens that are of particular importance in the area of hospital infections are *Acinetobacter baumannii*, *Stenotrophomonas maltophilia,* methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis* know as vancomycin resistant enterococci (VRE) (Madigan *et al.,* 2009).

Figure 40. Distribution of Costs incurred from resistant infections (Rowland *et al.,* 1999)



*A. baumannii* is a common gram-negative bacterium, which is an opportunistic nosocomial pathogen (Bergogne-Berezin and Towner, 1996). In figure 41 an electron micrograph of *A. baumannii* can be seen showing its coccobacilli structure.

Figure 41. A scanning electron micrograph of Acinetobacter baumannii magnified 13331x (CDC, 2004)

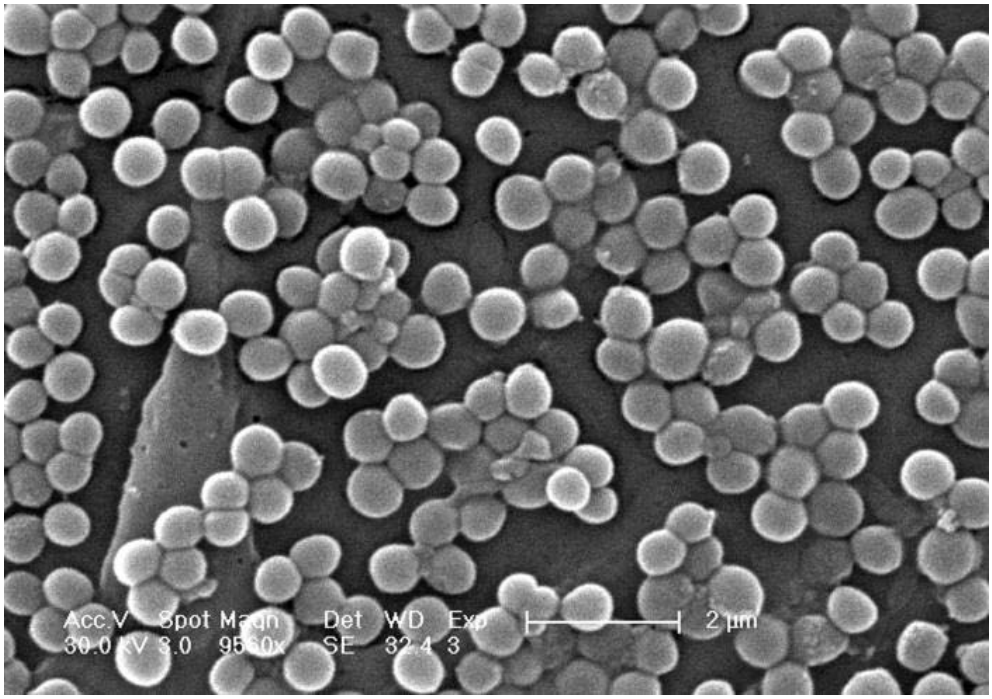



*A. baumannii* is of increasing medical importance due to its ability to colonise quickly and give resistance to many antibiotics (Dijkshoorn, Nemec and Seifert, 2007). The infections it causes can seriously affect immuno-suppressed/compromised individuals such as, the critically ill, burns patients and the elderly (Van Looveren *et al.,* 2004). With the increase in the number of individuals with HIV this could be of particular concern (CDC, 2002). Due to the increasing resistance of *Acinetobacter* spp. infections have become increasingly difficult to treat (Turton *et al.,* 2004). There have also been many reports of resistance of infections in military personnel (Jones *et al.,* 2006), and have shown a wide variety in types of infection caused by *Acinetobacter* (Davis *et al.,* 2005). Over the last 30 years the types of infection that *Acinetobacter*

*baumannii* cause has increased not only in hospitals but also in community-acquired infections (Joly-Guillou, 2005).

*Staphylococcus aureus* is a cocci shaped gram-positive bacteria (Prescott, Harley and Klein, 2007). *S. aureus* is part of the natural flora of the human body and is found in the nose of up to 40% of the population (Enright, 2006). Unlike MRSA, *S. aureus* causes infections that are often easily treated. MRSA, which can be seen in figure 42 is, like *A. baumannii* an opportunistic pathogen in hospitals and in the community (Bloomfield, 2006). MRSA emerged shortly after the introduction of the antibiotic methicillin in the early 1960's (Maltezou and Giamarella, 2006) and is now a problematic organism around the world. Like many opportunistic pathogens, MRSA infections can affect people who are immuno-compromised, elderly and those whom have undergone recent surgery (Kanerva *et al.,* 2007) and can be very costly to treat.

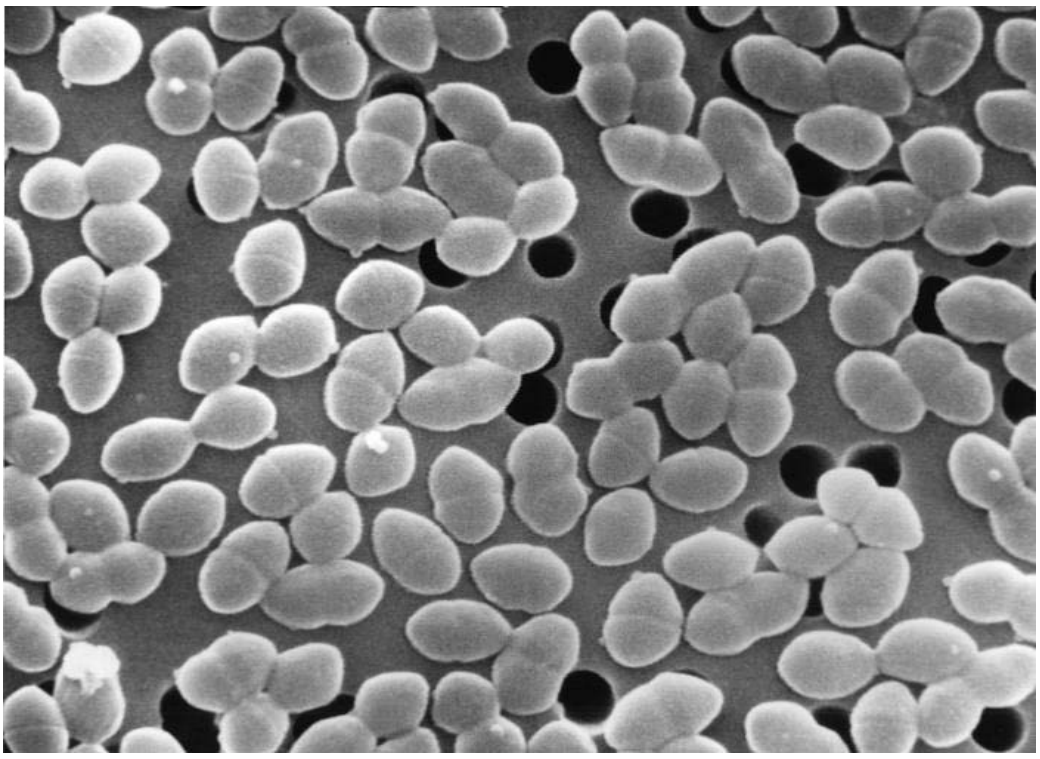
Figure 42. A scanning electron micrograph of MRSA magnified 9560x (CDC, 2005)




Vancomycin resistant enterococci are resistant strains of *Enterococcus* that are resistant to the antibiotic vancomycin and were identified as hospital-associated

pathogens in the middle of the 1980's (Tacconelli and Cataldo, 2008). VRE are cocci shaped (see figure 43), gram-positive opportunistic hospital pathogens (Madigan *et al.,* 2009). Like MRSA and *A. baumannii,* infections caused by VRE often affect individuals who are immuno-compromised, elderly or those whom have undergone recent surgery (Masterton, 2005). VRE are able to exchange genes with other gram-positive bacteria and therefore can transfer resistance (Albrich *et al.,* 1999). The number individuals with nosocomial infections caused by VRE increased by 55% between 1993 and 1997 in ICU departments in Taiwan (Hsueh *et al.,* 2002) and became increasingly difficult to treat. The resistance to vancomycin is not just a problem linked to VRE as resistance has occurred in many different organisms, often due to over prescribing of antibiotics and requires education to prevent further resistance (Weber *et al.,* 1999).

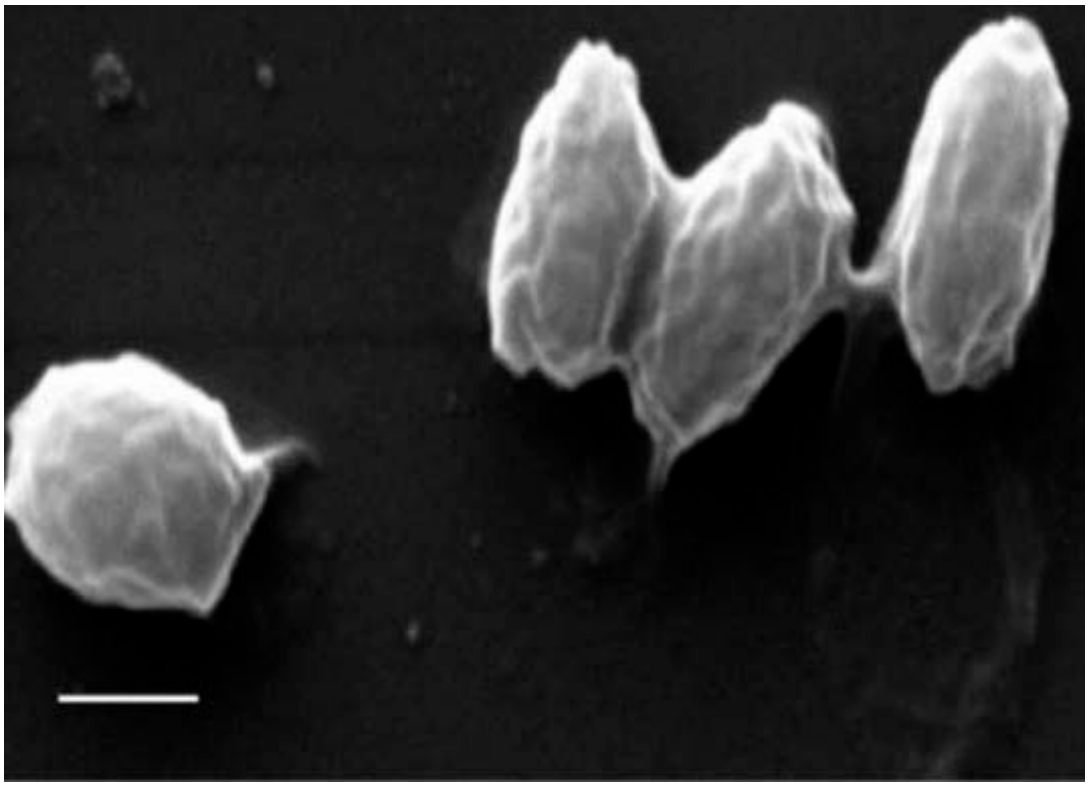
Figure 43. A Scanning electron micrograph of VRE magnified 12000x (CDC, 2005)



*Stenotrophomonas maltophilia* is a gram-negative bacilli and an opportunistic pathogen (figure 44), which can readily colonise epithelial cells in the human

respiratory tract and can cause problems when using medical devices such as respirators (Looney, Narita and Muhlemann, 2009). This can be especially dangerous in immunocompromised patients. Although rare, *S. maltophilia* has been shown to cause meningitis (Yemisen *et al.,* 2008), a condition, which can lead to the death of a patient. *S*. *maltophilia* has been shown to be highly resistant to many clinical antibiotics (Alonso and Martinez, 1997). There are various mechanisms attributed for antibiotic resistance in *S.* maltophilia including active efflux of the antibiotic. *S. maltophilia* can form biofilms, which is another key factor in drug resistance (Liaw, Lee and Hsueh, 2010).

Figure 44. A Scanning electron micrograph of *S. maltophilia* (Nangia *et al.,* 2009)



### 3.1.2 The use of natural compounds in antimicrobial drug discovery

In recent years there has been increasing research into the antimicrobial properties of natural compounds, especially into extracts from honey, herbs and tea (Al-Waili, 2004; Lai and Roy, 2004 and Taguri *et al.,* 2004). Many of the positive effects from these extracts are due to their content of phenolic and polyphenolic compounds (Estevinho *et al.,* 2008 and Hamilton-Miller, 1995).

Meyer *et al.,* (2008) found that catechins and epicatechin from grape seed extracts showed antibacterial activity against 10 different pathogens. An advantage of using natural compounds such as honey, herbs and tea is that there are often less side affects than antibiotics, which can kill off your natural flora and can lead to serious gastrointestinal infections caused by *Clostridium difficile* (Marciniak *et al.*, 2006).

Past research has shown that green tea extracts have significant antibacterial effects against antibiotic resistant isolates of *Acinetobacter* spp. (Jazani *et al*., 2007) and MRSA (Cho, Schiller and Oh, 2008). It was also shown by Osterburg *et al.,* (2009) that epigallocatechin gallate has a significant antibacterial effect against *A. baumannii*. Unfortunately there has been no research undertaken to show if other specific extracts from green tea or black tea have antibacterial properties against *Acinetobacter* spp. It is essential to undertake more susceptibility testing to discover if other catechins in green tea or theaflavins from black tea could potentially have even greater antibacterial effect against this organism. epigallocatechin gallate has been shown to have antifolate activity against *Stenotrophomonas maltophilia* (Navarro-Martinez *et al.,* 2005). Green tea polyphenols have shown to be effective against spore forming bacteria such as *Clostridium thermoaceticum* (Sakanaka, Juneja and Taniguchi, 2000) with the highest activity being form epigallocatechin gallate. Tea extracts have been shown to be effective as antibacterial agents against other organisms including *Listeria spp* (Mbata, Debiao and Saikia, 2006) and strains of *Escherichia coli*, *Shigella spp* and *Salmonella spp* (Bandyopadhyay *et al.,* 2005).

### 3.1.3 Susceptibility testing and determining minimum inhibitionary concentrations (MICs)

*A. baumannii* can be cultured in the laboratory using a variety of different media including susceptibility agar (ISO) and Columbia blood agar (CBA) (Oxoid, 2009). Susceptibility testing is performed to determine the effect of an antibacterial agent on a particular organism (Scorzoni *et al.,* 2007). This can lead to the minimum inhibitory concentration (MIC) of the compound against the

organism being discovered (Andrews, 2001). This can be determined by various methods including growth methods, whereby the growth performance of an organism is monitored with the addition of a compound. Another method is the diffusion method, where the investigated compound in impregnated onto a paper disc and gradually diffuses into agar, showing a circular zone around the disc, if an antibacterial effect occurs (Rios, Recio and Villar, 1988).

## 3.2 Aims

One aim of this study is to conduct a preliminary investigation into the susceptibility of important opportunistic pathogens to tea polyphenols and various antibiotics. The main aim of this study is to discover the potential antibacterial effect of theaflavin against hospital isolates of MRSA, *A.* baumannii and *S.* maltophilia and if any synergy exists with the green tea polyphenol epicatechin and to discover their minimum inhibitory concentrations against *S. maltophilia*. Another aim of this project is to identify if any synergy exists between either theaflavin, epicatechin or a combination of theaflavin and epicatechin with clinical antibiotics against these hospital isolates.

## 3.3 Preliminary antimicrobial testing against MRSA, VRE and *A. baumannii*

### 3.3.1 Microbiology methodologies

All methods were performed using aseptic procedures to reduce the risk of any contamination.

#### 3.3.1.1 Preparing stock cultures

Freeze dried cell cultures for *A. baumannii*, MRSA PLD46 and *Enterococcus faecalis* (VRE) were obtained from BSAC laboratories, UK. To each freeze dried culture 500µL of peptone broth was added and dissolved. Each dissolved culture was then used to inoculate an individual CBA (blood agar) plate and incubated at 37 °C for 24 hours. After this time the stock cultures were stored at 4 °C until further use.

### 3.3.1.2 Method of inoculating ISO plates from stock cultures

The method used for inoculating agar plates, from stock cultures, was based on the method by Moosdeen *et al.,* (1988). Using this method two *A. baumannii* colonies from a blood agar culture were added to 5 mL of a peptide broth and was mixed using a vortex machine. A blank tube containing only peptide broth was used to zero the spectrophotometer before a reading from the inoculated tube was taken. A reading of 0.155 nm was recorded and using the dilution chart 40 µL of the broth was added to 5 mL of sterile distilled water. Swabs were used to inoculate two ISO agar plates from the distilled water.

Two VRE colonies from the blood agar culture were added to 5 mL of a peptide broth and mixed. A blank tube containing only peptide broth was used to zero the spectrophotometer before a reading from the inoculated tube was taken. A reading of 0.232 nm was recorded and using the dilution chart 40 µL of the broth was added to 5 mL of sterile distilled water. Swabs were used to inoculate two ISO agar plates from the distilled water. This was repeated for *S. aureus* where a spectrophotometer reading of 0.814 nm was produced and as a result 20 µL of the peptide broth was added to 5 mL of sterile distilled water.

### 3.3.1.3 Preparation of polyphenol stock solutions

A 60 mM epicatechin solution was prepared by adding 0.174 g of epicatechin to 10 mL of ethanol and mixing for 5-10 min. A 100 mM epigallocatechin gallate was prepared by adding 0.458 g of epigallocatechin gallate to 10 mL of ethanol and mixing for 2 min. A 80 mM theaflavin solution was prepared by adding 0.415 g of theaflavin to 1 mL of ethanol and mixing for 5 min until the compound had dissolved. A 0.05 g/mL solution of mixed theaflavins containing theaflavin, theaflavin 3,3'-digallate and theaflavin monogallate (supplied by Unilever, China) was prepared by adding 0.5 g of the theaflavins powder to 10 mL of ethanol and mixing for 5 min.

All solutions were stored at 4 °C and were replaced every week to avoid any problems with compound stability/degradation.

### 3.3.1.4 Production of impregnated susceptibility discs

All discs were prepared by injecting 10 μL of compound solution at a time onto a blank susceptibility disc using a pipette. The discs were allowed to dry for 30 min before any further solution was added. This method was repeated until the required amount of compound was injected onto each disc. Ethanol control discs were prepared by injecting the same volume of ethanol as the maximum loaded polyphenol disc before allowing to dry. For example, if discs were prepared with a total of 60μL and 80μL of a polyphenol solution, the ethanol disc would be injected with 80 μL of ethanol. All susceptibility discs were dried prior to their addition to a culture plate to eliminate any effects from the ethanol solvent.

### 3.3.1.5 Antibiotic susceptibility testing

To test the organisms used for antibiotic resistance, various commonly used antibiotics were used. For MRSA and VRE susceptibility discs containing Rifampicin (2μg), Penicillin (1 unit – standard purchased concentration), Nitrofurantoin (200μg), Cefotaxime (30μg), Ciprofloxacin (1μg), Tetracycline (10μg), Gentamycin (10μg), Vancomycin (5μg) and Amoxicillin/clavulanic acid (30μg) 2:1 were added to inoculated ISO agar plates and incubated for 24hrs at 37°C. For *A. baumannii* susceptibility discs containing Rifampicin (2μg), Penicillin (1 unit), Nitrofurantoin (200μg), Ampicillin (25μg), Cefotaxime (30μg), Ciprofloxacin (1μg), Tetracycline (10μg), Gentamycin (10μg), Amoxicillin/clavulanic acid (30μg) 2:1 and Vancomycin (5μg) were added to inoculated ISO agar plates and incubated for 24hrs at 37°C.

After 24hrs the area surrounding each disc with no growth (zone of inhibition) was examined. The diameter across the zone was measured and noted. All antibiotic susceptibility tests were repeated to eliminate any abnormalities.

### 3.3.1.6 Susceptibility testing with tea compounds

To test the antibacterial activity of epicatechin against *A. baumannii* susceptibility discs containing 40 μL (0.7 mg of epicatechin), 60 μL (1.0 mg of epicatechin) and 80 μL (1.4 mg of epicatechin) of epicatechin, a 10 mg gentamycin disc and an ethanol control disc were added onto inoculated ISO agar. To test the antibacterial activity of epicatechin against MRSA and VRE susceptibility discs containing 10 μL (0.17 mg of epicatechin), 20 μL (0.35 mg of epicatechin), 40 μL (0.7 mg of epicatechin), 60 μL (1.0 mg of epicatechin) and 80 μL (1.4 mg of epicatechin) of epicatechin and an ethanol control disc were added onto inoculated ISO agar for each organism. All plates were incubated for 24hrs at 37°C.

After 24hrs the area surrounding each disc with no growth (zone of inhibition) was examined. The diameter across the zone was measured and noted. All antibiotic susceptibility tests were repeated to eliminate any abnormalities.

To test the antibacterial activity of epigallocatechin gallate against *A. baumannii*, MRSA and VRE, susceptibility discs containing 10 μL (0.46 mg of epigallocatechin gallate), 20 μL (0.92 mg of epigallocatechin gallate), 40 μL (1.8 mg of epigallocatechin gallate), 60 μL (2.7 mg of epigallocatechin gallate) and 80 μL (3.7 mg of epigallocatechin gallate) of epigallocatechin gallate and an ethanol control disc were added onto inoculated ISO agar for each organism. All plates were incubated for 24 h at 37 °C.

After 24 h the area surrounding each disc with no growth (zone of inhibition) was examined. The diameter across the zone was measured and noted. All antibiotic susceptibility tests were repeated to eliminate any abnormalities.

To test the antibacterial activity of theaflavin against *A. baumannii* and VRE susceptibility discs containing 25 μL (1.1 mg of theaflavin), 30 μL (1.4 mg of

theaflavin), 35 μL (1.6 mg of theaflavin) and 40 μL (1.8 mg of theaflavin) of theaflavin, gentamycin (10 mg) and an ethanol control disc were added onto inoculated ISO agar for each organism. All plates were incubated for 24 h at 37 °C. After 24 h the area surrounding each disc with no growth (zone of inhibition) was examined. The diameter across the zone was measured and noted. All antibiotic susceptibility tests were repeated to eliminate any abnormalities.

To test the antibacterial activity of mixed theaflavins against *A. baumannii,* MRSA and VRE, susceptibility discs containing 10 μL (0.5 mg of mixed theaflavins), 20 μL (1.0 mg of mixed theaflavins), 40 μL (2.0 mg of mixed theaflavins), 60 μL (3.0 mg of mixed theaflavins) and 80 μL (4.0 mg of mixed theaflavins) of mixed theaflavins and an ethanol control disc were added onto inoculated ISO agar for each organism. All plates were incubated for 24 h at 37 °C. After 24 h the area surrounding each disc with no growth (zone of inhibition) was examined. The diameter across the zone was measured and noted. All antibiotic susceptibility tests were repeated to eliminate any abnormalities.

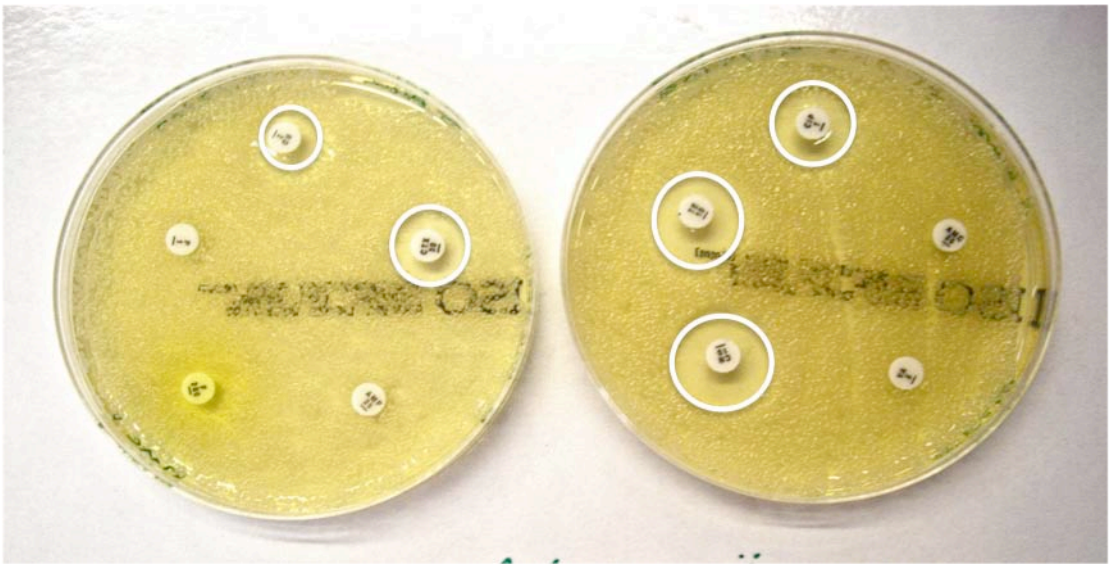
### 3.4 Results and discussion for preliminary antibacterial testing

During this study any chemical that created a zone of inhibition ≥7.5 mm was considered to have antibacterial activity.

#### 3.4.1 Antibiotic susceptibility testing

This results in figures 45 and table 7 show that the *A. baumannii* used was resistant to penicillin, nitrofurantoin, ampicillin, vancomycin and amoxicillin/clavulanic acid. It can be seen in figure 45 that their zones including the disc were not above 6.5 mm. All of these antibiotics are commonly used in treating a variety of infections. However, in the case of an infection caused by *A. baumannii* they would be very unsuccessful. In this study the antibiotic with the greatest effect against *A. baumannii* was gentamycin.

Figure 45. Antibiotic disc susceptibility testing for *A. baumannii* on ISO-agar.



Discs on the left petri dish from the top moving anti-clockwise are 2μg rifampicin, 1 unit penicillin, 200 μg nitrofurantoin, 25 μg ampicillin and 30 μg cefotaxime. Discs on the right petri dish from the top moving anti-clockwise are 1 μg ciprofloxacin, 10 μg Tetracycline, 10 μg gentamycin, 5 μg vancomycin and 30 μg amoxicillin/clavulanic acid 2:1. Zone outlines are highlighted.

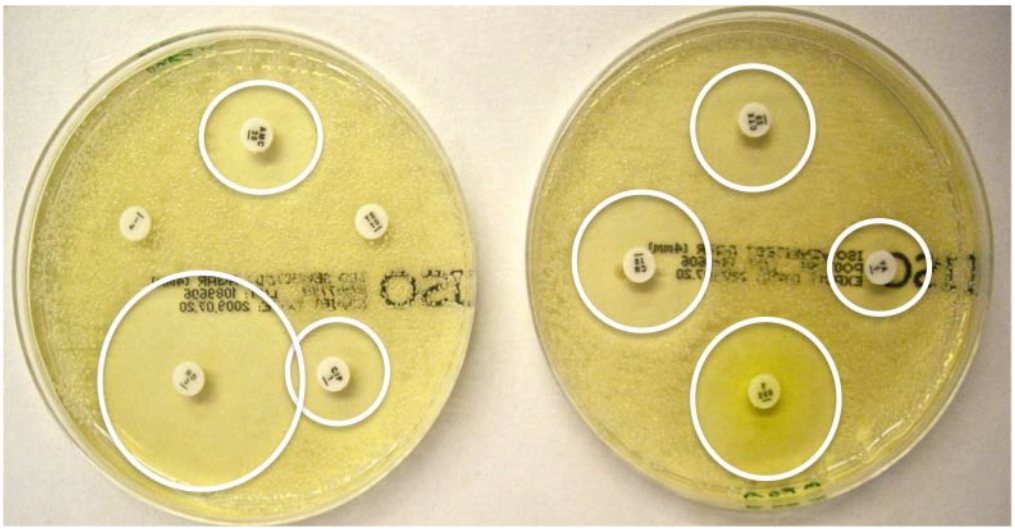
Table 7. Showing the zones of inhibition for a variety of antibiotics against the bacterium *A. baumannii*

| Antibiotic | Zone of inhibition (mm)* |
|---|---|
| Rifampicin (2 μg) | 14.5 |
| Penicillin (1 unit) | 6.5 |
| Nitrofurantoin (200 μg) | 8.5 |
| Ampicillin (25 μg) | 6.5 |
| Cefotaxime (30 μg) | 12.5 |
| Ciprofloxacin (1 μg) | 14.5 |
| Tetracycline (10 μg) | 16.5 |
| Gentamycin (10 μg) | 18.5 |
| Vancomycin (5 μg) | 6.5 |
| Amoxicillin/clavulanic acid (30 μg) 2:1 | 6.5 |

* note: the zone of inhibition includes the disc diameter of 6.5mm

Figure 46 and table 8 clearly show that MRSA in this case was resistant to penicillin, tetracycline and cefotaxime with zones not greater than 6.5 mm. The antibiotic with the greatest effect against MRSA in this study was nitrofurantoin an antibiotic commonly used to treat urinary tract infections (Lee *et al.,* 2008).

Figure 46. Antibiotic disc susceptibility testing for MRSA on ISO-agar.



Discs on the left petri dish from the top moving anti-clockwise are 30 μg amoxicillin/clavulanic acid 2:1, 1 unit penicillin, 2 μg rifampicin, 1 μg ciprofloxacin and 10 μg Tetracycline. Discs on the right petri dish from the top moving anti-clockwise are 30 μg cefotaxime, 10 μg gentamycin, 200 μg nitrofurantoin and 5 μg vancomycin. Zone outlines are highlighted.

From Table 8 gentamycin and ciprofloxacin would be useful oral antibiotics to treat an infection from this strain of MRSA, where often intravenous vancomycin would normally be given (Zvethkova, Laffeach and Goldstein, 2004).

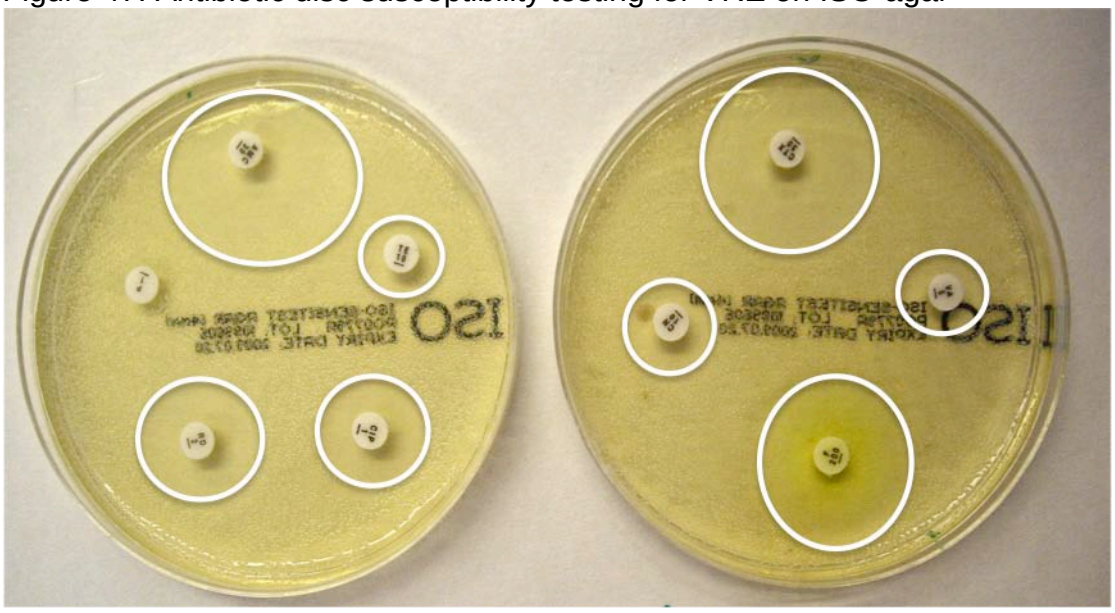
In this study VRE showed resistance to less antibiotics than MRSA and *A. baumannii* with the results in figure 47 and table 9 showing only resistance to penicillin and lower effectiveness for the antibiotics gentamycin and tetracycline. Surprisingly this strain of VRE showed no resistance against vancomycin (5 μg). However previous research found MIC values of vancomycin for VRE are greater than 128 μg/mL (CDC, 1999).

Table 8. Zones of inhibition for a variety of antibiotics against MRSA

| Antibiotic | Zone of inhibition (mm)* |
|---|---|
| Rifampicin (2 μg) | 40.5 |
| Penicillin (1 unit) | 6.5 |
| Nitrofurantoin (200 μg) | 27.5 |
| Cefotaxime (30 μg) | 22.5 |
| Ciprofloxacin (1 μg) | 21.5 |
| Tetracycline (10 μg) | 6.5 |
| Gentamycin (10 μg) | 26.5 |
| Vancomycin (5 μg) | 19.5 |
| Amoxicillin/clavulanic acid (30 μg) 2:1 | 20.5 |

* note: the zone of inhibition includes the disc diameter of 6.5mm

Figure 47. Antibiotic disc susceptibility testing for VRE on ISO-agar



Discs on the left petri dish from the top moving anti-clockwise are 30 μg amoxicillin/clavulanic acid 2:1, 1 unit penicillin, 2 μg rifampicin, 1 μg ciprofloxacin and 10 μg Tetracycline. Discs on the right petri dish from the top moving anti-clockwise are 30 μg cefotaxime, 10 μg gentamycin, 200 μg nitrofurantoin and 5 μg vancomycin. Zone outlines are highlighted.

Table 9. Zones of inhibition for a variety of antibiotics against VRE

| Antibiotic | Zone of inhibition (mm)* |
|---|---|
| Rifampicin (2 μg) | 20.5 |
| Penicillin (1 unit) | 6.5 |
| Nitrofurantoin (200 μg) | 28.5 |
| Cefotaxime (30 μg) | 30.5 |
| Ciprofloxacin (1 μg) | 18.5 |
| Tetracycline (10 μg) | 10.5 |
| Gentamycin (10 μg) | 11.5 |
| Vancomycin (5 μg) | 14.5 |
| Amoxicillin/clavulanic acid (30 μg) 2:1 | 32.5 |

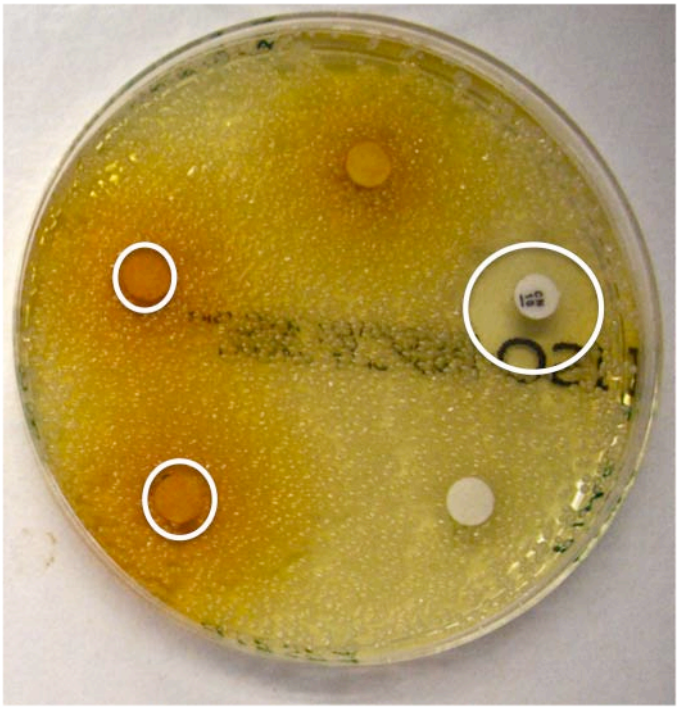
* note: the zone of inhibition includes the disc diameter of 6.5mm

### 3.4.2 Theaflavin, epicatechin and epigallocatechin gallate susceptibility testing

Interesting results were observed from the polyphenol testing. Colour diffusion into the agar that often was greater that the zone of inhibition. Other variations in colour are due to unintentional differences in photo exposure. Results from the EC susceptibility testing for *A. baumannii,* which can be seen in figure 48 and table 10 showed very little antibacterial activity. On the disc containing 0.7 μg no antibacterial action was seen.

In figure 48 a significant colour change can be seen where epicatechin has diffused into the ISO agar. This could be due to the compound being metabolised by the organism or that some decomposition is taking place. In the susceptibility discs containing 1.0 mg and 1.4 mg of epicatechin, little antibacterial action was seen against *A. baumannii*. However results shown in table 10 are encouraging and indicate that if the concentration was increased it could result in a much improved antibacterial activity. In this preliminary study the MIC of Epicatechin against *A. baumannii* was found to be 1.0 mg.

Figure 48. *A. baumannii* susceptibility testing with epicatechin (EC).



Discs on the petri disc are from the top moving anti-clockwise 0.7 mg EC, 1.0 mg EC, 1.4 mg EC, ethanol control disc and 10 μg gentamycin. Zone outlines are highlighted.

Table 10. Mean zones of inhibition for epicatechin (EC) at various concentrations against *A. baumannii*

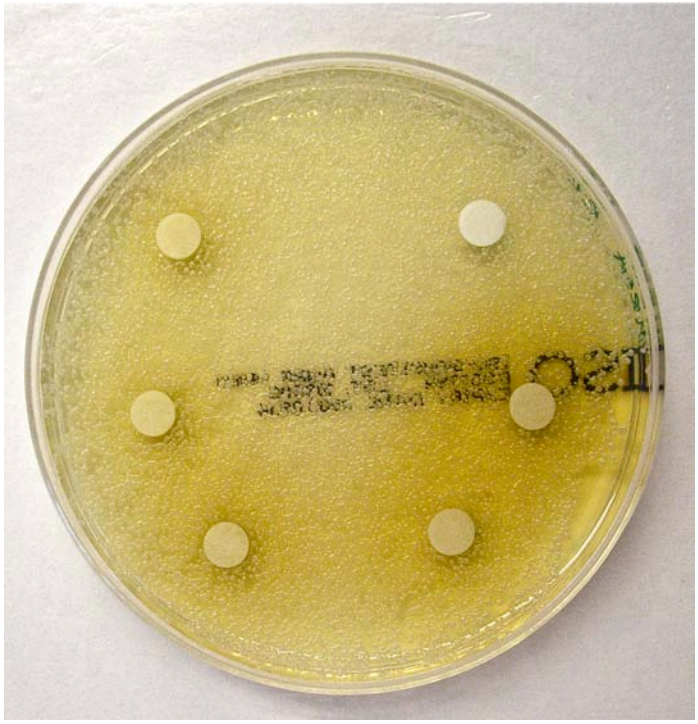
| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 0.7 mg EC | 6.5 |
| 1.0 mg EC | 8.5 |
| 1.4 mg EC | 9.5 |
| Ethanol control | 6.5 |
| 10μg gentamycin | 20.5 |

* note: the zone of inhibition includes the disc diameter of 6.5mm

In the epicatechin susceptibility testing against MRSA no antibacterial activity was seen with any of the compound amounts on the discs. Like with *A. baumannii* it is possible this could be improved by further increasing the concentration of epicatechin on the discs in a future investigation. In past investigations no antibacterial activity was seen with catechin (epicatechin's

stereo isomer) against *S. aureus* (Stapleton *et al.,* 2004). In figure 49 increasing decolouration of the agar can be seen which corresponds to an increase in the amount of epicatechin on the susceptibility discs. In table 11 the lack of antibacterial activity of the compound is shown as the zone of inhibition does not increase above 6.5 mm, which is the diameter of the disc. At these concentrations it is suspected that epicatechin would not be a viable agent to treat a MRSA infection either *in Vivo* or topically.

Figure 49. MRSA susceptibility testing with epicatechin (EC).



The discs from the top moving anti-clockwise on the petri dish are 0.17 mg EC, 0.35 mg EC, 0.7 mg EC, 1.0 mg EC, 1.4 mg EC and an ethanol control disc

Like in the case of MRSA it can be seen from figure 50 and table 12 that epicatechin had no antibacterial activity against VRE. The results from table 11 show that the compound on all the discs failed to achieve a zone of inhibition greater than 6.5 mm (the disc diameter). Like in the tests for *A. baumannii* and MRSA a small increasing colour change can be seen as the compound concentration increases, indicating its decomposition or that it has been metabolised giving a coloured by-product.

Table 11. Mean zones of inhibition for epicatechin (EC) at various concentrations against MRSA

| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 0.17 mg EC | 6.5 |
| 0.35 mg EC | 6.5 |
| 0.7 mg EC | 6.5 |
| 1.0 mg EC | 6.5 |
| 1.4 mg EC | 6.5 |
| Ethanol control | 6.5 |

* note: the zone of inhibition includes the disc diameter of 6.5 mm

Figure 50. VRE susceptibility testing with epicatechin (EC).

The discs from the top moving anti-clockwise on the petri dish are 0.17 mg EC, 0.35 mg EC, 0.7 mg EC, 1.0 mg EC, 1.4 mg EC and an ethanol control disc.

Due to this result it can be concluded that it is highly unlikely epicatechin will have a antibacterial effect even if the concentration is doubled. This is also

confirmed from previous research by Cushnie *et al.,* (2008), who found no antibacterial activity of epicatechin against VRE at similar concentrations.

Table 12. Mean zones of inhibition for epicatechin (EC) at various concentrations against VRE

| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 0.17 mg EC | 6.5 |
| 0.35 mg EC | 6.5 |
| 0.7 mg EC | 6.5 |
| 1.0 mg EC | 6.5 |
| 1.4 mg EC | 6.5 |
| Ethanol control | 6.5 |

* note: the zone of inhibition includes the disc diameter of 6.5mm

Epigallocatechin gallate showed a high antibacterial activity against *A. baumannii* and in figure 51 and table 13 zones of inhibition of up to 19 mm can be seen. These results exceed that of all the antibiotics in table 7 that were tested. The antibiotic with the highest antibacterial activity shown in table 7 was gentamycin with a zone of 18.5 mm. This shows that epigallocatechin gallate is a very strong antimicrobial agent as the 19 mm zone was created by only 3.7 mg of epigallocatechin gallate whereas 10 μg of gentamycin produced the 18.5 mm zone. The MIC of epigallocatechin gallate was found to be 0.92 mg in this preliminary study.

In figure 51 an increasing large coloured zone can be seen which corresponds to the increase in epigallocatechin gallate on the susceptibility discs.

Figure 51. *A. baumannii* susceptibility testing with epigallocatechin gallate (EGCG)

The discs from the top moving anti-clockwise on the petri dish are 0.46 mg EGCG, 0.92 mg EGCG, 1.8 mg EGCG, 2.7 mg EGCG, 3.7 mg EGCG and an ethanol control disc. Zone outlines are highlighted.

Table 13. Mean zones of inhibition for epigallocatechin gallate (EGCG) at various concentrations *A. baumannii*

| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 0.46 mg EGCG | 6.5 |
| 0.92 mg EGCG | 8.0 |
| 1.8 mg EGCG | 14.5 |
| 2.7 mg EGCG | 17.0 |
| 3.7 mg EGCG | 19.0 |
| Ethanol control | 6.5 |

* note: the zone of inhibition includes the disc diameter of 6.5 mm

From figure 52 and table 14 the high antibacterial effect of epigallocatechin gallate is observed against MRSA. From these results a maximum zone of 29 mm from 3.7 mg of epigallocatechin gallate can be seen which exceeds all the zones from antibiotics shown in table 8 with the exception of rifampicin. This confirms again that epigallocatechin gallate is a powerful antibacterial agent with an MIC in this study of 0.46 mg. The high antibacterial effect of epigallocatechin gallate against MRSA confirms the research of many groups including that of Cho, Schiller and Oh (2008). However, in that particular study the MIC ranged from 50-180 mg/mL which is higher than that found in this investigation.

Figure 52. MRSA susceptibility testing with epigallocatechin gallate (EGCG).

The discs from the top moving anti-clockwise on the petri dish are 0.46 mg EGCG, 0.92 mg EGCG, 1.8 mg EGCG, 2.7 mg EGCG, 3.7 mg EGCG and an ethanol control disc. Zone outlines are highlighted.

As with the *A. baumannii* test in figure 48 an increasing large coloured zone can be seen which corresponds to the increase in epigallocatechin gallate on the susceptibility discs

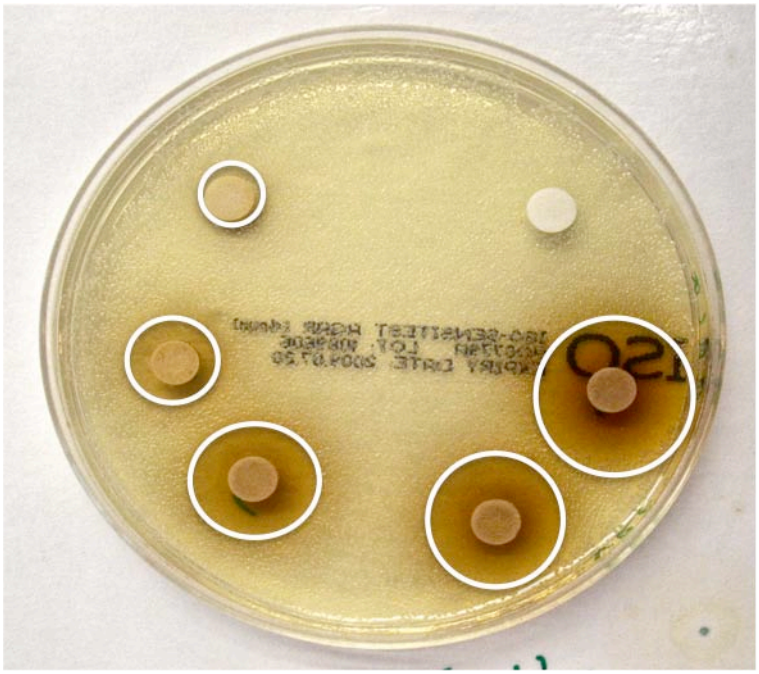
epigallocatechin gallate showed to be an effective antibacterial agent against VRE and in figure 53 and table 15 significant zones of inhibition can be seen. This suggests that in this study epigallocatechin gallate is a greater antibacterial agent that over 50% of the antibiotics tested in table 9.

Table 14. Mean zones of inhibition for epigallocatechin gallate (EGCG) at various concentrations against MRSA

| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 0.46 mg EGCG | 9 |
| 0.92 mg EGCG | 17 |
| 1.8 mg EGCG | 22 |
| 2.7 mg EGCG | 25 |
| 3.7 mg EGCG | 29 |
| Ethanol control | 7 |

* note: the zone of inhibition includes the disc diameter of 6.5mm

Figure 53 Showing VRE susceptibility testing with epigallocatechin gallate (EGCG).



The discs from the top moving anti-clockwise on the petri dish are 0.46 mg EGCG, 0.92 mg EGCG, 1.8 mg EGCG, 2.7 mg EGCG, 3.7 mg EGCG and an ethanol control disc. Zone outlines are highlighted.

The largest zone of inhibition was 19.5 mm from a disc containing 3.7 mg of epigallocatechin gallate, which is greater than that by ciprofloxacin, which created a zone of 18.5 mm, although this was from only 1 μg. This still proves that epigallocatechin gallate could be a very effective agent in treating infections caused by VRE.

Figure 15. Mean zones of inhibition for epigallocatechin gallate (EGCG) at various concentrations against VRE

| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 0.46 mg EGCG | 9 |
| 0.92 mg EGCG | 11 |
| 1.8 mg EGCG | 15 |
| 2.7 mg EGCG | 18 |
| 3.7 mg EGCG | 19.5 |
| Ethanol control | 6.5 |

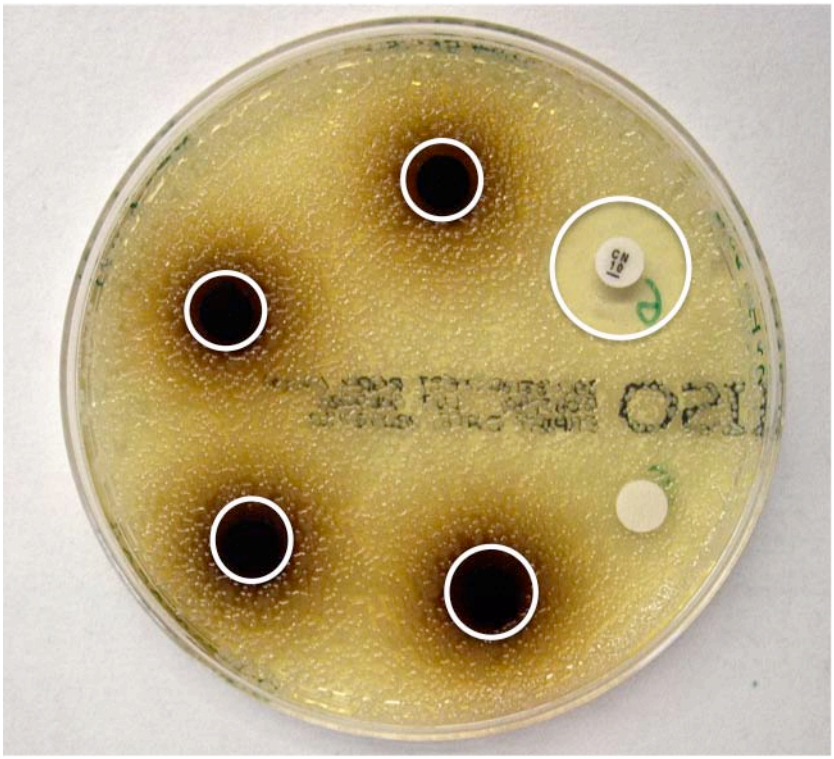
* note: the zone of inhibition includes the disc diameter of 6.5 mm

Epigallocatechin gallate has shown to be a much greater antibacterial agent than epicatechin. This is possibly be due to the gallate group and also could be related to the increase in the number of phenol groups, which have been found to have antibacterial properties.

Theaflavin also showed antibacterial effects against *A. baumannii,* which can be seen in figure 53 and table 16. Although the zones of inhibition produce by theaflavin are not as large as those by epigallocatechin gallate it still shows a significant antibacterial effect. In this study 1.8 mg of theaflavin produces a zone of 11.5 mm which is almost equal to that produced by 30 μg of Cefotaxime (see table 7). In figure 53 zone around the discs containing theaflavin can clearly be seen. This is the first time the antibacterial effects of pure theaflavin have been seen against *A. baumannii* and are encouraging that it could be used as a

future treatment against infections caused by the organism. The MIC of theaflavin against *A. baumannii* was shown in table 16 to be ≤1.1 mg.

Figure 53. *A. baumannii* susceptibility testing with theaflavin.



The discs from the top moving anti-clockwise on the petri dish are 1.1 mg theaflavin, 1.4 mg theaflavin, 1.6 mg theaflavin, 1.8 mg theaflavin, an ethanol control disc and 10 μg gentamycin. Zone outlines are highlighted.

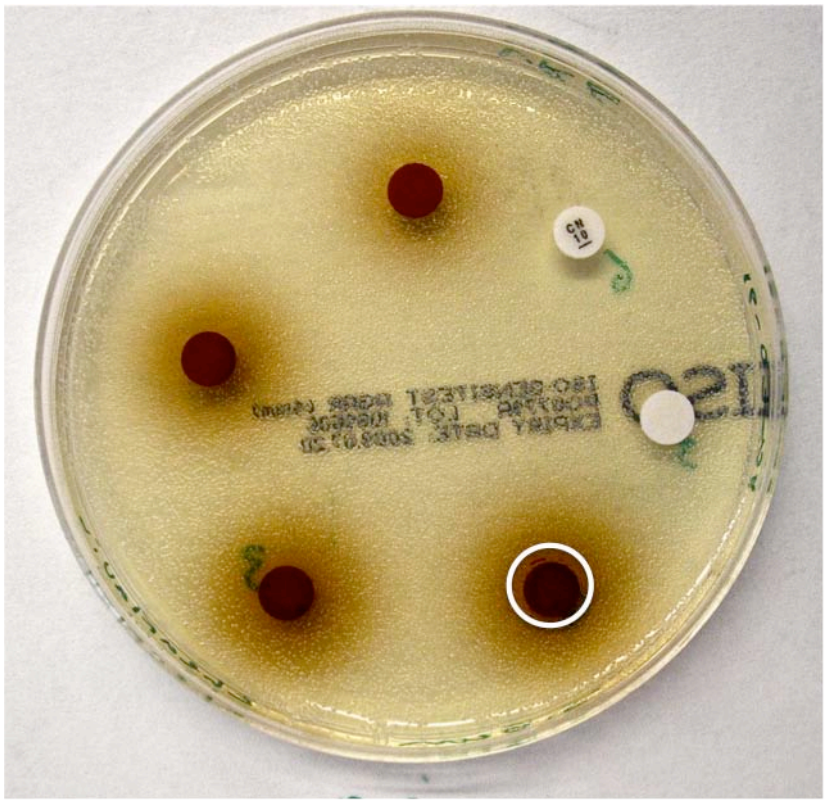
Table 16. Mean zones of inhibition for theaflavin at various concentrations against *A. baumannii*

| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 1.1 mg theaflavin | 8.5 |
| 1.4 mg theaflavin | 9.5 |
| 1.6 mg theaflavin | 10.5 |
| 1.8 mg theaflavin | 11.5 |
| Ethanol control | 6.5 |
| 10μg gentamycin | 19.5 |

* note: the zone of inhibition includes the disc diameter of 6.5 mm

Theaflavin showed some antibacterial effects against VRE at higher concentrations. From figure 54 and table 17 it can be seen that theaflavin only had an antibacterial effect with the disc containing 1.8 mg of the compound where it produced a zone of inhibition of 8.5 mm. Although this was still less than the effect of tetracycline (10.5 mm), it was still more effective than penicillin as can be seen from table 9. Diffusion of theaflavin into the ISO agar can be seen in figure 54. The MIC of theaflavin for VRE in this study was 1.8 mg. It is possible that if the concentration of theaflavin on the discs was increased a greater antibacterial effect would be seen against VRE.

Figure 54. VRE susceptibility testing with theaflavin.



The discs from the top moving anti-clockwise on the petri dish are 1.1 mg theaflavin, 1.4 mg theaflavin, 1.6 mg theaflavin, 1.8 mg theaflavin, an ethanol control disc and 10μg gentamycin. Zone outlines are highlighted.

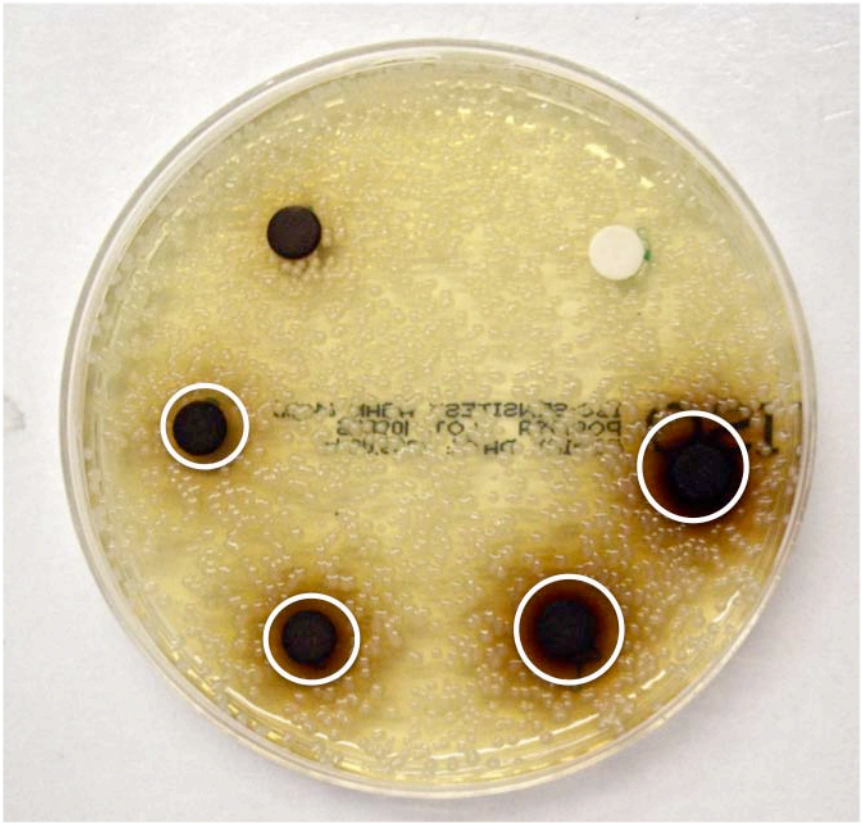
Table 17. Mean zones of inhibition for theaflavin at various concentrations against VRE

| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 1.1 mg theaflavin | 6.5 |
| 1.4 mg theaflavin | 6.5 |
| 1.6 mg theaflavin | 6.5 |
| 1.8 mg theaflavin | 8.5 |
| Ethanol control | 6.5 |
| 10μg gentamycin | 6.5 |

* note: the zone of inhibition includes the disc diameter of 6.5 mm

Mixed theaflavins showed a significant antibacterial effect against *A. baumannii* producing a maximum zone of inhibition in this study of 14 mm with 4.0 mg. This can be seen in figure 55 and table 18 where a MIC of 1.0 mg was discovered. Overall, mixed theaflavins showed to be a less effective antimicrobial agent against *A. baumannii* than EGCG. It was also less effect against *A. baumannii* than pure theaflavin where 2.0 mg of mixed theaflavins produced a zone of 10 mm (table 18) where as pure theaflavin produced a zone of 11.5 mm for 1.8 mg. This could be due to the mixed theaflavins containing 3 different theaflavins and therefore each one at a lower concentration that the pure theaflavin alone. This could suggest that there is little synergy between the different theaflavins against *A. baumannii*. In this case it is shown that mixed theaflavins have almost the same antibacterial effect as rifampicin and ciprofloxacin (Table 7).

Figure 55. *A. baumannii* susceptibility testing with mixed theaflavins.



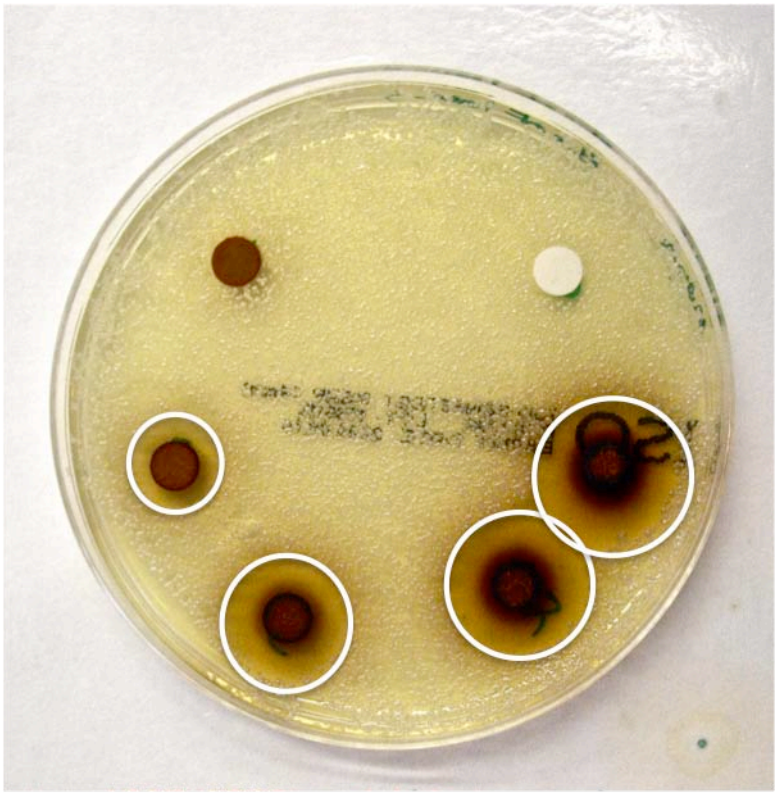
The discs from the top moving anti-clockwise on the petri dish are 0.5 mg mixed theaflavins, 1.0 mg mixed theaflavins, 2.0 mg mixed theaflavins, 3.0 mg mixed theaflavins, 4.0 mg mixed theaflavins and an ethanol control disc. Zone outlines are highlighted.

Figure 18. Mean zones of inhibition for mixed theaflavins at various concentrations against *A. baumannii*

| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 0.5 mg mixed theaflavins | 6.5 |
| 1.0 mg mixed theaflavins | 7.5 |
| 2.0 mg mixed theaflavins | 10 |
| 3.0 mg mixed theaflavins | 12 |
| 4.0 mg mixed theaflavins | 14 |
| Ethanol control | 6.5 |

* note: the zone of inhibition includes the disc diameter of 6.5 mm

Figure 56. MRSA susceptibility testing with mixed theaflavins.



The discs from the top moving anti-clockwise on the petri dish are 0.5 mg mixed theaflavins, 1.0 mg mixed theaflavins, 2.0 mg mixed theaflavins, 3.0 mg mixed theaflavins, 4.0 mg mixed theaflavins and an ethanol control disc. Zone outlines are highlighted.

Table 19. Showing the mean zones of inhibition for mixed theaflavins at various concentrations against MRSA

| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 0.5 mg mixed theaflavins | 6.5 |
| 1.0 mg mixed theaflavins | 12.0 |
| 2.0 mg mixed theaflavins | 16.0 |
| 3.0 mg mixed theaflavins | 18.0 |
| 4.0 mg mixed theaflavins | 19.0 |
| Ethanol control | 6.5 |

* note: the zone of inhibition includes the disc diameter of 6.5 mm

The results in figure 56 and table 19 indicated that mixed theaflavins have a significant effect against MRSA with a maximum zone of inhibition in this case of 19 mm for 4.0 mg of the compounds. This was comparable with 5μg of vancomycin as shown in figure 27. The MIC for mixed theaflavins was found to be 1.0 mg. Although mixed theaflavins showed significant antibacterial effects there were not as great as those from epigallocatechin gallate against MRSA.

From the information in figure 57 and table 20 it can be clearly seen that mixed theaflavins had a significant antibacterial effect against VRE. With a maximum zone of inhibition of 12 mm it was as effective against VRE as the antibiotic gentamycin (table 8). Mixed theaflavins were more also more effective against VRE than pure theaflavin with a zone of 9 mm with 1.0 mg of the compounds rather than the 8.5 mm for 1.8 mg of pure theaflavin (almost twice the amount).

Figure 57. VRE susceptibility testing with mixed theaflavins.

The discs from the top moving anti-clockwise on the petri dish are 0.5 mg mixed theaflavins, 1.0 mg mixed theaflavins, 2.0 mg mixed theaflavins, 3.0 mg mixed theaflavins, 4.0 mg mixed theaflavins and an ethanol control disc. Zone outlines are highlighted.

A possible reason for the greater antibacterial effect of mixed rather than pure theaflavin is due to the increase in OH groups on the gallate forms. This should be investigated in future studies.

An MIC of 1.0 mg was discovered for the mixed theaflavins against VRE in this study. This is over twice the quantity than the amount of epigallocatechin gallate required to produce the same zone for its MIC.

Table 20. Mean zones of inhibition for mixed theaflavins at various concentrations against VRE

| Disc content | Mean Zone of inhibition (mm)* |
|---|---|
| 0.5 mg mixed theaflavins | 6.5 |
| 1.0 mg mixed theaflavins | 9.0 |
| 2.0 mg mixed theaflavins | 10.0 |
| 3.0 mg mixed theaflavins | 11.0 |
| 4.0 mg mixed theaflavins | 12.0 |
| Ethanol control | 6.5 |

* note: the zone of inhibition includes the disc diameter of 6.5 mm

Although epigallocatechin gallate, theaflavin and mixed theaflavins show antibacterial effects against all three organisms in this study the effect *in Vivo* is not entirely known and the concentrations needed to be effective in treating an infection could also be toxic to the patient. However, even if this were so the compounds could be used topically to treat for example infections of the skin or abscesses.

## 3.5 Antimicrobial and synergistic testing against hospital isolates of MRSA, *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*

### 3.5.1 Clinical isolates

*A. baumannii* strains were isolated from sputum samples of respiratory patients at Northern General Hospital, Sheffield, UK. *S. maltophilia* isolates were obtained from sputum samples of respiratory patients at Hull Royal Infirmary, UK. All strains of *A. baumannii* and *S. maltophilia* were isolated on blood agar and identified via antibiotic susceptibility tests and biochemical profiling using API 20E testing kits (bioMérieux, France). Hospital isolates of MRSA were obtained from routine screening swabs at Northern General Hospital, Sheffield, UK. All isolates were identified using Chromogenic media, Staph latex kits, DNase and antibiotic susceptibility tests. Gram stains were performed on all received isolates to check for correct morphology and gram type. Control strains of *A. baumannii* (NCTC 13485) and *S. maltophilia* (NCTC 13014) were purchased from the Health Protection Agency Culture Collections, Porton Down, UK.

### 3.5.2 Tea polyphenols

Epicatechin (EC) with purity ≥ 90% was purchased from Sigma-Aldrich, UK. Theaflavin (TF) and epigallocatechin (EGC) samples each with a purity ≥ 95% were donated by Unilever, Shanghai, China.

### 3.5.3 Culture media, antibiotics and other materials

Antibiotic discs including ampicillin (AMP) 25 μg, gentamycin (CN) 10 μg, imipenem (IPM) 10 μg, tetracycline (TE) 10 μg, cefoxitin (FOX) 10 μg and ciprofloxacin (CIP) 1 μg were purchased from Oxoid, UK. Blank susceptibility discs were purchased from Mastdiscs, UK. Iso-sensitive agar and Iso-sensitive broth powders were purchased from Oxoid, UK. All media and materials were autoclaved before their use to ensure sterility.

### 3.5.4 Methodologies for hospital isolate testing

#### 3.5.4.1 Microbiology methodologies

All methods were performed using aseptic procedures to reduce the risk of any contamination.

#### 3.5.4.2 Gram stains

Gram stains were performed on all clinical isolates. For each isolate a colony was taken from a previously incubated culture on blood agar. The colony was emulsified onto a glass slide with 1 drop of sterile water and left to dry for 5 minutes. When dried the slide was passed through the blue flame of a Bunsen burner to fix the cells. The slide was then flooded with crystal violet to stain the bacteria and left for 1 minute before being briefly washed with water. The slide was then flooded with iodine and left for 1 minute and after which was washed with water and drained. Ethanol (95%) was then used as a decolourizer for 10 seconds before being washed off with water. Finally the slide was flooded with a counterstain (Safranin) for 30 seconds and then washed off with water. The slide was drained and blotted with blotting paper and observed under a microscope using a x100 oil immersion lens.

When observing the slide any cells stained purple were considered gram-positive due to the crystal violent stain adhering the cells thick peptidoglycan cell wall. Any cells stained pink were considered gram-negative due to their thin cell wall, which is stained pink by the counter stain.

#### 3.5.4.3 Stock culture preparation

All clinical isolates were inoculated into an individual CBA (blood agar) plates and incubated at 37 °C for 24 hours. After this time the stock cultures were stored at 4 °C until further use.

### 3.5.4.4 Method inoculating ISO plates from stock cultures

The method used for inoculating agar plates from stock cultures, was based on the method by Moosdeen *et al.,* (1988) and is the same as that described in the methodologies for the preliminary antimicrobial testing.

### 3.5.4.5 Preparation of polyphenol stock solutions

Two epicatechin stock solutions were prepared. One stock solution was a 25 mg/mL and the other which was a 50 mg/mL. The 25 mg/mL was prepared by adding 0.25 g of epicatechin to 10 mL of ethanol and mixed for 15-20 min. The 50 mg/mL was prepared by adding 0.5 g of epicatechin to 10 mL of ethanol and mixed for 25 min. A 50 mg/mL theaflavin stock solution was prepared by adding 0.5 g of theaflavin to 10 mL of ethanol and mixed for 5 min until the compound had dissolved. A stock solution of theaflavin and epicatechin was prepared by adding 0.5 g of the theaflavin and 0.25 g of epicatechin to 10 mL of ethanol and mixed for 5 min. This produced a combination solution of 50 mg/mL theaflavin:25 mg/mL epicatechin. All stock solutions were stored at 4 °C and were replaced every week to avoid any problems with compound stability/degradation.

### 3.5.4.6 Production of impregnated susceptibility discs

As in the preliminary testing, discs were prepared by injecting 10 μL of compound solution at a time onto a blank susceptibility disc using a pipette. The discs were allowed to dry for 30 min before any further solution was added. This method was repeated until the required amount of compound was injected onto each disc. Ethanol control discs were prepared by injecting the same volume of ethanol as the maximum loaded polyphenol disc before allowing to dry. For example, if discs were prepared with a total of 60 μL and 80 μL of a polyphenol solution, the ethanol disc would be injected with 80 μL of ethanol. The experimental organisation can be seen in table 21.

Table 21. Experimental organisation of inoculated discs on plates 1-6 for MRSA, *A. baumannii* and *S. maltophilia*, where EC = Epicatechin, TF = Theaflavin and TF:EC = Theaflavin:epicatechin combination (2:1)

| | Disc 1 | Disc 2 | Disc 3 | Disc 5 |
|---|---|---|---|---|
| Plate 1 | EC (4 mg) | EC (3 mg) | EC (2 mg) | Ethanol |
| Plate 2 | EC (1 mg) | EC (0.75 mg) | EC (0.5 mg) | Ethanol |
| Plate 3 | TF (4 mg) | TF (3 mg) | TF (2 mg) | Ethanol |
| Plate 4 | TF (1 mg) | TF (0.75 mg) | TF (0.5 mg) | Ethanol |
| Plate 5 | TF:EC (4 mg:2 mg) | TF:EC (3 mg:1.5 mg) | TF:EC (2 mg:1 mg) | Ethanol |
| Plate 6 | TF:EC (1 mg:0.5 mg) | TF:EC (0.75 mg:0.375 mg) | TF:EC (0.5 mg:0.25 mg) | Ethanol |

All susceptibility discs were dried prior to their addition to a culture plate to eliminate any effects from the ethanol solvent. All isolates were incubated at 37°C for 24 hours. At the end of the incubation period, zones of inhibition around each disc were measured (mm) and recorded. Results are presented as the Mean Value +/- Standard Error on the Mean.

### 3.5.5 Determination of minimum inhibitory concentrations (MICs)

To determine the MIC of epicatechin, theaflavin and a combination of theaflavin and epicatechin against each isolate of *S. maltophilia*, a microtiter assay was performed. For this assay 9 clinical isolates and 1 control strain (NCTC 13014) of *S. maltophilia* were used. Stock solutions of each compound were first prepared in sterile dimethyl sulfoxide (DMSO). A 80 mg/ml stock solution of TF was produced by adding 160 mg of theaflavin to 2 mL of DMSO and mixed to dissolve for 5 minutes. To make a 80 mg:40 mg/mL of TF:EC 160 mg of theaflavin and 80 mg of epicatechin were added to 2 mL of DMSO and mixed to dissolved for 5-10 minutes. A 100 mg/mL stock solution of EC was produced by dissolving 200 mg of EC into 2 mL of DMSO and mixed for 5-10 minutes.

A serial dilution of each stock solution was then performed into sterile ISO-sensitive broth. For this 40 μL of stock solution was added to a sterile tube containing 2 mL of ISO-sensitive broth. From this solution a double dilution was performed 4 times, giving 5 different concentrations of the polyphenol in ISO-sensitive broth and a tube containing just ISO-sensitive broth, used as a control.

For each concentration of theaflavin in broth, 75 μL was pipetted into 10 horizontal wells of a 96 well cell culture plate (1 well per concentration per isolate). To each well 75 μL of a 0.5 MacFarland solution of an isolate of *S. maltophilia* was pipetted. The organisation of the 96 well plate can be seen in Figure 58. The procedure was repeated for each compound giving final well concentrations, which can be seen in table 22. Each plate was incubated in a rocking incubator at 30°C for 24 hours.

Figure 58. Organisation of a 96 well cell culture plate for MIC testing where C = Control strain and B = Blank (no isolate).

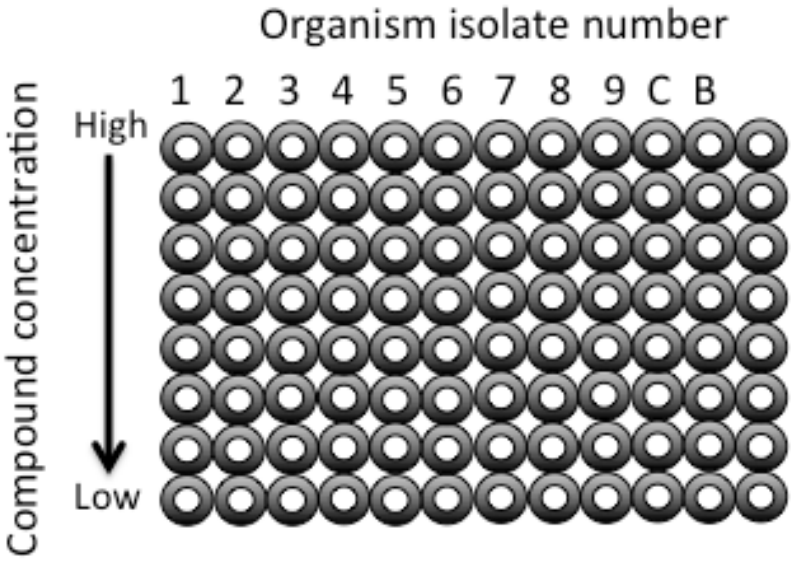


Table 22. Final compound concentrations in the 96 well plate

| Final compound concentration | | |
|---|---|---|
| TF | TF:EC combi (2:1) | EC |
| 800 μg/mL | 800:400 μg/mL | 1000 μg/mL |
| 400 μg/mL | 400:200 μg/mL | 500 μg/mL |
| 200 μg/mL | 200:100 μg/mL | 250 μg/mL |
| 100 μg/mL | 100:50 μg/mL | 125 μg/mL |
| 50 μg/mL | 50:25 μg/mL | 62.5 μg/mL |
| 0 μg/mL control | 0 μg/mL control | 0 μg/mL control |

After incubation wells were observed for turbidity and 2 μL from each well was spotted onto ISO-sensitive agar and incubated for a further 18 hours. After this period, if any growth was observed from any of the spots it was deemed that the concentration of compound was ineffective. The lowest concentration to show no growth was considered the minimum inhibitory concentration for that isolate.

### 3.5.6 Production of polyphenol impregnated antibiotic susceptibility discs

For each of the 6 clinical isolates and control strain of *A. baumannii* and for the 6 clinical isolates and control strain of *S. maltophilia* used in this test, 7 ISO-sensitive agar plates were made up. Plate 1 was inoculated Discs 1-4 containing 0.25 mg epicatechin, 0.5 mg TF, 0.25 mg epigallocatechin and an ethanol control, respectively. Plate 2 was inoculated with Discs 1-5 containing ampicillin (AMP) (25 μg), AMP + epicatechin (0.25 mg), AMP + theaflavin (0.5 mg), AMP + epigallocatechin (0.25 mg) and an ethanol control, respectively. Plates 3-7 were similar to Plate 2 but AMP was substituted with either gentamycin (CN) (10 μg), imipenem (IPM) (10 μg), tetracycline (TE) (20 μg), cefoxitin (FOX) (10 μg) or ciprofloxacin (CIP) (1 μg). The experimental organization is summarized in Table 23. All isolates were incubated at 37°C for

24 hours. At the end of the incubation period, zones of inhibition around each disc were measured (mm) and recorded. Each experimental plate was replicated 6 times. Results are presented as the Mean Value +/- Standard Error on the Mean. Different types of antibiotic were chosen to determine if synergy or antagonism is antibiotic type specific. Ampicillin is a type of Beta-lactam, gentamycin is a type of aminoglycoside, Imipenem is a type of carbapenem, tetracycline in a type of polyketide, cefoxitin is a type of cephalosporin and ciprofloxacin is a type of fluoroquinolone. With the exception of a sulphonamide, all relevant antibiotic types were used.

Table 23. Experimental organisation of inoculated discs and antibiotic disc on plates 1-7 for *A. baumannii* and *S. maltophilia*. Where AMP = ampicillin, CN = gentamycin, IPM = imipenem, TE = tetracycline, FOX = cefoxitin, CIP = ciprofloxacin, EC = epicatechin, TF = theaflavin and EGC = epigallocatechin

| | Disc 1 | Disc 2 | Disc 3 | Disc 4 | Disc 5 |
|---|---|---|---|---|---|
| Plate 1 | - | EC | TF | TF:EGC | Ethanol |
| Plate 2 | AMP | AMP+EC | AMP+TF | AMP+EGC | Ethanol |
| Plate 3 | CN | GEN+EC | GEN+TF | GEN+EGC | Ethanol |
| Plate 4 | IPM | IPM+EC | IPM+TF | IPM+EGC | Ethanol |
| Plate 5 | TE | TE+EC | TE+TF | TE+EGC | Ethanol |
| Plate 6 | FOX | FOX+EC | FOX+TF | FOX+EGC | Ethanol |
| Plate 7 | CIP | CIP+EC | CIP+TF | CIP+EGC | Ethanol |

For each of the 4 clinical isolates MRSA a different selection of antibiotics was used. Penicillin (a beta lactam) and clarithromycin (a macrolide) replaced cefoxitin, tetracycline and ciprofloxacin.

### 3.5.7 Statistical analysis

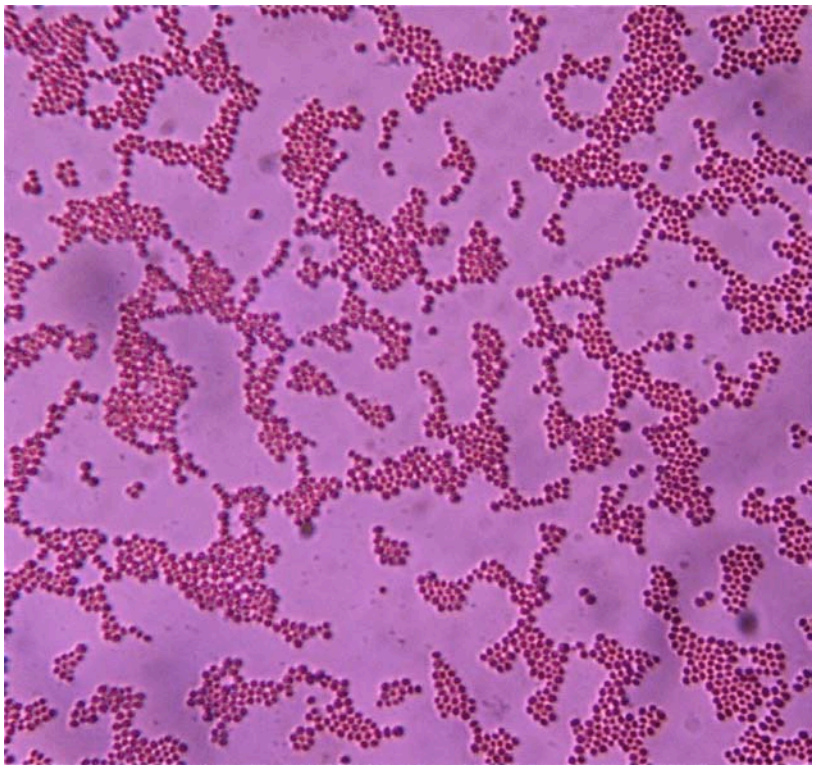
A student T-test (paired, 2 tailed) was performed to ascertain the significance of the results collected with, the null hypothesis for the statistical analysis being that there will be no significant difference between the antibacterial effect of polyphenols and combinations of polyphenols and that there will be no significant difference between the antibacterial effect of antibiotics and antibiotics with the addition of polyphenols. The test was performed using Graphpad InStat 3.00 for PC. Results with a P value > 0.05 were considered to prove the null hypothesis and results with a P value of $\leq$ 0.05 were considered to disprove the null hypothesis.

### 3.6 Results and discussion for hospital isolates

#### 3.6.1 Gram staining results

The result from the gram stains confirmed the correct morphology and gram types for each clinical isolate. The MRSA clinical isolates were stained purple and confirmed to be gram-positive cocci (Figure 59). The *A. baumannii* clinical isolates were stained light pink and confirmed to be gram-negative coccobacilli (Figure 60) and the *S. maltophilia* clinical isolates were stained pink and confirmed to be gram-negative bacilli (Figure 61).

Figure 59. An example of a gram stain undertaken for a MRSA clinical isolate



Control strains of *A. baumannii* and *S. maltophilia* were also confirmed to have the correct morphology and gram type.

Figure 60. An example of a gram stain undertaken for a clinical isolate of *A. baumannii*

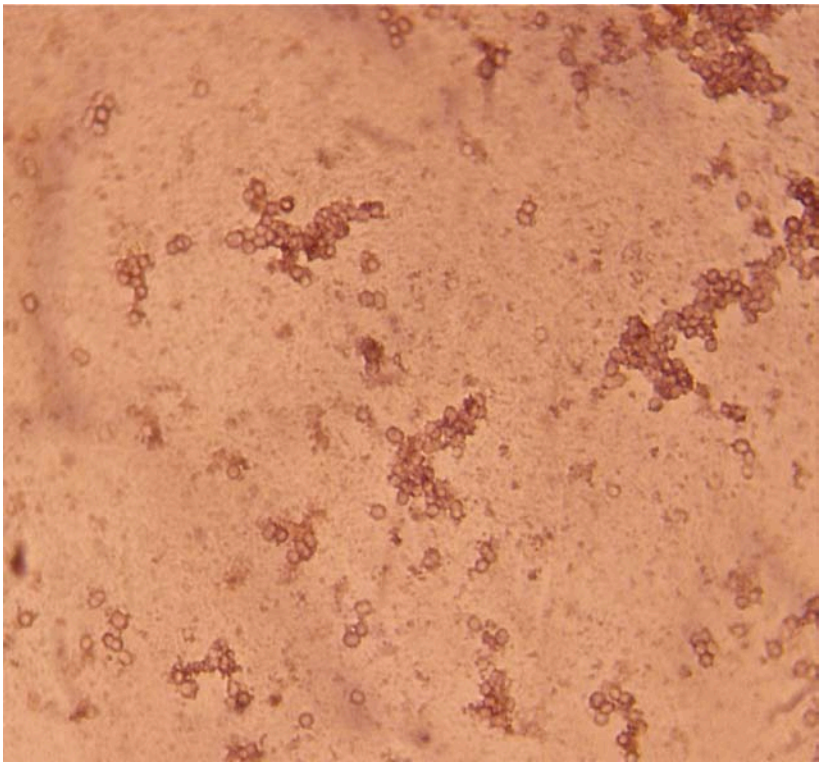
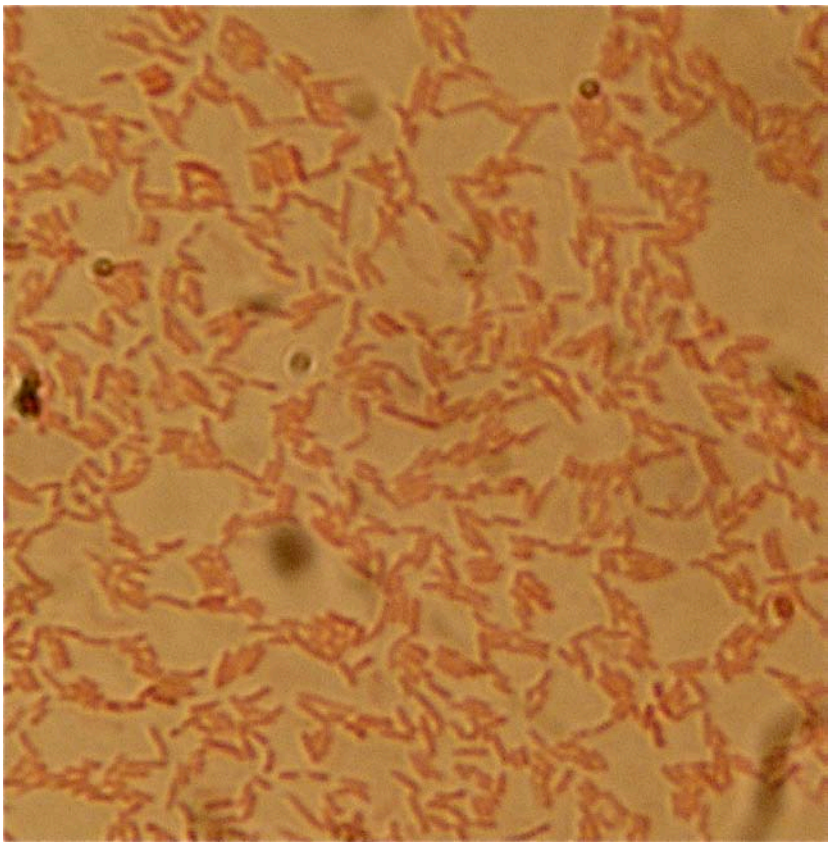


Figure 61. An example of a gram stain undertaken for a clinical isolate of *S. maltophilia*



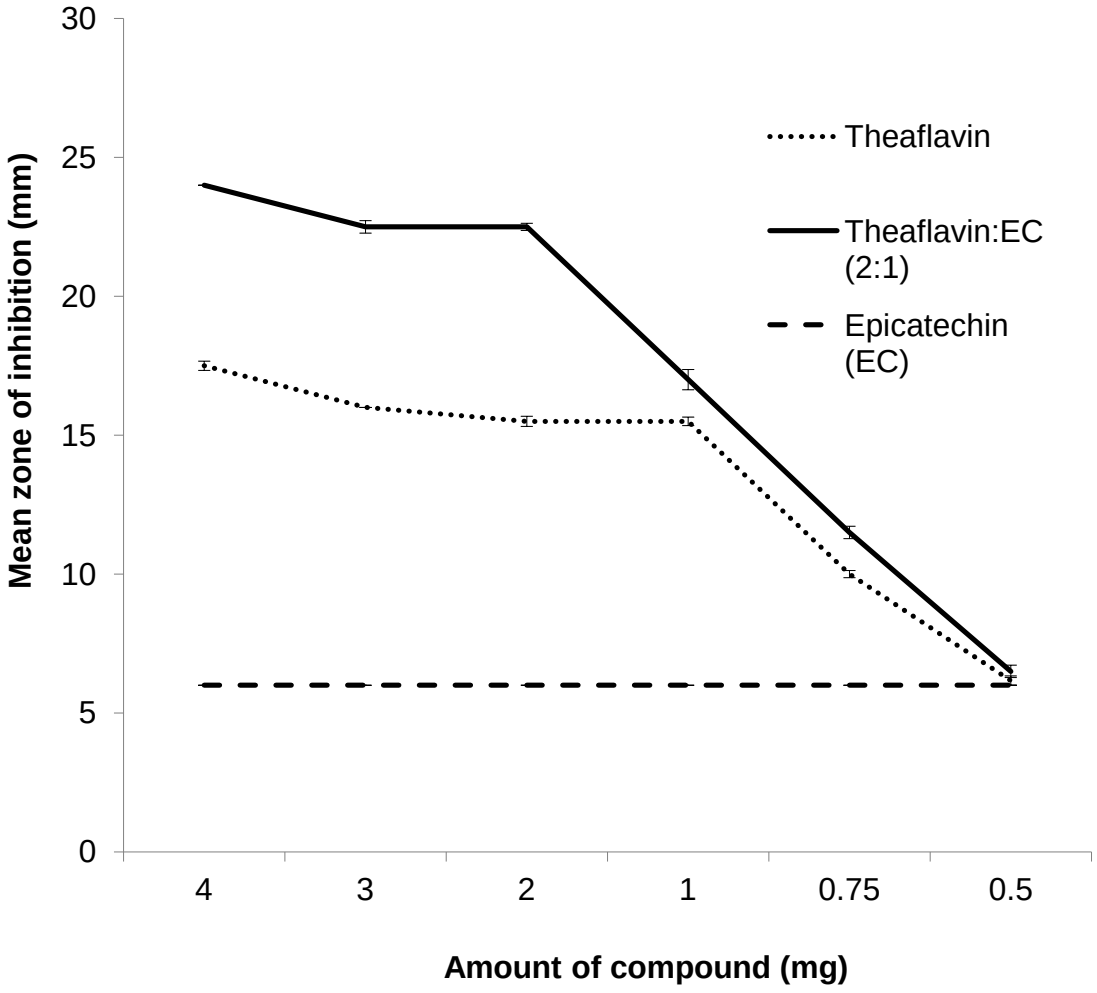
### 3.6.2 Results for antimicrobial and polyphenol synergy testing

It is clear from figures 62-73 that epicatechin shows no antibacterial activity against all isolates of MRSA, *A. baumannii* and *S. maltophilia*.

In figures 62-73 theaflavin shows antibacterial activity against all isolates of MRSA, *A. baumannii* and *S. maltophilia*. This was significantly higher ($P \geq 0.05$) than that of epicatechin (Tables 24, 25 and 26). However, against *A. baumannii* and *S.* maltophilia there is a significant reduction in antibacterial activity when disc concentrations are less than 3 mg as mean zones of inhibitions reduce in size. Against MRSA this reduction in activity occurs with disc concentrations of less than 2 mg.

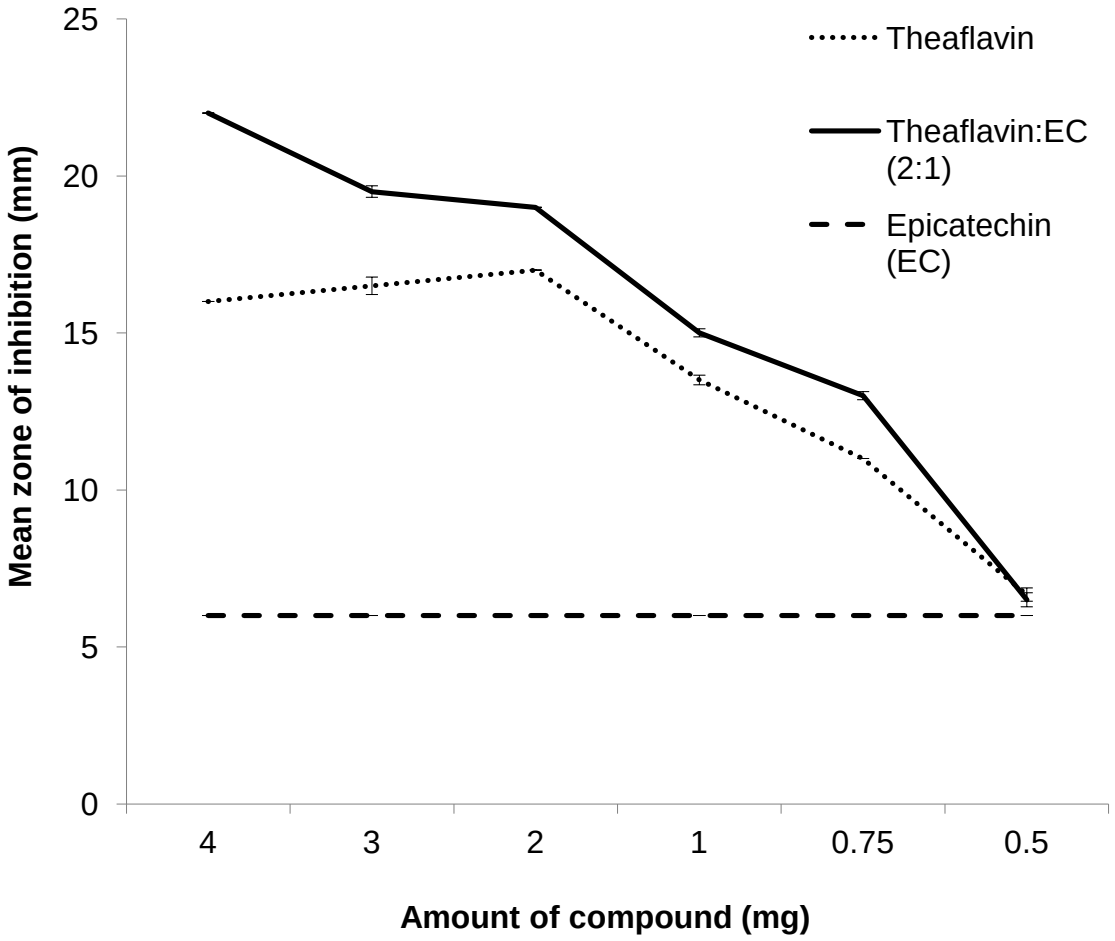
The theaflavin:epicatechin combination shows a significant increase in antibacterial activity ($P \leq 0.05$) compared with theaflavin alone against all but one isolate of *A. baumannii* and *S. maltophilia* above disc concentrations of 1 mg. The exception was isolate 2 of *A. baumannii* where the increase was above disc concentrations of 2 mg. In figures 63-65 and figure 70 the antibacterial activity of the theaflavin:epicatechin combination dramatically reduced when the disc concentrations was lower than 3 mg, whereas in figures 66 and 68-69 the large reduction in antibacterial effect is observed when the combination disc concentration was lower than 2 mg. In figures 62-65 significant antibacterial activity of the theaflavin:epicatechin combination can be seen against all MRSA isolates. Unlike with *A. baumannii* and *S. maltophilia* the theaflavin:epicatechin combination shows significantly more antibacterial activity than theaflavin ($P \leq 0.05$) at all disc concentrations above 0.5 mg.

Figure 62. Mean zones of inhibition of theaflavin, theaflavin:epicatechin combination and epicatechin against the MRSA isolate 1. Zones include the 6 mm disc diameter.



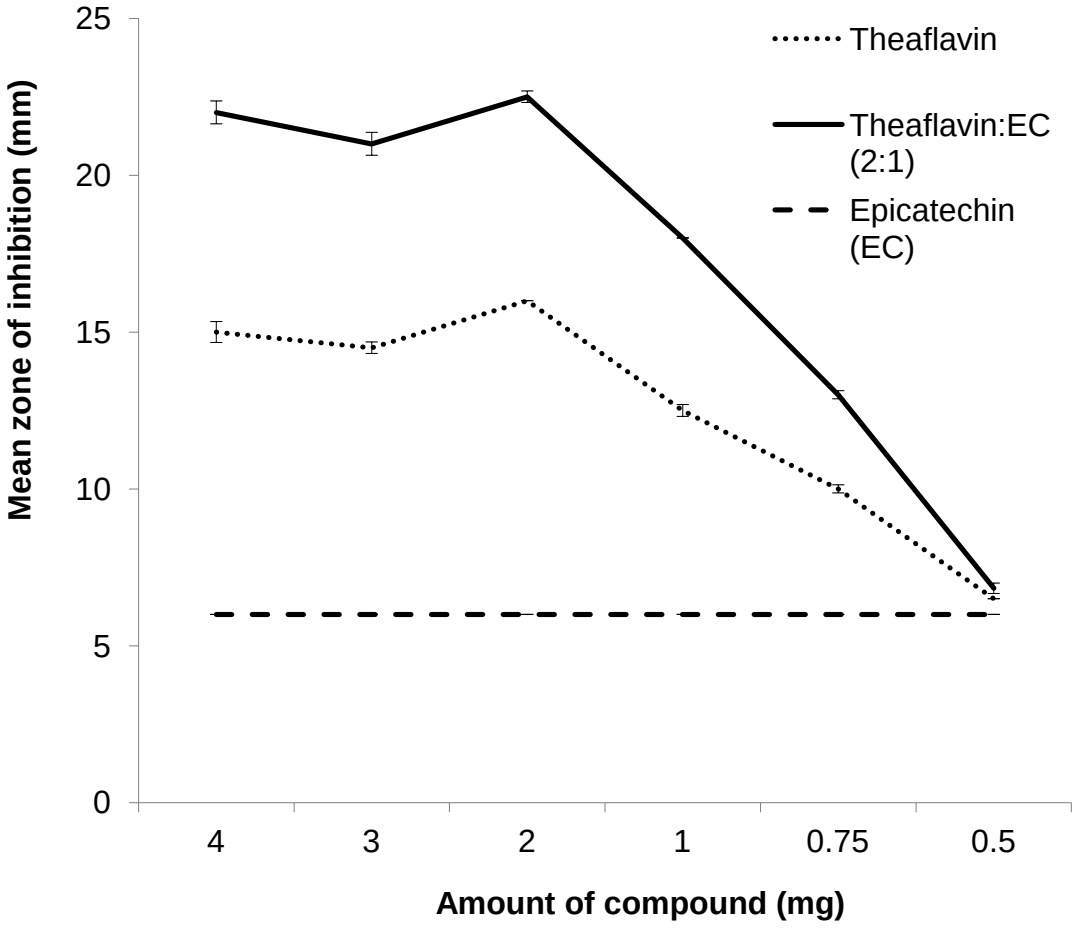

In figure 62 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination can be seen to reduce as there disc concentration is also reduced. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity against this isolate of MRSA.

Figure 63. Mean zones of inhibition for the MRSA isolate 2. Zones include the 6 mm disc diameter.



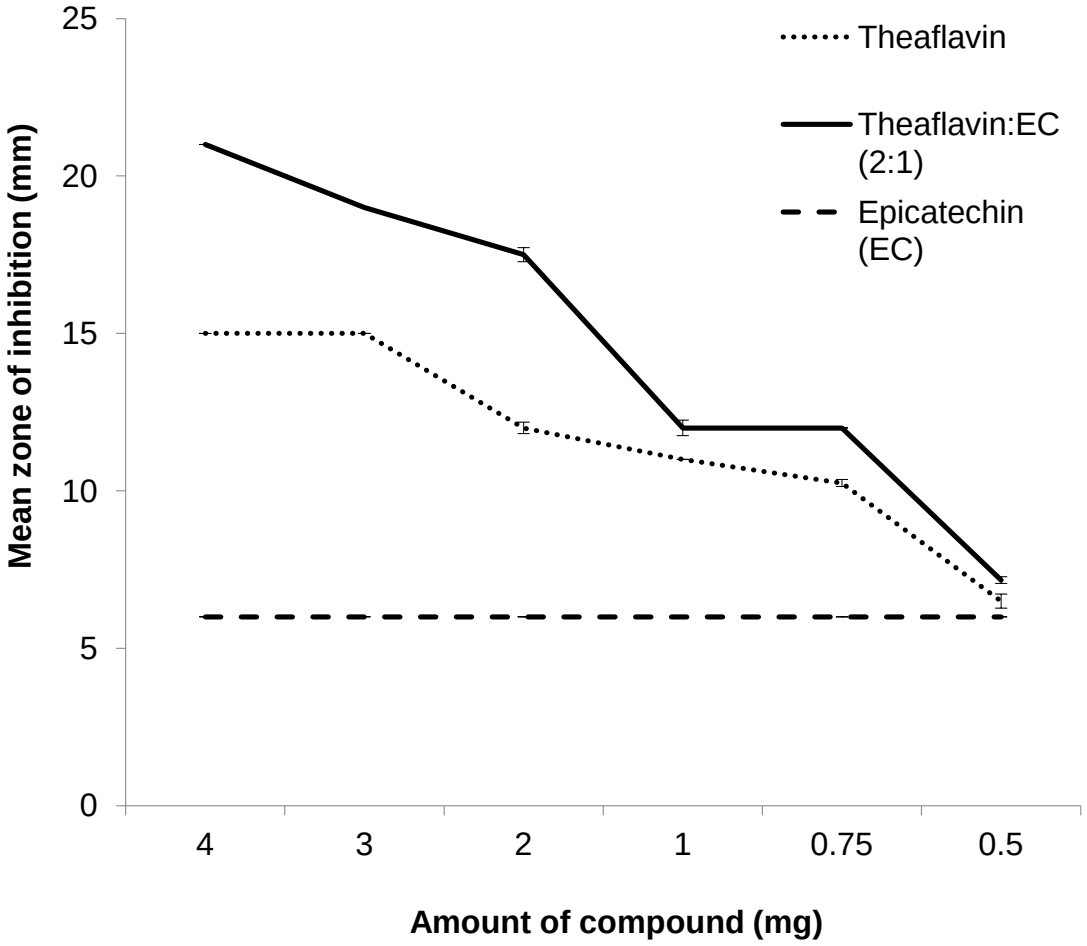

In figure 63 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination can be seen to reduce as the disc concentration is also reduced. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity against this isolate of MRSA.

Figure 64. Mean zones of inhibition for the MRSA isolate 3. Zones include the 6 mm disc diameter.



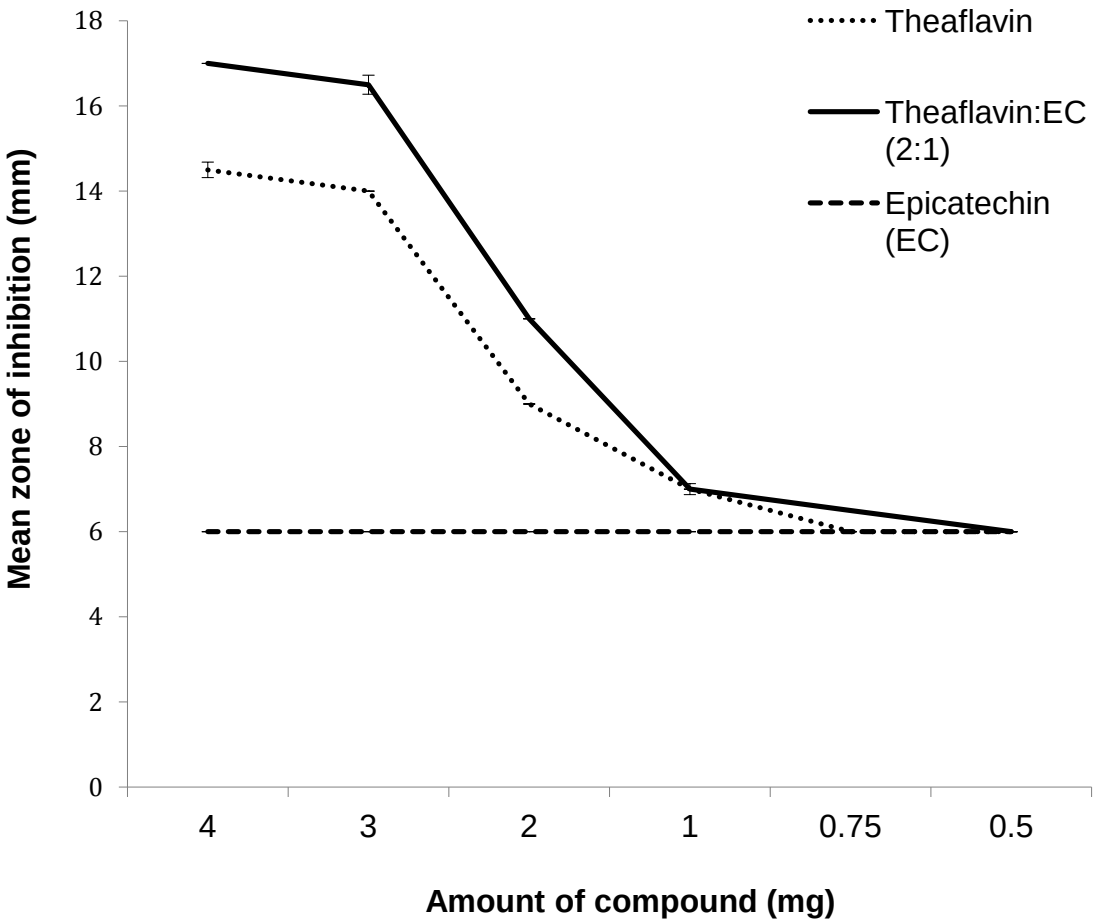

In figure 64 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination can be seen to reduce after the disc concentration is also reduced below 2 mg. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity against this isolate of MRSA.

Figure 65. Mean zones of inhibition for the MRSA isolate 4. Zones include the 6 mm disc diameter.



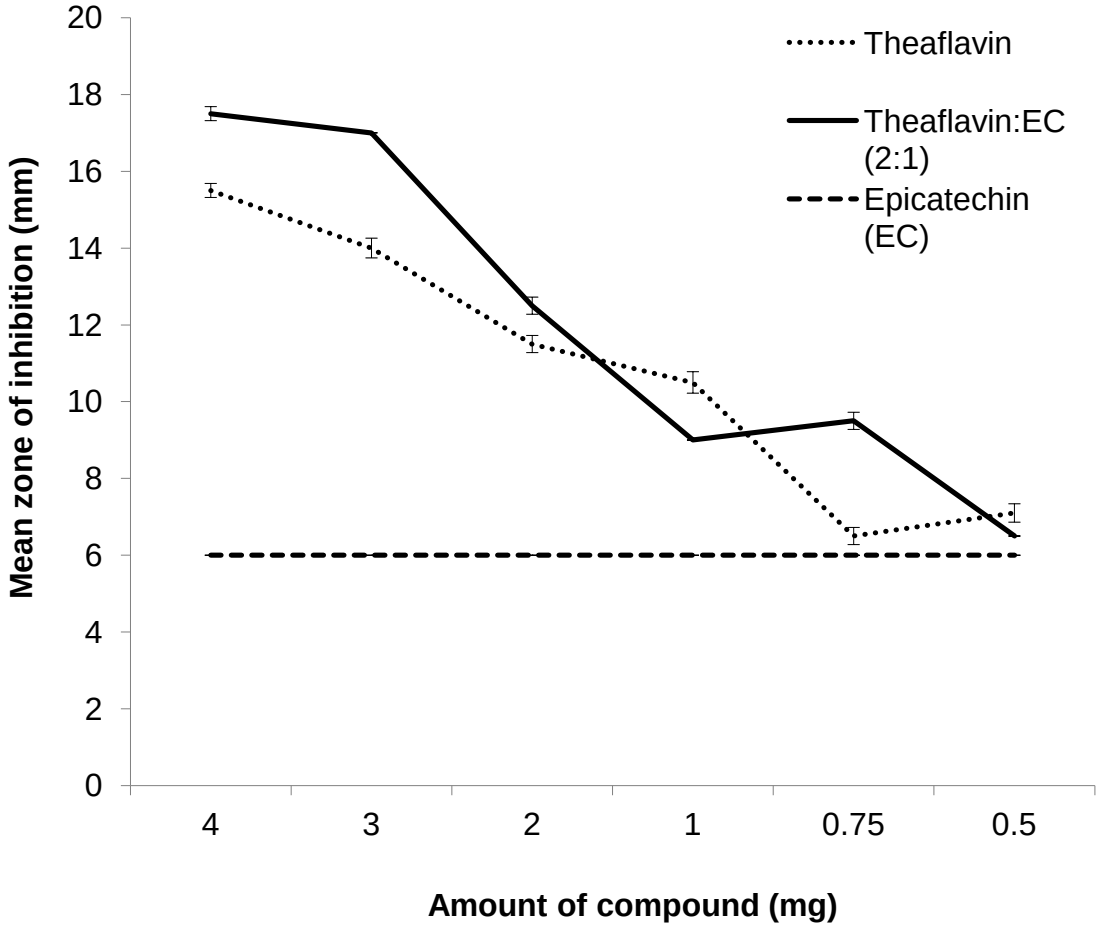

From the results seen in figure 65 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination reduces as the disc concentration is also reduced. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity against this isolate of MRSA.

Figure 66. Mean zones of inhibition for the *A. baumannii* isolate 1. Zones include the 6 mm disc diameter.



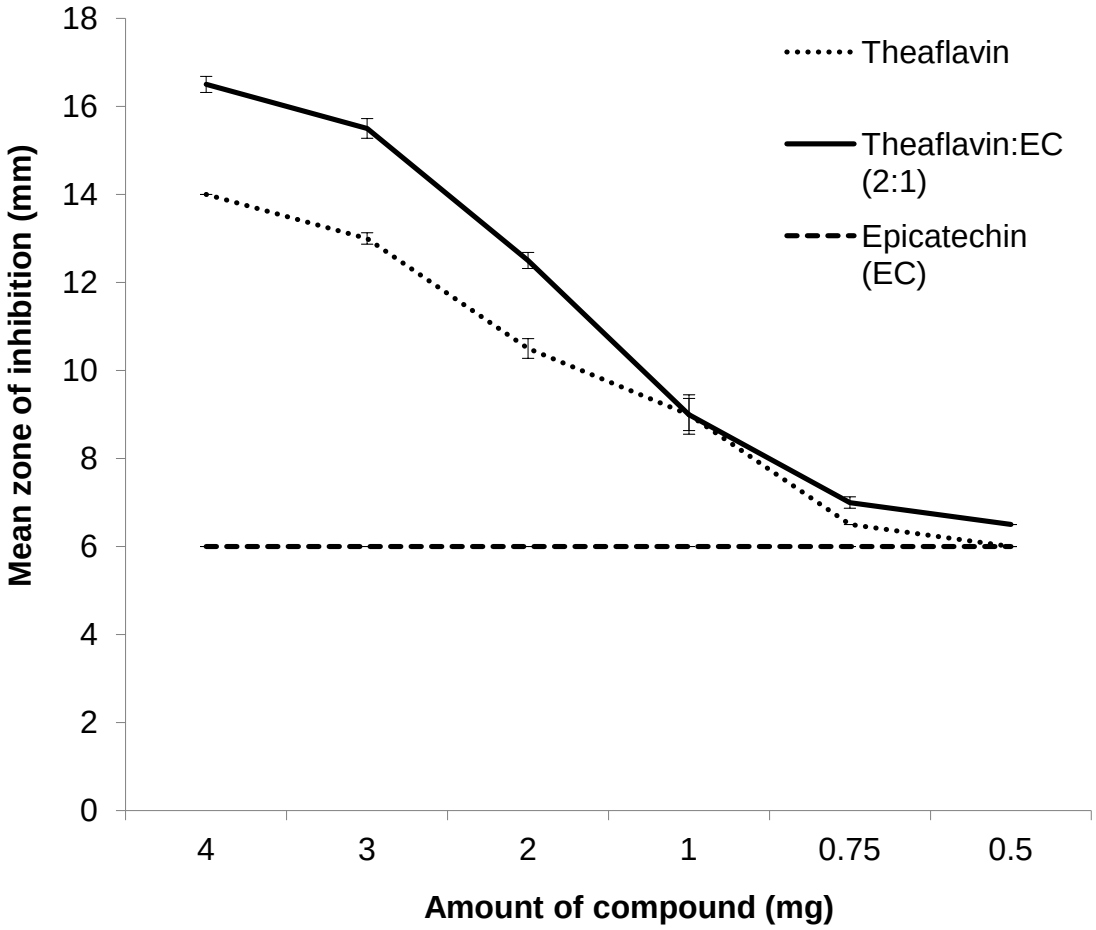

From the results seen in figure 66 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination reduces as the disc concentration is also reduced. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity against this isolate of *A. baumannii*.

Figure 67. Mean zones of inhibition for the *A. baumannii* isolate 2. Zones include the 6 mm disc diameter.



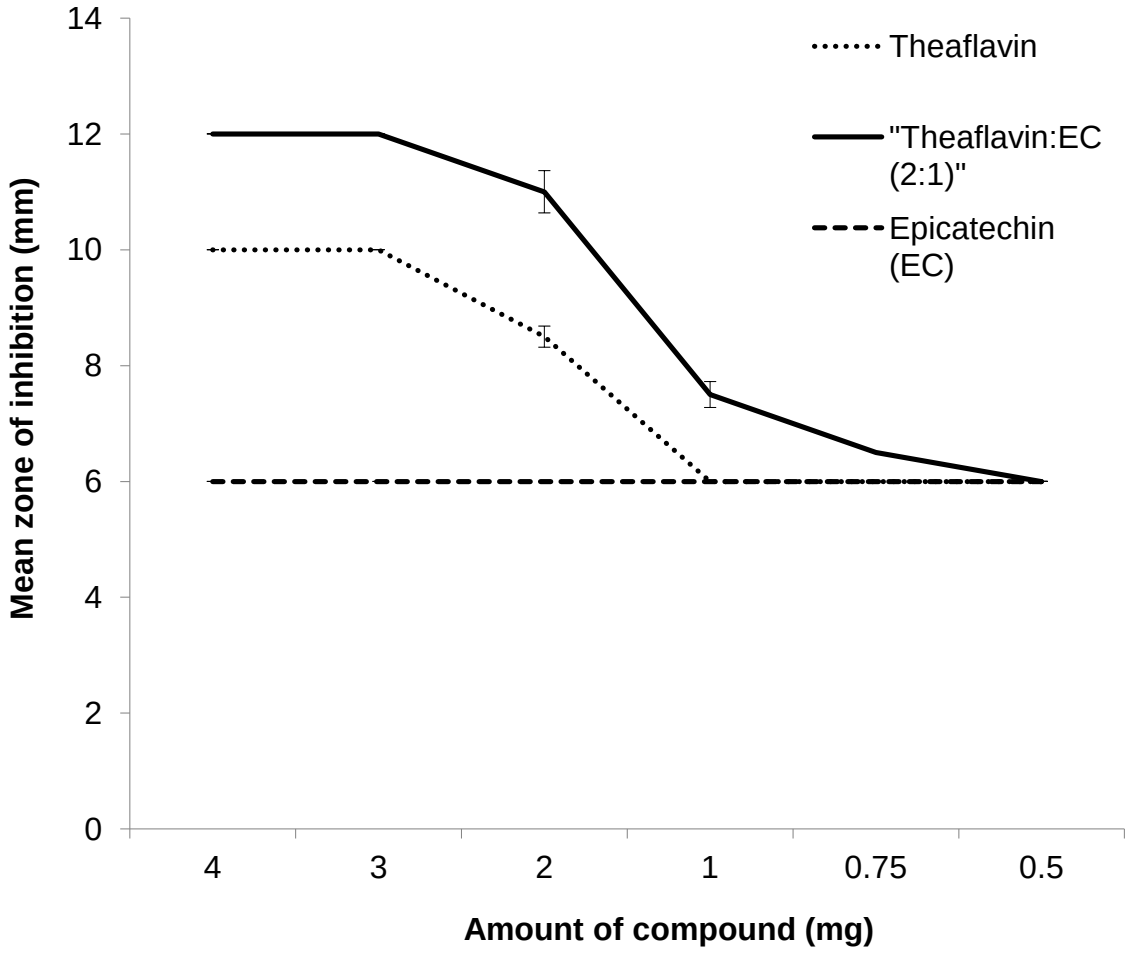

From figure 67 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination can be seen to reduce as the disc concentration is also reduced. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity for this isolate of *A. baumannii*.

Figure 68. Mean zones of inhibition for the *A. baumannii* isolate 3. Zones include the 6 mm disc diameter.



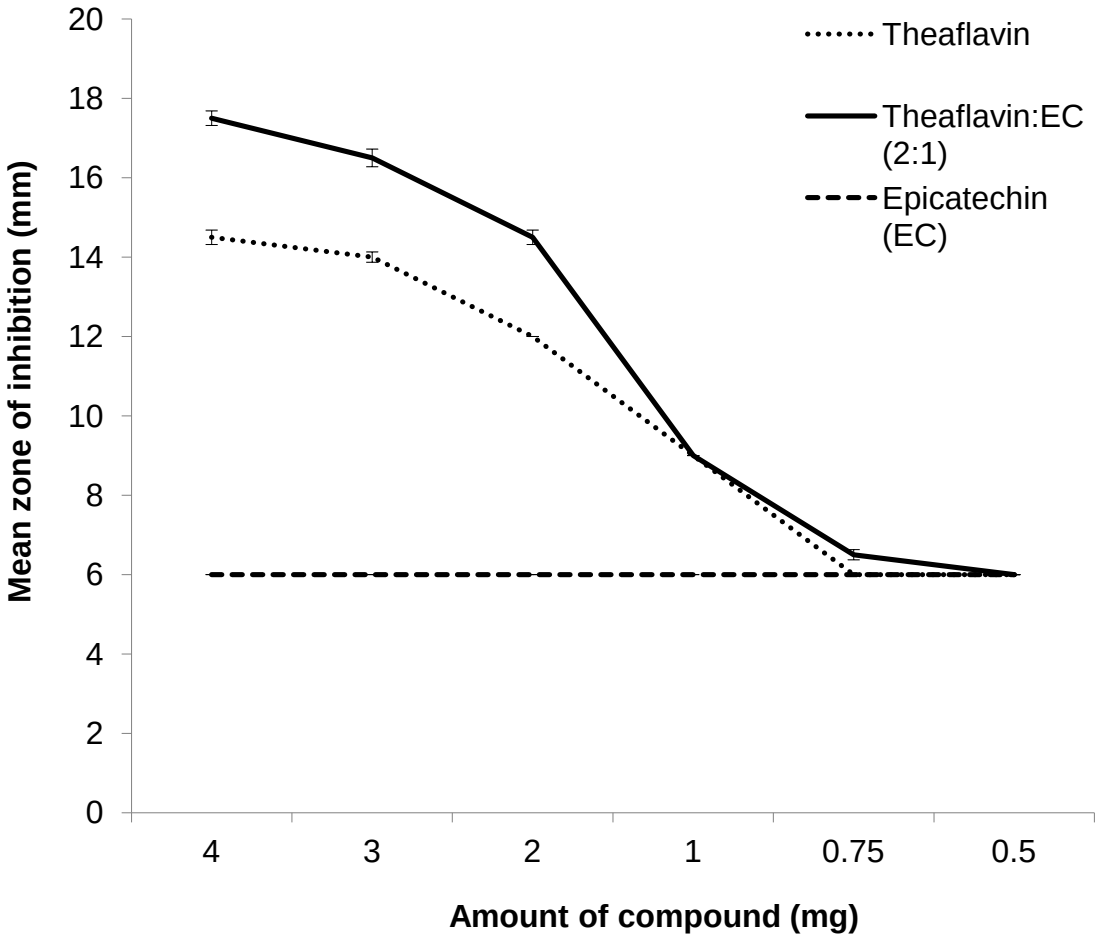

The mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination can be seen to reduce in figure 68 as the disc concentration is also reduced. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This again confirms epicatechin has no antibacterial activity against this isolate of *A*. *baumannii*.

Figure 69. Mean zones of inhibition for the *A. baumannii* isolate 4. Zones include the 6 mm disc diameter.



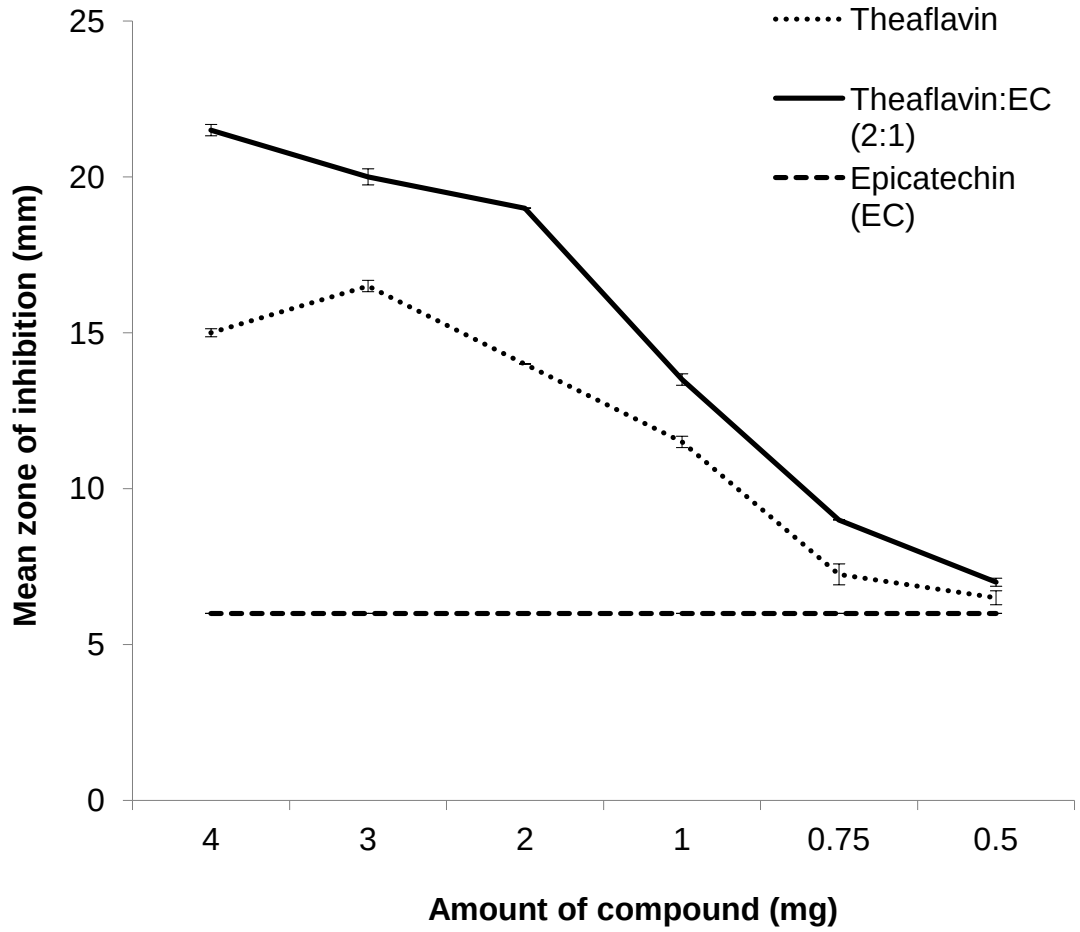

From the results seen in figure 69 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination reduces as the disc concentration is also reduced. No zone of inhibition beyond the 6 mm disc diameter was seen for theaflavin below a disc concentration of 1 mg for this isolate. However, for all concentrations of epicatechin on the disc the zone of inhibition did not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity against this isolate of *A. baumannii*.

Figure 70. Mean zones of inhibition for the *S. maltophilia* isolate 1. Zones include the 6 mm disc diameter.



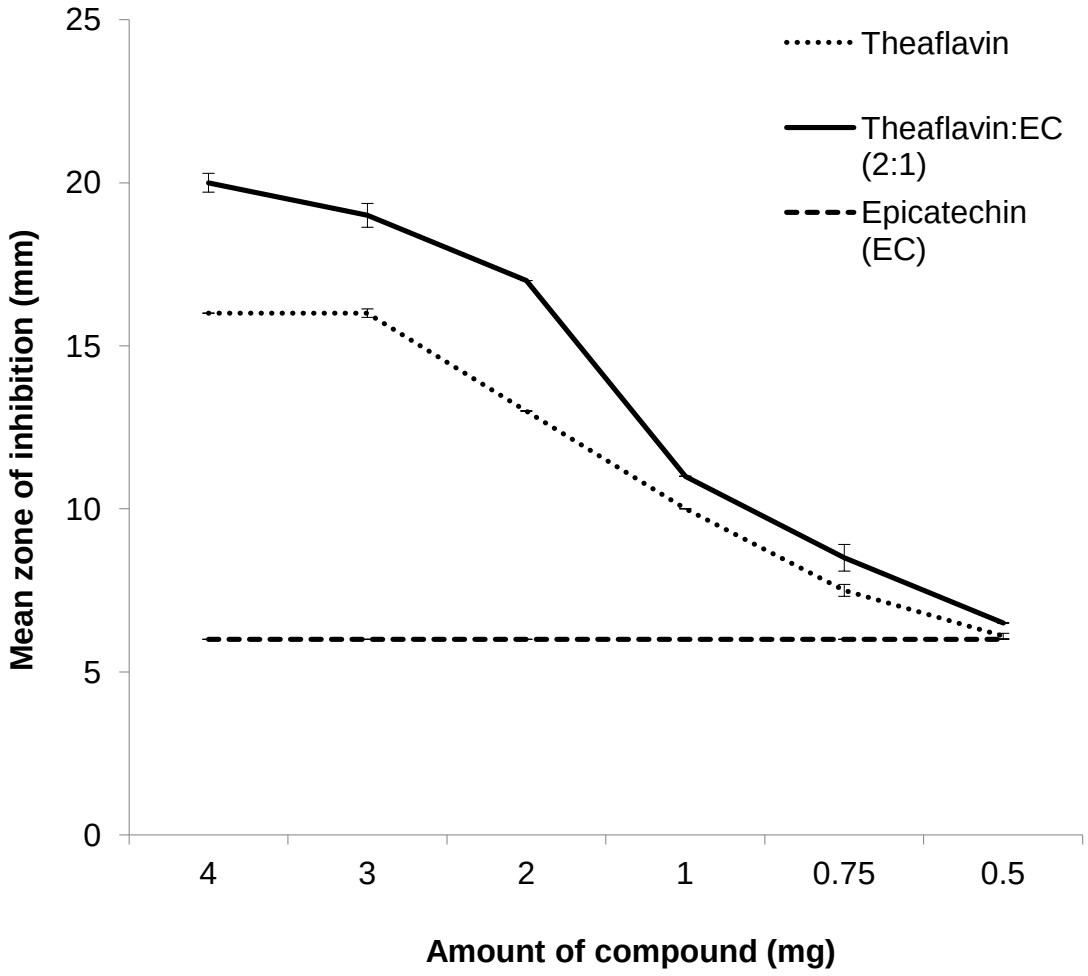

From the results seen in figure 70 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination reduces as the disc concentration is also reduced up until 0.5 mg where no antibacterial activity it seen. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity against this isolate of *S. maltophilia*.

Figure 71. Mean zones of inhibition for the *S. maltophilia* isolate 2. Zones include the 6 mm disc diameter.



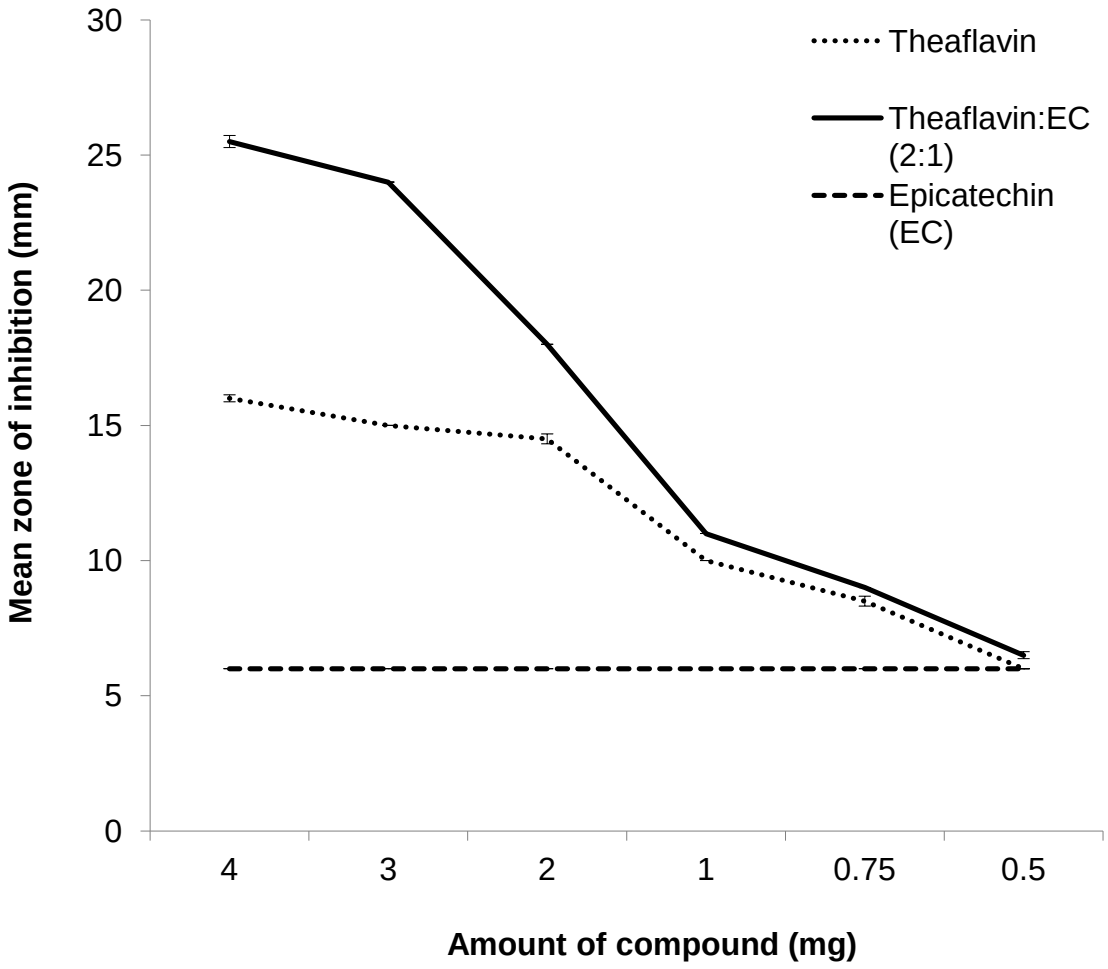

In figure 71 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination reduces as the disc concentration is also reduced. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity against this isolate of *S. maltophilia*.

Figure 72. Mean zones of inhibition for the *S. maltophilia* isolate 3. Zones include the 6 mm disc diameter.




In figure 72 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination reduces as the disc concentration is also reduced where at 0.5 mg almost no antibacterial activity is seen. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity against this isolate of *S. maltophilia*.

Figure 73. Mean zones of inhibition for the *S. maltophilia* isolate 4. Zones include the 6 mm disc diameter.




In figure 73 the mean zones of inhibition for theaflavin and the theaflavin:epicatechin combination reduces as the disc concentration is also reduced where at 0.5 mg almost no antibacterial activity is seen. However, for all concentrations of epicatechin on disc the zone of inhibition does not increase above the actual 6mm disc diameter. This confirms epicatechin has no antibacterial activity against this isolate of *S. maltophilia*.

Table 24. Results of a student T-test (paired, 2 tailed) to show significant difference between antibacterial effects of polyphenols and polyphenol combinations against isolates of MRSA.

| | Organism | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MRSA | | | | | | | | | | | |
| | [EC] vs. [TF] | | | | [EC] vs. [TF:EC] | | | | [TF:EC] vs. [TF] | | | |
| Isolate no. | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 4 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S |
| 3 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S |
| 2 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S |
| 1 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0144 S | P = 0.0004 S | P = 0.0001 S | P = 0.0001 S |
| 0.5 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0021 S | P = 0.0001 S | P = 0.1019 S | P = 0.0010 S |
| 0.75 mg | P = 0.3632 N | P = 0.0250 S | P = 0.0001 S | P = 0.0761 N | P = 0.0756 N | P = 0.0912 N | P = 0.0041 S | P = 0.0001 S | P = 0.3632 N | P = 0.6109 N | P = 0.0001 N | P = 0.0103 S |

Where epicatechin = EC, theaflavin = TF and epigallocatechin gallate = EGC. S = significant difference (Null hypothesis rejected) and N= no significant difference (Null hypothesis accepted). P = 2 tailed P value.

Table 24. Results of a student T-test (paired, 2 tailed) to show significant difference between antibacterial effects of polyphenols and polyphenol combinations against isolates of MRSA.

| | Organism | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *A, baumannii* | | | | | | | | | | | |
| | [EC] vs. [TF] | | | | [EC] vs. [TF:EC] | | | | [TF:EC] vs. [TF] | | | |
| Isolate no. | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 4 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S |
| 3 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0005 S | P = 0.0001 S |
| 2 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0409 S | P = 0.0010 S | P = 0.0025 S |
| 1 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0005 S | P = 0.3632 N | P = 0.0006 S | P = 0.0001 S | P = 0.0012 S | P = 0.0010 S | P = 0.9999 N | P = 0.0305 S | P = 0.9999 N | P = 0.0010 S |
| 0.5 mg | P = 0.3632 N | P = 0.0912 N | P = 0.0001 S | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.0006 S | P = 0.0001 S | P = 0.3632 N | P = 0.0004 S | P = 0.0117 S | P = 0.0001 S |
| 0.75 mg | P = 0.3632 N | P = 0.0068 S | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N | P = 0.3632 N | P = 0.0583 N | P = 0.0001 S | P = 0.3632 N |

Where epicatechin = EC, theaflavin = TF and epigallocatechin gallate = EGC. S = significant difference (Null hypothesis rejected) and N= no significant difference (Null hypothesis accepted). P = 2 tailed P value.

Table 24. Results of a student T-test (paired, 2 tailed) to show significant difference between antibacterial effects of polyphenols and polyphenol combinations against isolates of MRSA.

| | Organism | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *S. maltophilia* | | | | | | | | | | | |
| | [EC] vs. [TF] | | | | [EC] vs. [TF:EC] | | | | [TF:EC] vs. [TF] | | | |
| Isolate no. | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 4 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S |
| 3 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0005 S | P = 0.0001 S | P = 0.0009 S | P = 0.0001 S |
| 2 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S |
| 1 mg | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N | P = 0.0015 S | P = 0.0001 S | P = 0.0001 S |
| 0.5 mg | P = 0.3632 N | P = 0.0156 S | P = 0.0004 S | P = 0.0001 S | P = 0.0138 S | P = 0.0001 S | P = 0.0018 S | P = 0.0001 S | P = 0.0138 S | P = 0.0034 S | P = 0.1357 N | P = 0.0409 S |
| 0.75 mg | P = 0.3632 N | P = 0.0912 N | P = 0.4838 N | P = 0.3632 N | P = 0.3632 N | P = 0.0006 S | P = 0.0001 S | P = 0.0138 S | P = 0.3632 N | P = 0.1106 N | P = 0.0041 S | P = 0.0001 S |

Where epicatechin = EC, theaflavin = TF and epigallocatechin gallate = EGC. S = significant difference (Null hypothesis rejected) and N= no significant difference (Null hypothesis accepted). P = 2 tailed P value.

In the present study epicatechin shows no antibacterial activity against any clinical isolate tested, whereas previous research (Hirao *et al.,* 2010 and Toda *et al.,* 1992) did find activity against periodontal bacteria and *Vibrio cholera*. However, the work here parallels another (Cushnie *et al.,* 2008) were epicatechin was found to be an ineffective antibacterial agent, in their case against *Staphylococcus aureus*. This variation in antibacterial activity is often observed when testing antimicrobial agents against various bacterial species.

When examining the results for theaflavin, it shows strong antibacterial effects that could potentially be of clinical relevance when dealing with microorganism's resistance to conventional antibiotics. In this study MRSA and *S. maltophilia* isolates are most susceptible to the antibacterial action of theaflavin. When examining previous research is thought that the potential mechanism is related to membrane interaction (Sirk, Friedman and Brown, 2011). Further research is required to determine whether this is a bactericidal or bacteriostatic effect and what other mechanisms are involved.

It is apparent from the results reported here that significant synergism exists between theaflavin and epicatechin against all isolates of MRSA. *A. baumannii* and *S. maltophilia*. It is suggested that the mechanism behind the synergy is similar to that shown for ascorbic acid and epigallocatechin gallate (Hatano *et al.,* 2008). In this study it is believed that epicatechin inhibits the oxidation of theaflavin and prolongs its antibacterial effect. Although synergy is observed when low concentrations of the compounds are used, the greatest levels of synergy are observed when the compounds are used in concentrations in excess of 2 mg per susceptibility disc. This could be due to either diffusion rate limitations of the compounds into the media, the impact of polyphenol concentration on bacterial osmolality or a saturation point of any polyphenol expulsion by the bacteria.

It is believed this is the first occasion where the antibacterial effects of theaflavin against clinical isolates of *A. baumannii* and *S. maltophilia* are reported. It is

also believed to be the first report of antibacterial synergy between theaflavin and epicatechin against hospital isolates of MRSA, *A. baumannii* and *S. maltophilia*.

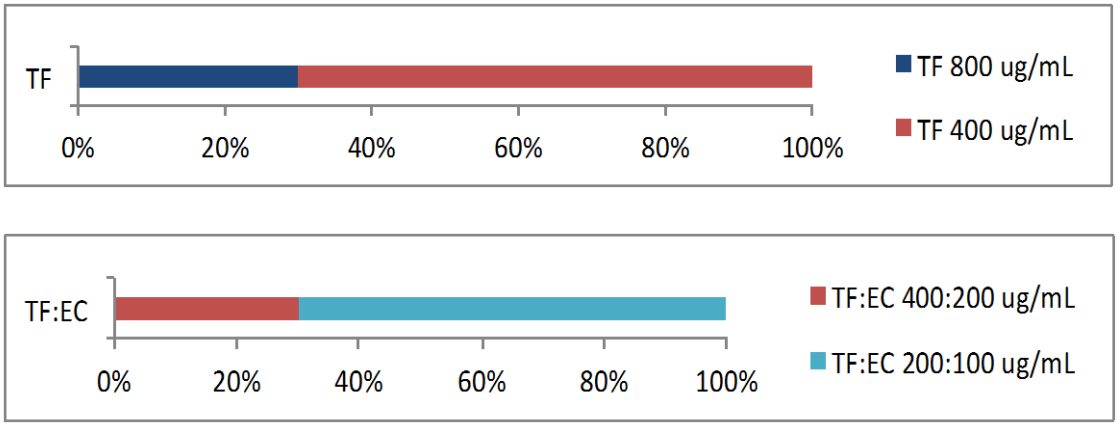
### 3.6.3 Results for the minimum inhibitory concentration testing against clinical isolates of *S. maltophilia*

The results from the MIC testing confirm that epicatechin has no antibacterial effect against the strains of *S. maltophilia* used in this study. Concentrations of up to 1 mg/mL of epicatechin did not inhibit the growth of *S. maltophilia* and therefore no MIC was achieved.

The MIC results for theaflavin confirmed that the polyphenol produced antibacterial effects even when used in a broth culture. The MIC of theaflavin ranged between 400 μg/mL and 800 μg/mL. This can be seen in figure 74. The *S. maltophilia* had a MIC for theaflavin of 400 μg/mL.

For the theaflavin:epicatechin combination the MIC value ranged between 200 μg/mL and 400 μg/mL (see figure 74). This was significantly lower than when using just theaflavin. The *S. maltophilia* control strain (NCTC 13014) had a MIC of 200 μg/mL for the theaflavin:epicatechin combination.

Figure 74. Graphs representing the percentage of isolate MICs for theaflavin (TF) and theaflavin:epicatechin combination (TF:EC)



The result confirms that a significant synergistic relationship exists between theaflavin and epicatechin. This would also present a large reduction in cost if the combination treatment was used. The reason for this is that half of the more expensive theaflavin would be required to treat an isolate of *S. maltophilia* in comparison with theaflavin alone.

Although the mechanisms underlying the antibacterial activity and synergy are not yet clear, the findings indicate that there might be important clinical potential for these polyphenols, especially when used in combination. Further studies are recommended to determine the mechanisms involved and the potential synergy of theaflavin or epicatechin with other polyphenols from natural sources.

### 3.6.4 Results and discussion for antibiotic synergy testing

In this study all isolates of MRSA, *A. baumannii* and *S. maltophilia* showed resistance to ampicillin (Figures 75-92). Epicatechin showed significant $P \leq 0.05$ synergistic effects with ampicillin against every isolate and control strain of MRSA, *A. baumannii* and *S. maltophilia* as did theaflavin Table (27–29). Epigallocatechin showed synergy with ampicillin against most isolates with the exception to those in figures 77, 79, 81, 83-86 and 89.

As expected, all clinical isolates of MRSA showed resistance to penicillin G. Epicatechin gave no additional antibacterial effect against any isolate of MRSA, indicating no synergy occurred. However, theaflavin showed significant synergy with penicillin G against all isolates of MRSA (Figures 75-78) and epigallocatechin showed synergy with isolates 1 and 4 of MRSA.

No bacterial isolates in this investigation were resistant to gentamycin. Gentamycin appeared to have strong antibacterial activity against MRSA, *A. baumannii* and *S. maltophilia*. However, when gentamycin was used in

combination with theaflavin or epigallocatechin a significant reduction in antibacterial effect was recorded at $P \leq 0.05$ (Table 27-29, figures 75-80, 82-83 and 85-91). With the exception of isolate 3 of *A. baumannii* where no effect was seen (Table 28, figure 81) with TF and isolate 6 of *A. baumannii* and the *S. maltophilia* control when used with epigallocatechin. In most cases epicatechin showed no effect when used with gentamycin, with the exception of MRSA isolates 1 and 2 where antagonism and synergy was seen respectively (Figures 77-78, table 27), isolate 3 of *A. baumannii* where synergy was seen (Figure 81, table 28) and isolate 1 of *S. maltophilia* (Figure 86, table 29) where antagonism was seen.

In this study imipenem was an effective antibacterial agent against all clinical isolates of MRSA, clinical isolates of *A. baumannii* and its control NCTC 13485 (Tables 27 and 28, figures 75-85). However all isolates of *S. maltophilia* and its control NCTC 13014 were resistant to imipenem (Table 29, figures 86-93) with the exception of isolate 6 which showed sensitivity (Figure 91). When used in conjunction with epicatechin synergy ($P \leq 0.05$) was seen for isolate 1 of MRSA, 6 of *A. baumannii* and 5 and 6 of *S. maltophilia* (Figures 75, 84, 90 and 91). However with isolates 1 and 3 of *A. baumannii* significant antagonism was observed (Figures 79 and 81). When imipenem was used with theaflavin significant synergy was produced with isolates 1 and 2 of MRSA (Figures 75 and 76) and isolates 5 and 6 of *S. maltophilia* (Figures 90 and 91). However, significant antagonism was produced when the combination was used against isolate 6 of *A. baumannii* (Figure 84). When Imipenem was used with epigallocatechin mixed results were also produced. Against MRSA synergy was seen against isolates 1 and 2 and no effects against isolates 3 and 4 (Figures 75-78). However, against *A. baumannii* mixed results were produced as antagonism was seen against isolates 1 and 3 (Figures 79 and 81) and significant synergy ($P \leq 0.05$) against isolates 4-6 (Figures 82-84, table 28). Against *S. maltophilia* the combination of imipenem and epigallocatechin showed no difference with the exception of isolate 4 were significant synergy was produced (Figure 89, table 29).

Clarithromycin was seen to be effective against clinical isolates 3 and 4 of MRSA with no effect seen against isolates 1 and 2. When epicatechin was used with clarithromycin no effect was seen indicating it produces no synergy or antagonism when used with this antibiotic. Mixed results were seen when clarithromycin was used with epigallocatechin. With isolates 1 and 2 of MRSA significant synergy was produced ($P \leq 0.05$), with isolate 3 antagonism was seen and with isolate 4, no significant difference was observed (Figures 75-78, table 27). However, against all isolates of MRSA used, significant synergy ($P \leq 0.05$) was produced between theaflavin and clarithromycin (Figures 75-78, table 27).

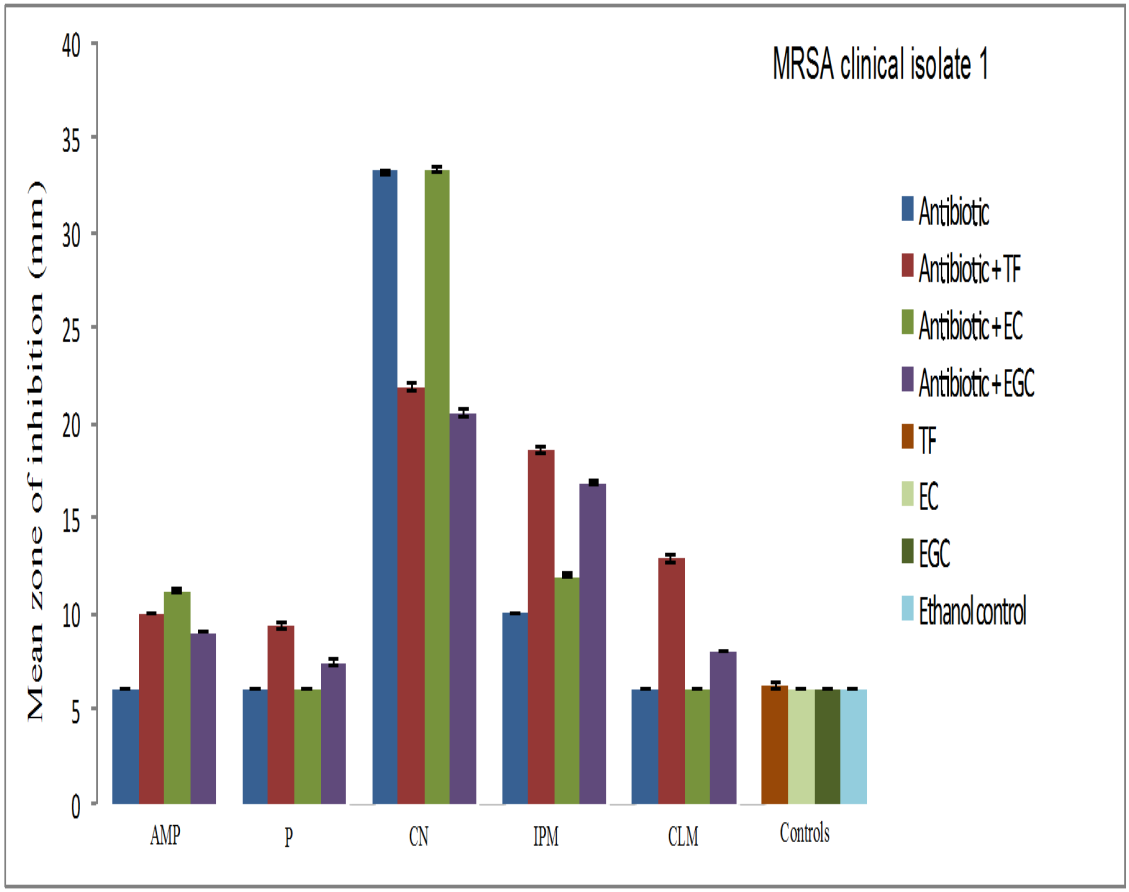
Tetracycline was shown to be an effective antibacterial agent against all clinical isolates and the control of *A. baumannii* and also effective for clinical isolates 1,4 and 6 of *S. maltophilia* (Figures 79-86, 89 and 91). Clinical isolates 2, 3 and 5 and the control strain (NCTC 13014) of *S. maltophilia* were resistant to tetracycline (Figures 87, 88, 90 and 92). With the exception of *A. baumannii* isolates 3 and 5 where synergy was seen and isolate 1 of *S. maltophilia* where antagonism was shown, epicatechin produced no other synergy or antagonism when combined with tetracycline. Theaflavin shown only significant antagonism ($P \leq 0.05$) or no effect against all isolates (Figures 79-92, table 28 and 29). When tetracycline was used in combination with epigallocatechin mixed results were seen. With *A. baumannii* isolate 1 and the control strain, antagonism was produced, but with isolate 6, synergy was observed (Figures 79-85). With *S. maltophilia* isolate 1 gave antagonism, whereas isolates 2 and 6 produced significant synergy ($P \leq 0.05$).

Cefoxitin only showed an antibacterial effect against isolates 1, 4 and 5 of *A. baumannii* and with the control strain of *S. maltophilia*. With all other isolates, cefoxitin showed no action. A synergistic effect was observed when epicatechin was used with cefoxitin against isolates 2 and 5 of *A. baumannii*, antagonism with isolate 4 and no effect with all other isolates of *A. baumannii* (including control) and also no effect with all isolates and control of *S. maltophilia*. However, when cefoxitin was used with theaflavin significant synergy was

produced against all isolates of *S. maltophilia* and all but isolate 1 of *A. baumannii* (Figures 79-92, tables 28 and 29). The combination of epigallocatechin and cefoxitin gave similar results with significant synergy observed with all isolates with the exception of isolate 1 and the control strain of *A. baumannii* and control strain of *S. maltophilia*.

Ciprofloxacin showed strong antibacterial activity against all isolates of *A. baumannii* (Figures 79-85) and the control strain (NCTC 13485). Against clinical isolates of *S. maltophilia* the effect was mixed, with isolates 1, 2, 5 and the control strain showing resistance (Figures 86, 87, 90 and 92). When used with epicatechin mixed results were produced with significant synergy ($P \leq 0.05$) shown for isolates 2, 3, 5 and 6 of *A. baumannii* and isolates 6 0f *S. maltophilia* (Figures 80, 81, 83, 84, and 91, table 28 and 29), antagonism for isolate 4 of *A. baumannii* and isolates 2 and 4 of *S. maltophilia* (Figures 82, 88 and 89, tables 28 and 29) and no difference with all other isolates. When ciprofloxacin was used in combination with theaflavin significant antagonism ($P \leq 0.05$) was seen with all isolates of *A. baumannii* (Figures 79-85, table 28) and isolates 4 and 6 of *S. maltophilia* (Figures 89 and 91, table 29). However, significant synergy was produced against isolates 1-3 and 5 of *S. maltophilia* (Figures 86-88 and 90, table 29). The combination of epigallocatechin and ciprofloxacin produced a antagonistic effect with the majority of *A. baumannii* isolates and control strain (Figures 81-85) and mixed results against *S. maltophilia.* Against isolates 2, 3 and 5 of *S. maltophilia* an significantly increased antibacterial effect with the combination of epigallocatechin and ciprofloxacin was seen (Figures 87, 88 and 90), whereas against isolate 6 the antibacterial effect was reduced meaning antagonism between the 2 compounds took place.

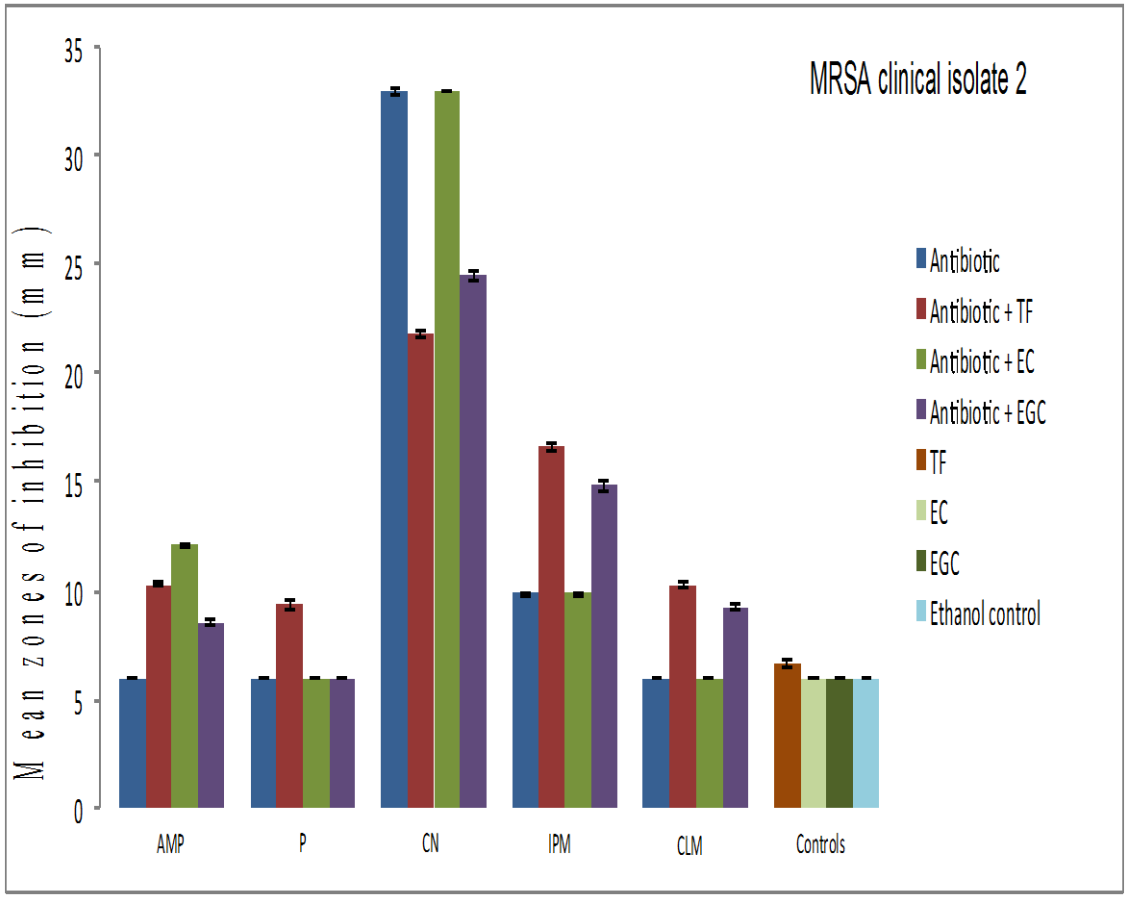
Figure 75**.** Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 1 of MRSA.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, penicillin G = P, gentamycin = CN, imipenem = IPM and clarithromycin = CLM. Zones include the 6 mm disc diameter.

In figure 75, isolate 1 of MRSA is seen to be resistant to ampicillin, penicillin G and clarithromycin although very susceptible to gentamycin and susceptible to Imipenem. With the exception of ampicillin and imipenem epicatechin shows no significant synergy or antagonism with any other antibiotic. Both TF and the epigallocatechin show significant antagonism with gentamycin but significant synergy with all other antibiotics used ($P \leq 0.05$). As expected the control concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

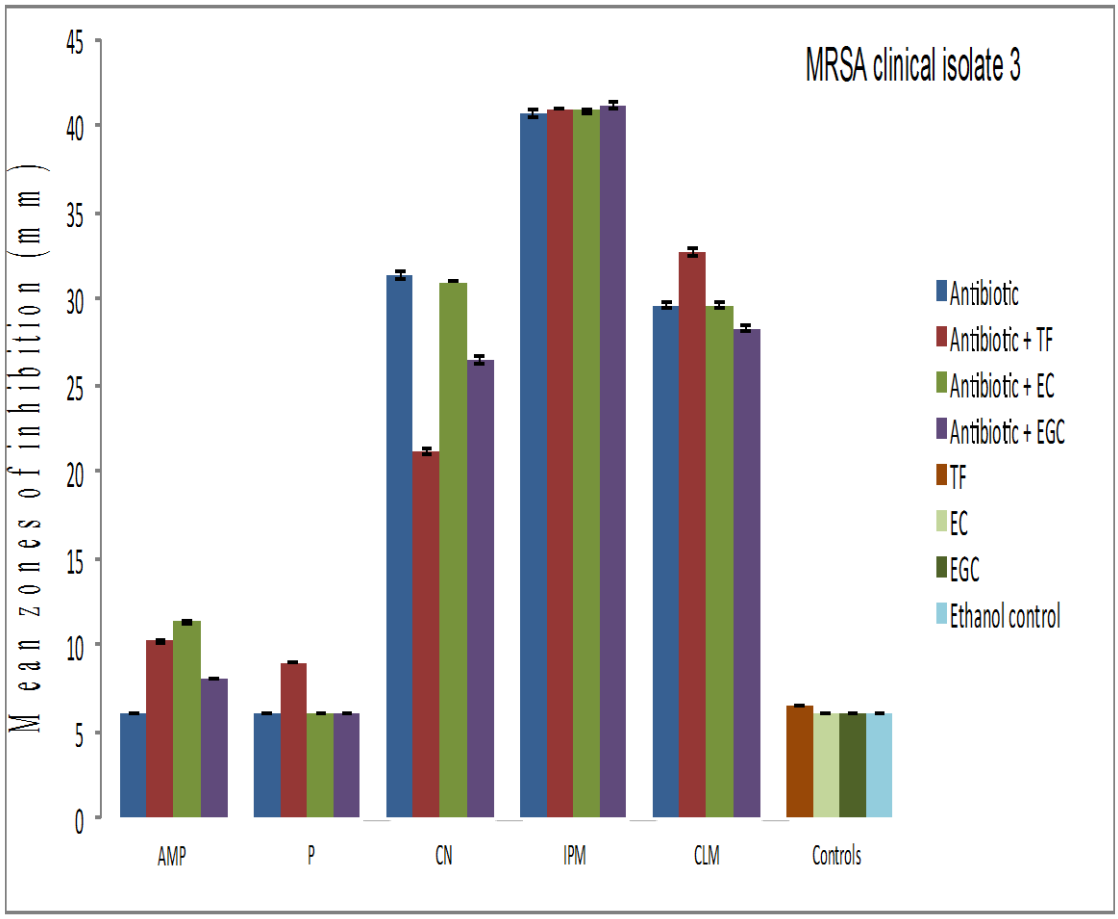
Figure 76**.** Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 2 of MRSA.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, penicillin G = P, gentamycin = CN, imipenem = IPM and clarithromycin = CLM. Zones include the 6 mm disc diameter.

In figure 76, isolate 2 of MRSA is seen to be resistant to ampicillin, penicillin G and clarithromycin although very susceptible to gentamycin and susceptible to Imipenem. epicatechin shows no significant synergy or antagonism with any antibiotic with the exception of ampicillin where significant synergy is seen for this isolate. Both theaflavin and epicatechin show significant antagonism with gentamycin but significant synergy with all other antibiotics used ($P \leq 0.05$) with the exception of epigallocatechin with penicillin G where no difference is seen. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

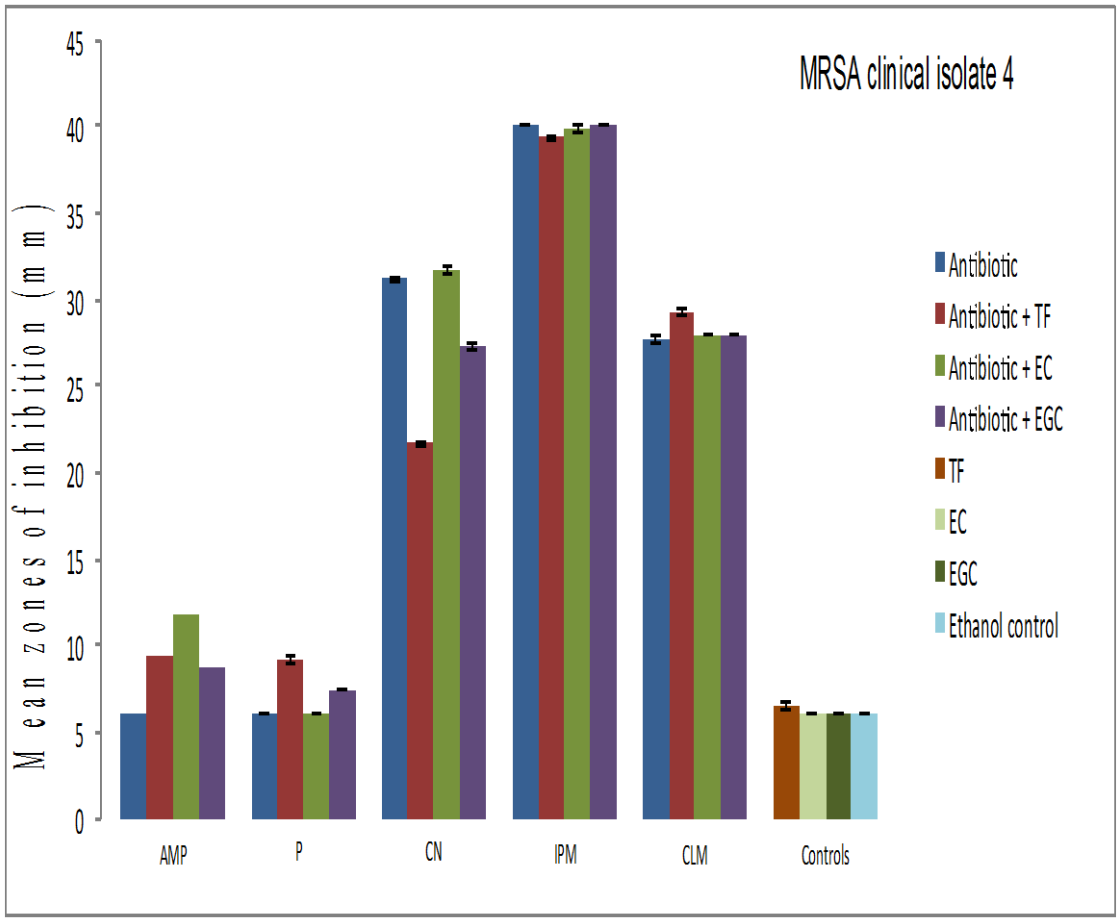
Figure 77. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 3 of MRSA.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, penicillin G = P, gentamycin = CN, imipenem = IPM and clarithromycin = CLM. Zones include the 6 mm disc diameter.

In figure 77, isolate 3 of MRSA is seen to be resistant to penicillin G and ampicillin, although it is highly susceptible to gentamycin, imipenem and clarithromycin. With this isolate of MRSA epicatechin only shows significant synergy with ampicillin but no antagonism with any antibiotic. Both theaflavin and the epigallocatechin show significant antagonism with gentamycin and clarithromycin ($P \leq 0.05$) but significant synergy with ampicillin ($P \leq 0.05$) and no significant effect with imipenem. Theaflavin also shows significant synergy with penicillin G. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

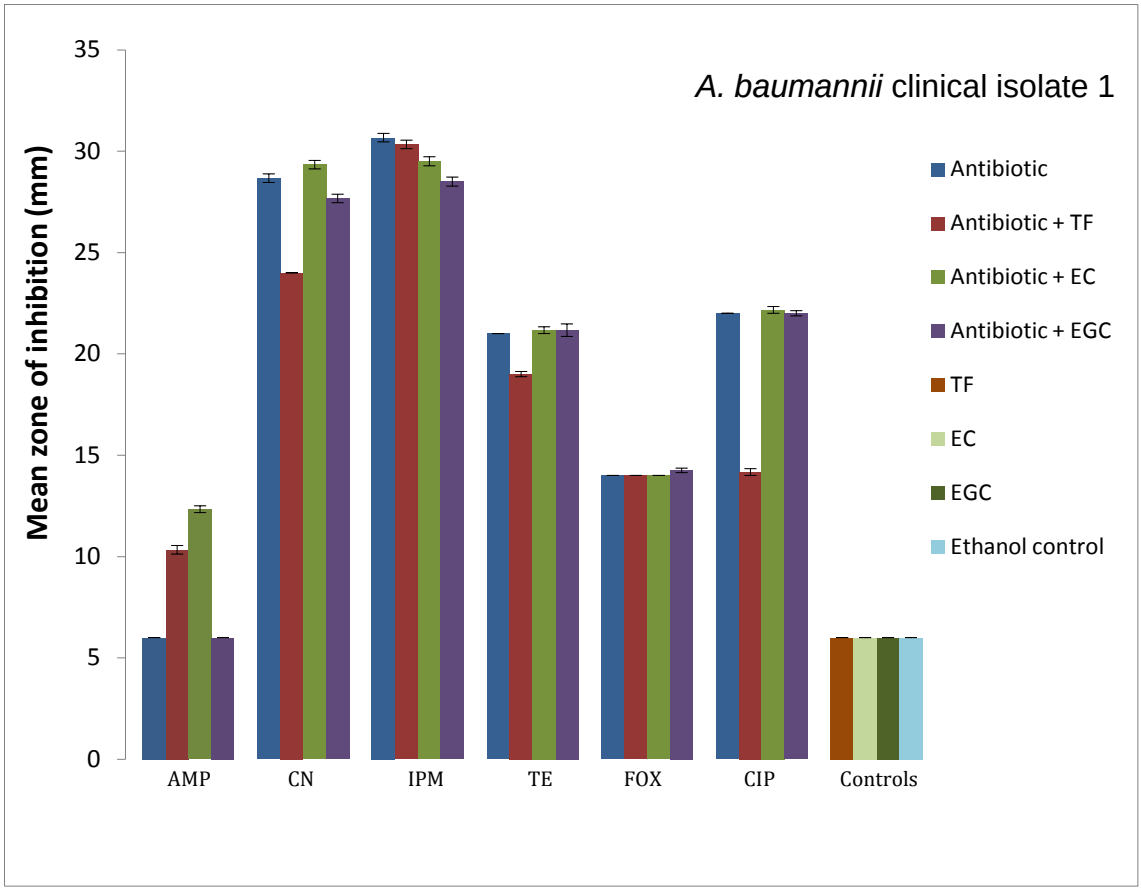
Figure 78. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 4 of MRSA.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, penicillin G = P, gentamycin = CN, imipenem = IPM and clarithromycin = CLM. Zones include the 6 mm disc diameter.

In figure 78, isolate 4 of MRSA is seen to be resistant to ampicillin and penicillin G although it is highly susceptible to gentamycin, imipenem and clarithromycin. With this isolate of MRSA epicatechin only shows significant synergy with ampicillin. With all other antibiotics no significant synergy or antagonism is seen. Both theaflavin and the epigallocatechin show significant antagonism with gentamycin. Significant synergy between theaflavin and clarithromycin was observed and antagonism with imipenem ($P \leq 0.05$). Both theaflavin and epigallocatechin showed significant synergy with penicillin G ($P \leq 0.05$). As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

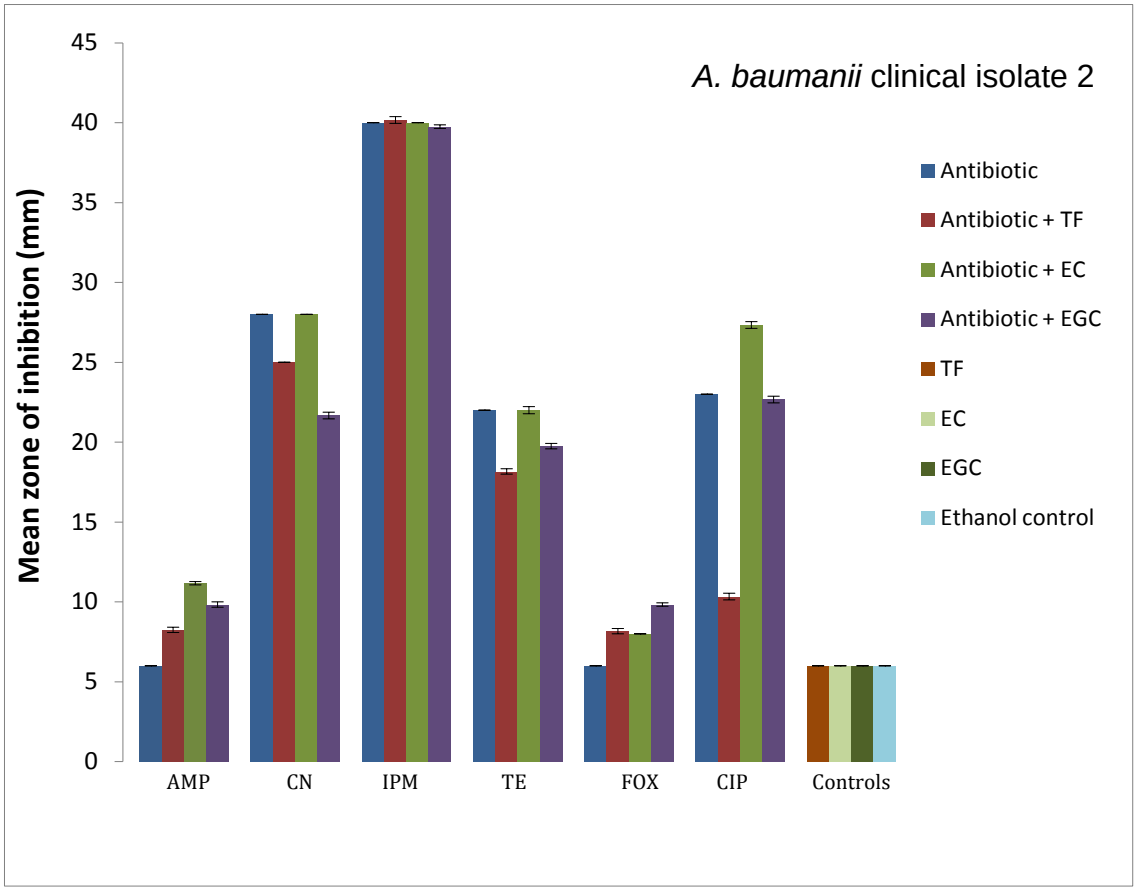
Figure 79. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 1 of *A. baumannii*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 79, isolate 1 of *A. baumannii* is seen to be resistant to ampicillin although it is highly susceptible to all other antibiotics tested. With this isolate of *A. baumannii* epicatechin only shows significant synergy with ampicillin and gentamycin. Both theaflavin and the epigallocatechin show significant antagonism with gentamycin ($P \leq 0.05$) but theaflavin show significant synergy with ampicillin ($P \leq 0.05$). Theaflavin produces significant antagonism with ciprofloxacin and tetracycline. No significant synergy or antagonism is observed between theaflavin and imipenem or cefoxitin. No significant synergy or antagonism was observed between epigallocatechin and tetracycline, cefoxitin or ciprofloxacin and As expected the concentrations used for the epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

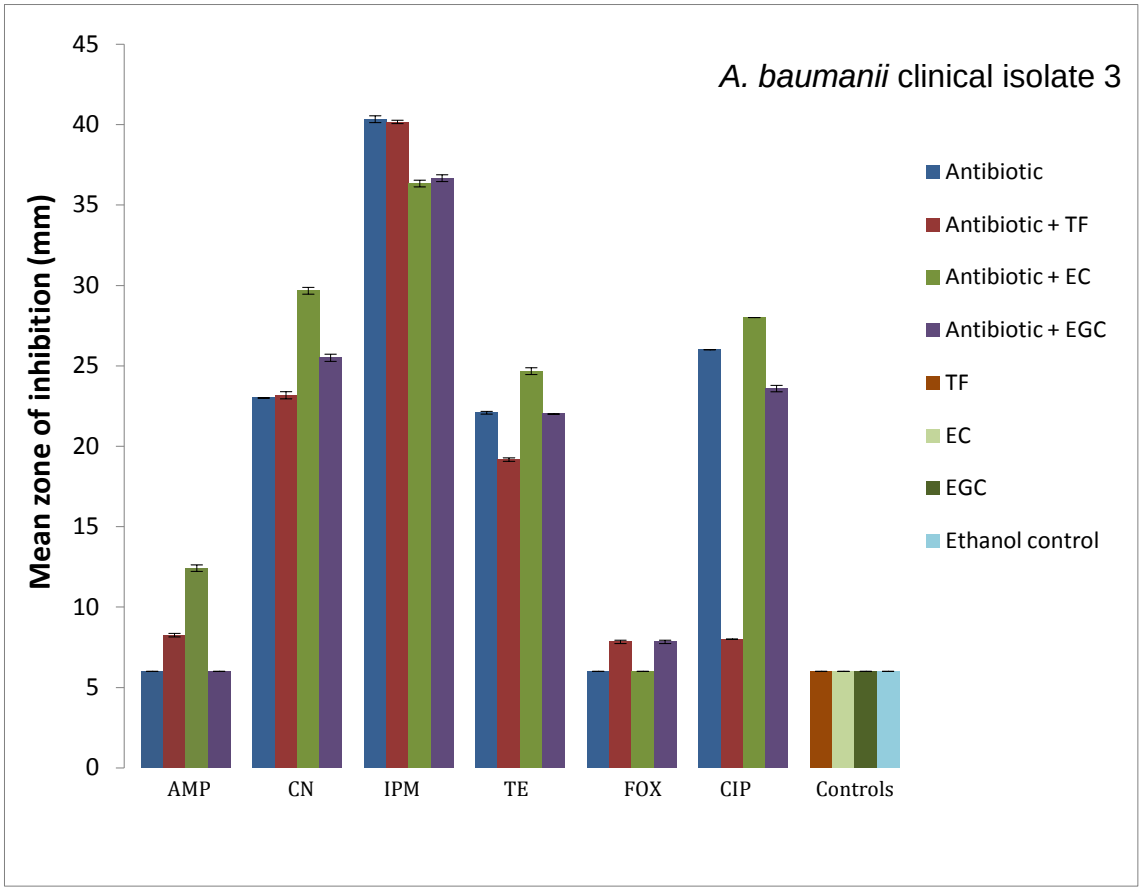
Figure 80. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 2 of *A. baumannii*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 80, isolate 2 of *A. baumannii* is seen to be resistant to ampicillin and cefoxitin. However, it is highly susceptible to all other antibiotics used. With this isolate of *A. baumannii* epicatechin shows significant synergy with ampicillin, cefoxitin and ciprofloxacin and no significant difference with any other antibiotic. When used with gentamycin and tetracycline, epigallocatechin and theaflavin show significant antagonism ($P \leq 0.05$) but both show significant synergy with ampicillin and cefoxitin ($P \leq 0.05$). No significant synergy or antagonism is observed between imipenem and theaflavin or epigallocatechin. However significant antagonism is seen between theaflavin and ciprofloxacin. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

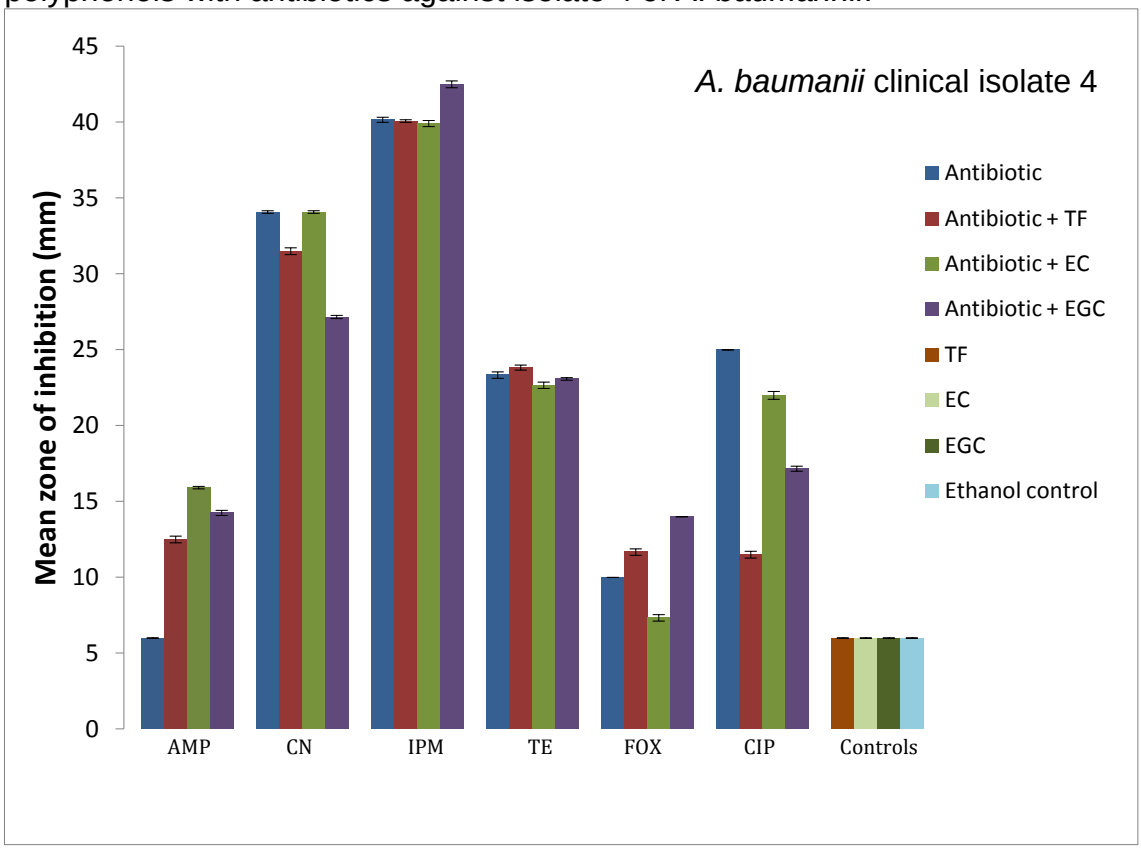
Figure 81. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 3 of *A. baumannii*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 81, isolate 3 of *A. baumannii* is seen to be resistant to ampicillin and cefoxitin. However, it is highly susceptible to all other antibiotics used. With this isolate of *A. baumannii* epicatechin shows significant synergy with ampicillin, gentamycin, tetracycline and ciprofloxacin, no significant difference with cefoxitin and significant antagonism with imipenem. When used with ciprofloxacin, epigallocatechin and theaflavin show significant antagonism ($P \leq 0.05$) but both show significant synergy with ampicillin and cefoxitin ($P \leq 0.05$). No significant synergy or antagonism is observed between imipenem and theaflavin. However significant antagonism is seen with epigallocatechin and theaflavin against ciprofloxacin. epigallocatechin also shows antagonism when used with imipenem. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

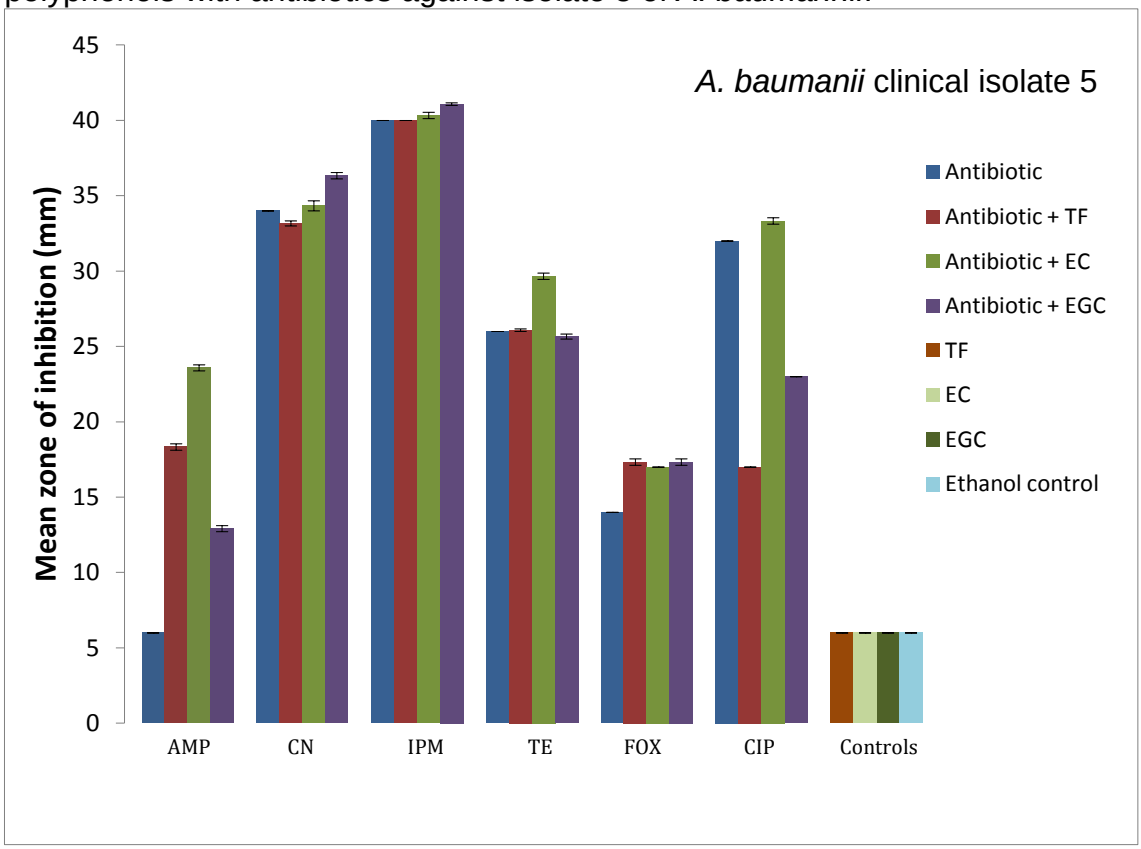
Figure 82. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 4 of *A. baumannii*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 82, isolate 4 of *A. baumannii* is seen to be resistant to ampicillin and is highly susceptible to all other antibiotics used. With this isolate of *A. baumannii* epicatechin shows significant synergy with ampicillin, no significant difference with imipenem, tetracycline and gentamycin and significant antagonism with cefoxitin and ciprofloxacin. When used with ciprofloxacin and gentamycin, epigallocatechin and theaflavin show significant antagonism ($P \leq 0.05$) but both show significant synergy with ampicillin and cefoxitin ($P \leq 0.05$). No significant synergy or antagonism is observed between imipenem and theaflavin. Epigallocatechin also shows synergy when used with imipenem. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

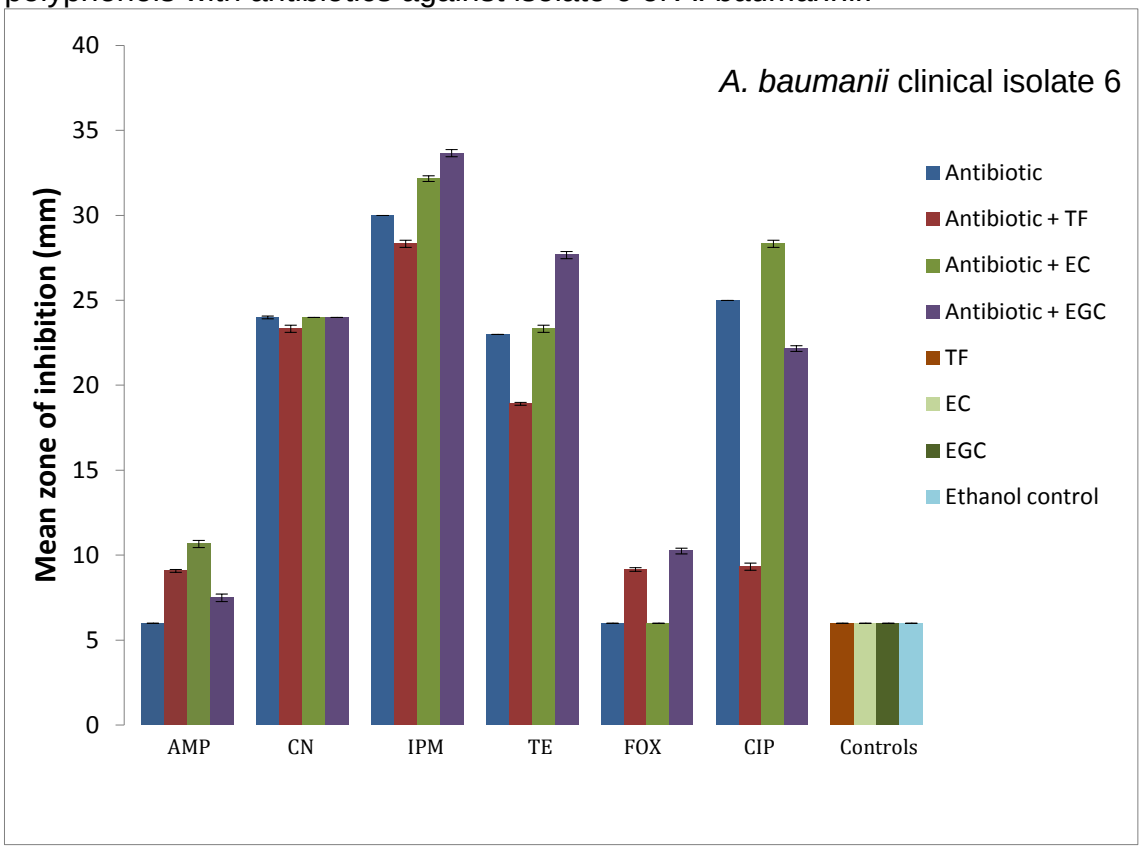
Figure 83. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 5 of *A. baumannii*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 83, isolate 5 of *A. baumannii* is seen to be resistant to ampicillin and is highly susceptible to all other antibiotics used. With this isolate of *A. baumannii* epicatechin shows significant synergy with ampicillin, tetracycline, cefoxitin and ciprofloxacin and no significant difference with imipenem and gentamycin. When used with ciprofloxacin, epigallocatechin and theaflavin show significant antagonism ($P \leq 0.05$) but both show significant synergy with ampicillin and cefoxitin ($P \leq 0.05$). No significant synergy or antagonism is observed between imipenem or tetracycline and theaflavin or epigallocatechin. When gentamycin when used with epigallocatechin synergy seen but when used with theaflavin antagonism is shown. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

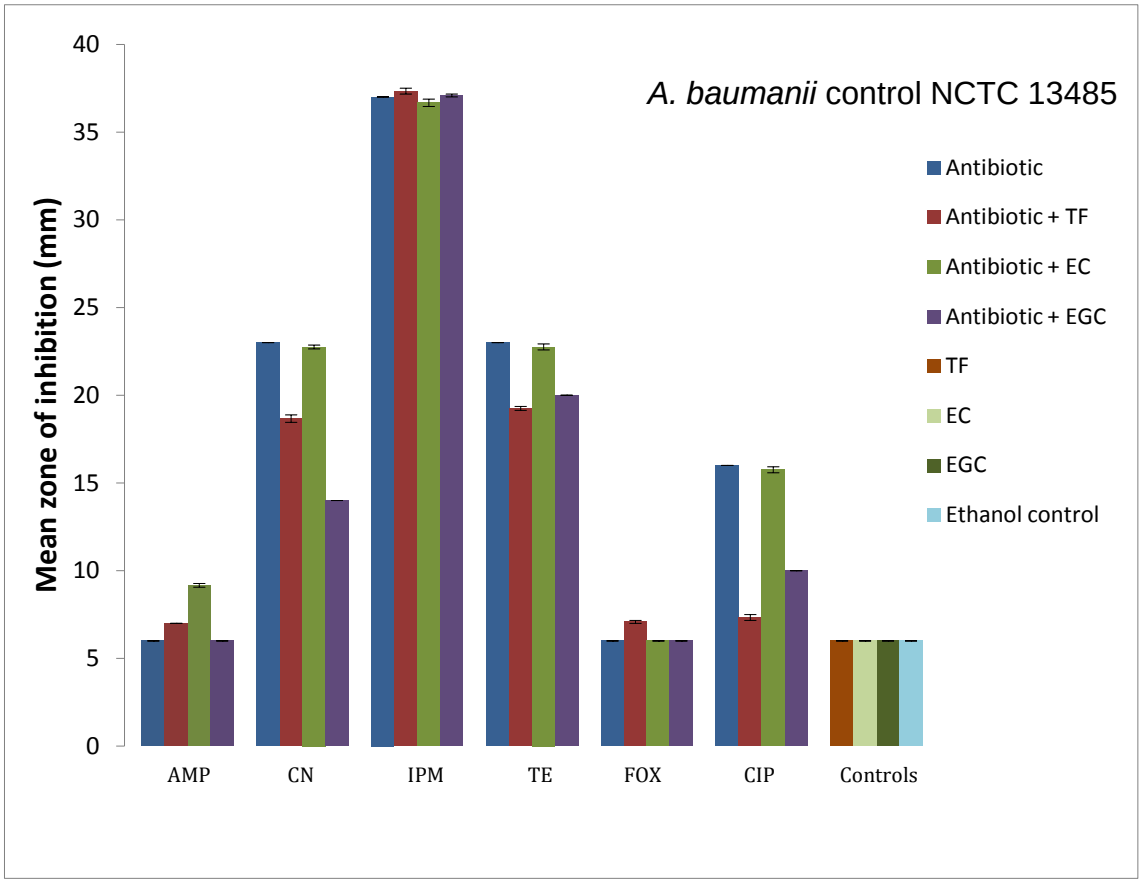
Figure 84. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 6 of *A. baumannii*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 84, isolate 6 of *A. baumannii* is seen to be resistant to ampicillin and cefoxitin, but is highly susceptible to all other antibiotics used. With this isolate of *A. baumannii* epicatechin shows significant synergy with ampicillin, imipenem and ciprofloxacin and no significant difference with gentamycin and cefoxitin. When used with ciprofloxacin, Eepigallocatechin and theaflavin show significant antagonism ($P \leq 0.05$) but both show significant synergy with ampicillin and cefoxitin ($P \leq 0.05$). Antagonism is observed between theaflavin and gentamycin, imipenem and tetracycline. No significant synergy or antagonism is observed between gentamycin epigallocatechin. Combinations of tetracycline or imipenem with epicatechin show synergy. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

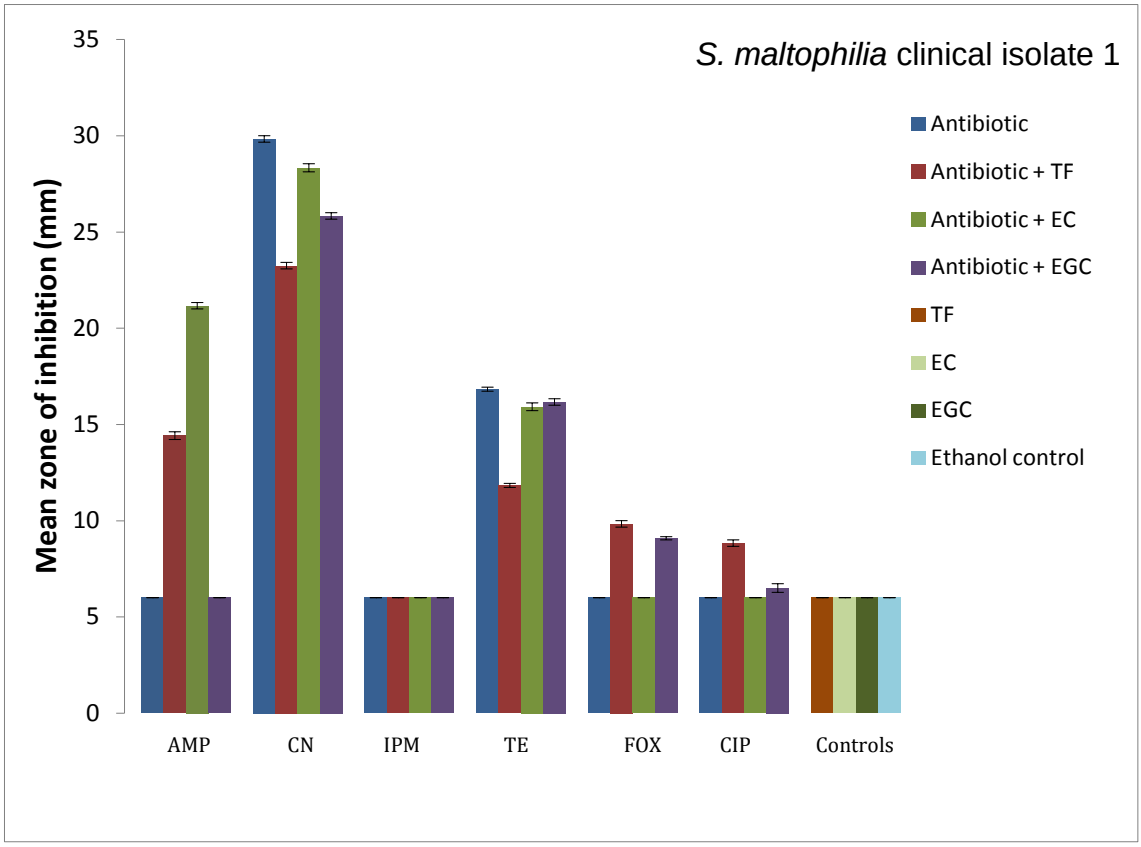
Figure 85. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against the control strain (NCTC 13485) of *A. baumannii*. Zones include the 6 mm disc diameter.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 85, control NCTC 13485 of *A. baumannii* is seen to be resistant to ampicillin and cefoxitin and is highly susceptible to all other antibiotics used. With this isolate of *A. baumannii* epicatechin shows significant synergy with ampicillin and no significant difference with any other antibiotic. When used with gentamycin and ciprofloxacin, epigallocatechin and theaflavin show significant antagonism ($P \leq 0.05$) but theaflavin shows significant synergy with ampicillin ($P \leq 0.05$). No significant synergy or antagonism is observed between imipenem and theaflavin or epigallocatechin. When used with theaflavin antagonism is shown for tetracycline but synergy is observed with cefoxitin. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

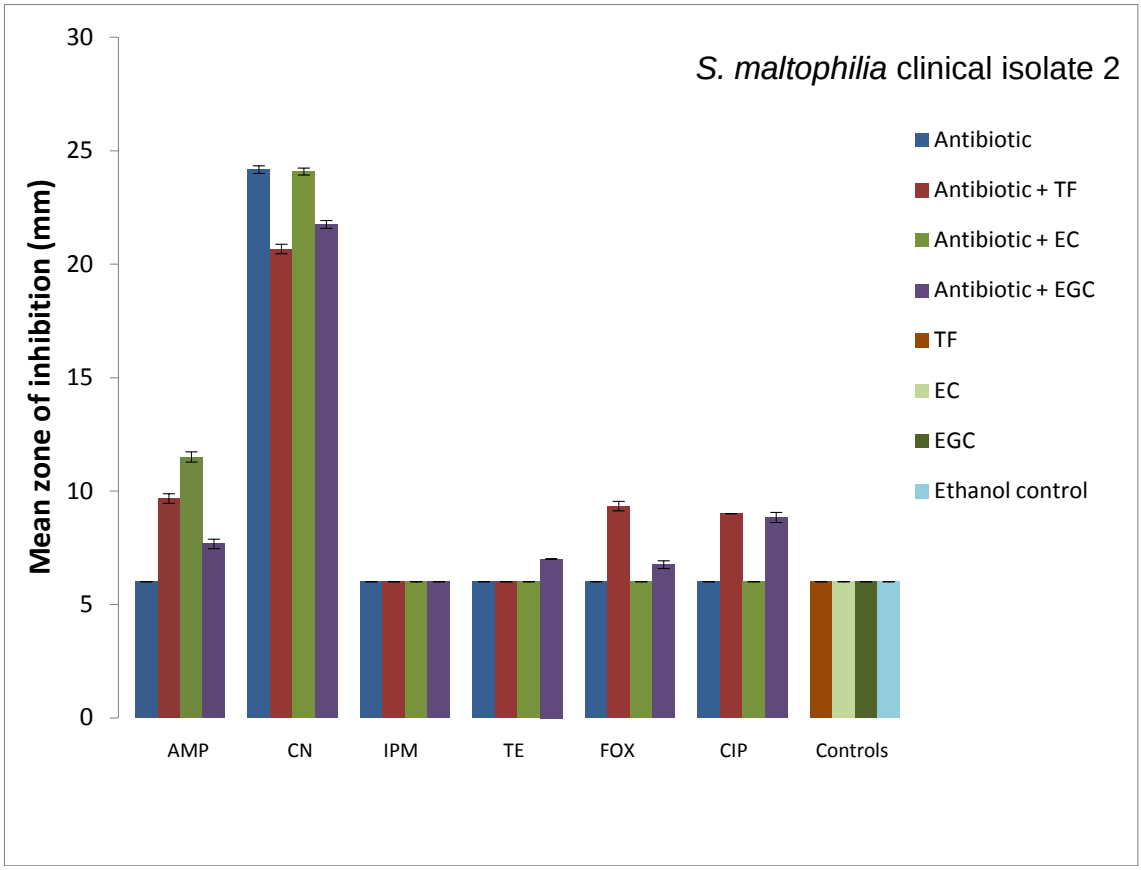
Figure 86. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 1 of *S*. *maltophilia*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 86, isolate 1 of *S. maltophilia* is seen to be resistant to ampicillin, Imipenem, cefoxitin and ciprofloxacin. However, it is highly susceptible to gentamycin and tetracycline. With this isolate of *S. maltophilia* epicatechin shows significant synergy with ampicillin, antagonism with gentamycin and tetracycline and no effect with all other antibiotics used. Both theaflavin and the epigallocatechin show significant antagonism with gentamycin ($P \leq 0.05$) and tetracycline but significant synergy with cefoxitin ($P \leq 0.05$) and no effect with imipenem. theaflavin also showed significant synergy with ampicillin and ciprofloxacin. As expected the concentrations used for the epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

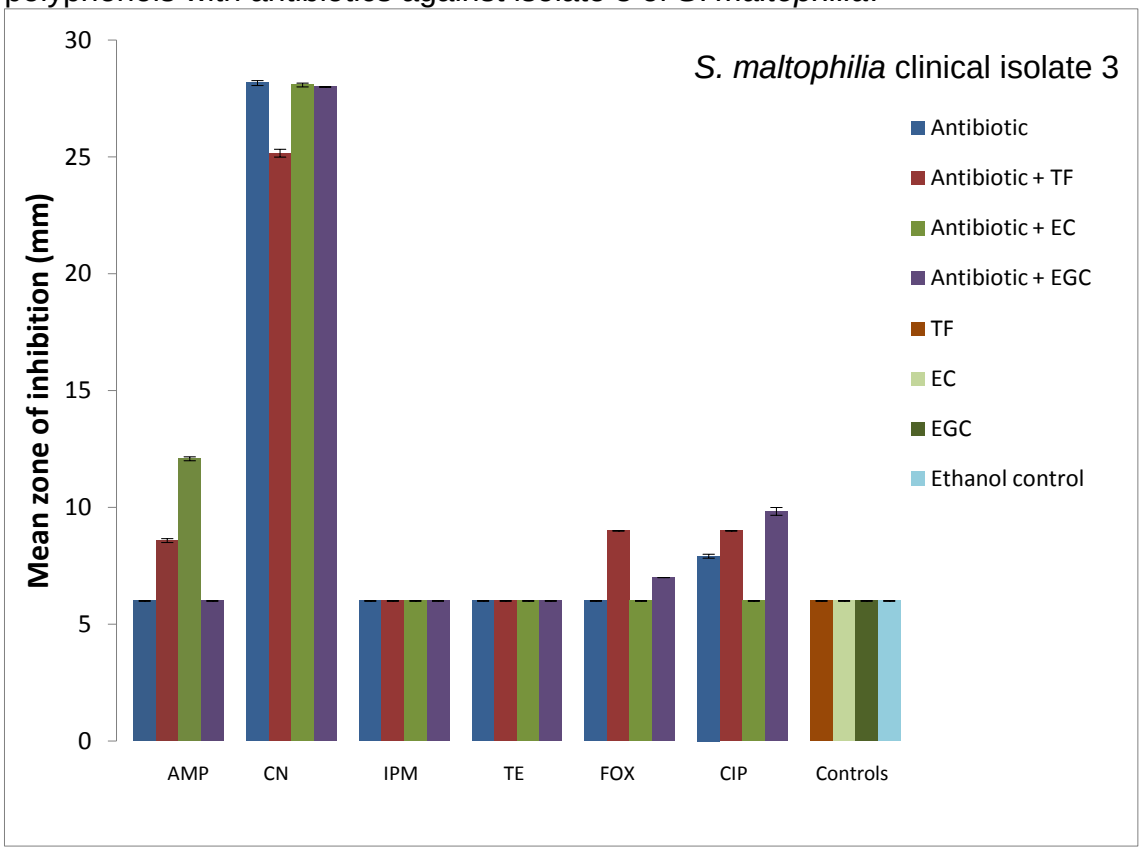
Figure 87. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 2 of *S*. *maltophilia*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 87, isolate 2 of *S. maltophilia* is seen to be resistant to all antibiotics used in this study with the exception of gentamycin. With this isolate of *S. maltophilia* epicatechin shows significant synergy with ampicillin and no effect with any other antibiotic used. Both theaflavin and the epigallocatechin show significant antagonism with gentamycin ($P \leq 0.05$), significant synergy with ampicillin and ciprofloxacin ($P \leq 0.05$) and no effect with imipenem and tetracycline. theaflavin also showed significant synergy with cefoxitin. As expected the concentrations used for the epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

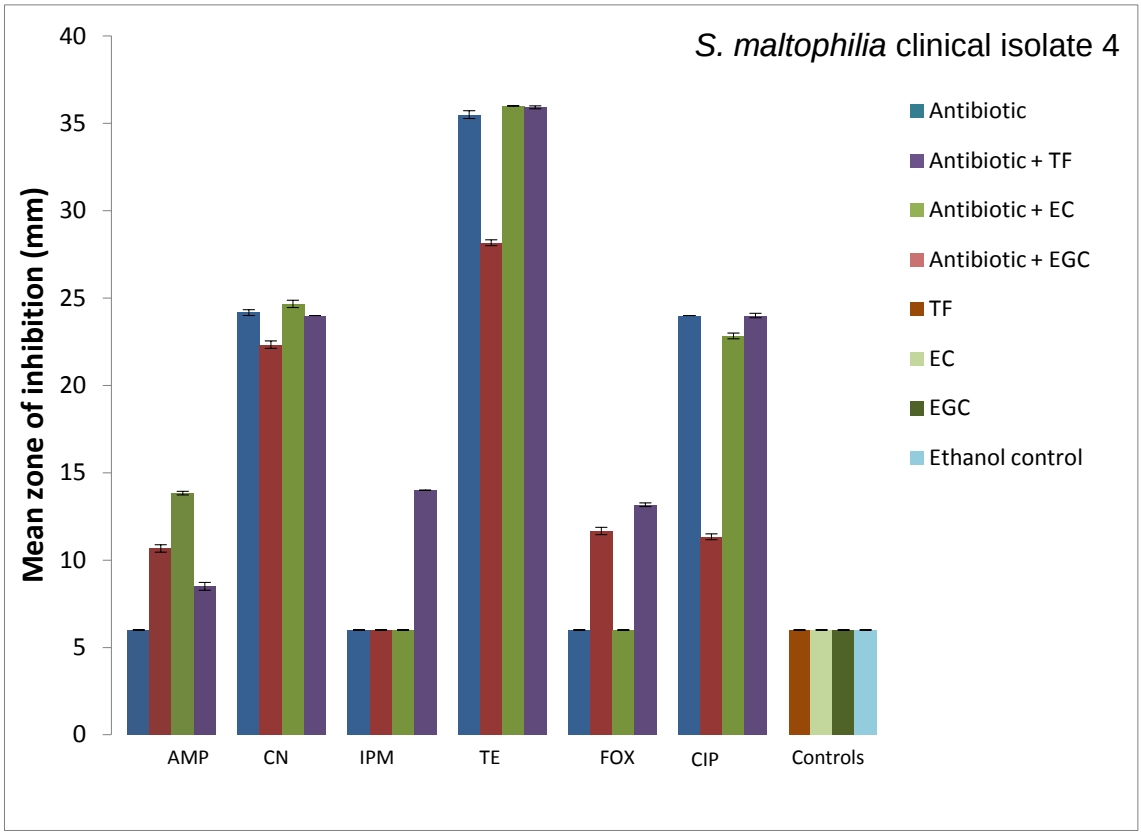
Figure 88. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 3 of *S. maltophilia*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 88, isolate 3 of *S. maltophilia* is seen to be resistant to all antibiotics used in this study with the exception of gentamycin and ciprofloxacin. With this isolate of *S. maltophilia* epicatechin shows significant synergy ($P \leq 0.05$) with ampicillin and no effects with any other antibiotic used. Both theaflavin and epigallocatechin show significant synergy ($P \leq 0.05$) with ampicillin, cefoxitin and ciprofloxacin, however theaflavin shows significant antagonism with gentamycin ($P \leq 0.05$). As expected the concentrations used for the epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

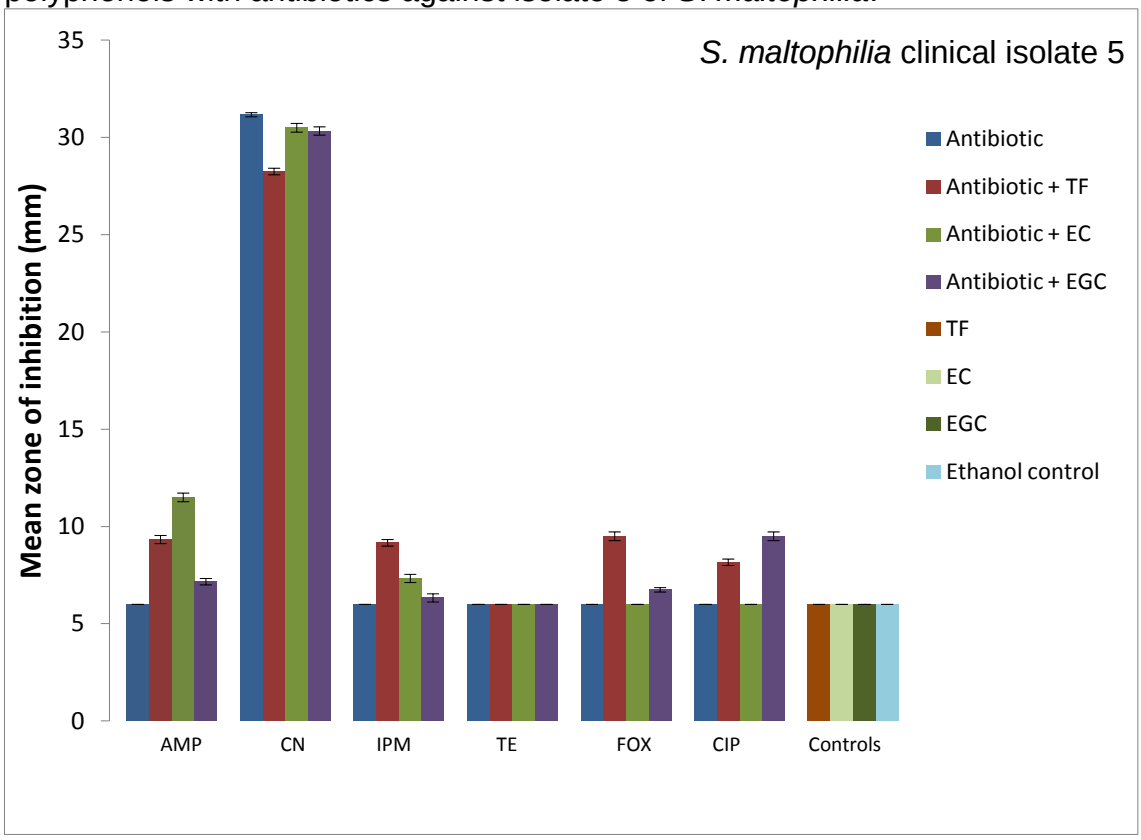
Figure 89. S Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 4 of *S. maltophilia*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 89, isolate 4 of *S. maltophilia* is seen to be resistant to ampicillin, imipenem and cefoxitin. However, it is highly susceptible to gentamycin, tetracycline and ciprofloxacin. With this isolate of *S. maltophilia* epicatechin shows significant synergy ($P \leq 0.05$) with ampicillin, antagonism with ciprofloxacin and no effect with any other antibiotic used. Both theaflavin and epigallocatechin show significant synergy ($P \leq 0.05$) with ampicillin and cefoxitin and antagonism with gentamycin ($P \leq 0.05$). Theaflavin shows antagonism with ciprofloxacin and tetracycline and epigallocatechin shows significant synergy with imipenem and no effect with ciprofloxacin or tetracycline. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

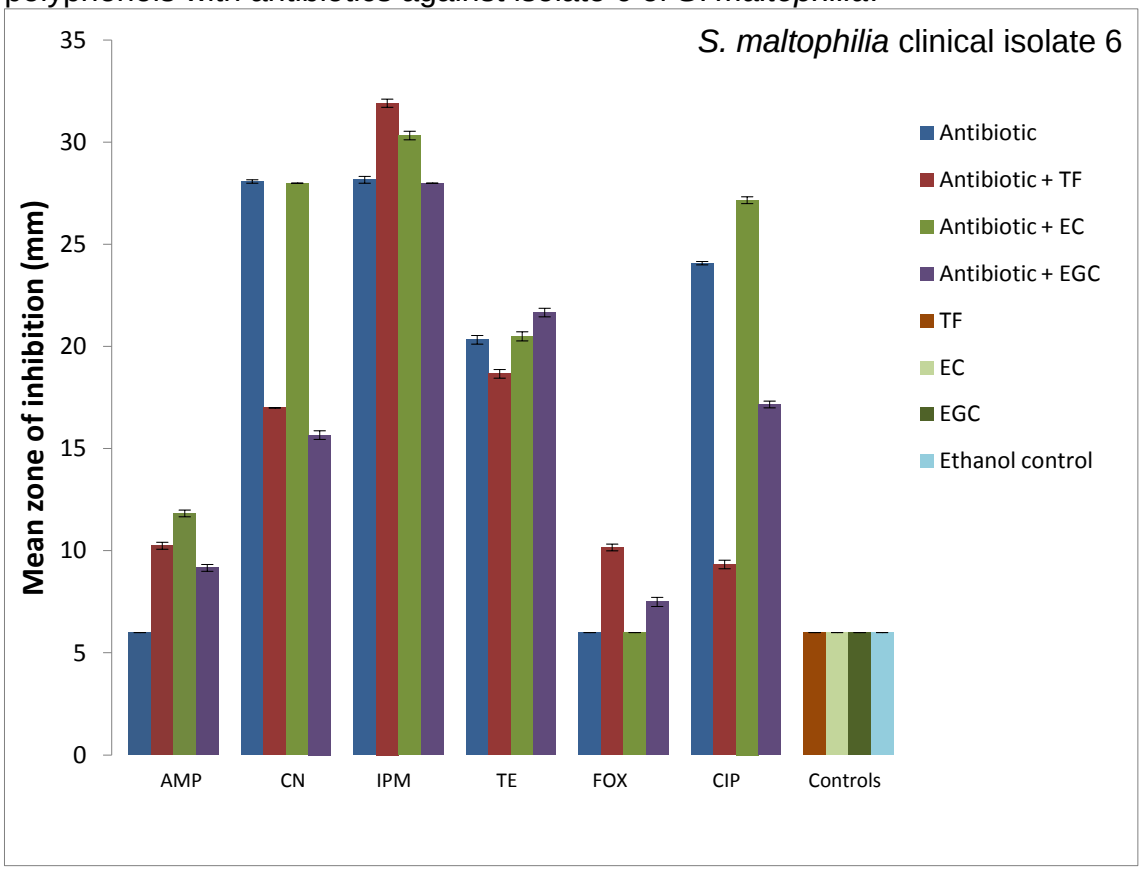
Figure 90. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 5 of *S. maltophilia*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 90, isolate 5 of *S. maltophilia* is seen to be resistant to all antibiotics used in this study with the exception of gentamycin. With this isolate of *S. maltophilia* epicatechin shows significant synergy ($P \leq 0.05$) with ampicillin and imipenem, antagonism with gentamycin and no effect with any other antibiotic used. Both theaflavin and epigallocatechin show significant synergy ($P \leq 0.05$) with ampicillin, cefoxitin and ciprofloxacin, antagonism with gentamycin ($P \leq 0.05$) and no effect with tetracycline. Theaflavin shows significant synergy with imipenem ($P \leq 0.05$) whereas epigallocatechin shows no effect. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

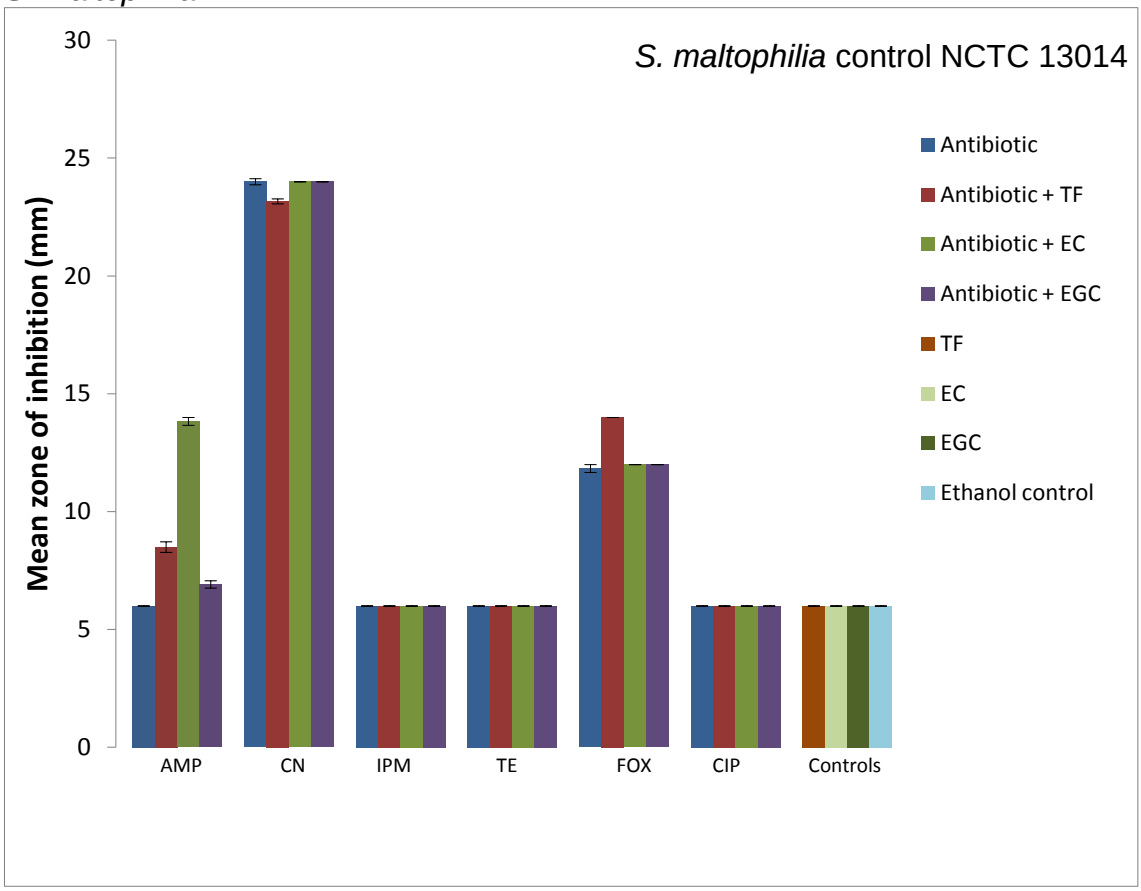
Figure 91. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against isolate 6 of *S. maltophilia*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 91, isolate 6 of *S. maltophilia* is seen to be resistant to ampicillin and cefoxitin and susceptible to all antibiotics used in this study. With this isolate of *S. maltophilia* epicatechin shows significant synergy ($P \leq 0.05$) with ampicillin, imipenem and ciprofloxacin and no effect with any other antibiotic used. Both theaflavin and epigallocatechin show significant synergy ($P \leq 0.05$) with ampicillin and cefoxitin, but antagonism with gentamycin, and ciprofloxacin ($P \leq 0.05$). Theaflavin shows significant synergy with imipenem ($P \leq 0.05$) whereas epigallocatechin shows no effect. Significant antagonism exists between theaflavin and tetracycline whereas with epigallocatechin synergism occurs. As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

Figure 92. Graph showing the synergistic and antagonistic effects of polyphenols with antibiotics against the control strain (NCTC 13014) of *S. maltophilia*.




Where theaflavin = TF, epicatechin = EC, epigallocatechin = EGC, ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP. Zones include the 6 mm disc diameter.

In figure 92, control strain NCTC 13014 of *S. maltophilia* is seen to be resistant to all antibiotics used in this study with the exception of gentamycin and cefoxitin. With this strain of *S. maltophilia* epicatechin shows significant synergy ($P \leq 0.05$) with ampicillin and no effect with any other antibiotic used. Both theaflavin and epigallocatechin show significant synergy ($P \leq 0.05$) with ampicillin and no effect with ciprofloxacin, tetracycline or imipenem. Theaflavin shows significant antagonism with gentamycin ($P \leq 0.05$) and significant synergy with cefoxitin ($P \leq 0.05$). As expected the concentrations used for epicatechin, theaflavin, epigallocatechin controls and the ethanol control show no significant antibacterial effect.

Table 27. Results of a student T-test (paired, 2 tailed) to show significant difference between antibacterial effects of antibiotics and antibiotic-polyphenol combinations against MRSA isolates.

| | Organism | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MRSA | | | | | | | | | | | |
| | [Antibiotic] vs. [Antibiotic + EC] | | | | [Antibiotic] vs. [Antibiotic + TF] | | | | [Antibiotic] vs. [Antibiotic + EGC] | | | |
| Isolate no. | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| AMP | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S |
| PEN | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S |
| CN | P = 0.3632 N | P = 0.3632 N | P = 0.4838 N | P = 0.1019 N | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S |
| IPM | P = 0.0001 S | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N | P = 0.0429 S | P = 0.0001 S | P = 0.0001 S | P = 0.1747 N | P = 0.1747 N |
| CLM | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.4838 N | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N |

Where ampicillin = AMP, penicillin G = PEN, gentamycin = CN, imipenem = IPM and clarithromycin = CLM, epicatechin = EC, theaflavin = TF and epigallocatechin gallate = EGC. S = significant difference (Null hypothesis rejected) and N= no significant difference (Null hypothesis accepted). P = 2 tailed P value.

Table 28. Results of a student T-test (paired, 2 tailed) to show significant difference between antibacterial effects of antibiotics and antibiotic-polyphenol combinations against *A. baumannii* isolates.

| | Organism | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *A. baumannii* | | | | | | | | | | | | | | | | | | | |
| | [Antibiotic] vs. [Antibiotic + EC] | | | | | | | [Antibiotic] vs. [Antibiotic + TF] | | | | | | | [Antibiotic] vs. [Antibiotic + EGC] | | | | | | |
| Isolate no. | 1 | 2 | 3 | 4 | 5 | 6 | C | 1 | 2 | 3 | 4 | 5 | 6 | C | 1 | 2 | 3 | 4 | 5 | 6 | C |
| AMP | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N | P = 0.0001 S | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.0011 S | P = 0.3632 N |
| CN | P = 0.025 S | P = 0.3632 N | P = 0.0001 S | P = 0.999 N | P = 0.3632 N | P = 0.4838 N | P = 0.0756 N | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N | P = 0.0002 S | P = 0.0041 S | P = 0.0301 S | P = 0.0001 S | P = 0.0409 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N | P = 0.0001 S |
| IPM | P = 0.0004 S | P = 0.3632 N | P = 0.0001 S | P = 0.3632 N | P = 0.1747 N | P = 0.0001 S | P = 0.1747 N | P = 0.1747 N | P = 0.4650 N | P = 0.2374 N | P = 0.6952 N | P = 0.3632 N | P = 0.0005 S | P = 0.1019 N | P = 0.0001 S | P = 0.0756 N | P = 0.0001 S | P = 0.0009 S | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N |
| TE | P = 0.3148 N | P = 0.999 N | P = 0.0002 S | P = 0.1886 N | P = 0.0001 S | P = 0.1747 N | P = 0.2031 N | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0756 N | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.6109 N | P = 0.0001 S | P = 0.3632 N | P = 0.3632 N | P = 0.1019 N | P = 0.0001 S | P = 0.0001 S |
| FOX | P = 0.3632 N | P = 0.0001 S | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.0005 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0756 N | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N |
| CIP | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0015 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.999 N | P = 0.1747 N | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0005 S |

Where ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP, epicatechin = EC, theaflavin = TF and epigallocatechin gallate = EGC. S = significant difference (Null hypothesis rejected) and N= no significant difference (Null hypothesis accepted). P = 2 tailed P value.

Table 29. Results of a student T-test (paired, 2 tailed) to show significant difference between antibacterial effects of antibiotics and antibiotic-polyphenol combinations against *S. maltophilia* isolates.

| | Organism | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *S. maltophilia* | | | | | | | | | | | | | | | | | | | |
| | [Antibiotic] vs. [Antibiotic + EC] | | | | | | | [Antibiotic] vs. [Antibiotic + TF] | | | | | | | [Antibiotic] vs. [Antibiotic + EGC] | | | | | | |
| Isolate no. | 1 | 2 | 3 | 4 | 5 | 6 | C | 1 | 2 | 3 | 4 | 5 | 6 | C | 1 | 2 | 3 | 4 | 5 | 6 | C |
| AMP | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N | P = 0.0005 S | P = 0.3632 N | P = 0.0001 S | P = 0.0009 S | P = 0.0001 S | P = 0.0019 S |
| CN | P = 0.011 S | P = 0.79 N | P = 0.3632 N | P = 0.0756 N | P = 0.0429 S | P = 0.3632 N | P = 0.999 N | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0041 S | P = 0.0001 S | P = 0.0001 S | P = 0.1747 N | P = 0.42 N | P = 0.0305 N | P = 0.0001 S | P = 0.999 N |
| IPM | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.0015 S | P = 0.0009 S | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.0001 S | P = 0.1747 N | P = 0.42 N | P = 0.3632 N |
| TE | P = 0.019 S | P = 0.3632 N | P = 0.3632 N | P = 0.0628 N | P = 0.3632 N | P = 0.6109 N | P = 0.3632 N | P = 0.0001 S | P = 0.3632 N | P = 0.3632 N | P = 0.0001 S | P = 0.3632 N | P = 0.0041 S | P = 0.3632 N | P = 0.0429 S | P = 0.0001 S | P = 0.3632 N | P = 0.1852 N | P = 0.3632 N | P = 0.0015 S | P = 0.3632 N |
| FOX | P = 0.3632 S | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0429 S | P = 0.0071 S | P = 0.0001 S | P = 0.0001 S | P = 0.0011 S | P = 0.0011 S | P = 0.3632 N |
| CIP | P = 0.3632 N | P = 0.3632 N | P = 0.0001 S | P = 0.0009 S | P = 0.3632 N | P = 0.0001 S | P = 0.3632 N | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N | P = 0.0756 S | P = 0.0001 S | P = 0.0002 S | P = 0.999 N | P = 0.0001 S | P = 0.0001 S | P = 0.3632 N |

Where ampicillin = AMP, gentamycin = CN, imipenem = IPM, tetracycline = TE, cefoxitin = FOX and ciprofloxacin = CIP, epicatechin = EC, theaflavin = TF and epigallocatechin gallate = EGC. S = significant difference (Null hypothesis rejected) and N= no significant difference (Null hypothesis accepted). P = 2 tailed P value.

The results in this study have demonstrated that the tea polyphenols epicatechin and theaflavin had significant synergy with ampicillin against every isolate tested with epicatechin giving the highest increase of antibacterial activity. Epigallocatechin also showed significant synergy with ampicillin against all but 5 isolates. The proposed mechanism for this synergy is the modification or destruction of the Beta-lactamases produced by the organisms Hemaiswarya, Kruthiventi and Doble, 2008). This work clearly shows the potential for polyphenol-ampicillin combination drugs to treat infections caused by ampicillin resistant bacteria.

From the results with penicillin G it was clear that theaflavin showed significant synergy against MRSA with epigallocatechin giving either synergy or indifference. As in the study by Zhao *et al.* (2001), who used epigallocatechin gallate against *Staphylococcus aureus*, it is clear that the addition of polyphenols can enable penicillin to be once again an effective antibiotic. However, further investigations should take place as previous studies have found that some polyphenols can also produce antagonistic effects with penicillin (Denny, West and Mathew, 2008).

In the case of gentamycin in combination with theaflavin or epigallocatechin, significant antagonism was produced when used against almost all the isolates. It is unclear of the mechanism of the antagonism. However, it is possible that the polyphenols either blocked the site of action for gentamycin or interacted with the structure of the antibiotic to reduce its effectiveness. Another proposal to explain the antagonism is that theaflavin somehow reduced the ability of the antibiotic to diffuse into the agar, therefore reducing its apparent antimicrobial effect. However, from a previous study it was discovered that pomegranate extracts, comprised of high levels of polyphenols, produced significant synergy with gentamycin against MRSA (Braga *et al.,* 2005). In their research they demonstrated the extracts inhibit the bacteria's efflux pump NorA or enhanced the efflux of the drug. The variation in the chemical structures of the polyphenols in the extract could account for the differences in the results achieved in the previous study to this study. From this research it is clear that

using epicatechin, theaflavin and epigallocatechin with gentamycin would be of little clinical use against isolates of MRSA, *A. baumannii* and *S. maltophilia*.

When using imipenem with the addition of epicatechin mixed results were seen. Further research should be undertaken to confirm whether overall epicatechin could be a useful addition to increase the antibacterial action of imipenem. However, when imipenem is used in conjunction with theaflavin or the epigallocatechin synergy was produced against MRSA $\geq$ 50% indicating that a future duel treatment could be possible. Future research should investigate this further with a larger number of MRSA isolates. This was also found by Hu *et al.,* (2002), when epigallocatechin gallate was used in combination with imipenem against MRSA. In combination with theaflavin imipenem showed antagonism or no difference against *A. baumannii* and although further work into the mechanisms behind the relationship should take place, the preliminary results suggest a combination treatment would not be beneficial. However, against *S. maltophilia* there is potential due to the synergy observed although the reason for this is unclear and further investigation is required for confirmation. This variation between isolates might also indicate that the change is related to the site of antimicrobial attachment and that in certain strains the theaflavin reduces the ability of imipenem to inhibit cell wall synthesis. When imipenem is used in combination with epigallocatechin mixed results suggest that synergy or antagonism varies between isolates. Further investigations should investigate if any interactions between polyphenols and imipenem occur.

The results for clarithromycin indicated that there is no synergy with epicatechin but there is overall synergy with theaflavin. Previous research supports polyphenol synergy with clarithromycin (Yanagawa *et al.,* 2003) and it was concluded that the polyphenol epigallocatechin gallate had a synergistic effect with the antibiotic. Although the previous work investigated synergy between polyphenols and clarithromycin against gram-negative bacteria, this study confirms that the synergy also exists against gram-positive bacteria. As with their work it is suggested here that the clinical application of theaflavin and

clarithromycin should be investigated further as a method of treating resistant infections.

The *A. baumannii* isolates used in this investigation were all susceptible to tetracycline unlike the isolates of *S. maltophilia* where only ≤ 50% were affected by the antibiotic. Previous research has shown polyphenols interact well with this antibiotic whereby the polyphenol epigallocatechin to produces synergy with tetracycline against *S. aureus* by inhibiting the efflux of the antibiotic (Roccaro *et al.,* 2004). Unlike the previous investigation this research found no overall synergy between epicatechin, theaflavin or epigallocatechin and tetracycline. However, unlike the previous research this investigation investigated the effects against gram-negative bacteria. The different outer structure of the gram-negative bacteria used could be a possible reason for the antagonism or the polyphenol either preventing uptake of the antibiotic due to interacting with the cells mechanism of active transport. Further *in vitro* studies should investigate this mechanism to highlight the cause of the antagonism.

In this investigation it has be shown that synergies exist between polyphenols and cefoxitin with theaflavin and epigallocatechin showing synergy against almost all isolates used. This indicates a huge potential for polyphenols to be used in combination with this antibiotic to treat bacterial infections caused by gram-negative bacteria. Further research should look into the interaction of theaflavin and cefoxitin with gram-positive bacteria. With epicatechin, synergy was shown against 2 of 14 isolates and antagonism in 1 isolate. It could be concluded that the benefits of using epicatechin with this antibiotic are unclear and further research has been conducted. Present indications are that it would be unsuitable for combination therapy with cefoxitin. In a previous investigation, when using polyphenols with cefoxitin, no antagonism was observed (Denny, West and Mathew, 2008) showing that overall polyphenols do not lower the antibacterial activity of this antibiotic. Further research should be conducted to discover the mechanism behind the synergy of polyphenols and cefoxitin.

The *A. baumannii* isolates used in this investigation were all susceptible to Ciprofloxacin unlike the isolates of *S. maltophilia* where only ≤ 50% were

affected by the antibiotic. Against all *A. baumannii* isolates theaflavin and ciprofloxacin combinations showed significant antagonism and with epigallocatechin this was shown against 5 of the 7 isolates. It can be concluded that these 2 polyphenols would not be good candidates for a duel treatment of infections caused by *A. baumannii*. A more positive result was seen when these combination were used against *S. maltophilia* with ≥ 50% synergy produced. A previous investigation showed that when leaf extracts, containing polyphenols were used with ciprofloxacin against various gram-positive and gram-negative bacteria, a synergy rate of ≥ 60% was found (Aiyegoro, Afolayan and Okoh, 2009). With epicatechin a potential for a duel treatment exists as against 4 of the 7 isolates of *A. baumannii* synergy was shown. Further investigations to check the synergy should be undertaken.

Epicatechin had mixed synergy, antagonism and indifference with all other antibiotics against *A. baumannii* and *S. maltophilia*. Although results by Martins *et al.,* (2011) saw synergy of epicatechin and Ciprofloxacin against *Salmonella* strains, against *A. baumannii* and *S. maltophilia* mixed results were seen, with synergy and indifference occurring more times than antagonism. Overall this research cannot totally confirm or disagree with data previously presented by Stapleton *et al.,* (2004) where it was found that due to the lack of a gallate group epicatechin lacks synergy with antibiotics.

The mechanisms of the synergy between polyphenols, particularly theaflavin and clinical antibiotics are not yet fully understood. However, previous studies have proposed that synergy when using polyphenols and flavanols could be due to inhibition of β-lactamases (Zhao *et al.,* 2002) and destabilisation of the bacterial cytoplasmic membrane (Wang *et al.,* 2010).

From the antibiotic testing it was clear that the isolates with the highest level of antibiotic resistance were *S. maltophilia*. In future cases, the effectiveness of antibiotic therapies to treat infections caused by this bacterium will continually decline. To combat its high level of resistance, combination therapies could be

used to slow the rate of resistance and also increase the effectiveness of the treatment. Antibiotics often have problematic side effects, using lower dosages in combination with other natural compounds such as polyphenols could be the answer.

It is clear from the results in this study that synergy and antagonism between polyphenols and clinical antibiotics varies between bacterial genera and isolates of species. If clinical antibiotic/polyphenol combinations were to be considered as treatments for bacterial infections, there is an obvious requirement for preliminary susceptibility testing beforehand, in order to check the efficacy as is done with all infections in hospitals.

### 3.7 Conclusions

In the preliminary testing the antibacterial effects of epigallcatechin gallate, theaflavin and mixed theaflavins against *A. baumannii*, MRSA and VRE are very encouraging and hopefully in the future these compounds will be used as an alterative to antibiotics for treating infections. This investigation found epigallocatechin gallate to be the most effect antimicrobial agent against all three organisms. However, the results from theaflavin show it is an antimicrobial agent that is as effective against *A. baumannii* organisms as many antibiotics. Mixed theaflavins showed a higher antibacterial effects against VRE but less of an effect against *A. baumannii* than pure theaflavin. This, like in the case of epicatechin and epigallocatechin gallate could be due to the extra gallate group possessed by theaflavin 3,3'-digallate and theaflavin monogallate in the theaflavins mixture. Mixed theaflavins also showed a significant antibacterial effect against MRSA. Further tests will need to be performed to identify other organisms that theaflavin and mixed theaflavins can be used as antimicrobial agents against.

Due to the large difference between the antibacterial effects of epicatechin and epigallocatechin gallate a further investigation using labelled epicatechin and epigallocatechin gallate could be performed to identify how the compounds

interact within bacteria. This would lead to increased knowledge and potential modifications to the compounds to further increase their beneficial properties.

When testing the antibacterial effects of flavonoids against hospital isolates it was found that not only did theaflavin show significant antibacterial activity, but also this activity was significantly increased with the addition of epicatechin. As epicatechin had no obvious antibacterial action by itself, the results showed that a synergistic effect had occurred. It is the first time that the synergy between theaflavin and epicatechin has been reported against hospital isolates of *A. baumannii,* MRSA and *S. maltophilia*. These research findings are significant in that the results could be used to develop new antimicrobial combination therapies to treat infections caused by resistant microorganisms. This study also reported for the first time the MICs of theaflavin and a theaflavin:epicatechin combination against hospital isolates of *S. maltophilia*. Future work should look into the mechanisms behind the synergy and undertake time-kill studies against not only *S. maltophilia* but also against other important hospital pathogens.

This is also believed to be the one of the first reports providing evidence of synergistic or antagonistic properties of theaflavin, epicatechin and EGC when used with antibiotics against clinical isolates of MRSA, *A. baumannii* and *S. maltophilia*. From the results in this study it is clear that there is huge potential for polyphenols to be used in combination with traditional antibiotics to treat infections caused by resistant organisms. Further work is recommended to investigate the mechanisms underlying the interactions of these antimicrobial agents and their effectiveness in the wider bacterial spectrum. Further work should also be conduction *in vivo* to understand if the poor bioavailability of polyphenols affects the synergistic benefits seen *in vitro*, if this can be overcome and if this limits their medial uses to topical treatments.

## 3.8 References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# Chapter 4

Production of theaflavin in a microreactor and the road to integrated antimicrobial testing

## 4.1 Introduction

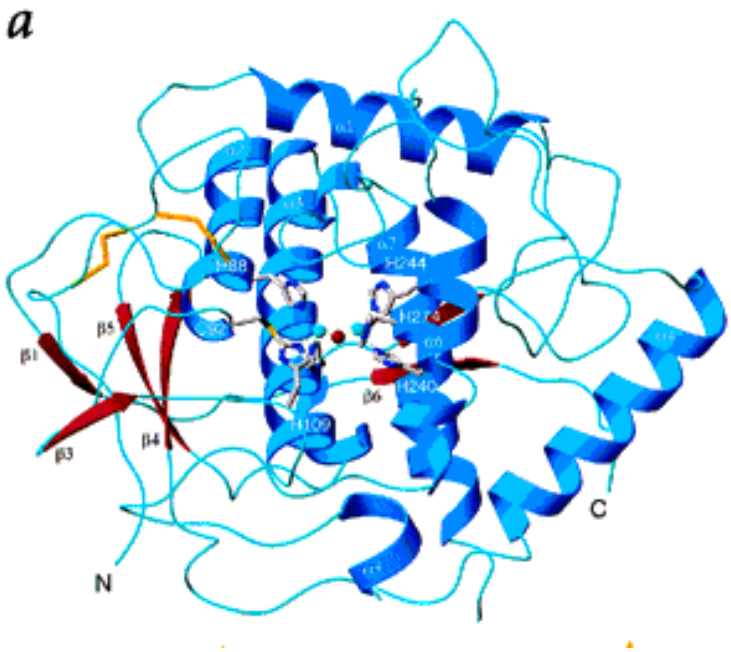
### 4.1.1 Synthesis of theaflavin

Theaflavin is a flavanoid found in black tea where it is produced during a fermentation process (Tu *et al.,* 2005) and was first identified in 1954 (Cartwright and Roberts). The synthesis of theaflavin does however occur naturally in the leaves of *Camellia sinensis* in the presence of oxygen and is often due to leaf damage (Jhoo *et al.,* 2004). The reaction involves the coupling of the polyphenols epicatechin and epigallocatechin (Wang *et al.,* 2008). Epicatechin and epigallocatechin are found abundantly in green tea where no oxidation has taken place. The reaction by which the enzyme polyphenol oxidase (PPO), catalyses the coupling of the two polyphenols is shown in figure 93.

Figure 93. Enzyme coupling of epicatechin and epigallocatechin to form theaflavin

Polyphenol oxidase is found in the cell vacuoles of the leaf cells and is released when disruptions in the intracellular compartments occur (Subramanian *et al.,* 1999). In the food industry this occurs due to the maceration of the tea leaves leading to fermentation (UK tea council, 2011). Polyphenol oxidases, sometimes known as catechol oxidases are members of the type-3 copper proteins (Klabunde *et al.,* 1998), which contain a dinuclear copper centre. An example of the structure of a catechol oxidase can be see in figure 94.

Figure 94. Example of the structure of catechol oxidase (Klabunde *et al.,* 1998)




The mechanism is similar to that of a wound response, where it is proposed that via one of its oxygen atoms, the polyphenol substrate binds to the second catalytic copper of the enzyme. In the co-oxidation reaction the benzotropolone skeleton in theaflavin is formed from the selected pair of catechins when polyphenol oxidase is present. In this reaction one catechin must have a *ortho*-dihydroxyphenyl structure and the other a *vic*-trihydroxyphenyl moiety (Sang *et al.,* 2004). The same polyphenol oxidase enzyme will also catalyse reactions between different polyphenols to form compounds other than theaflavin. Another example of this is when epigallocatechin gallate and epicatechin form theaflavin-3'-gallate in the presence of oxygen and polyphenol oxidase (Jhoo *et al.,* 2004). The enzyme has been found to be relatively unstable and is

susceptible to conditions of extreme pH and high temperatures that can lead to its inactivation (Sharma, Bari and Singh, 2008).

The production of theaflavin has been previously undertaken in batch reactions (Peng and Yao, 2009; Sang *et al.,* 2004; Jhoo *et al.,* 2004), in which epicatechin and epigallocatechin where reacted with polyphenol oxidase in the presence of oxygen. In the work by Peng and Yao (2009) a crude polyphenol oxidase extract from tea leaves was used producing a theaflavin yield of 43 %. In the study by Jhoo *et al.,* (2004) polyphenol oxidase was extracted from banana flesh and produced a poor theaflavin yield of 15.2 %. However, in the reaction described by Sang *et al.,* (2004) horseradish peroxidase was used to form the same theaflavin product producing a yield of 25 %.

In previous research, polyphenol oxidase has been immobilized to try and increase the yield of theaflavin produced and to prolong the use of the enzyme (Sharma, Bari and Singh, 2008; Tu *et al.,* 2005). In the work by Sharma, Bari and Singh (2008) a cellulose matrix is used to immobilize the PPO. By doing this the group claims that the enzyme still had 94 % activity after being used on 15 occasions and that an 85 % conversion of catechins to theaflavins was achieved. In the method by Tu *et al.,* (2005) the PPO was immobilized on a microporous metal plate to form a membrane that was cross-linked using glutaraldehyde. In this study the enzyme still had 73 % activity after 75 days of storage at 4 °C. Another method of PPO immobilization is to use a cross-linked chitosan-$SiO_2$ gel. This has previously been undertaken to remove aqueous phenol (Shao, Ge and Yang, 2007). In this study it was shown that unlike free PPO, the immobilized PPO could be easily removed from the reaction solution and be reused for a subsequent reaction.

Due to the increased surface area to volume ratio in a microreactor, transferring this reaction to a continuous flow system could potentially increase the low yields often obtained in this biosynthesis.

### 4.1.2 Synthesis of deuterium labelled theaflavin

The synthesis of deuterated compounds is very important as their application in toxicological, metabolite and mechanistic studies is vital for the understanding of how compounds interact with living organisms. Whilst the synthesis of theaflavin and deuterated theaflavin has been demonstrated in batch (Peng and Yao, 2009), yields are often poor and reactants can be expensive. By transferring the reaction to a microreactor, the synthesis could be made more efficient and linked to bioreactors for direct use. As in the study by Peng and Yao (2009) the methodology to produce deuterated theaflavin would be the same as that to produce theaflavin with the substitution of one of the substrates one has been previously labelled (Figures 20 and 21). A schematic of the proposed reaction can be seen in figures 95.

Figure 95. Enzyme coupling of deuterated epicatechin and epigallocatechin to form deuterated theaflavin

### 4.1.3 Enzymatic synthesis in a microreactor

There have been many reports of the use of enzymes in microreactors for biosynthesis with the enzymes usually requiring immobilization in a support layer or monolith (Kataoka *et al.,* 2009), although this is not always the case (Shaw *et al.,* 2010).

In the literature there are 3 mains methods for immobilizing enzymes in microreactors (Asanomi *et al.,* 2011) these are:

- Enzyme immobilization within microchannels
- Immobilization of enzymes on the microchannel surface
- Enzyme immobilization onto a membrane within the microchannel

The method of immobilizing enzymes within channels has proven to be very successful producing high enzyme reactivities (Matsuura *et al.,* 2011). One example of this is the use of a silica monolith to immobilize enzymes. In this method, enzymes such as proteases have been successfully immobilized and proven to give significantly higher conversion rates than corresponding reactions in batch (Kawakami *et al.,* 2005). Another example of this type of immobilization was shown in research by Kataoka *et al.,* (2009), where a lipase was immobilized onto a mesoporous silica support layer. This study found that the immobilized enzyme was stable and showed good activity over a 24 hour period whilst catalysing continuous ester hydrolysis reactions. Although using a monolith has advantages, many other groups use an agarose complex to which the enzyme is bound. Previous research, hydroxylase enzymes were immobilized onto Ni-NTA agarose beads and loaded into a microreactor (Srinivasan *et al.,* 2004). In this study it was found that reaction times were not only short but the volumes and enzyme amounts required for the reaction were far smaller than in conventional methods.

The method of immobilizing enzymes onto the surface of a microreactor have been shown to be advantageous as they use the large surface area present in a microreactor system (Asanomi *et al.,* 2011; Schwarz, Thompson and Nidetzky, 2009). An example of this method is using fused silica to immobilize the enzyme required onto the surface of the microreactor via Ni-complex (Miyazaki *et al.,* 2005). In the research by Miyazaki *et al.,* (2005) enzymes were immobilized on microcapillary walls. This was found to be an efficient methodology for immobilization. Whilst covalent immobilization of enzymes to silanised walls of PDMS microchannels by coupling with glutardialdehyde (Thompson, Polt and Nidetzky, 2007) has also been described. Using this method a reaction conversion of 60 % was achieved and although not high it presents an alternative method of immobilization.

The final method of immobilizing enzymes in a microreactor is with the use of a membrane within a microchannel. This method has shown to be easy to prepare but less efficient than other methods of immobilization (Asanomi *et al.,* 2011). In a previous study a functional polymer membrane was produced in a microreactor via a interfacial polycondensation reaction. The research showed that creating this membrane allowed horseradish peroxidase to be immobilized on one side of the surface allowing chemical transformations to take place.

Each of the methods discussed, can produce more efficient and cost effective reactions and also allow a greater level of safety than reactions performed in batch models. The use of enzymes in microreactors can also lead to a higher degree of reaction control and a continuous-flow output (Haswell and Skelton, 2000).

### 4.1.4 Antibacterial testing and monitoring cell viability and metabolites

#### 4.1.4.1 Polyphenol metabolites

Polyphenols have been shown to have strong antimicrobial effect against many different bacteria. However, as bacteria can metabolise polyphenols, studies into their metabolites are of great interest. A large amount of polyphenol

metabolism occurs in the gut where bacteria produce enzymes, which lead to their hydrolysis (Bokkenheuser, Shackleton and Winter, 1987). Enzymes such as alpha-rhamnosidase and beta-galactosidase produced by bacteria hsuch as *Bacteriodes spp* have been reported to hydrolyse polyphenols such as naringin and hesperidin. This metabolism has been reported to alter their bioavailability and thus can reduce or increase their beneficial effects (Rooks and Garrett, 2001). Previous research has also shown these metabolites to be more effective than their parental compounds giving rise to greater anti-platelet activity and cytotoxicity (Kim *et al.,* 1998).

Using a labelled theaflavin would therefore allow clear identification of metabolites and give an indication to how the polyphenol was metabolised. Previous studies have used labelled polyphenols to determine metabolites. An examples of this is in the research study by Kohri *et al.,* (2001) where labelled epigallocatechin gallate (EGCG) was synthesized in batch to discover its metabolic rate in rats. In this study is was discovered that the major excretion route of EGCG was in bile within 4 hours of dosing with polyphenol. Metabolites in this investigation were identified by HPLC where 4‘,4‘‘-di-*O*-methyl-EGCG was found to make up 14.7% of the administered EGC.

#### 4.1.4.2 Antibacterial testing

Antibacterial testing is used to identify new antibacterial agents and the susceptibility to pathogens to antibacterial agents. Determining the minimum inhibitory concentrations (MICs) of antimicrobials is very important in order to judge their effectiveness. MICs are considered as the ‘gold standard’ for determining antimicrobial susceptibilities (Andrews, 2001) and methods range from the disc diffusion method to the microtiter assay.

New methods include assays in microfluidic devices for detection of bacteria (Qiu *et al.,* 2009) and determining susceptibilities (Chen *et al.,* 2010). These methodologies are more rapid and cost efficient that traditional methods with a greater range of control. Using a microreactor, antibiotic susceptibilities of

bacterial biofilms can also be monitored. In a recent study (Kim *et al.,* 2010) minimum biofilm eradication concentrations were determine against biofilms of *Pseudomonas aeruginosa* and were reported as higher than those for the minimum inhibition of planktonic cells. In a different study the use of plug-based microreactor technology for the rapid detection antibiotic susceptibilities (Boedicker *et al.,* 2008) has been described. In this investigation droplets with a volume of nano litres were created to isolate individual bacteria and where confinement increases bacterial cell density eliminating any incubation step usually required. Due to this and the high throughput multiple tests were performed simultaneously giving a more rapid result.

These new microfluidic methodologies could generate data regarding antimicrobial susceptibilities significantly faster than current methods.

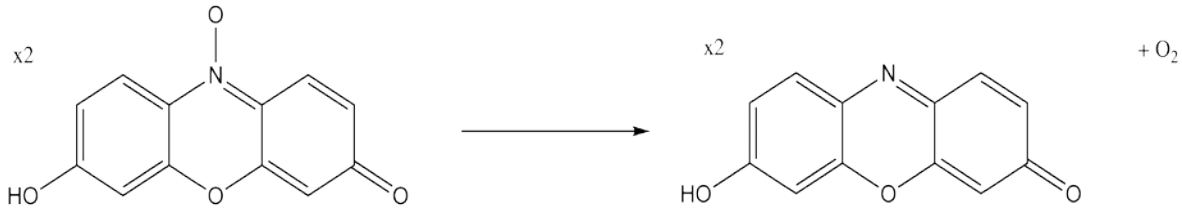
### 4.1.5 Monitoring cell viability

There are a variety of methods to determine bacteria cell viability. The detection of biomarkers for cell viability related to transcriptional activity is one method of determining bacterial cell death (Kort *et al.,* 2008). In the investigation by Kort *et al.,* (2008) indentification of transcriptional biomarkers and the rapid expression of small numbers of competence genes were monitored after lethal heat stress. These competence genes are known to be active in the stationary growth phase of bacteria. The presence of living cells was indicated due to the expression of these genes after the heat treatment.

Another simple method of monitoring bacterial cell viability is the use of resazurin (Borra *et al.,* 2009). Resazurin is a blue dye with the ability to show cell viability as in the presence of NADPH + $H^+$ it is reduced to resorufin compound pink in colour (Figure 96). As the transfer of elections from NADPH to resazurin occurs due to the presence of mitochondrial enzymes, it can indicate active metabolism and the presence of living cells. Its use was first described in 1963 by Moyer and Campbell, to show bacterial counts in milk. This method has been used in several studies to show the presence of living

bacteria. In a investigation by Perrot *et al.,* (2002) resazurin was successfully used to indicate living bacteria on the cornea of pigs.

Figure 96. Breakdown of resazurin to resorufin



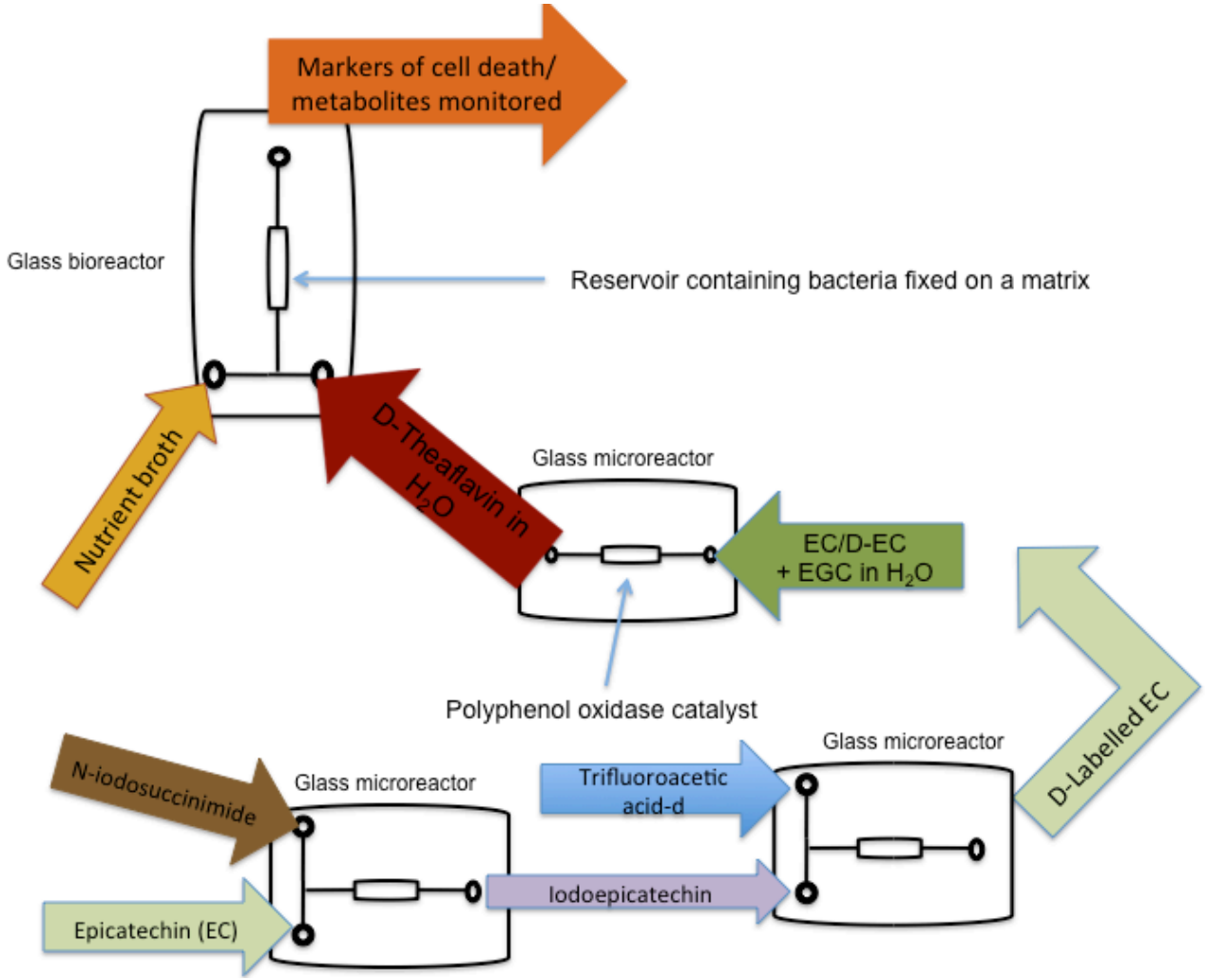

In another study using resazurin the dye was found to provide a good indication if living cells in a biofilm (Sandberg *et al.,* 2009). However, it was also mentioned that the quality of the stains was dependant on the biofilm concentration and the length of the staining procedure.

### 4.1.6 Links between synthesis and antimicrobial testing

Microreactor synthesis of antimicrobial agents could be potentially linked to bioreactors for the determination of antimicrobial susceptibility. This would offer a rapid method of reporting antibacterial activities of newly synthesized compounds. Not only would this increase the speed of determining susceptibilities but also the amount of control would be greater.

A proposed link between synthesis of labelled theaflavin and antimicrobial testing/monitoring of bacterial metabolites can be seen in figure 97. In this proposed methodology, a 3 step process of producing deuterium labelled theaflavin or 1 step process of synthesizing non-labelled theaflavin is linked to a bioreactor containing bacteria trapped on a matrix/filter to enable antimicrobial testing, monitoring of the markers of cell death/cell viability or the determination of polyphenol metabolites.

Figure 97. Proposed integration between microreactor synthesis and antimicrobial testing/monitoring of bacterial metabolites



## 4.2 Aims

The aims of this investigation are to produce theaflavin and deuterated theaflavin in a biochemical microreactor. A further aim of this study was to link the deuterium synthesis with immobilized bacteria to monitor metabolites/markers of cell death and to create a method linking synthesis to antibacterial testing.

## 4.3 Methods

### 4.3.1 Method 1 for the microreactor synthesis of theaflavin

#### 4.3.1.1 Preparation of substrates

It was decided that a 10 mM solution of epicatechin and epigallocatechin would be suitable, as this concentration was found to be a good balance between sufficient quantity of reactants and their solubility in 10% methanol. To prepare the substrates 0.0291 g of epicatechin and 0.0304 g of epigallocatechin were weighed out and then dissolved in 1mL of methanol in a 15 mL tube. To this 9 mL of distilled water was added and then vortexed for 5-10 minutes to mix. The tube was then wrapped in aluminium foil to prevent light degradation and was kept in the fridge. The catechins prepared were used within 1 day of production to minimise any risk of degradation/oxidation.

#### 4.3.1.2 Tea leaf catalyst preparation

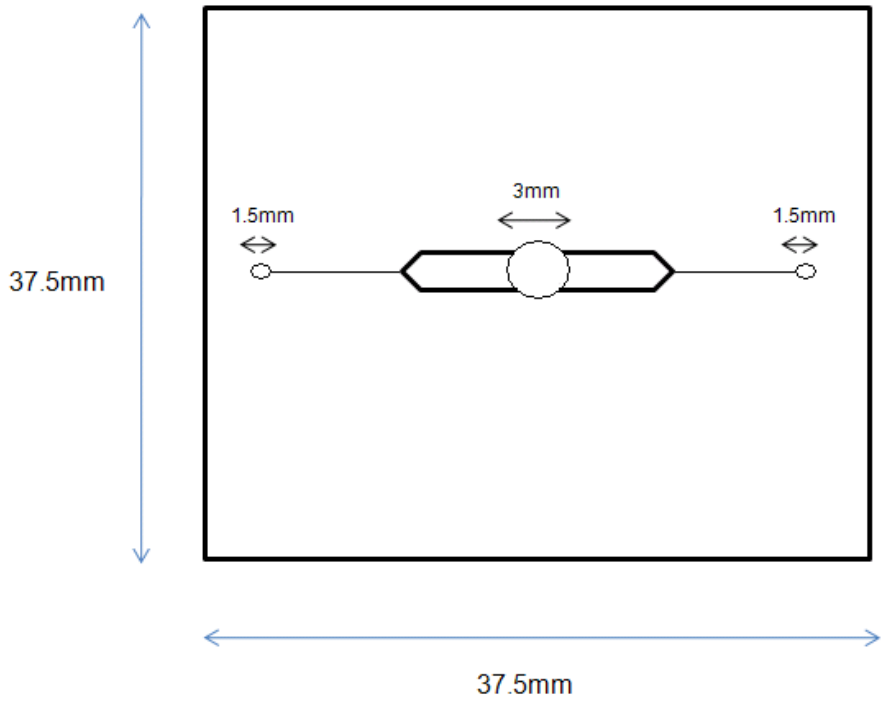
1.5 mL of $H_2O$ was added to 0.1 g of fresh green tea leaf in a pestle and then ground for 30 seconds using a mortar. When the leaf was ground into a paste the mixture including any liquid was transferred to an Eppendorf tube. The tube was placed in a centrifuge and spun at 4000 rpm for 1 minute. Excess supernatant was then removed from the tube using a syringe and disposed off leaving a pellet. To remove as many natural catechins naturally found in the tea leaf the pellet was washed with distilled water three times by adding 1.5 mL of distilled water to the tube, vortexing and centrifuging at 4000 rpm before removing the excess water. The remaining cleaned residue was stored at 4°C and used within 24 hours.

#### 4.3.1.3 Microfluidic reactor design and preparation

A specific chip was designed and produced in glass, which would take in to consideration various factors including loading of a green tea catalyst and also adequate flow of catechins across the catalyst giving sufficient time for their reaction and oxidation.

The chip designed had a reservoir to allow the green tea catalyst to be added which can be seen in figure 98. The channels in the microreactor had a diameter of 60 µm

Figure 98. Microreactor design used for the synthesis of theaflavin



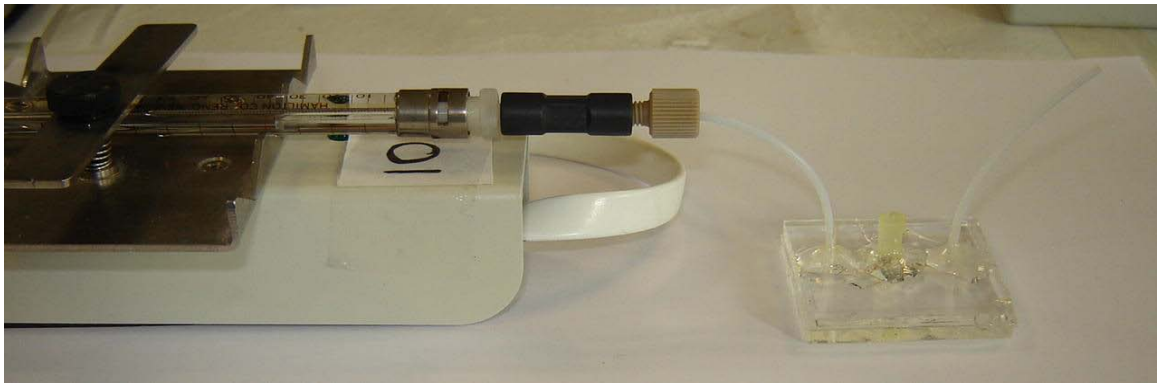

Capillary tubes were attached to the 1.5 mm inlet and outlet holes using an adhesive epoxy resin. These were left to dry for approximately 1 hour. When dry, 1 mL of $H_2O$ was pushed through each capillary to check for any blockages. After this, 0.01 g of green tea catalyst was added to the reservoir via its inlet using a micro spatula and a needle. It was important that the catalyst covered the whole width of the reservoir to ensure reactants would pass across it. A PDMS plug was then placed into the reservoir inlet and sealed in place with

an adhesive epoxy resin and left to dry for at least 1hr. The PDMS bung allowed gas exchange to occur which was required for the reaction.

#### 4.3.1.4 Experimental setup

Before any reaction took place the microreactor channels were washed with 1 mL of methanol and 1 mL of $H_2O$. This was to allow any residual catechins or potential theaflavin naturally in the leaf to be washed away. The epicatechin:epigallocatechin stock solution was then flowed across the fresh green tea catalyst in the microreactor at either 1, 2, 5 or 10 µL/min using a BabyB syringe pump. A photograph showing the reaction setup can be seen in figure 99. All products were collected into a syringe and using a separating funnel washed and extracted into ethyl acetate. The ethyl acetate layers were dried over $MgSO_4$ and concentrated under reduced pressure.

Figure 99. Experimental setup for the microreactor synthesis of theaflavin



To determine if theaflavin had been produced, product samples were analysed using MS. MS was set to look at compounds in the range of 150 – 800 m/z. The elutants used were acetonitrile and water in a 50:50 ratio flowing at 800 µL/min. Each sample was analysed within 1 hour of the completed experiment and chromatographs were produced from the data acquired using LCQ tune software.

### 4.3.2 Method 2 for the microreactor synthesis of theaflavin

#### 4.3.2.1 Preparation of substrates

A 10 mM solution of epicatechin and epigallocatechin was prepared by dissolving 0.0291 g of epicatechin and 0.0304 g of epigallocatechin in 1 mL of acetone in a 15 mL tube. To this 9 mL of distilled water was added then vortexed for 5 minutes to mix. The tube was then wrapped in aluminium foil to prevent light degradation and was kept in the fridge. The catechins prepared were used within 1 day of production to minimise any risk of degradation/oxidation.

#### 4.3.2.2 Crude polyphenol oxidase preparation

Into a pestle, 1 g of tea leaves were added and then ground for 30 seconds using a mortar. The leaf paste created was transferred to a Büchner funnel containing filter paper were it was washed with acetone (5 mL x 5) to remove any polyphenols (Sharma, Bari and Singh, 2009). The remaining powder was resuspended in 5 ml of PBS (phosphate buffered saline) and centrifuged at 10000 rpm for 20 minutes. The clear supernatant was removed and washed with ice-cold acetone (Jhoo *et al.,* 2004). The solution was centrifuged again at 10000 rpm for 20 minutes to precipitate out the proteins. The pellet was washed with PBS and the crude polyphenol oxidase (PPO) extract was dried under reduced pressure for 1 hour to give a beige powder. This was stored at 4°C and used within 24 hours. The PPO extract was mixed with silica gel 40 purchased from SigmaAldrich, UK (particle size 75-150 µm, pore size 40 Å) prior to loading into the microreactor.

#### 4.3.2.3 Experimental setup for method 2

For this reaction a single inlet/outlet glass microreactor was used with a central reservoir (Figure 98). To prepare the microreactor the side of the reservoir next to the outlet channel was packed with a piece of moist cotton wool and the side nearest the inlet channel was packed with the crude polyphenol oxidase power. The cotton wool was used to eliminate the risk of any crude polyphenol power being washed out of the reservoir. The reservoir opening was then sealed with

a PDMS bung. The bung was kept in place using epoxy resin, which was allowed to harden for up to 1 hour before the reaction took place.

In the reaction 1 mL of the 10 mM epicatechin:epigallocatechin stock solution was flowed across the through the microreactor at room temperature. The reaction was controlled by a syringe pump at a flow rate of 5 µL/min. All products were collected into a syringe. Using a separating funnel, products were washed and extracted into ethyl acetate (4 x 5 mL). The ethyl acetate layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The resulting brown/orange oil was weighed before being analysed via MS and $^1$H-NMR.

### 4.3.3 The microreactor synthesis of deuterium labelled theaflavin

In this reaction 0.0292 g of deuterium labelled epicatechin produced via the 2-step method described in chapter 3 (Figures 20 and 21) and 0.0304 g of epigallocatechin was dissolved in 10 mL of water in a 10 mL tube and vortexed for 5-10 minutes to mix.

To prepare the microreactor (Figure 98) the reservoir was packed with crude polyphenol oxidase as in method 2 of theaflavin production and was sealed with a PDMS bung with was glued in place with epoxy resin.

In the reaction 2 mL of the 10 mM epicatechin-d:epigallocatechin stock solution was flowed across the through the microreactor at room temperature. The reaction was controlled by a syringe pump at a flow rate of 5 µL/min. All products were collected into a syringe. Using a separating funnel, products were washed and extracted into ethyl acetate (4 x 5 mL). The ethyl acetate layers were dried over $MgSO_4$, filtered and concentrated under reduced pressure. The resulting brown/orange oil was weighed before being analysed via MS and $^1$H-NMR.

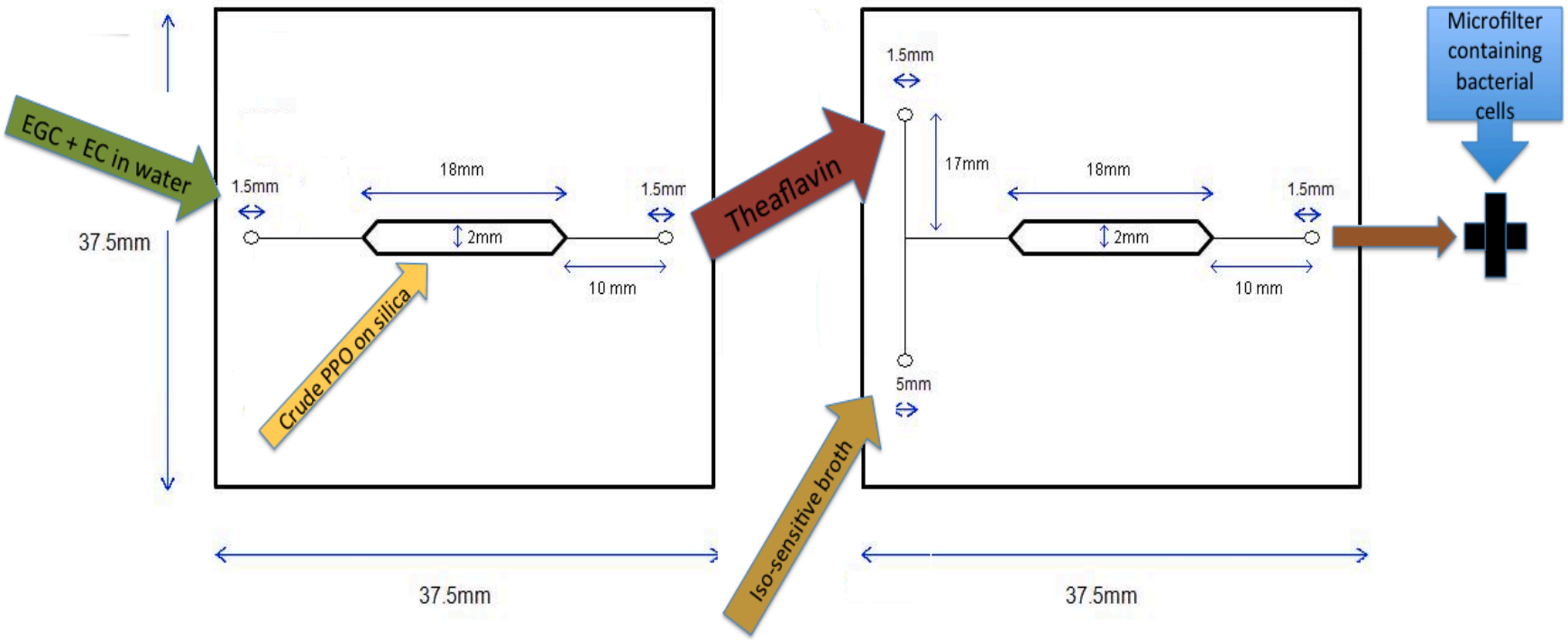
### 4.3.4 Method for linking microreactor synthesis products to a method for antibacterial testing

#### 4.3.4.1 Experimental setup

The setup for the synthesis was the same as method 2 for the synthesis of theaflavin, with the exception that the outlet tube was connected to a second t-shaped microfluidic device (Figure 100). In the second device the theaflavin products were passed with iso-buffer, also controlled by a syringe pump at a flow rate of 5 μL/min increasing the overall flow rate to 10 μL/min. The outlet of this device was connected to a membrane filter, which had been inoculated with 100 μL of a 0.5 MacFarland solution of *Acinetobacter baumannii* NCTC 13485 in 900 μL of nutrient broth. *Acinetobacter baumannii* was prepared based on the method by Moosdeen *et al.,* (1988) by taking 1 large colony of *A. baumannii* using a flamed loop and adding to 2 mL of peptone water. This was vortexed and turbidity was measured using a spectrophotometer. A reading of 0.345 nm was recorded and using the dilution chart 25 μL of the broth was added to 5 mL of sterile distilled water giving a 0.5 MacFarland solution. The reaction products were flown across the cell membrane for 1, 2, 4 or 6 hours. This meant bacterial cells were exposed to either 600 μL (0.15 mg), 1200 μL (0.3 mg), 2400 μL (0.6 mg) or 3600 μL (0.9 mg) of theaflavin.

The setup was repeated but a solution of sterile water containing no epicatechin:epigallocatechin was flowed through microreactor 1. This acted as a control. All apparatus/reagents were sterilized with ethanol/autoclaved prior to use.

Figure 100. Microreactor - microbial filter setup



#### 4.3.4.2 Cell viability testing

After treatment with synthesized theaflavin at each time period, the filter containing the bacteria was washed with sterile PBS buffer. The cells were then back flushed out of the filter into an Eppendorf tube containing 1 mL of PBS buffer. The tube was centrifuged at 4000 rpm for 5 minutes (Sarker *et al.,* 2007). The supernatant was removed and the pellet was re-suspended in 1 mL of PBS buffer before the sample was centrifuged again at 4000 rpm for 5 minutes. The supernatant was removed and the pellet was again re-suspended in 1 mL of peptone water. This procedure was repeated for the control samples for each time period used.

To each well of a 24 well pate 500 μL of PBS buffer was added. To each of the first 8 wells 100 μL of the bacterial suspension from the 20 minutes theaflavin treatment was added. To each of the next 8 wells 100 μL of the bacterial suspension from the 20 minutes treatment with water was added. To the final 8 wells 100 μL of sterile peptone water was added. To test the viability of the bacteria, 100 μL of resazurin (0.05 %) was added. Any colour changes in the wells were noted and photographs were taken at 0, 3 and 10 minutes post resazurin addition.

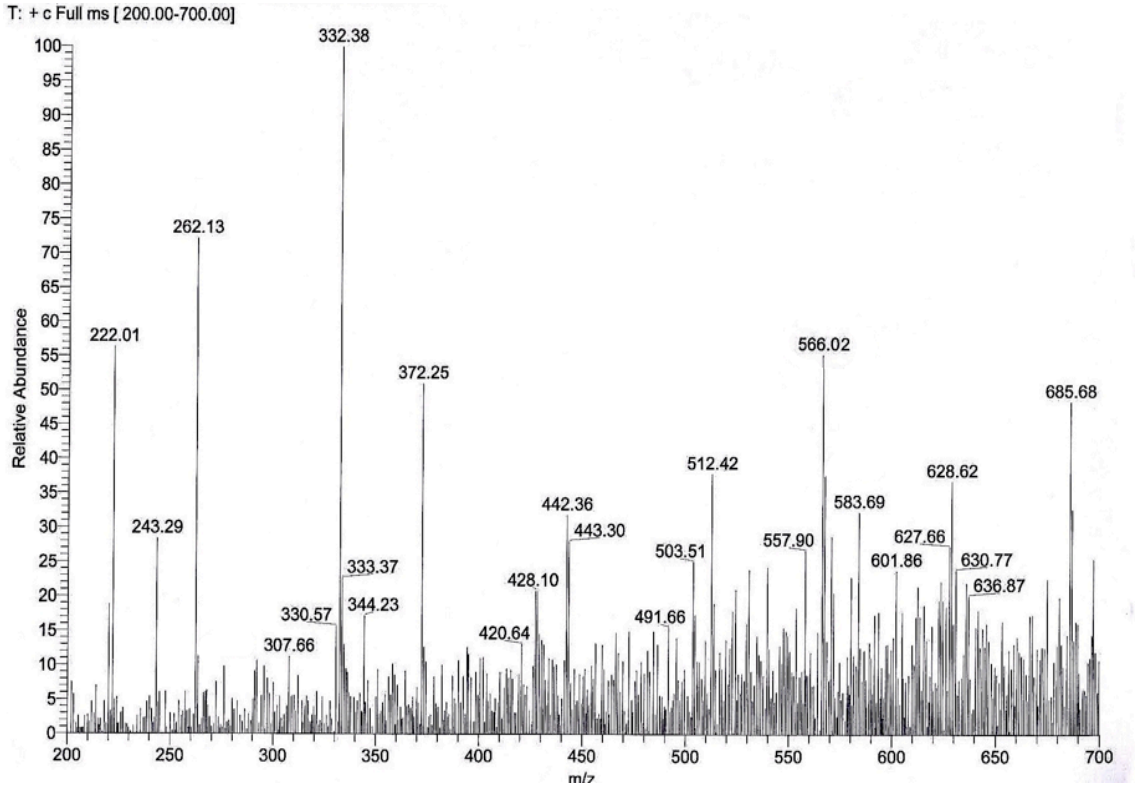
### 4.4 Results and discussion

#### 4.4.1 Results of method 1 for the microreactor synthesis of theaflavin

The results from this reaction show that the crude leaf catalyst was successful in producing theaflavin, although it was unsuccessful at producing the polyphenol alone. From the MS data (Figure 101) the peak from theaflavin can be seen at 566.02 m/z. Whilst the mass of theaflavin is 564.13 g, the higher reading is likely to occur due to an interaction with 2 hydrogen atoms from the elutant. From the MS spectra it can be clearly seen that multiple peaks not associated with theaflavin exist. These could be from polyphenols and other compounds from the leaf catalyst that were not originally washed away during preparation. It is clear that the polyphenol oxidase catalyst required for this reaction should be extracted from the tea leaves to avoid multiple peaks from other compounds present. This would increase the purity of the theaflavin

produced enabling the continuous flow microreactor to be directly linked to a bioreactor for antimicrobial/metabolic testing.

Figure 101. MS spectra for method 1 products of microreactor theaflavin synthesis

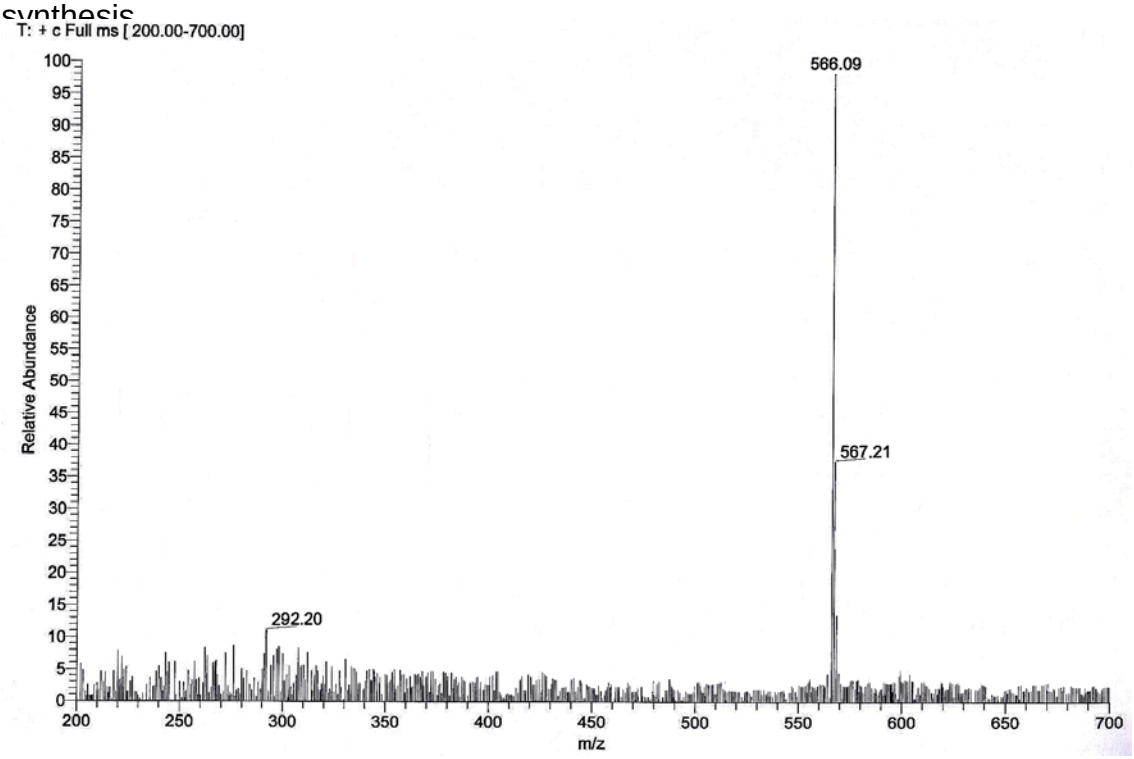



### 4.4.2 Results method 2 for the microreactor synthesis of theaflavin

The results from method 2 of the microreactor synthesis of theaflavin show that the reaction was successful in producing theaflavin. The brown/orange oil (0.05 g) collected was a good indicator that theaflavin was present in the product. From the MS spectra peaks can be seen at 292.2 m/z, 566.09 m/z and 567.21 m/z. The peak at 292.2 m/z represents epicatechin that has not been used up in the reaction, the peaks at 566.09 and 567.21 represent theaflavin. The peaks values for these compounds are slightly higher than their mass values, which could be due to proton interaction from the elutant or the presence of a natural carbon/oxygen/hydrogen isotope e.g. Carbon$^{13}$. Due to the relevant abundance of the peak at 566.09 m/z it is clear that the reaction was very successful and that almost all epicatechin was used up. However, as a small peak for

epicatechin is present one might have expected that a peak for epigallocatechin also to be present. Its absence suggests it was either completely oxidised during the reaction or decomposition has taken place. When the products were analyzed by $^{1}$H'NMR they appeared to be of high purity due to the absence of any peaks from epicatechin or epigallocatechin.

Figure 102. MS spectra for method 2 products of microreactor theaflavin synthesis




The $^{1}$H'NMR data below compares well with previous data (Peng and Yao, 2009; Tu *et al.,* 2005; Jhoo *et al.,* 2004) with variations most likely being due to the different deuterated solvents used in the $^{1}$H'NMR method.

$^{1}$H'NMR (400 MHz, $CD_3CD$) 7.97 (1H, s), 7.84 (1H, s), 7.21 (1H, d, J=8.4Hz, H-5'), 7.33 (1H, s), 6.05 (1H, d, J=2.2 Hz), 6.02 (1H, d, j=2.2 Hz), 5.99 (1H, d, j=2.2 Hz), 5.96 (1H, d, J=2.2 Hz), 5.63 (1H, s), 4.90 (1 H, s), 4.46 (1H, m), 4.37 (1H, m), 2.89 (2H, d, J=3.4 Hz), 2.81 (1H, s), 2.79 (1H, s).

From this data it is clear that the production of theaflavin in a microreactor is successful using a crude polyphenol oxidase on silica gel. The estimated yield from this reaction is approximately 88% with this being higher than previous batch reactions from the production of theaflavin (Jhoo *et al.,* 2004). It is believed this is the first occasion where theaflavin has been produced in a microfluidic system. The result also shows that the reaction is successful enough to use products directly from the continuous flow system to a biological system for metalbolic/antibacterial testing.

### 4.4.3 Results for the microreactor synthesis of d-labelled theaflavin

From the results of this reaction it is clear that the synthesis of deuterium labelled theaflavin was not successful. From the MS spectra one major product peak was visible at 566.09 m/z, which was the same as in the production of non-labelled theaflavin. This indicates that the addition of a deuterium did not occur. Results from the $^{1}$H'NMR shows an almost identical spectra to that of non-labelled theaflavin. This confirmed that the reaction was unsuccessful. The possible reason for this is that proton transfer occurred during the enzymatic reaction resulting in the loss of deuterium from the labelled epicatechin substrate.

$^{1}$H'NMR (400 MHz, $CD_3CD$) 7.97 (1H, s), 7.85 (1H, s), 7.21 (1H, d, J=8.4Hz, H-5'), 7.33 (1H, s), 6.05 (1H, d, J=2.2 Hz), 6.01 (1H, d, j=2.2 Hz), 5.99 (1H, d, j=2.2 Hz), 5.96 (1H, d, J=2.2 Hz), 5.63 (1H, s), 4.90 (1 H, s), 4.46 (1H, m), 4.37 (1H, m), 2.89 (2H, d, J=3.4 Hz), 2.81 (1H, s), 2.79 (1H, s).

From this result it can be concluded that the labelled epicatechin produced in chapter 1 (Figures 20 and 21) is not stable enough to be used in the synthesis of deuterium labelled theaflavin. Future studies could investigate the synthesis of a more stable deuterium labelled theaflavin.

### 4.4.4 Results for monitoring bacterial metabolites from deuterated theaflavin

As the reaction to synthesize deuterium labelled theaflavin was unsuccessful it was decided to carry out metabolic studies due to time restraints. Further investigations could use a more stable deuterium labelled theaflavin to monitor bacterial metabolites or those produced by labelled epicatechin.

### 4.4.5 Results for linked microreactor theaflavin synthesis-cell viability testing

The results from this investigation indicate that the continuous flow reactor and antibacterial testing has been successful. *Acinetobacter baumannii* isolates collected after 1 hour of treatment with theaflavin from the continuous flow reactor turned from blue at 0 minutes post resazurin addition to light purple after 10 minutes to purple/pink after 15 minutes (Figure 103). This indicates that the amount of theaflavin the bacteria were exposed to (0.15 mg in 600 µL over 1 hour), from the microreactor, was insufficient to cause cell death. The control isolates exposed to no theaflavin turned pink after 10 minutes post reazurin addition indicating, metabolism of the resazurin to resorufin by live bacterial cells. The control wells containing no cells remained blue indicating no metabolism of resazurin.

The bacterial isolates collected after 2 hours of treatment with theaflavin from the continuous flow reactor also turned from blue at 0 minutes post resazurin addition to light purple after 10 minutes to dark purple/pink after 15 minutes (Figure 104). This is also evidence to suggest that the amount of theaflavin the bacteria were exposed to (0.3 mg in 1200 µL over 2 hours), from the microreactor, was insufficient to cause cell death. The control isolates exposed to no theaflavin also turned pink after 10 minutes post reazurin addition, indicating metabolism of the resazurin to resorufin by live bacterial cells. The control wells containing no cells remained blue indicating no metabolism of resazurin.

*A. baumannii* isolates collected after 4 hours of treatment with theaflavin from the continuous flow reactor remained blue from 0 minutes post resazurin addition to 15 minutes post resazurin addition (Figure 105). This is evidence to suggest that the amount of theaflavin from the microreactor that the bacteria were exposed to (0.6 mg in 2400 µL over 4 hours) was sufficient to cause cell death. However, the control isolates exposed to no theaflavin turned pink after 10 minutes post reazurin addition indicating metabolism of the resazurin to resorufin by live bacterial cells. The control wells containing no cells remained blue indicating no metabolism of resazurin.

*A. baumannii* isolates collected after 6 hours of treatment with theaflavin from the continuous flow reactor remained blue from 0 minutes post resazurin addition to 15 minutes post resazurin addition (Figure 105). This is evidence to suggest that the amount of theaflavin from the microreactor that the bacteria were exposed to (0.9 mg in 3600 µL over 60 minutes), was sufficient to cause cell death. However, the control isolates exposed to no theaflavin turned pink after 10 minutes post reazurin addition indicating metabolism of the resazurin to resorufin by live bacterial cells. The control wells containing no cells remained blue indicating no metabolism of resazurin.

From the results of this investigation a minimum concentration of theaflavin to cause inhibition of *A. baumannii* NCTC 13485 was found to be 0.6 mg/2400 µL over 4 hours. The continuous exposure to theaflavin most likely accumulated in the bacterial cells leading to their inhibition after 4 hours. It is believed that this study demonstrates for the first time the link between enzymatic synthesis and antibacterial testing and unlike traditional methods such as the disc diffusion and the microtiter assay, is a faster and less expensive methodology.

Figure 103. Resazurin assay to test for cell viability for *A. baumannii* treated for 1 hour with synthesized theaflavin. Photographs taken at 0, 3, 10 and 15 minutes after the addition of resazurin. In each image the first 8 wells contain treated cells (T), the second 8 wells contain untreated cells (U) and the last 8 wells contain no cells (N).

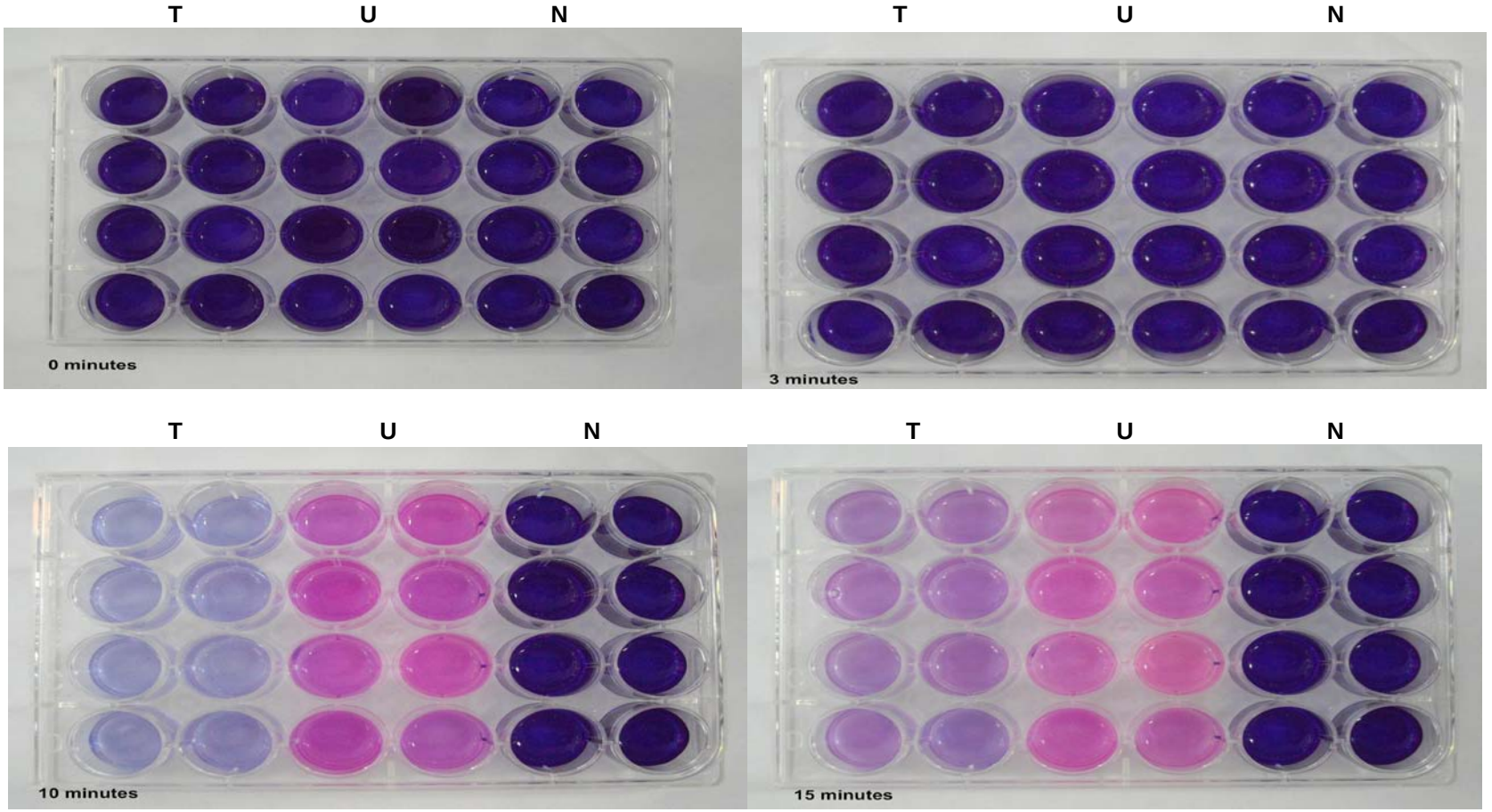


Figure 104. Resazurin assay to test for cell viability for *A. baumannii* treated for 2 hours with synthesized theaflavin. Photographs taken at 0, 3, 10 and 15 minutes after the addition of resazurin. In each image the first 8 wells contain treated cells (T), the second 8 wells contain untreated cells (U) and the last 8 wells contain no cells (N)**.**

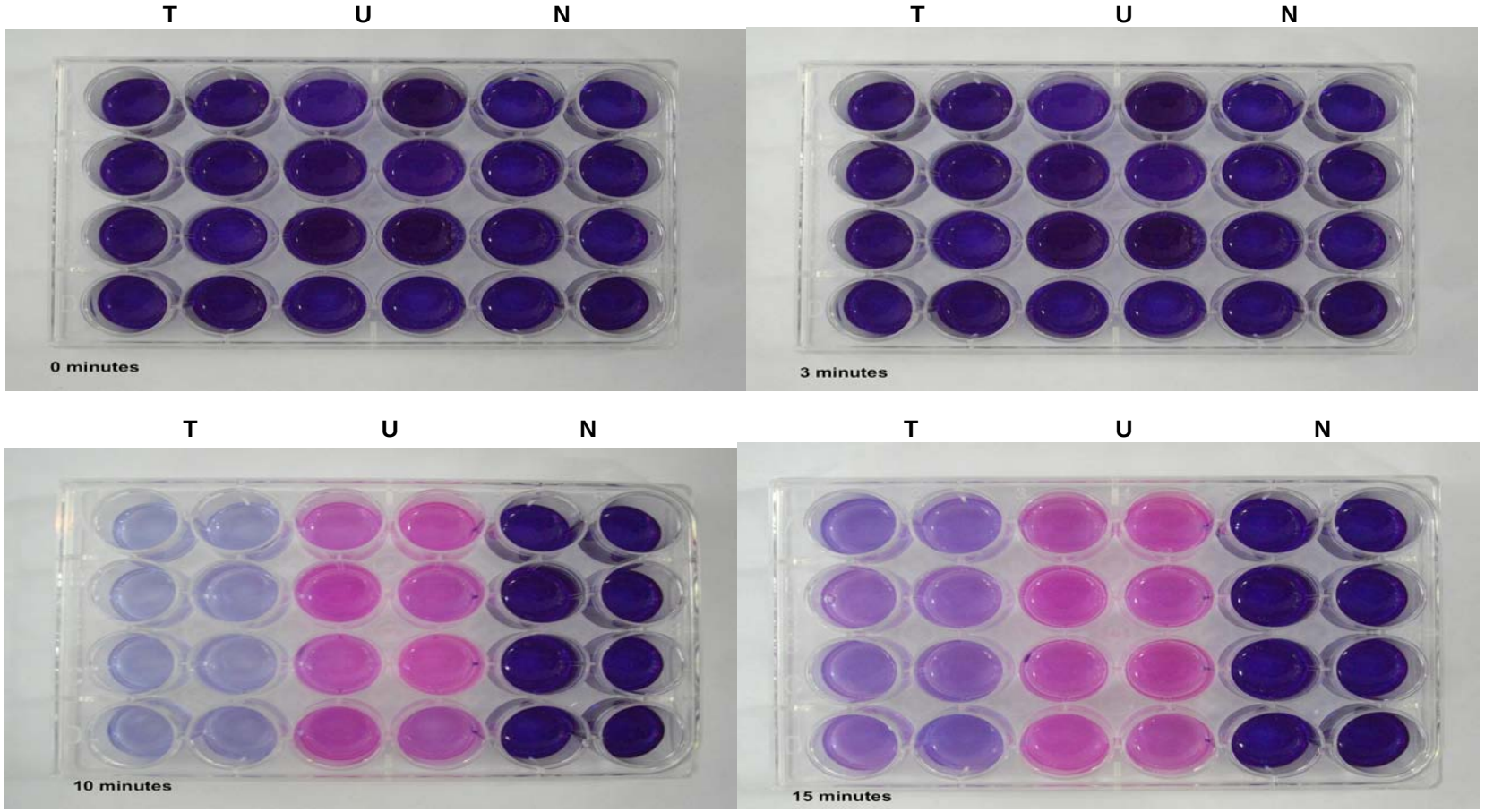


Figure 105. Resazurin assay to test for cell viability for *A. baumannii* treated for 4 hours with synthesized theaflavin. Photographs taken at 0, 3, 10 and 15 minutes after the addition of resazurin. In each image the first 8 wells contain treated cells (T), the second 8 wells contain untreated cells (U) and the last 8 wells contain no cells (N).

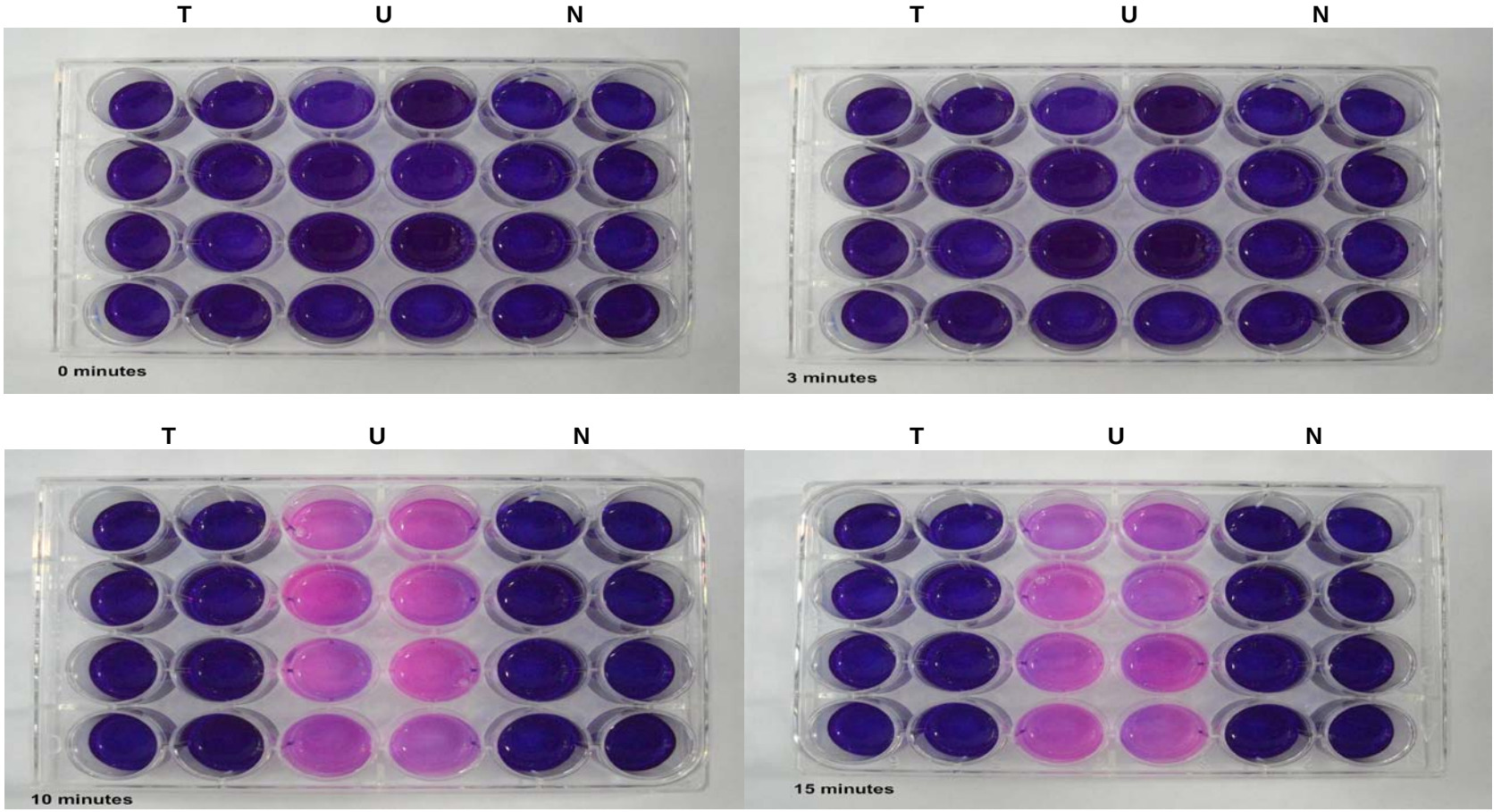
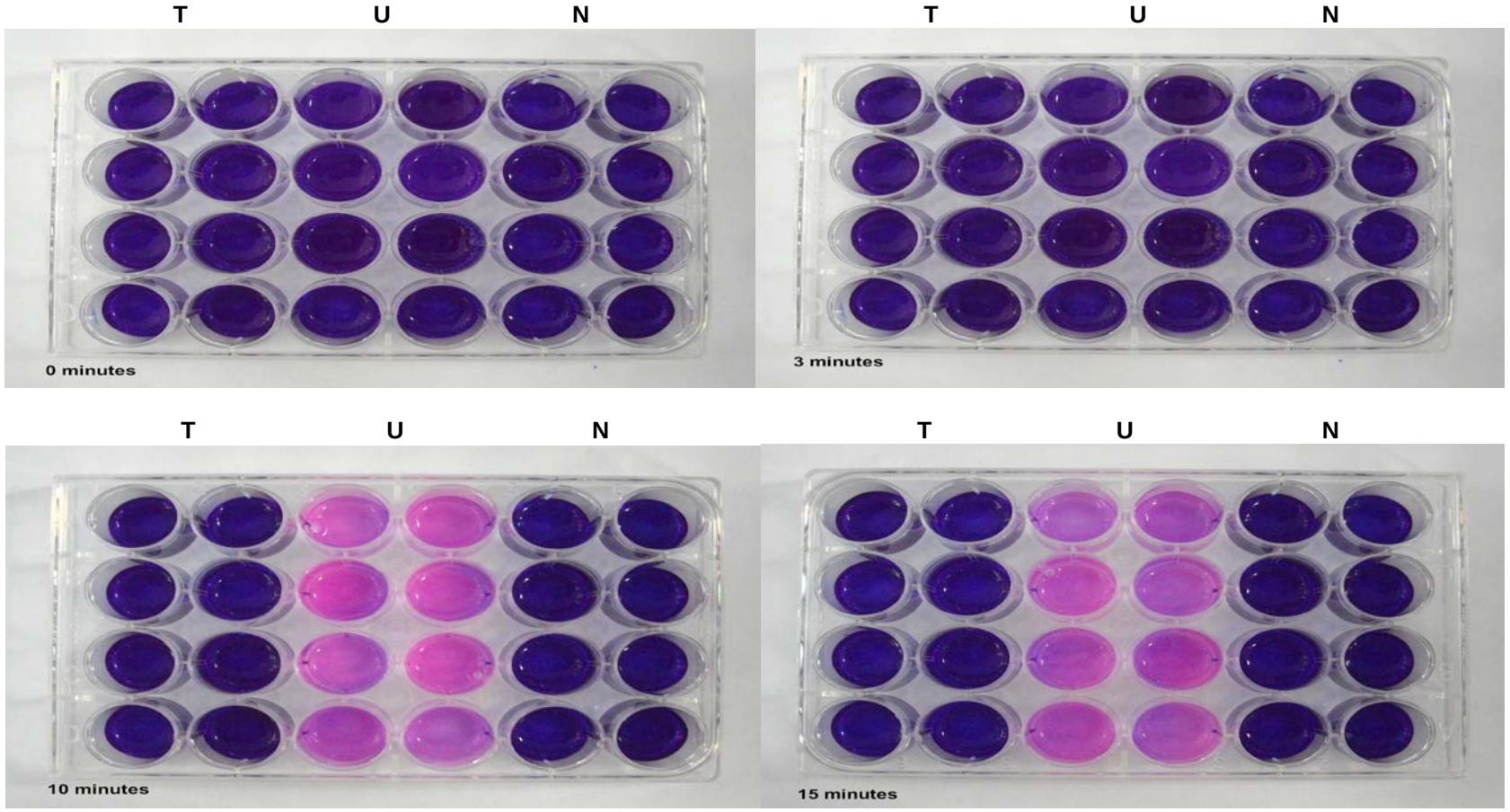


Figure 106. Resazurin assay to test for cell viability for *A. baumannii* treated for 6 hours with synthesized theaflavin. Photographs taken at 0, 3, 10 and 15 minutes after the addition of resazurin. In each image the first 8 wells contain treated cells (T), the second 8 wells contain untreated cells (U) and the last 8 wells contain no cells (N).



## 4.5 Conclusions

In conclusion it has been shown in this study that the synthesis of theaflavin in a microreactor is not only possible, but produces high yields of pure product when compared to batch methods. It is believed that this is the first occasion that the microreactor enzymatic synthesis of theaflavin has been reported.

Due to the stability of deuterium labelled epicatechin during the enzymatic synthesis of theaflavin is has been shown that this methodology although successful for producing theaflavin, could not produce labelled theaflavin and thus metabolic investigations did not take place. Future methodologies to produce more stable deuterium labelled epicatechin in a microreactor should be investigated to hopefully lead to a stable microreactor synthesized deuterium labelled theaflavin for use in metabolic studies.

In this study it has also been shown that microreactor synthesis and antimicrobial susceptibility methodologies can be linked to produce more rapid chemical production to antimicrobial testing times. It is believed it is the first occasion where methods for continuous flow microreactor synthesis and antimicrobial testing have been linked together.

In future research the bacterial filter will be integrated into a microfluidic device and linked to the microreactor for compound synthesis. This would give a fully integrated system to conduct antibacterial testing. There is also potential for linking the system for microreactor synthesis with other rapid antimicrobial determining methods such as bioluminescence.

## 4.6 References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# Chapter 5

Overall conclusions and future research

## 5.1 Overall PhD conclusions

Overall the research conducted in this PhD was very successful. The direction of the PhD changed from the original goal of monitoring human metabolites from polyphenols into something more clinically important, involving the antibacterial actions of polyphenols and how they can interact with current antibiotics.

The results proved that microreactors are an important tool in chemical synthesis. Using a microreactor system the research in this thesis has shown for the first time that not only could epicatechin could be labelled with deuteratium, but this could be achieved efficiently, producing very high yields.

Research from this thesis has also shown for the first occasion that the enzymatic synthesis of theaflavin could be achieved in a microreactor. This method could be a cost effective alternative for producing small quantities of theaflavin for laboratory testing. The method developed is a more rapid approach of acquiring small quantities of theaflavin that of extracting theaflavin directly from tea leaves.

Antimicrobial results in this thesis show the potential use of polyphenols as antimicrobial agents against clinical isolates of antibiotic resistant bacteria. For the first occasion the antimicrobial synergy between polyphenols against problematic bacteria has been observed. The research here has also shown, that synergy can occur between polyphenols and antibiotics. This results in the resistance of bacteria to specific antibiotics being reversed and the antibiotic again having some clinical use.

The major difficulties encountered, concerned the labelling of epicatechin and theaflavin using a microreactor. Difficulties included blocking of the microreactor from precipitating products and bubbles, solvent selection, which needed to be

carefully chosen as compound solubility and reaction yields were massively effected by the solvent and stability of materials produced.

### 5.2 Future work

Future research projects might investigate the production of epicatechin with a more stable deuterium label, which can withstand the coupling reaction with epigallocatechin to form deuterium labelled theaflavin. A possible reaction scheme could be based on that used in figure 24, with the use of different protecting groups to allow for a more stable reaction when using Dess-Martin reagent. The production of stable, labelled theaflavin is still an important task that should be undertaken to discover more about how it is metabolised in the human body and how its toxicological effects differ between human and bacterial cells.

Future work might investigate the immobilization of polyphenol oxidase on a monolith for the microreactor synthesis of theaflavin. This might increase the efficiency of the reaction and also enable a more commercial system.

Futher work concerning the antimicrobial effects of polyphenols might investigate the mechanisms behind the synergism between polyphenols and polyphenols and antibiotics. Future work might also investigate if the synergism between polyphenols occurs when they are used purely as antioxidants. If this is the case they could be used as additives into various products including suncreams, to prevent DNA damage caused by free radicals resulting from sun exposure.

Further work might continue to develop the system to link a chemical synthesis microreactor and a bioreactor for the growth of bacteria. The end goal would be to produce a commercial system for rapid antimicrobial testing.