Purification and Antimicrobial Properties of Oleuropein

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Dedicated to my parents and my wife.

Contents

Abstract

Olive leaves contain substantial amounts of phenolic substances, including the polyphenol oleuropein. This compound has been reported to possess antimicrobial properties. The purpose of this study was to develop an improved method for the extraction and purification of oleuropein from olive leaves and characterise its activity as well as the mode of action against a range of bacteria.

Phenolic compounds from olive leaves were extracted in methanol and oleuropein was separated from the mixture by countercurrent chromatography (CCC). Separation was confirmed by high performance liquid chromatography. CCC processing resulted in a purity of oleuropein of 60% and this was later improved to 90% (compared to 83% in a commercially available oleuropein product). Flash chromatography was successfully introduced as an additional purification step and this eliminated some of the surfactants in the extract.

Fifteen strains of bacteria and one yeast, including species commonly associated with hospital infections, were tested for their sensitivity to oleuropein in agar supplemented with oleuropein and by disc diffusion on agar media. Most of the bacteria used in this study were inhibited by oleuropein but the amount of oleuropein required for inhibition varied from 0.25 to 3.0%. The two most sensitive strains were *Enterobacter faecalis* and one strain of group A Streptococci in agar supplemented with oleuropein. *Staphylococcus* spp. were inhibited by oleuropein concentrations of 0.5 to 1.5%. Gram-negative species, such as *Escherichia coli* and *Pseudomonas aeruginosa*, the Gram-positive *Micrococcus luteus* and *Bacillus subtilis* as well the fungus *Candida albicans* were not inhibited in the same assay. In the disc diffusion test, 10% oleuropein inhibited Gram-negatives (4-15 mm) whereas 5 and 10% oleuropein resulted in inhibition zones from 12 to 30 mm in Gram-positives and *C. albicans*. Four strains of *S. aureus* were subjected to further studies. In bacterial-time kill assays, exposure to 2% oleuropein resulted in reductions of up to 6 log cfu mL^{-1} in 4 hours and 6 hours for two methicillin resistant and two methicillin susceptible strains, respectively.

A methicillin susceptible and a methicillin resistant *S. aureus* were investigated using transmission electron microscopy following exposure to 2% oleuropein. Cells of both types showed leakage of cell contents and ultimately lysis within two and four hours of exposure. Further work on leakage of cell constituents based on absorbance measurements was inconclusive due to interference by coloured compounds formed by the oxidation of oleuropein.

Leakage of amino acids from cells treated with oleuropein was investigated using ninhydrin and Bradford assays. It was observed that 12 to 38% of amino acids leaked from *S. aureus* treated with oleuropein. The results were confirmed by sodium dodecyl acrylamide electrophoresis where several bands were absent from treated cell extracts. In addition, fluorescent microscopy of lectin labelled *S. aureus* cells was attempted to investigate damage of glycoproteins attached on the extracellular cell wall. Lectin binding was unsuccessful and was replaced by fluorescein isocyanate, which selectively binds to lysine groups; the latter indicated reduced fluorescence in treated cells.

In conclusion this work demonstrated the application of a novel purification method based on countercurrent chromatography to obtain oleuropein with improved purity. The antimicrobial studies showed that oleuropein has the potential to eliminate bacteria. The mode of action studies showed that denaturation of proteins by oleuropein occurred, resulting in irreversible cell degradation. Oleuropein might be contemplated as a cleaning agent in environments where strong acids and bases are harmful for equipment.

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Abbreviations

ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BHA	Butylated Hydroxyanisole
BHI	Brain Heart Infusion
BSAC	British Society for Antimicrobial Chemotherapy
CCC	Countercurrent Chromatography
CCD	Countercurrent Distribution
CJA	Cucumber Juice Agar
CPC	Centrifugal Partition Chromatography
DCCC	Droplet Countercurrent Chromatography
DSC	Differential Scanning Calorimetry
FITC	Fluorescein 5, (6)- isothiocyanate
GAS	Group A *Streptococci*
GC	Gas Chromatography
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MRS	Man-Rogosa-Sharpe
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Susceptible *Staphylococcus aureus*
NCIMB	National Collection of Industrial and Marine Bacteria
NCYC	National Collection of Yeast Cultures
NRRL	Northern Regional Research Laboratory (Culture Collection)
NZA	N-Z amine
OD	Optical Density
PBS	Phosphate Buffered Saline
Q_m	Quantity of solute in mobile phase
Q_s	Quantity of solute in stationary phase

rev/min	Revolutions per minute
SCV	Small Colony Variants
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SPN	*Streptococcus pneumoniae*
TEM	Transmission Electron Microscopy
TFA	Trifluoro Acetic Acid
TLC	Thin Layer Chromatography
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
W	Watt
WGA	Wheat Germ Agglutinin
UV	Ultra Violet
μ_{max}	Maximum growth rate
v/v	volume per volume
v/w	volume per weight
w/w	weight per weight

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Chapter One

1 Introduction and Literature Review

1.1 Introduction

Natural plant materials have served as an important source of pharmaceutical products since ancient times (Pérez-Bonilla *et al.,* 2006). The group of compounds known as polyphenols are the main class of secondary metabolites in plants. Polyphenols are essential for plant physiology, being involved in diverse functions such as pigmentation, pollination and enhanced predator resistance (Kondratyuk & Pezzuto, 2004). Reports on polyphenols suggest that polyphenolic plant extracts, including those found in fruits and vegetables, have beneficial medicinal attributes including antileishmanial (Rocha *et al.,* 2005), antimalarial (Caniato & Puricelli, 2003) and anticarcinogenic activity (Matsui *et al.,* 2005), as well as enhanced suppression of HIV (Uchiumi *et al.,* 2003) and prevention of Alzheimer's disease (Dai *et al.,* 2006). The traditional 'Mediterranean diet', which consists of a versatile intake of fruits and vegetables and in particular the consumption of olives and olive oil, was associated with low occurrence of cardiovascular diseases and reduced risk of certain cancers (Soler-Rivas *et al.,* 2000; Visioli & Galli, 2002).

Much research has been aimed at identifying phytochemicals (polyphenols) of plant-derived products because of their beneficial properties. Bourquelot & Vintilesco (1908) discovered oleuropein from olives and olive leaves. Panizzi *et al* (1960) investigated that oleuropein is present throughout the olive tree *(Olea europaea)* and also in olive oil. Oleuropein is the bitter principle of olives and confers resistance to disease and to insect infestation of the olive tree (Soler-Rivas *et al.,* 2000). The secoiridoid oleuropein exists as most phenolic compounds in nature in the conjugated form, mainly with a sugar molecule

attached (Figure 1.1). Secoiridoids in the plant family *Oleaceae* are compounds with a structure that comprises an exocyclic 8,9-olefinic functionality, a combination of elenolic acid and a glucosidic residue. All are derived via secondary metabolism of monoterpenes as precursors of various indole alkaloids (Ryan *et al.,* 2002).

The phenomenon of antibiotic resistance led to renewed interest in plant derived products as alternative agents to tackle bacterial diseases and infections, such as those caused by Methicillin-resistant *Staphylococcus aureus* (MRSA). Natural compounds may act, in contrast to antibiotics, by several mechanisms on various target sites within the bacterial cell (Russell, 2003; 2004). The literature published to date suggests that phenolic substances have a broad spectrum of activity and therefore may be suitable for use in disinfection and cleaning as well as in nutraceutical and pharmaceutical products. The mode of action of most of these compounds on the bacterial cell envelope is still not clear and findings from this work may contribute to a better understanding about the functionality of natural compounds.

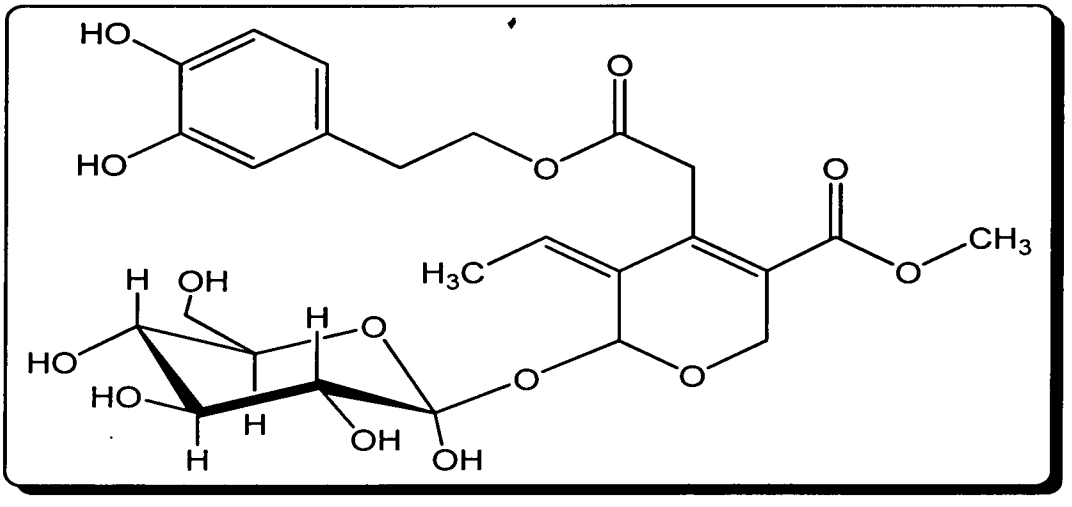

Figure 1.1: Chemical structure of oleuropein

1.2 Phenolic compounds in nature and their applications

Phenols are a class of chemical compounds consisting of a hydroxyl group (-OH) attached to a benzene ring. Polyphenols are compounds comprising at least two hydroxyl groups attached to the same benzene ring (Belitz *et al.*, 2004). The term phenolic described compounds that have one or more hydroxyl groups attached to an aromatic ring (Vermerris & Nicholson, 2006). Polyphenols show antioxidant capacity *in vitro*, whereas phenols have little or none (Rice-Evans *& Miller,* 1996).

Phenolic substances are found in many food products (Scalbert & Williamson, 2000; Manach *et al.,* 2004). For instance, fruits and plant-derived beverages such as fruit juices, coffee, cocoa, tea and red wine contain polyphenols (Table 1.1). Dai *et al* (2006) linked in an epidemiological study the consumption of polyphenols through fruit and vegetable juices with reduced occurrence of Alzheimer's disease in Japanese-Americans. Findings such as these have generated great interest in investigating the mode of action of polyphenols as well as their possible application as anti-cancer drugs, health-promoting and/or disease-preventing foods (known as functional foods), and as pharmaceutical and cosmetic additives (Ariga, 2004).

Table 1.1: Polyphenol composition of common food products

(Adapted from Manach *et al.,* 2004)

Food product*	Polyphenol	Weight mg L^{-1}
Blackberry	Cyanidin	1000 – 4000
Strawberry	Malvidin	75 – 375
Red Wine	Malvidin	200 – 350
Chocolate	Epicatechin	920 – 1200
Cherry	Chlorogenic acid	90 – 575
Kiwi	Caffeic acid	600 – 1000

*Weight of polyphenol extracted from equivalent of 100 g food product

1.2.1 Phenolic compounds in Oleaceae

The olive tree belongs to the *Oleaceae* plant family. Table 1.2 shows the phenolic compounds found in the olive fruit, olive oil, and olive leaves (Romani *et al.,* 1999; Savournin *et al.,* 2001; Tuck & Hayball, 2002). The main phenolic fraction in olive oil contains hydroxytyrosol, oleuropein and tyrosol whereas oleuropein is the main constituent in the leaves followed by verbascoside and oleosides. The olive fruit contains oleuropein and its derivatives as well as verbascoside and traces of oleosides. The phenolic composition of the olive fruit is qualitatively and quantitatively affected by the cultivar (Luque de-Castro & Japón-Luján, 2006; Ranalli *et al.,* 2006).

There is much interest in the potentially beneficial properties of olives, olive oil, olive leaves and purified compounds extracted from various constituents of the olive plant in the fight against cardiovascular diseases, cancer, obesity and diabetes (Table 1.3). The lower incidence of coronary heart disease (CHD) in the Mediterranean basin has been linked with the regular consumption of olive oil. Pharmacological studies done *in vitro* have shown that oleuropein extracted from olive oil inhibits oxidation of low density lipoproteins (Visoli & Galli, 1994, 1998, 2001; Tuck & Hayball *et al.,* 2002; Leenen *et al.,* 2002). Oxidised low density lipoproteins are undesirable as they may damage blood vessel cells, alter blood coagulation and promote atherosclerotic plaque formation. Wiseman *et al* (1996) indicated with *in vivo* studies in rabbits that olive oil polyphenols increased the resistance of low density lipoproteins to oxidation, a process that is believed to play a role in the development of CHD.

Table 1.2: Predominant polyphenol composition in olive fruit, olive leaf and olive oil

Polyphenol	Olive fruit mg g^{-1} [a]	Olive oil mg kg^{-1} [b]	Leaves mg g^{-1} [c]
Oleuropein	2.4	7.7*	140.0
Hydroxytyrosol	0.5	1.3	ND
Tyrosol	0.1	2.1	ND
Verbascoside	3.2	ND	5.0
Demethyloleuropein	0.14	ND	ND
Apigenin-7-glucoside	0.40	ND	0.5
Luteolin-7-glucoside	0.13	ND	4.4
Oleoside-11-methylester	0.5	4.0	4.8

Compiled from: [a]Romani *et al.*, 1999; [b]Gómez-Alonso *et al.*, 2002; [c]Savournin *et al.*, 2001); ND = Not detected; *value for oleuropein aglycone

It is unlikely that oleuropein could be used as a food preservative because of its unpleasant bitterness. However, it may be possible to mask the bitterness and conserve the beneficial properties via encapsulation (Gibbs *et al.*, 1999). Efmorfopoulou & Rodis (2004) investigated the possible formation of inclusion complexes of oleuropein with cyclodextrins, a process considered as molecular encapsulation. Cyclodextrins are hydrophilic on the exterior with a nonpolar central cavity allowing the inclusion of hydrophobic compounds. The appearance of turbidity was considered as evidence of inclusion complex formation. In neutral medium, haze production in an aqueous solution of cyclodextrins was indicating possible inclusion of oleuropein. Nevertheless, more research in this field is needed, especially in terms of selecting the coating materials, activity as preservative and metabolism of encapsulated substances. Oleuropein might be used in medicinal and cosmetic products but there are relatively few studies in this area.

Table 1.3: The possible role of olive plant polyphenols in common human disorders

Adapted from Luque de Castro & Japón-Luján (2006)

Polyphenol	Disease	Metabolic target	Reference
Oleuropein and derivatives	Coronary Heart Disease	Prevention of lipid membrane oxidation	Salami *et al.*, 1995; Saija *et al.*, 1998; Visioli *et al.*, 2002; Caturla *et al.*, 2005
	Cancer	Protection of enzymes and prevention of cell death; scavenging peroxy radicals, hydroxyl radicals and superoxide anions	Visioli & Galli 1994; *et al.*, 1998; Owen *et al.*, 2004
	Obesity	Improvement of lipid metabolism by inhibiting the activity of hydroxy-methylglutaryl-CoA enzyme	Bellosta *et al.*, 2000
Oleuropein hydrolysis products	Coronary Heart Disease	Protection of human erythrocytes; increase of plasma antioxidant capacity	Petroni *et al.*, 1995; Manna *et al.*, 1997
	Cancer	Inhibition of cell proliferation and induction of apoptosis in human tumours	Visioli *et al.*, 2000
Oleocanthal	Inflammation	Anti-inflammatory (Ibuprofen-like activity by inhibiting cyclooxygenase enzymes)	Beauchamp *et al.*, 2005
Olive oil polyphenols	Coronary Heart Disease	Inhibition of lipoprotein oxidation	Visioli *et al.*, 1995; Visioli & Galli, 2002

1.2.2 Phenolic compounds in processed olive fruit

Olive fruits are usually processed to obtain edible and palatable table olives and also to produce olive oil (Soler-Rivas, *et al.,* 2000). Bianchi (2003) and Soler-Rivas *et al* (2000) have described all stages of olive fruit processing. The ripe olive fruit contains between 2 and 14 mg g^{-1} oleuropein conferring bitterness to the raw fruit (Briante *et al.,* 2002; Malik & Bradford, 2006). In a study by Amiot *et al* (1986) green olives were reported to contain as much as 140 mg g^{-1} oleuropein.

Green olives are chemically treated followed by lactic acid fermentation before they are ready for sale. Two distinct methods of processing green olives are described below: In the Sevillian or Spanish method, olives are immersed for 8-12 h in a 2-2.5% sodium hydroxide solution in order to debitter the fruit. After the NaOH has penetrated a large proportion of the flesh, the alkali treatment is interrupted by a water wash cycle, which neutralises the medium. The washing step alters the sugar and salt content but the oil content remains unchanged. The final stage is fermentation in brine (an aqueous solution of 5-9% sodium chloride).

In the Californian method, green olives are successively treated with 1-2% sodium hydroxide after keeping the olives in brine under anaerobic conditions for several months (Bianchi, 2003; Solver-Rivas *et al.,* 2000). The olives are then neutralised in water or diluted brines, through which air is bubbled. This aerobic alkali treatment tends to cause dramatic changes in the texture of the flesh. Consequently, the fruits become brown and dark due to polymerisation of phenolic compounds. Finally, the olives are mixed in an aqueous solution of ferrous gluconate or lactate in order to balance the black colour.

None of the numerous processes utilised for the preparation of black table olives undergo sodium hydroxide treatment (Bianchi, 2003). In the Greek method, the olives are kept for a specific period of time (three to six months) in 8% brine solution. *Lactobacillus plantarum,* used as a starter culture to degrade oleuropein, may substitute for the chemical treatment of olives. In studies by Marsilio & Lanza (1998; Marsilio *et al.,* 1996), the lactic acid bacteria first hydrolysed oleuropein by means of β-glucosidase to form oleuropein aglycone that was further degraded by esterase action to hydroxytyrosol and elenolic acid. The quantity of hydrolysis products from oleuropein might be considered as a qualitative feature for the maturation of olives (Esti *et al.,* 1998).

Many changes occur during table olive processing, and they affect the chemical and organoleptic properties of the product (Romero *et al.,* 2004a; 2004b). In the Spanish method, sodium hydroxide hydrolyses oleuropein into hydroxytyrosol and elenolic acid glucoside (Brenes *et al.,* 1995). Hydroxytyrosol then diffuses from the flesh into the brine. Oleuropein content thus diminishes during the fermentation process (Soler-Rivas *et al.,* 2000).

Ruiz-Barba *et al.,* (1993) reported that non-alkali treated olive brines contained hydroxytyrosol, tyrosol, vanillic acid, verbascoside, luteolin-7-glucoside and oleuropein. Alkali treated olive brines contained hydroxytyrosol, tyrosol and vanillic acid. Ryan *et al* (1999; 2003) showed the phenolic changes in olives during maturation and compared the phenolic content from olive fruits after hydrolysis with an acid and a base (Ryan *et al.,* 2001). Acid hydrolysis affected the breakdown of oleuropein into hydroxytyrosol glucoside, whereas after base hydrolysis oleuropein

was not found at all. Tyrosol was detected at higher concentrations after base than following acid hydrolysis. This supports the suggestion that a large proportion of the phenolic fraction is either transferred into the brine and/or destroyed under acidic/basic conditions. The chemical structures and metabolite products of main phenolic compounds in the olive plant before processing are shown in Figure 1.2.

Cardoso *et al* (2005) explored phenolic compounds in olive pomace and olive pulp (olive fruit without seed) by extracting the phenolic fractions with methanol. The recovery of the phenolic fraction with methanol:water (1:1) was 111 mg g^{-1} in olive pulp and 106 mg g^{-1} in olive pomace determined by the Folin-Ciacalteau assay (Cardoso *et al.*, 2005).

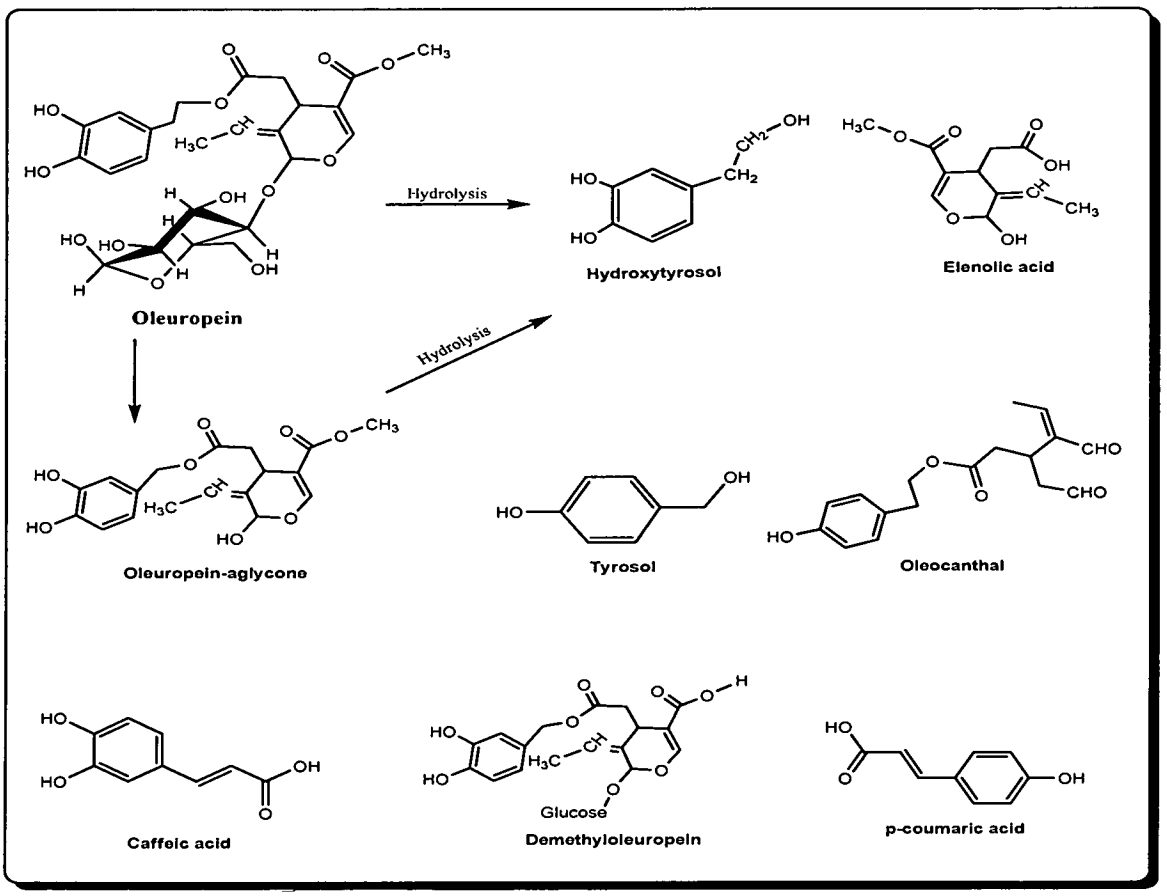

Figure 1.2: Chemical structures of predominant olive plant polyphenols

1.2.3 Phenolic compounds in olive oil

Olive oil is obtained either by centrifugation or hydraulic pressing of malaxed olive fruits, which are denoted as drupes (the ground flesh and pits are denoted as pomace). Malaxation is the action of slowly churning or mixing milled olives in a specially designed mixer. This mixing allows the agglomeration of smaller droplets of oil into larger ones in order to facilitate separation. The polyphenol profile of olive oil contains mainly elonic acid, hydroxytyrosol, ligstroside aglycone, oleuropein aglycone and tyrosol (Gómez Alonso *et al.,* 2002).

Carrasco-Pancarbo *et al* (2005) have summarised the literature for the analysis of phenolic compounds from olive oil using Capillary electrophoresis, GC and HPLC. In the HPLC process, a gradient of water and methanol mixtures or acetonitrile with methanol were frequently used as mobile phases.

Phenolic content in olive oil is influenced by the cultivar, location, degree of ripeness of the fruits and the type of extraction employed (Esti *et al.,* 1998). The total phenolic content in olive oil was reported by Visioli & Galli (2002) to vary between 150 mg kg^{-1} and 700 mg kg^{-1}. Extra-virgin olive oil contained higher amounts of oleuropein, hydroxytyrosol, and tyrosol than refined olive oil. Owen *et al* (2004) reported that the average content of total phenolics in olive oil was 196 ± 19 mg kg^{-1}. The oleuropein, hydroxytyrosol and tyrosol content in olive oil reported in the literature is variable. The concentration of hydroxytyrosol in olive oil was reported to be 1.4 - 5.6 mg L^{-1} (Montedoro *et al.,* 1992) and 11.66 - 22.13 mg kg^{-1} (Owen *et al.,* 2004) whilst oleuropein concentration varied between 2.3 and 9.0 mg L^{-1}. Tyrosol concentrations were reported between 2.98 - 27.45 mg L^{-1} (Coni *et al.,* 2000; Owen *et al.,* 2004).

1.2.4 Phenolic compounds in olive leaves

Although not used as food, olive leaves contain substantial amounts of phenolic compounds. The levels of oleuropein vary from 1 to 14% (w/w) in different cultivars (Amiot *et al.,* 1986; Savournin *et al.,* 2001; Priego-Capote *et al.,* 2004). Amiot *et al* (1986) reported an oleuropein content of 140 mg g^{-1} in green olives with successive petroleum-ether extractions to remove pigments and lipids followed by three extractions with ethyl acetate containing ammonium sulphate (20%), metaphosphoric acid (2%) and methanol (20%).

The oleuropein content in olive leaves was reported by Savournin *et al* (2001) at 140 mg g^{-1} using microwave-dried leaves extracted with 50% methanol followed by chloroform, salt saturation and ethyl acetate treatment. Le Tutour & Guedon (1992) extracted oleuropein from olive leaves with heated methanol and obtained 190 mg g^{-1} oleuropein. Sivakumar *et al* (2005) investigated three different green olive cultivars and showed oleuropein values from 10.1 to 14.6 mg g^{-1}. For the extraction of oleuropein, 15 g freeze-dried fruits were homogenised with methanol-acetone (1:1) prior to further processing (Sivakumar *et al.,* (2005).

Malik & Bradford (2006) extracted oleuropein from powdered fruits and leaves by duplicate extraction with 80% methanol and reported 14 mg g^{-1} oleuropein in olives and around 35 mg g^{-1} in mature leaves. Results published by Romani *et al* (1999) and Esti *et al* (1998) reported oleuropein contents of green olives of 2.4 mg g^{-1} and 1.5-3.5 mg g^{-1}, respectively. In the study by Romani *et al* (1999), 100 olives were extracted with 80% ethanol, which seemed to extract lower amounts of oleuropein than the extraction with methanol.

Because of their richness in antioxidant phenols, olive leaves are mixed with overripe olives before processing to produce oils with a more marked flavour and higher resistance to oxidation (Ranalli *et al.,* 2003). Olive leaves are an inexpensive source of oleuropein and other phenolic compounds, such as rutin, verbascoside, luteolin-7-glucoside, apigenin-7-glucoside and oleosides. Ranalli *et al* (2006) investigated the oleuropein content in seven Italian olive cultivars in different seasons of the year and found that overripe green leaves have a higher oleuropein content than unripe material.

1.3 Extraction and purification of phenolic compounds

The isolation of phenolic compounds from a mixture can be complex and time consuming. Most common methods consist of multiple leaching stages in a solvent system of acetone, dichloromethane, methanol, ethyl acetate or hexane with the addition of sodium carbonate (or sodium bicarbonate) resulting in the formation of phenolate salts of the phenolics, that are soluble in water (Vermerris & Nicholson, 2006). The choice of solvent depends on the crude material. For olives and olive oil, the solvent system tends to be a mixture of polar and unpolar solvents, whereas for leaves, unpolar solvents are preferable.

The methods used to determine the phenolic content of the olive plant are the Folin-Ciocalteau colourimetric assay and High Performance Liquid Chromatography (Gutfinger, 1981). The Folin-Ciocalteau method determines the total phenolic content only. In this method, the reduction of a mixture of phosphotungstic and phosphomolybdic reagent by the phenolic hydroxyl group (from a wide range of sources) leads to the formation of a blue product (Folin &

Denis, 1912; Folin & Ciocalteau, 1927; Vermerris & Nicholson, 2006). An aliquot of the aqueous-methoanolic extract is mixed with water and Folin-Ciocalteau reagent before sodium carbonate solution is added and finally mixed with water (Carrasco-Pancorbo *et al.,* 2005). The absorbance at 750 nm of the mixture is measured after two hours (Carrasco-Pancorbo *et al.,* 2005). A standard curve can be prepared using oleuropein in the concentration range of 0.01 to 1 mg mL^{-1}. The advantage of the Folin-Ciocalteau method is its speed and simplicity. However, the low specificity means that the colour reaction can occur with any oxidisable phenolic hydroxyl group (Carrasco-Pancorbo *et al.,* 2005). The Folin-Ciocalteau reagent can also be mixed with copper ions to form copper-protein complexes (Lowry method) for the spectrophotometric determination of proteins (Holme & Peck, 1998).

High Performance Liquid Chromatography (HPLC) currently represents the most popular and reliable technique for phenolic analysis. Phenolic compounds show high absorption in the UV region, so the most commonly used detector for the HPLC is a multi-wavelength UV or UV-vis detector. Single wavelength detectors are inappropriate as phenolics display absorbance maxima at different wavelengths i.e. oleuropein 280 nm, verbascoside 330 nm, apigenin-7-glucoside 340 nm and luteolin-7-glucoside 350 nm. In principle, HPLC analysis provides an accurate picture of the phenolic fraction in the sample matrix if the right conditions, such as solvent selection and UV-range are applied. In the following paragraphs, the extraction, separation and purification of phenolic compounds are reviewed in detail.

1.3.1 Extraction of phenolic compounds from Oleaceae

A range of processes is available for the extraction of phenolic substances from foods. The conditions employed should be as mild as possible to avoid oxidation, thermal degradation and chemical and biochemical changes in the sample. The ultimate goal is the preparation of an extract, where the proportion of the components is a reflection of the composition of the plant material and gives a reasonable extraction yield of the component of interest.

Walter *et al* (1973) isolated oleuropein from frozen raw olives homogenised in methanol followed by hexane to remove lipids before extraction with ethyl acetate. By using hexane as secondary extraction solvent, some of the surfactant components were removed but inevitably, some phenolic content would also have been lost.

Nychas *et al* (1990) mixed 500 g olives with 1 L water and brought the mixture to the boil. The aqueous extract was then filtered and the filtrate freeze-dried and kept at -20 °C. A quantity of 10 g of the freeze-dried olive powder was suspended in 60 mL water and extracted with petroleum-ether followed by ethyl acetate before the extract was concentrated. Tassou & Nychas (1994) crushed olives, which were frozen in liquid nitrogen. The mixture was twice homogenized in 80% aqueous ethanol. Four successive petroleum ether extractions followed to remove pigments and most of the lipids. The phenolic compounds were then extracted with ethyl acetate before the solvent was evaporated under vacuum.

Savournin *et al* (2001) described a rapid method for separation of the major phenolic compounds from powdered olive leaf material by treating them in an ultrasonic bath (30 mL methanol:deionised water 60:40 w/w) for 15 minutes. The

solution was then filtered and mixed with 100 mL of the same solvent. The disadvantage was that there was still a range of phenolic and non phenolic compounds left in the extract.

Japón-Luján *et al* (2006b) described an extraction process using microwave energy to heat solvents (ethanol:deionised water 80:20) in contact with olive leaves. Extraction time for one gram of milled leaves with 8 mL extractant was set to 8 min by microwave irradiation at 200 W followed by centrifugation of the suspension. It is noteworthy that pH 7 was reported as the optimum pH value (tested between pH 3 and 13) for the extraction of oleuropein, apigenin-7-glucoside, luteolin-7-glucoside and verbascoside. The microwave-assisted extraction resulted in lower amounts of phenolic compounds (0.03 mg g^{-1}) compared to literature data (Table 1.2). Therefore, it still needs to be optimised to increase phenolic contents.

A novel technique that could be tested on oleuropein is the process of extraction using Colloidal Gas Aphrons (CGA). CGAs are a gas-liquid dispersion of microbubbles generated by stirring a surfactant solution at high speed. Depending on the surfactant used to form CGA (cationic, anionic, or non ionic), the outer surface of the gas bubble may be positively, negatively or non charged to which oppositely charged or non charged molecules will adsorb resulting in their effective separation from the liquid pool (Spigno & Jauregi 2005). For example, a foam created from a cationic surfactant may absorb the negatively charged phenolic compounds from a solution.

In summary, most extraction procedures are based on the solubility of the phenolic material in polar and non-polar solvent mixtures, in particular methanol and water. Lipid fragments are removed by successive treatments with hexane or chloroform. However, use of those solvents is also linked with the loss of phenolic material due to the protic (can donate H^+) and aprotic (cannot donate H^+) nature of those compounds in solution. Additional drawbacks of these solvents are the adverse effects on human health and the environment. There is an interest in finding improved methods for extracting natural compounds with similar features as oleuropein. In selecting an appropriate solvent, judgements need to be made to balance the efficiency of the process against the complexity and cost of the procedure as well as suitability for scale-up.

1.3.2 Separation and purification of phenolic compounds by chromatography

Chromatography is a separation technique that partitions the components of a mixture between a stationary phase and a mobile phase. The basic process of chromatography is illustrated in Figure 1.3.

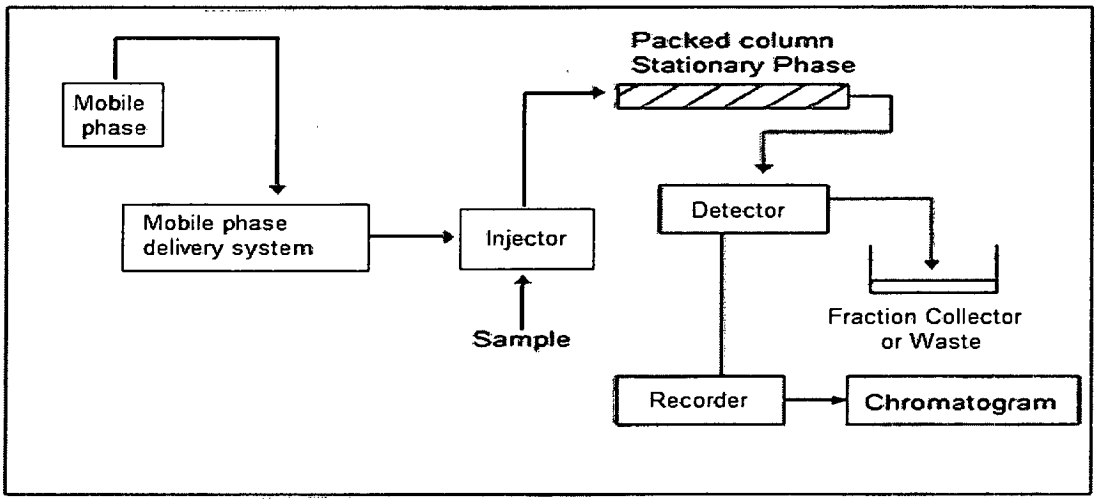

Figure 1.3: Flowchart of chromatography

There are two main types of chromatography, liquid chromatography and gas chromatography, depending upon the nature of the mobile phase. With the stationary phase being either a solid or a liquid, this makes the four main chromatographic groups as shown in Figure 1.4. In liquid chromatography, a liquid is used as the mobile phase to separate compounds based on hydrophilic and hydrophobic properties, electrostatic interactions, charged ion exchange, size exclusion or other techniques (McMaster, 2007). In gas chromatography (GC) the mobile phase is an inert carrier gas, whereas the stationary phase is a solid or a viscous liquid coating the walls of a long capillary tube. Supercritical fluid chromatography uses compressed CO_2 as mobile phase. Thin-layer chromatography (TLC) uses an absorbent material on flat glass or metal plates, whereas paper chromatography uses a strip of paper as the stationary phase. Capillary action is used to pull the solvents up through the paper and separate the solutes which were previously adsorbed onto the stationary phase.

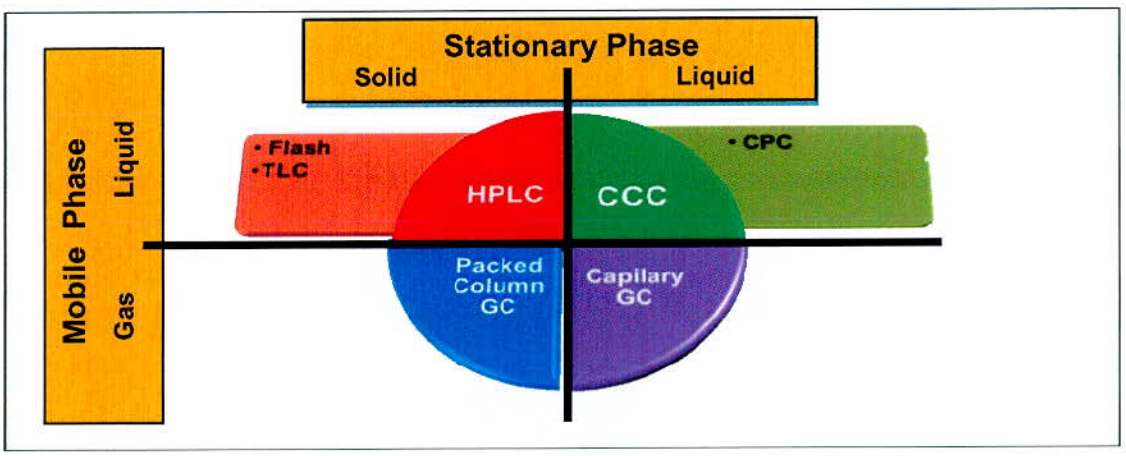

Figure 1.4: Chromatography options in natural product identification

High performance liquid chromatography (HPLC) is a separation technique that uses a liquid as mobile phase to separate components of a mixture. The injected sample is carried under high pressure by the mobile phase through a column,

containing particles of the stationary phase. In reverse phase HPLC (the most prevalent technique) the stationary phase consists of spherical particles of silica as a base support to which straight chain alkyl groups such as $C_{18}H_{37}$ are attached. This makes the stationary phase non-polar with the mobile phase being a moderately polar, aqueous-based solvent. Non-polar components introduced to the column are therefore retained longer on the stationary phase and elute after the more polar components, with hydrophobic interaction being the predominant retention mechanism of the technique. The elution strength of the mobile phase is increased by making it less polar and in gradient mode this is done by reducing the water content and increasing the organic solvent content.

In contrast, normal phase chromatography has a column consisting of a polar stationary phase based on silica or alumina. The mobile phase consists of organic solvents and is moderately non-polar. Compounds elute from normal phase columns in order or polarity, the most non-polar components eluting first. Normal phase chromatography is not commonly used today as water and other protic solvents change the hydration state of the stationary phase, causing irreproducible retention times.

Flash chromatography, also known as column chromatography, is an inexpensive and simple technique for purifying organic compounds. In flash chromatography, a column is filled with silica particles, immersed in a solvent, which is flowing down the column. The separation of the compounds occurs due to retention by the silica and affinity with the solvent mobile phase flowing down under gravity. Equilibrium is established between the solute adsorbed and the eluting solvent flowing down

the column. Due to the different chemical properties of the components in the mixture, the interactions with the stationary and mobile phase differ for each component. They are carried along with the mobile phase to varying degrees and undergo a separation. The individual components are collected as fractions as the solvent elutes from the bottom of the column. A frit, or filter is required at the base of the column to prevent the silica from descending out of the column. Kefalas (2007) claimed that flash chromatography, after pre-treatment of the crude olive leaves would give an oleuropein purity of up to 98% when using a mixture of methylene chloride, methanol and water.

Gel exclusion chromatography is used to separate a mixture on the basis of particle size. In gel exclusion chromatography smaller particles are driven into small pores of silica (e.g. Sephadex) and are retained as bigger particles flow down the column. This method is used extensively for protein and polymer purifications but has not been used for oleuropein separation before.

Counter Current Distribution (CCD) is the precursor of Counter Current Chromatography (CCC). The separation is similar to CCC (described in the section below) with a two phase system containing a train of test tubes, which are successively shaken and allowed to settle (Meltzer, 1958). Walter *et al* (1973) used CCD for the extraction of oleuropein from olive fruits with a mobile phase of ethyl acetate and stationary phase of 0.1 M potassium phosphate. CCD extract was subjected to Thin Layer Chromatography (TLC) and using light absorption wavelength of 280 nm to collect the major band. Solvents were removed by evaporation before further purification by a second process of CCD using ethyl

acetate as the mobile and distilled water as the stationary phases. Mass spectra verified the molecular weight of oleuropein as 540 g mol^{-1} (Walter *et al.*, 1973).

Kubo *et al* (1985) extracted oleuropein and ligstroside by placing olive fruits in methanol for more than six months prior to extraction with Droplet CCC (DCCC) with the solvent system Chloroform-Methanol-Water (13:7:4 v/v). Another solvent system for DCCC was Hexane-Chloroform-Methanol-Water (5:5:7:2 v/v), which allowed a better separation of ligstroside. In DCCC, droplets of mobile phase are passed through a test tube containing stationary phase. The entire process is motionless and mobile phase elutes through a capillary into the next test tube separating its components on the basis of affinity to the stationary phase. The disadvantage of DCCC is its low flow rate and long separation times.

1.3.2.1 Counter Current Chromatography (CCC)

CCC is a sophisticated method for separating individual components from a mixture in solution and is based on a two-phase immiscible system. In contrast to HPLC where the stationary phase is a solid column, the stationary phase in CCC is a liquid consisting of one of the immiscible solvents. One of the immiscible solvents is held in place by rotation of the coil whereas the other is pumped through the CCC coil. After injecting a sample, the individual components within the sample settle either in the mobile phase or in the stationary phase. The Archimedean screw force leads to a push-out effect of mobile phase through the CCC coil. Due to the spinning of the tubing, liquids are forced in a certain direction. This technique was used for centuries to pump water up against the force of gravity.

In a CCC machine, the screw force makes the coil work as a pump. The spinning motion produces a force that pushes the contained liquid towards one end of the coil. One of the phases dominates and pushes the other phase towards the opposite end of the coil. As part of the push-out effect, the compounds in the mobile phase are constantly experiencing a series of mixing and settling steps resulting in a separation. The flow rate of the mobile phase is one factor to determine the efficiency of the separation and collection of fractions, which can be subjected to further analysis.

CCC is a versatile technique applicable for a range of natural materials. Its main advantages are (i) no irreversible adsorption onto a solid stationary phase, (ii) total recovery of injected sample, (iii) low risk of sample denaturation, (iv) low solvent consumption and (v) no requirement for expensive columns (Marston & Hostettmann, 2006). Figure 1.5 displays the individual process steps during a CCC operation.

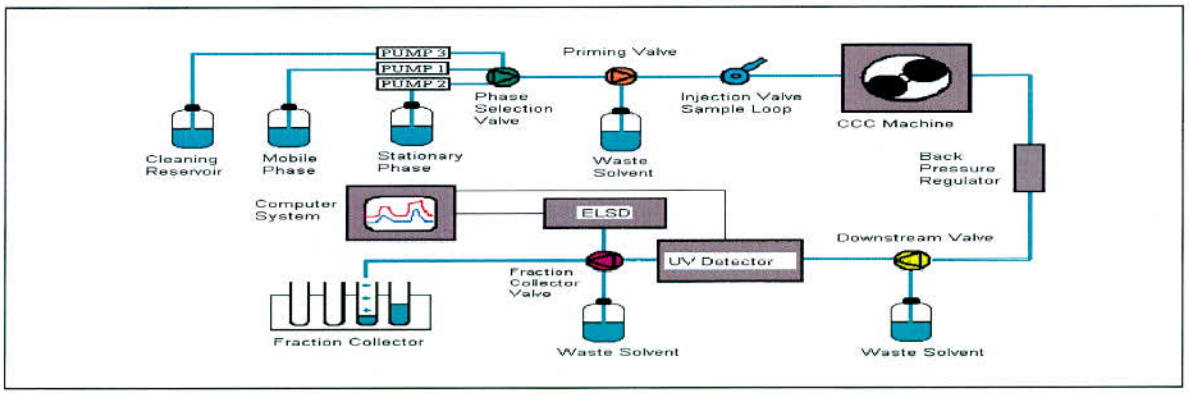

Figure 1.5: Flowchart countercurrent chromatography
(Garrard, 2005)

A successful CCC separation depends on the proper selection of an appropriate solvent system. CCC requires that the component of interest in a solvent mixture is distributed between two immiscible phases in order to permit a reasonable

separation of the compounds in the mixture defined as distribution coefficient or D-value. The distribution coefficient of a sample is determined by dividing the HPLC peak area in the stationary phase by the peak area of mobile phase of the compound of interest. A D-value between 0.2 and 5 is considered appropriate for CCC separation.

$$D = \frac{Concentration\ of\ component\ in\ stationary\ phase\ (in\ upper\ phase)}{Concentration\ of\ component\ in\ mobile\ phase\ (in\ lower\ phase)}$$

Equation 1: Distribution ratio calculation

The lower the D-value, the faster the compound of interest is eluted. However, a faster elution often results in a lower resolution of peaks in the chromatogram. Hence, distribution ratios in the order of 1 are selected to achieve a separation in an acceptable length of time. Adjustments to the solvent system are made according to whether the sample favours the less polar or more polar phase. HPLC peak area is frequently used to determine the D-values, though other methods could be used, such as HPLC peak height, UV absorbance, GC, TLC or even bio-activity.

If the sample is principally in the more polar phase, then a more polar solvent system should be tested. If the sample is in the less polar phase, then a less polar system should be tested (Garrard, 2005). The ratios of the solvents that were used to select the solvent system in this study are given in Table 1.5 (Garrard, 2005). There are five criteria for selecting a CCC solvent system described below. The first four criteria are essential in order to successfully operate a countercurrent chromatograph. The last criterion is desirable, especially if the separation is being done on a larger scale and fractions are collected.

- The solvent system must consist of two immiscible phases.
- The selectivity of the phases must be sufficient for good resolution of the components to be separated in the sample.
- The density difference between the phases must be at least 0.1g mL^{-1} in order to retain the stationary phase in the column (Martin, 1986).
- The settling time should be less than about 60 seconds in order for the mixing and settling steps inside the CCC column to operate efficiently (Ito, 1984). Note that this is an approximate rating as the settling time will vary with the selected end-point and the size of the vessel used.
- The mobile phase should be volatile so it can easily be removed from collected fractions.

In Table 1.4 a comparison of CCC and HPLC has been illustrated. Depending on the usage both methods show advantages in the field of sample analysis.

Table 1.4: Summary of advantages and disadvantages of HPLC and CCC (Garrard, 2005)

	HPLC	CCC
Advantages:	• Speed of analysis • Excellent resolution • Highly sensitive • Robust technique	• Versatile technique • Cross contamination rare • Economical • Particulates & crude samples accepted • Predictable & reproducible • High sample loading • Total sample recovery
Drawbacks:	• Particulates not tolerated • Undesirable interactions with stationary phase • Permanently retained compounds • Degradation of solutes on column surface	• Lower efficiency • Narrow polarity range • Difficult optimisation • Labour-intensive operation

The purification of natural compounds with CCC was applied in a wide range of studies (Hostettmann & Marston, 2001; Qizhen *et al.,* 2005). Fisher *et al* (2005) demonstrated the purification (greater than 98%) of glucoraphinin from broccoli seed extracts using CCC and also showed the scale up of the process. CCC is used extensively in natural product purification in Asia primarily because CCC is considered as a suitable technique for the purification of traditional Chinese medicine products. Current research is also focused on CCC applications in the purification of monoclonal antibodies and proteins (Sutherland *et al.,* 2008). However, the CCC process is still in the development phase and needs more test trials to establish it as a mainstream technique. Whether CCC will play any role in the industrial production of medicines, drugs, or natural compounds in the future is unknown but most likely it will find its niche.

Table 1.5: Solvent selection table consisting of heptane, ethyl acetate, methanol, butanol and water for the countercurrent operation given in ratios analysed by the liquid handling robot

(Garrard, 2005)

No		Heptane	EtOAc	MeOH	Butanol	Water
1		0	0	0	5	5
2		0	1	0	4	5
3		0	2	0	3	5
4		0	3	0	2	5
5		0	4	0	1	5
6		0	1	0	0	1
7	More	1	19	1	0	19
8	Polar	1	9	1	0	9
9		1	6	1	0	6
10		1	5	1	0	5
11		1	4	1	0	4
12		1	3	1	0	3
13		2	5	2	0	5
14		1	2	1	0	2
15		2	3	2	0	3
16		5	6	5	0	6
17		1	1	1	0	1
18		6	5	6	0	5
19		3	2	3	0	2
20		2	1	2	0	1
21		5	2	5	0	2
22		3	1	3	0	1
23		4	1	4	0	1
24		5	1	5	0	1
25	Less	6	1	6	0	1
26	Polar	9	1	9	0	1
27		19	1	19	0	1
28		1	0	1	0	0

1.4 Antimicrobial properties of oleuropein and olive plant derivatives

Several authors have examined the antimicrobial activity of olive plant polyphenols and their findings are summarised in Table 1.6. A more detailed review of a selection of papers in Table 1.6 is given below.

Initial interest in oleuropein was in the inhibition of lactic acid bacteria used as starter cultures in olive fermentation (Fleming, *et al.,* 1973; Juven *et al.,* 1970). Fleming *et al* (1973) reported that oleuropein aglycone and elenolic acid but not undegraded oleuropein, inhibited growth of four lactic acid bacteria after 16 h incubation in Cucumber-Juice Agar (CJA, pH 5.3) and Tryptone Soya Agar (TSA, pH 6.9) as determined by disc diffusion tests (temperature not indicated). Although the work by Fleming *et al* (1973) was advanced for its time, it lacked analytical detail afforded by current techniques such as HPLC. Instead, the phenolic compounds were separated by CCD (described in section 1.3.2) and their absorption wavelengths were determined at 280 nm followed by mass spectral analysis (Walter *et al.,* 1973). The total phenolic fraction was determined by the Folin-Denis method, which was a precursor of the Folin-Ciocalteau method (Swain & Hillis, 1959). The excessive pre-treatments used to obtain the compounds of interest most likely had an adverse effect on phenolic recovery. Moreover, the use of CJA is questionable due to the lack of nutrients and the low pH, which may have hindered bacterial growth.

Kubo *et al* (1985) reported that methanol extracted oleuropein (0.5 mg mL^{-1}) from olives inhibited the growth of *Bacillus subtilis* (inoculum size 10^5 cfu mL^{-1}) and

Saccharomyces cerevisiae (inoculum size 10^4 cfu mL^{-1}) producing zones of inhibition of 8 and 14 mm diameter, respectively, in disc diffusion tests supplemented with 0.5 mg mL^{-1} β-glucosidase in the growth medium. Without β-glucosidase the results showed no inhibition of the same microorganisms. The enzyme β-glucosidase generated in the fermentation process of olives seemed to promote the antimicrobial activity of oleuropein. The enzyme might be responsible for the splitting of oleuropein into its components hydroxytyrosol and elonic acid.

Nychas *et al* (1990) investigated the growth of *Staphylococcus aureus* in Brain Heart Infusion (BHI) and N-Z amine in the presence of 10% olive fruit extract without shaking at 37°C after 24 h. BHI broth contains substantial amounts of proteins that may mask antibacterial properties. The olive extract retarded the growth of the organism as measured by absorbance at 600 nm. However, in N-Z amine and BHI broth, secretion of exoprotein into the medium (measured by sodium dodecyl sulphate polyacrylamide gel electrophoresis) was accentuated. The olive extract used in this study was crude without identifying the components of the extract by HPLC and their purity. The antimicrobial effect against *S. aureus* needed further details such as range of measured time points and statistical evaluation.

Ruiz-Barba *et al* (1991) tested the bactericidal effect of alkali-treated, heat-treated and untreated oleuropein solutions extracted from green olives in distilled water (4 mg mL^{-1}) against *Lactobacillus plantarum* strains in Man-Rogosa-Sharpe broth. The inoculum size was approximately 10^6 cfu mL^{-1} with an incubation temperature of 30°C. Heat-treated oleuropein (121°C for 15 min) decreased viable colonies of

nine strains of *L. plantarum* by up to 6 log cfu mL^{-1} within 1 h incubation. In contrast, alkali-treated oleuropein allowed growth of *L. plantarum.*

Ruiz-Barba *et al* (1993) reported that hydroxytyrosol (1.2 mg mL^{-1}) reduced the numbers of viable *L. plantarum* by 7 log cfu mL^{-1} in Man-Rogosa-Sharpe broth within two hours. Hydroxytyrosol in combination with 24 μg mL^{-1} oleuropein resulted in a similar reduction. Verbascoside (87 μg mL^{-1}) exhibited antimicrobial properties against *L. plantarum* only in combination with hydroxytyrosol (1 mg mL^{-1}) or oleuropein (24 μg mL^{-1}). Inoculum size was 8 log cfu mL^{-1} and cells were incubated at 30°C for 48 h. All phenolic fractions were dissolved in acetate buffer (0.05 M, pH 4). Luteolin-7-glucoside, oleuropein, verbascoside, vanillic acid and tyrosol (67 μg mL^{-1}; 24 mg mL^{-1}; 87 μg mL^{-1}; 6.25 μg mL^{-1}; 21 μg mL^{-1}) were individually ineffective against *L. plantarum.*

The study by Tassou & Nychas (1994) showed a reduction of 1.5 log cfu mL^{-1} in *S. aureus* in the presence of 2% olive fruit extract within 21 h in a non fat milk model system (10% w/v). However, conductance observations showed that oleuropein at 1.5% drastically reduced the conductance of *S. aureus.* The reduction of 1.5 log cfu mL^{-1} by viable counting indicates that low conductance was not equivalent to bacterial killing when tested in a milk model system. In a similar experiment with *Salmonella enteritidis*, the conductance was measured in the presence of 2 mg mL^{-1} oleuropein combined with 0.5% NaCl (Tassou & Nychas, 1995). NaCl reduced the conductance with oleuropein and 0.5% oleuropein only delayed the detection time by two hours compared to the control in the milk model system.

Capasso *et al* (1995) investigated the antibacterial activity of extracts from olive mill wastewater against *Pseudomonas syringae* pv. Savastanoi (causal agent of knot disease in the olive plant) and *Corynebacterium michiganense* (causal agent of a tomato plant disease). Hydroxytyrosol (150 mg mL^{-1}) was found to be active against *P. syringae* pv. Savastanoi (73 % growth inhibition) but inactive against *C. michiganense* (Tryptose Broth for 48 h at 20°C; 10^6 cfu mL^{-1}). Other phenolic compounds in olive oil waste water showing an antimicrobial effect were o-quinone, catechol, 4-methylcatechol, guaiachol, and diaceethyl-1,2-tyrosol. There is interest in better exploitation of olive mill wastewater because of its high content of hydroxytyrosol and other phenolic compounds.

Surprisingly, *L. plantarum* was able to grow at 30°C in olive brines in the presence of 2% hydroxytyrosol, 1% oleuropein and 1% p-coumaric acid supplemented with NaCl from 0 to 10%, respectively (Marsilio & Lanza, 1998). In this study, 100 μL of culture in MRS broth was diluted with 900 μL distilled water and absorbance was measured at 660 nm. The combination of NaCl and oleuropein increased the absorbance of *L. plantarum,* whereas hydroxytyrosol and p-coumaric acid were similar to the NaCl only sample. NaCl was ineffective in reducing absorbance, only delayed the onset of growth and most likely was ineffective in retarding oxidation of oleuropein. Absorbance measurements with oleuropein are in general problematic as oxidation and browning of the sample occur. Therefore, it is useful to shake and suspend the sample during the incubation period to provide an equal distribution of contents, which was not done in the work by Marsilio & Lanza (1998). The authors suggested that the higher permeability of the olive fruits restrain the antimicrobial activity of phenolic components, which led to diffusion of hydroxytyrosol into the

brines. Secondly, organic cell constituents, such as sugars, amino acids and fatty acids from the olive fruit were released and favoured the growth of *L. plantarum*. The starter culture *L. plantarum* used in a range of food products, such as meat, bread and dairy products seemed to degrade phenolic compounds (Landete *et al.*, 2008).

Growth inhibition of *Klebsiella pneumoniae, Bacillus cereus, Aspergillus parasiticus* and *Escherichia coli* exposed to oleuropein was measured by the disc diffusion method by Aziz *et al* (1998). Nutrient Agar for bacteria and Czapek-Dox Agar for fungi were adjusted to pH 7 and used as growth media. Bacteria were incubated for five days and fungi for 10 days at 30°C. Oleuropein (dissolved in water) at 0.2 mg mL^{-1} resulted in inhibition zones of 60 mm for *A. parasiticus*, 30 mm for *A. flavus* and 60 mm for *B. cereus*, respectively. An oleuropein solution of 0.3 mg mL^{-1} showed an inhibition zone of 50 mm for *E. coli* and 0.4 mg mL^{-1} showed a 60 mm zone of inhibition for *K. pneumoniae* (Aziz *et al.*, 1998). It is hard to believe that extracts from dried olive cake showed such high inhibition zones. The authors claimed that the extracts were pure but this needs further documentation.

Bisignano *et al* (1999) investigated the antimicrobial activity of oleuropein and hydroxytyrosol compared to ampicillin and erythromycin against several bacterial strains using the disc diffusion method in Mueller Hinton Agar (MHA) and the microdilution test in Mueller-Hinton Broth (MHB) incubated at 37°C for 18 h. The activity of antimicrobial agents is often expressed as Minimum Inhibitory Concentration (MIC) that is required to inhibit the growth of the target organisms or as Minimum Bactericidal Concentration (MBC) that leaves no detectable survivors

after a specified contact time (Bondi *et al.,* 1947). This was the first attempt to study the antimicrobial activity of oleuropein and hydroxytyrosol on bacteria resistant to therapeutic antibiotics. It was shown that hydroxytyrosol (0.97 $\mu g\ mL^{-1}$) and oleuropein (500 $\mu g\ mL^{-1}$) significantly inhibited the growth of *Haemophilus influenzae* (eight penicillin resistant strains) and *S. aureus* (hydroxytyrosol: 3.9 - 31.25 $\mu g\ mL^{-1}$; oleuropein: 31.25 - 125 $\mu g\ mL^{-1}$; six penicillin resistant strains). Oleuropein, hydroxytyrosol, other olive plant phenolics and extracts from the olive plant were also tested against ten Gram-positive as well as Gram-negative strains (see Table 1.6) with the main observation that hydroxytyrosol showed a stronger antimicrobial activity than oleuropein. The MICs for hydroxytyrosol ranged from 0.24 to 7.85 $\mu g\ mL^{-1}$ for culture collection strains and between 0.97 and 31.25 $\mu g\ mL^{-1}$ for clinically isolated strains. Oleuropein inhibited the growth of several bacterial strains with MICs of 62.5 – 500 $\mu g\ mL^{-1}$ for culture collection strains and 31.25 - 250 $\mu g\ mL^{-1}$ for clinical isolates (Bisignano *et al.,* 1999).

Furneri *et al* (2002; 2004) has tested the antimicrobial action of oleuropein and hydroxytyrosol against *Mycoplasma* strains in a microdilution test. MICs for oleuropein were reported as 20 mg mL^{-1} (at 37°C for 24 - 48 h) against *M. hominis, M. fermentans* and 160 and 320 mg mL^{-1} against *Mycoplasma pneumoniae and M. pirum* (3 - 5 days at 37°C) in *Mycoplasma* broth. *S. aureus* was included as a control strain and showed a MIC of 40 $\mu g\ mL^{-1}$. MIC was defined as the lowest concentration of antimicrobial that inhibited a colour change in the broth compared to the colour of the control (Furneri *et al.,* 2002).

Tranter *et al* (1993) reported that oleuropein at 0.6% reduced *S. aureus* by up to 7 log cfu mL^{-1} in N-Z amine and BHI medium within 46.5 hours. Growth was monitored with a conductance analyser, which measured the electrical conductance of the medium, and viable counting of treated cells. A gradual but low increase in conductance was detected in the presence of 0.6 and 0.4% oleuropein indicating that the growth of *S. aureus* was delayed but the organism was not killed.

Markin *et al* (2003) tested olive leaves extracted in water against *E. coli, P. aeruginosa, S. aureus, B. subtilis, K. pneumoniae* (10^6 cfu mL^{-1}), dermatophytes (*Trichophyton mentagrophytes, Microsporum canis, Trichophyton rubrum* (10^5 cfu mL^{-1}) and yeast (*Candida albicans*; 10^5 cfu mL^{-1}). Incubation time was 24 h for bacteria, 48 h for dermatophytes and yeast at 37°C. All bacteria were killed within three hours of exposure to 6 mg mL^{-1} olive leaf extract except *B. subtilis*, which was inhibited only at 200 mg mL^{-1} leaf extract. Dermatophytes were all inhibited by 12.5 mg mL^{-1} leaf extract. *C. albicans* cells were killed in the presence of 150 mg mL^{-1} leaf extract. As with many other studies using an extract of olive plant material, the active component in the mixture was not identified. However, a reduction of 6 log cfu mL^{-1} of bacteria within three hours exposure suggests that high levels of oleuropein may have been present in the olive leaf extract.

Korukluoglu *et al* (2008) showed that different extracts from olive leaves and purchased olive plant polyphenols including oleuropein, caffeic acid, p-coumaric acid and tyrosol inhibited fungi. In this study the paper disc assay was used and extracts in different solvents (distilled water, acetone, methanol and diethyl ether)

were compared with purchased phenolic compounds. As with most papers, the active component in the extract was not determined. The solvent acetone was used to dissolve the purchased compounds whereas methanol was used for extracts. Twelve *Aspergillus* spp., two *Fusarium* spp., and seven *Penicillium* spp. were among the test fungi. All fungi were inhibited to a zone of inhibition of at least 10 mm in the presence of purchased 1% oleuropein. The plant extracts showed similar results between acetone and methanol, whereas diethyl ether extracts gave rise to lower inhibition zones. Acetone and methanol most likely evaporated after three days incubation at 30°C. These solvents may have contributed to the inhibition zone or might have reacted with the antimicrobial reducing the antimicrobial activity.

Antimicrobial studies on phenolic plant extracts are difficult to compare because of different methodologies used. The composition and purity of the extract is not defined in most studies and invites speculation on the active compound in the extract. The culture medium, pH, temperature conditions, inoculum size, concentration of the compound and method used to investigate the antimicrobial activity are often very different and prevent direct comparisons. The results from some studies with oleuropein are contradictory. For example, Ruiz-Barba *et al* (1991; 1993) reported that *L. plantarum* was killed by oleuropein, whereas Marsilio & Lanza (1998) disagreed and showed that *L. plantarum* degraded oleuropein and was ineffective. The use of different antimicrobial assays was probably the reason for the contrary results.

Recently, it has been reported that MRSA and MSSA were inhibited by commercial olive leaf extract containing 4.4 mg mL^{-1} oleuropein (Sudjana *et al.*, 2009). In this work a wide range of bacteria was tested by the microdilution assay in Man Rogosa Sharpe broth incubated for 3 days at 35°C with 5% CO_2. MICs were determined as the lowest concentration of olive leaf extract resulting in an optically clear microtitre tray well. Minimal bactericidal concentrations & minimal fungicidal concentrations were determined from microdilution assays by sub-culturing 10 μL on Rogosa & Mueller Hinton Agar prior to colony counting. Low MICs (0.3 to 125 mg mL^{-1}) of olive leaf extract have been reported against *Helicobacter pylori*, *Campylobacter jejuni*, MSSA and MRSA. However, this study is missing details on inoculum size and composition of the extract.

In another study, Bisignano *et al* (1999) used DMSO to dissolve oleuropein before application onto paper discs. Furneri *et al* (2002) observed a colour change in oleuropein containing media but used absorbance measurements to determine MIC values. Since DMSO probably contributed to the antimicrobial activity of oleuropein and oxidation reactions interfered with absorbance readings, the MICs reported in both studies are neither comparable nor reliable.

From the literature it can be summarised that the activity of oleuropein was dose-dependent: the more oleuropein the greater the antimicrobial activity. Several studies have reported that oleuropein showed a stronger activity against Gram-positive than Gram-negative bacteria. Fungi and yeast were to some extent also sensitive to oleuropein (Korukluoglu *et al.*, 2008).

With respect to pH and temperature, oleuropein showed higher antimicrobial activity at low pH levels and higher temperatures. Tassou & Nychas (1994; 1995) used conductance measurements and indicated that lower conductance occurred at pH 5.15 compared to pH 6.15 and 7.15. Ruiz-Barba *et al* (1993) showed that heat-treated oleuropein reduced *L. plantarum* by 6 log cfu mL^{-1} compared to alkali treated oleuropein. However, the temperatures used in many studies on oleuropein were selected according to the growth requirements of the target microorganism. The possible temperature dependence of oleuropein activity has not been considered in the literature.

Table 1.6: The antimicrobial properties of oleuropein and other phenolic compounds from the olive plant

Source/form of phenolic compound	Conditions Inoculum	Microorganisms	Conc. Anti-microbial (mg mL^{-1})	Inhibition/Inactivation reported	Reference
Phenolic compounds isolated from olive cake dissolved in deionised water	After 5 days Zone of inhibition at 30°C Inoculum = 10^6 cfu mL^{-1}	- *E. coli* - *K. pneumoniae* - *B. cereus* - *A. flavus* - *A. parasiticus*	Oleuropein from 0.1 to 0.4 mg mL^{-1}	0.2 mg mL^{-1} zones of inhibition *for A. parasiticus* 60 mm, 30 mm for *A. flavus* and 60 mm for *B. cereus;* 0.3 mg mL^{-1} 50 mm for *E. coli*; 0.4 mg mL^{-1} 60 mm for *K. pneumoniae*	Aziz *et al.*, 1998
Oleuropein (Extrasynthese); Hydroxytyrosol	Disc diffusion test and Microdilution assay; Incubation at 37°C and 24 h; Incubation: 37°C for 18 h; 10^7-10^8 cfu mL^{-1} and 10^5 - $5*10^5$ cfu mL^{-1} for microdilution assay;	- *H. influenzae* - *S. enterica* serovar Typhimurium - *V. parahaemolyticus* - *M. catarrhalis* - *Salmonella* spp. - *V. alginolyticus* - *V. cholerae* - *S. aureus*	In disc diffusion test 100 μg mL^{-1} oleuropein; 500 µg mL^{-1} dissolved in dimethyl-sulphoxide	MIC with hydroxytyrosol between 0.24 - 7.85 µg mL^{-1}; oleuropein 62.5 – 500 µg mL^{-1}; For clinical isolates 0.97 – 31.25 µg mL^{-1} hydroxytyrosol; oleuropein 31.25 - >500 µg mL^{-1} Penicillin resistant *S. aureus* strains were reported with MIC of 31.25-125 µg mL^{-1} compared to penicillin susceptible *S. aureus* with 62.5-125 µg mL^{-1}	Bisignano *et al.*, 1999
Olive oil mill waste-waters and individual phenolic compounds extracted according to Capasso (*et al.*, 1992, 1994) and suspended in DMSO	Incubation at 20°C for 24-48 h; MIC and MBC determined by turbidity; 10^6 cfu mL^{-1};	*Pseudomonas syringae; Corynebacterium michiganense*	10^{-2}-10^{-4} mol L^{-1};	*P. syringae* was to 63% inhibited by hydroxytyrosol (10^{-4} mol L^{-1})	Capasso *et al.*, 1995

Extract containing (1) oleuropein (2) acid hydrolysate of oleuropein (3) extract of frozen olives (4) β-3,4 dihydroxyphenyl ethyl alcohol (5) methyl-o-methyl elenolate (6) elenolic acid all extracts dissolved in methanol	Synergy effects of (1) aglycone of (1), (4), (5), and (6) with NaCl (5%) in cucumber juice broth; turbidity examined at 650 nm	- *Lactobacillus spp.* - *P. cerevisiae* - *L. mesentorides* - *S. aureus* - *B. subtilis* - *Escherichia spp.* - *S. typhimurium* - *Pseudomonas spp.* - *Erwinia spp.* - *X. vesicatoria* - *C. michiganese* **Yeasts** - *C. krusei* - *P. and D. membranaefaciens* - *H. subpelliculosa* - *K. apiculata* - *S. rosei* - *S. cerevisiae*	All micro-organisms tested with: - Oleuropein 10 mg; - Oleuropein hydrolysate 7.5 mg; - Extract of frozen olives 3.5 mg	Extracts (1-3) showed inhibition zones against most of the strains. No inhibition zones. Aglycone of (1), (6) with NaCl (5%) delayed lag phase of *L. plantarum* and completely inhibited at 150 μg mL^{-1}. No delay or inhibition of growth with (1), (4), (5), combined with NaCl (5%).	Fleming *et al.*, 1973
Oleuropein (Extrasynthese) in distilled water; Hydroxytyrosol in 0.1 M phosphate buffer	Broth microdilution assay; MIC defined as the lowest concentration that inhibit a colour change in the broth;	- *S. aureus* - *M.hominis* - *M. pneumoniae* - *M. fermentans* - *M. pirum* $10^3 > 10^5$ cfu mL^{-1}	20 mg mL^{-1} for oleuropein; hydroxytyrosol not given	MIC ranged from 20 - 320 μg mL^{-1} for oleuropein; *S. aureus* MIC was 40 μg mL^{-1}; MICs with hydroxytyrosol ranged from 0.03-0.5 μg mL^{-1}; 4 μg mL^{-1} for *S. aureus*	Furneri *et al.*, 2002; 2004
Oleuropein (Extrasynthese); tyrosol, caffeic acid, p-coumaric acid, hydroxy benzoic acid syringic acid and vanillic acid from Merck (Germany)	Olive leaves extracted in methanol, acetone and diethyl ether; Incubation for 3 days in Saboraud Dextrose Agar at 30°C; 10^4 cfu mL^{-1}	- *Alternaria sp.* - *12 Aspergillus spp.* - *7 Penicillium spp.* - *2 Fusarium spp.* - *Rhizopus sp.*	1–10 mg mL^{-1} dissolved in acetone; Extracts dissolved in methanol;	All fungi showed at least 10 mm inhibition zones; Oleuropein (1%) showed highest inhibition zones than other phenolic compounds.	Korukluoglu *et al.*, 2008

Oleuropein (Extrasynthese) in distilled water	BHI broth, pH 5-8; Temp. 22-42°C; Conductance measurements; 10^9 cfu mL^{-1}	*S. enterica* ss Enteritidis	0-8 mg mL^{-1}	Detection time increased approx. 40% at 22°, doubled at 28° and quadrupled at 37°C at all pH values tested (except pH 5.5 at the two lower temperatures) μ_{max} decreased approx. 30-90% overall;	Koutsoumanis *et al.*, 1998
Analysis of oleuropein from olives, ligstroside and dimethyl ester from olive leaves	Extracted for six months with methanol; 10^4-10^5 cells mL^{-1}	*S. cerevisiae* *B. subtilis* *E. coli*	Up to 2 mg per disc	Inhibition zones observed at pH 7 for *S. cerevisiae* and *B. subtilis* only when supplemented with 0.5 units mL^{-1} β-glucosidase	Kubo *et al.*, 1985
Olive leaves	Extracted with deionised water	*P. aeruginosa* *S. aureus* *B. subtilis* *K. pneumoniae* *T. mentagrophytes* *M. canis and* *T. rubrum* *C. albicans*	For bacteria 1.5-6 mg mL^{-1}; Yeast 100-300 mg mL^{-1}; Dermatophytes 1.5-100 mg mL^{-1}	Except *B. subtilis* were killed with 0.6% oleuropein after 3 hours exposure. *B. subtilis* killed with 20% olive leaf extract; MBC was 0.13% for *P. aeruginosa*, 0.3% for *K. pneumoniae* & *E. coli* and 0.6% for *S. aureus*. *C. albicans* killed with 15% olive leaf extract; MFC for the three dermatophytes was 1.25%.	Markin, *et al.*, 2003
Oleuropein (Extrasynthese) - Hydroxytyrosol	MRS broth incubated at 30°C for 24 h; NaCl added from 0-100 mg mL^{-1}; Absorbance measured at 660 nm	*L. plantarum*	Oleuropein 10 mg mL^{-1}; hydroxytyrosol 2 mg mL^{-1}; p-coumaric acid 1 mg mL^{-1}	Glucose (50 mg mL^{-1}) increased bacterial growth and reduced the hydrolysis of oleuropein to hydroxytyrosol and oleuropein aglycone; oleuropein in combination with NaCl showed no inhibition of *L. plantarum*	Marsilio & Lanza, 1998
Olives extracted in boiled water, petroleum ether, ethyl acetate; dissolved in distilled water	N-Z amine and BHI; turbidity at 600 nm; SDS-PAGE	*S. aureus*	0.1 g mL^{-1}	Electrophoretic pattern of treated *S. aureus* with olive extract showed less accentuations than the untreated sample; Olive extract delayed the growth in both media but less in N-Z amine; Glucose (0.2%) reduced the effect of inhibition by olive extract	Nychas *et al.*, 1990

Powdered olive leaves Boiled for 45 min in water then filtered	Incubated for 24h (fungi 48h) at 37°C in Nutrient Broth - Absorbance 540 nm; - Concentration inhibiting 25% of growth; - 10^8 cfu mL^{-1}	*B. cereus*, *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumonia*, *C. albicans* *C. neoforman*	0.05-5 mg mL^{-1} olive leaf extract	*B. cereus* and *C. albicans* were the most sensitive microorganisms *E. coli* > *S. aureus* > *C. neoformans* ~ *K. pneumoniae* ~ *P. aeruginosa*; *B. subtilis* was the most resistant microorganism	Pereira *et al.*, 2007
Oleuropein obtained from green olives dissolved in deionised water	pH adjusted to 5; 10^6 cfu mL^{-1}	*L. plantarum*	4 mg mL^{-1}	*L. plantarum* used as control strain; Oleuropein combined with NaCl increased bacterial growth (same for hydroxytyrosol)	Ruiz-Barba *et al.*, 1991
Phenolics of NaOH-treated and untreated green olive brines; Oleuropein (Extrasynthese)	Dissolved in acetate buffer 0.05 mol pH 4; 10^8 cfu mL^{-1}	*L. plantarum*	Hydroxytyrosol 1.2 mg mL^{-1}; Oleuropein 24 µg mL^{-1}; Verbascoside 6.25 µg mL^{-1}	Hydroxytyrosol reduced 7 log cfu mL^{-1} of *L. plantarum* within 2 hours; 8 log cfu mL^{-1} with hydroxytyrol + oleuropein within 2 hours, and 8 log cfu mL^{-1} with hydroxytyrosol + verbascoside and oleuropein + verbascoside	Ruiz-Barba *et al.*, 1993
Commercial olive leaf extract dissolved in agar and broth media	Agar method in Rogosa Agar; microdilution assay in MRS broth incubated for 3 days at 35°C with 5% CO_2; MICs determined by subculturing into solid media followed by colony counting	*Bacillus spp.* *C. jejuni* *C. albicans* *E. faecalis* *E. coli* *H. pylori* *K. pneumoniae* *K. rhizophilia* *Lactobacillus spp.* *Listeria spp.* *M. luteus* *P. aeruginosa* *S. enterica* *Serratia sp.* *MRSA* *MSSA* *Staphylococcus spp.*	Oleuropein 4.4 mg mL^{-1} in extract; Concentrations for microdilution assay ranging from 0.02 mg mL^{-1} to 500 mg mL^{-1}; Agar method 0.16 mg mL^{-1} mg mL^{-1}	MICs: between 125 to 500 mg mL^{-1} for most of the strains; - MSSA: 8 to 62 mg mL^{-1} - MRSA: 8 to 125 mg mL^{-1} - *E. coli*: 250 to 500 mg mL^{-1} - *C. albicans* 500 mg mL^{-1} - *C. jejuni*, *H. pylori* & *S. epidermidis* showed lowest MIC between 3 to 31 mg mL^{-1}	Sudjana *et al.*, 2009

Oleuropein (Extrasynthese) and extracted oleuropein from olives with ethanol, petroleum ether, ethyl acetate	Potato dextrose agar; spores examined by phase-contrast microscopy; spores detected by fall in optical density; viable count assay in TSA; Incubation 24h/30°C	*B. cereus* T spores	Up to 10 mg mL^{-1}	- Change from phase bright to dark occurred within 7 minutes - Oleuropein 1% has slow down the germination process by 60%	Tassou *et al.*,1991
Oleuropein (Extrasynthese) dissolved in distilled water	Conductance and viable counts	*S. aureus*	0-6 mg mL^{-1}	Growth in N-Z amine despite a long delay in the lag phase occurred (after 30 h); 4 mg mL^{-1} showed a 8 log cfu mL^{-1} reduction within 46 h Oleuropein delayed detection time	Tranter *et al.*, 1993
Oleuropein (Extrasynthese) Phenolic compounds extracted from olives with ethanol, petroleum ether and ethyl acetate	Dissolved in deionised water	*S. aureus*	1 & 6mg mL^{-1}; milk system 0.05, 0.1, 15, and 20 mg mL^{-1}	Conductance delayed and lower at pH 5.15 than 6.15, 7.15 with commercial oleuropein; in milk sytem reduction of 1.5 log cfu mL^{-1} after 11 hours incubation with olive extract	Tassou & Nychas, 1994
Oleuropein (Extrasynthese) dissolved in deionised water	10^9 cfu mL^{-1}	*S.enterica* ss Enteritidis	1 mg mL^{-1}; 2 mg mL^{-1} 6 mg mL^{-1}	Conductance measurements were 30% lower with oleuropein & NaCl (0.2%, 0.5%) at lower pH value 5.5 compared to pH 6.0, 7.0, and 7.8	Tassou & Nychas, 1995
Oleuropein (Extrasynthese) dissolved in deionised water;	Medium: Plate count agar using disc diffusion test incubated at 30°C for 24 h	*S. aureus*	50 mg mL^{-1}	25-35 mm zone of inhibition in diameter; In combination with hydrogen peroxide (0.03, 0.3 and 3%) inhibition zones were 11, 36 and 53 mm	Zanichelli *et al.*, 2005

1.4.1 Mode of action of oleuropein

Few attempts have been made to elucidate the precise mode of antimicrobial action of oleuropein. The precise mechanism of action is unclear but it has been proposed that the oleosides, characterised by an exocyclic 8,9-olefinic functionality (Ryan *et al.*, 2002), have surface-active properties, which are able to disrupt cell membranes (Juven *et al.,* 1972). Disruption of bacterial membrane functions appears to be a common feature of the action of phenolic compounds (Hugo & Bloomfield, 1971b). Membrane disruption could entail alterations in membrane permeability and loss of membrane-associated enzyme functions. Hugo & Bloomfield (1971b, c) reported that the initial rapid loss of low molecular weight metabolites is often accompanied by initiation of autolytic enzymes causing a gradual breakdown of protein and nucleic acids to give soluble products which are leaking out of the cell.

Juven *et al* (1972) observed that oleuropein (2 mg mL^{-1}) caused leakage of potassium and inorganic phosphate from *L. plantarum* and also decreased its ATP content. Phosphate leakage was measured by liquid scintillation spectrophotometry measuring a radioactively labelled potassium isotope in supernatant from cells treated with oleuropein. Inorganic phosphate was determined by absorbance at 852 nm whereas ATP was enzymatically measured. Oleuropein (2 mg mL^{-1}) also induced changes in the membrane of red blood cells and ultimately caused haemolysis by up to 60% of cells.

Lipid constituents of the cell membrane such as phospholipids and cholesterol were considered as a target for surface-active agents. Kroll (1981) proposed that potassium leakage was a consequence of membrane injury caused by phenols. In

agreement with Hugo & Bloofield (1971b) and Juven *et al* (1972), the major target site of phenolic compounds appears to be the lipidic fraction. Lattanzio *et al* (1994) stated that lipophilic properties of phenolic compounds allow penetration of biological membranes while hydroxyl groups help to uncouple oxidative phosphorylation.

Kubo *et al* (1985) and Trombetta *et al* (2002) hypothesized that unsaturated aldehydes from olive leaves act first on the plasma membrane by disrupting the lipids and then act on intracellular target sites in food-borne bacteria. Trombetta *et al* (2002) examined selected aliphatic plant aldehydes from olives and tested them on model phospholipid membranes (liposomes of phosphatidylcholine). Release of carboxyfluorescein was demonstrated via fluorescence spectrophotometry indicating damage of the cell membrane. Trombetta *et al* (2002) also scrutinized membrane permeation by olive plant aldehydes using differential scanning calorimetry (DSC) and speculated that interaction with intracellular sites may come into force. The DSC findings confirmed the introduction of lipophilic molecules within the lipid bilayer, suggesting that interstitial impurities interfere with bilayer functions.

Zanchelli *et al* (2005) investigated the mode of action of oleuropein and concluded that ortho-diphenols such as oleuropein need an activating factor to initiate antimicrobial activity. This trigger element was ascribed to hydrogen peroxide, which is thought to be involved in an oxidative reaction before the active inhibitory compound is formed. Tryptone, a peptide formed by the metabolism of casein, was utilized in the medium to investigate its presumed inhibitory contribution. It was assumed that oleuropein and tryptone interacted with one another and generated

hydrogen peroxide. However, hydrogen peroxide at 0.03% did not inhibit *S. aureus;* instead, it was able to trigger the mechanism that presumably induced inhibition due to oleuropein. Hence, the role of tryptone in the medium was suggested to serve as a source for hydrogen peroxide. In disc diffusion tests on a range of bacteria, only *S. aureus* was inhibited by oleuropein. Therefore, Zanchelli *et al* (2005) hypothesized that other factors in addition to hydrogen peroxide may contribute to the inhibitory action of oleuropein. However, Juven *et al* (1972) had observed many years previously that the antibacterial activity was reduced by enhanced concentrations of tryptone and/or yeast extract in the medium inferring that factor other than tryptone are involved. In summary, there is scant evidence about the mode of action of oleuropein.

1.5 Staphylococcus aureus

S. aureus is a ubiquitous Gram-positive spherical bacterium. The organism is both a commensal and versatile pathogen in humans and animals, causing a broad range of diseases in humans including septicemia, toxic shock syndrome and food poisoning (Kloos & Lambe, 1991). Staphylococci are widespread in nature and found mainly on the skin, skin-glands and upper respiratory tracts. Colonies of *S. aureus* are usually 6-8 mm in diameter after three days of incubation at 37°C, have a smooth appearance and are translucent (Kloos & Bannerman, 1995).

Gov *et al* (2004) stated that *S. aureus* pathogenesis is based on a wide range of cell wall associated and extracellular proteins that are regulated by the accessory gene regulator *agr.* Regulation of virulence determinants occurs through the quorum sensing mechanisms, where molecules (autoinducers) produced and secreted by

the bacteria accumulate as a function of cell density (Gov *et al.,* 2004). The molecules are called autoinducers because they are produced by the same cell whose metabolism they regulate (DeKievit & Iglewski, 2000). Once the autoinducers reach threshold concentration they may activate signal transduction pathways leading to the expression of genes that code for virulence factors (Gov *et al.,* 2004). Novick & Jiang (2003) stated that *S. aureus* strains produce a large number of extracellular proteins including virulence factors, which enables the organism to adapt to various external factors. These extracellular proteins are accessory proteins and are not required for basic growth and multiplication. External protein production occurs when the cell density is increased, which activates the agr-locus responsible for encoding most extracellular proteins.

1.5.1 The cell wall of S. aureus

Many of the cell wall features in *S. aureus* were discovered by transmission electron microscopy (Amako *et al.,* 1982; Beveridge & Matias 2006). The cytoplasmic membrane serves as an osmotic barrier for the cell and contains complex proteins, lipids and carbohydrates (Figure 1.6). The outermost layer of staphylococcal cells can be covered with a polysaccharide capsule which protects the cell from phagocytosis (Murray *et al.,* 2005). A loosely bound water soluble film (slime layer) consisting of monosaccharides, proteins and small peptides is produced by most staphylococci in varying amounts depending on genetic factors and the growth conditions. The extracellular slime layer can bind the cells to tissues and foreign bodies permitting further spread of bacteria. The peptidoglycan layer in Gram-positive bacteria is thick and lacks the outer membrane layer. Murray *et al* (2005) reported that half of the cell wall by weight is peptidoglycan, which consists of layers

of glycan chains and subunits of *N*-acetylmuramic acid and *N*-acetylglucosamine. Amako *et al* (1982) extracted the cell wall with sodium dodecyl sulphate and trichloroacetic acid. The amount of peptidoglycan (approximately 200 μg mg^{-1}) in the cell wall fraction was estimated from the amount of N-acetylglucosamine measured by absorbance at 585 nm (Reissig *et al.,* 1955).

From electron microscopic analysis it was established that oligopeptide side chains are attached to the *N*-acetylmuramic acid subunits and cross-linked with peptide bridges (Fischetti, 2006). The glycan chains are cross-linked with pentaglycine bridges that are attached to L-lysine in one oligopeptide and to D-alanine in an adjacent chain (Murray *et al.,* 2005). Teichoic acids are phosphate containing polymers and a major component of the cell wall. They are covalently bound to *N*-acetylmuramic acid residues in the peptidoglycan layer through lipophilic linkage to the cytoplasmic membrane (lipoteichoic acids). Seltmann & Holst (2002) elucidated the principal function of teichoic acids which seems most likely the binding of metal cations and the regulation of the activity of autolytic enzymes necessary for growth and division of the cell.

Bacterial cell surfaces differ from those of higher organisms by their anionic net charge. Weidenmaier *et al* (2003) investigated the susceptibility of *S. aureus* to antimicrobial agents by mutagenic incorporation of D-alanine into teichoic acid. The incorporation of positively charged amino groups into teichoic acids led to partial neutralisation of the cell surface and so limited the interaction of cationic antimicrobial molecules with the bacterial cell wall leading to decreased susceptibility (Peschel *et al.,* 1999; Weidenmaier, 2003).

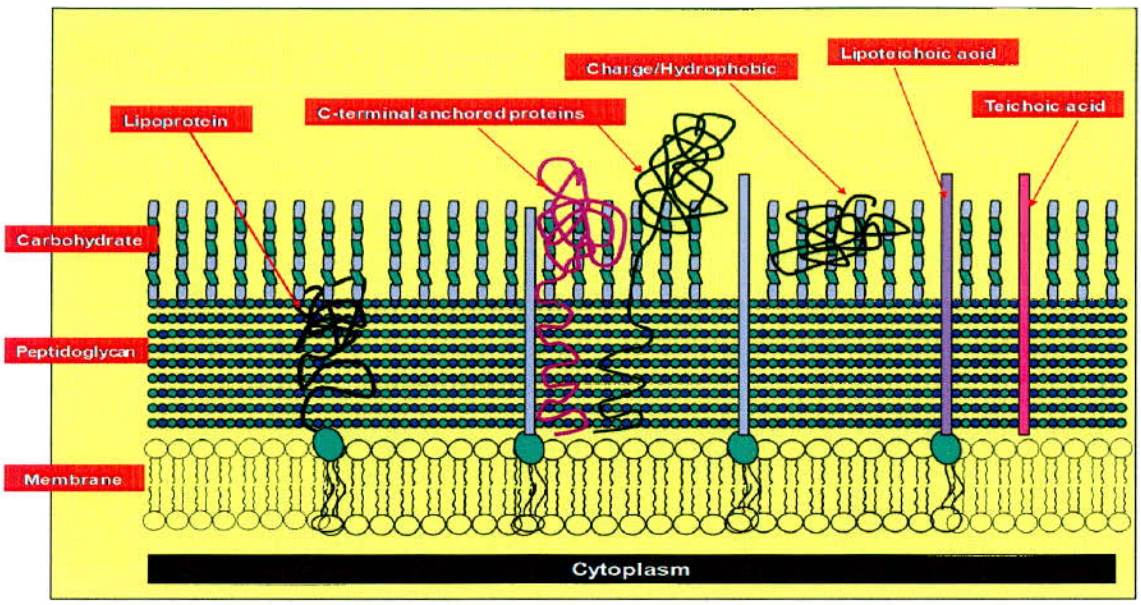

Figure 1.6: Cell wall structure of Gram-positive bacteria

Surface proteins linked to the surface of the peptidoglycan layer by three mechanisms: i) lipoproteins; ii) C-terminal anchored and iii) hydrophobic and charge interactions (Adapted from Fischetti *et al.*, 2006)

Bacterial cell wall polymers such as teichoic acids (Gram-positive bacteria), the lipid part of the lipopolysaccharide (LPS Gram-negative bacteria), peptidoglycan and most of the phospholipids are negatively charged (Weidenmaier *et al.*, 2003). Conversely, virtually all cationic antimicrobial molecules are positively charged (Weidenmaier *et al.*, 2003; Fedtke *et al.*, 2004). Modulation of the anionic cell envelope by introducing positively charged groups seems to be a strategy bacteria evolved to resist cationic host defence molecules. Using TLC to analyse cell surfaces, Peschel *et al* (2001) reported that a virulence gene in *S. aureus* reduced its negative net charge via the introduction of L-lysine to the bacterial phospholipids. Mutants lacking the gene showed interactions with cationic peptides up to three fold higher than in the wild type as determined by fluorescent spectrophotometry (Peschel *et al.*, 2001).

Current knowledge of the cell wall of *S. aureus* suggests the existence of a range of cell surface proteins with specific binding functions and enzymatic activity combined with the ability to generate energy (Fischetti *et al.*, 2006). Fischetti *et al* (2006) & Seltmann & Holst (2002) reported that more than 25 different proteins may be present on the Gram-positive cell surface, each protein ascribed with a potential of up to three functional domains. Sjöquist *et al* (1972) showed that protein A becomes solubilised with lysostaphin and reported the protein content with the Lowry assay of up to 1.7 μg mg^{-1} of protein A. Seltmann & Holst (2002) stated that the surface of most *S. aureus* strains is coated with protein A and is bound either to the peptidoglycan layer or the cytoplasmic membrane and has a unique affinity for binding to a receptor of immunoglobulin. This effectively prevents antibody-mediated clearance of the organism by deploying a hiding initiator from the innate immune system in the early stages of infection when bacterial cell numbers are low. Seltmann & Holst (2002) also suggested that protein A (molecular mass about 42kDa) could also be used for the detection and identification of *S. aureus.*

Fibronogen is a 340-kDa glycoprotein that is present at a concentration of 9 mM in the blood. FnbA and FnbB mediate the binding of *S. aureus* to fibronectin. Fibronectin-Fibrinogen binding proteins are abundant in the extracellular matrix and are important for wound healing. The outer surface of most *S. aureus* strains encodes two distinct fibrinogen binding proteins, known as clumping factors (ClfA and ClfB). This protein, exploited for diagnostic purposes, is an important virulence factor that binds fibrinogen and converts it to insoluble fibrin causing the organism to clump or aggregate in the presence of human plasma (Coagulase test).

Glycoproteins are located on the extracellular surface (Figure 1.7). They act as receptors and bind to certain molecules serving as vehicles of transport (Madigan *et al.,* 2003). Moreover, they bring other cells and proteins (collagen) together giving strength and support to a matrix. The binding of these carbohydrate groups in membranes can be indicated by lectin labelling techniques followed by fluorescence microscopy or with SDS-PAGE. Lectins are plant proteins with high affinity for sugar residues and can reveal subtle alterations in glycosylation between otherwise indistinguishable cells (Leathem & Brooks, 1998).

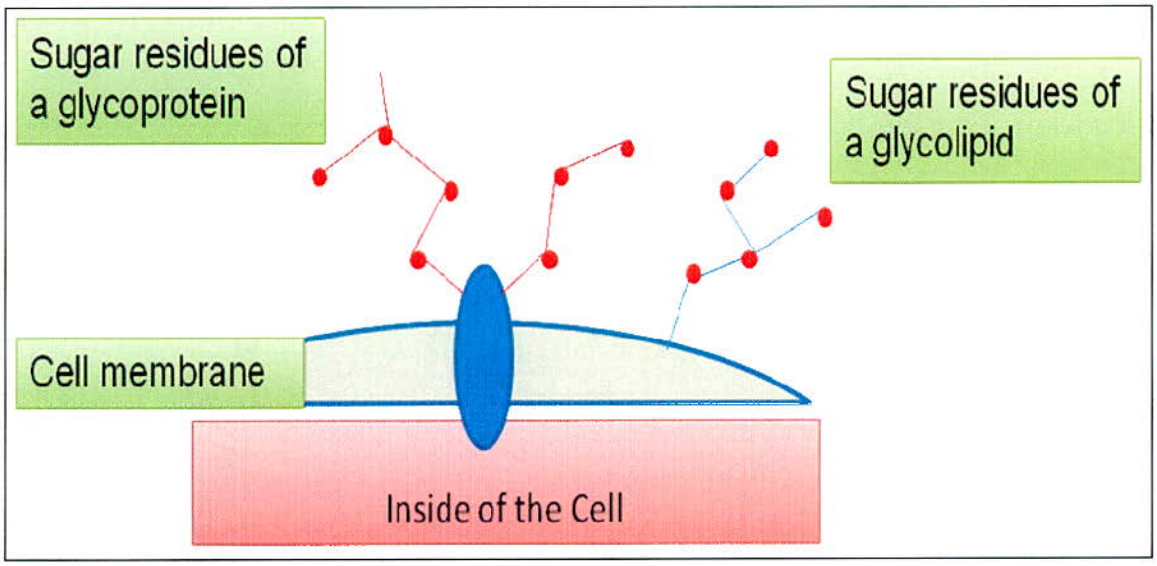

Figure 1.7: Glycoprotein and glycolipid location of mammalian membranes
(Adapted from Stryer, 1988)

Conjugated fluorescein, peroxidase, or colloidal gold-lectins may be used as probes to identify and localize specific carbohydrate residues in microorganisms by light or electron microscopy (Slifkin & Doyle, 1990). The lectin binding assay is also frequently used to characterise polysaccharides in bacterial biofilms (Strathmann *et al.,* 2002). Different lectins are readily available commercially and the specific bond to particular microorganisms restricts the use of each lectin. For instance, Wheat

Germ Agglutinin (WGA) lectin binds specifically to asparagine linked oligosaccharides through sialic acid, *N*-acetylglucose and *N*-acetylgalactosaminyl residues (Gerrard, 1990; Van Damme *et al.,* 1998). Fluorescein-isothiocyanate (FITC) is a compound that can be linked covalently with lectins, which selectively binds glycoprotein residues (Figure 1.7). Fluorescence occurs when a molecule struck by light emitting a longer wavelength than its originally exposed to by the fluorescence microscope. This is achieved by filtering light with long wavelengths and passing short wavelength to a dark field condenser to produce a dark background and then into the specimen. If fluorochrome in the specimen is present the short light is absorbed and long wavelength fluorescence light is emitted through a filter which removes short light wavelengths (Chapin-Robertson & Edberg, 1991). FITC is able to bind only to amino groups of lysyl residues. The use of FITC conjugation to amino acids of proteins treated with oleuropein might indicate the loss of attachment sites and therefore denaturation of proteins.

1.5.2 Methicillin-resistant Staphylococcus aureus

Bacteria resistant to multiple classes of antibiotics have emerged in the past several decades making many infections difficult to treat. Russell (2004) has reviewed bacterial adaptation and concluded that the development of general resistance in bacteria is not a new phenomenon. Microorganisms are able to overcome the activity of antimicrobial agents especially when their site of action is specific. However, development of resistance to broad-spectrum antimicrobials including many potent biocides, such as phenolic compounds and disinfectants is less likely than seen with narrow-spectrum antibiotics (e.g. penicillin).

Kluytmans *et al* (1997) reported that the difference between MRSA and methicillin-susceptible *S. aureus* (MSSA) is the resistance to ß-lactamase stable ß-lactam antibiotics. MRSA expresses methicillin-resistance by producing a specific penicillin-binding protein that has a decreased binding affinity to ß-lactam antibiotics and is encoded by the *mecA* Gene (Kitahara *et al.,* 2004). Colonisation with MRSA is not necessarily dangerous in healthy individuals but in immuno-compromised patients or the elderly and terminally ill it may lead to severe infections and complications. In addition, MRSA leads to patients staying longer in hospitals and consequently increased treatment costs. In the past, most serious *S. aureus* infections were treated with antibiotics closely related to penicillin but these are no longer used due to resistant cases. Currently, MRSA is treated with vancomycin and teicoplanin, which must be administered by intravenous infusion or injection, and for this reason they are used for treatment in hospitalised patients only (Ang *et al.,* 2004). Vancomycin disrupts peptidoglycan synthesis by perturbing the formation of bridges between the peptidoglycan chains. It is widely used to treat infections caused by Gram-positive organisms and penicillin-allergic patients, but its use is associated with a number of clinically adverse side-effects such as nephrotoxicity, ototoxicity and allergic reactions such as rash and drug fever (Liu *et al.,* 1996).

Phenolic compounds such as oleuropein and hydroxytyrosol have not been tested against MRSA. Tsuchiya *et al* (1996) tested the antimicrobial activity of flavanones extracted from the plant family *Leguminosae* against MRSA and stated that hydroxyl groups at the 2' position were important for anti-MRSA activity because similar structural compounds without the hydroxyl group were resulting in MIC values over 200 µg mL^{-1} compared to less than 12.5 µg mL^{-1} (Figure 1.9). The

presence of a hydroxyl group at position 5' of flavanones and flavones was also suggested as important against MRSA, whereas the -OCH_3 group had the reverse effect (Alcaraz *et al.*, 2000).

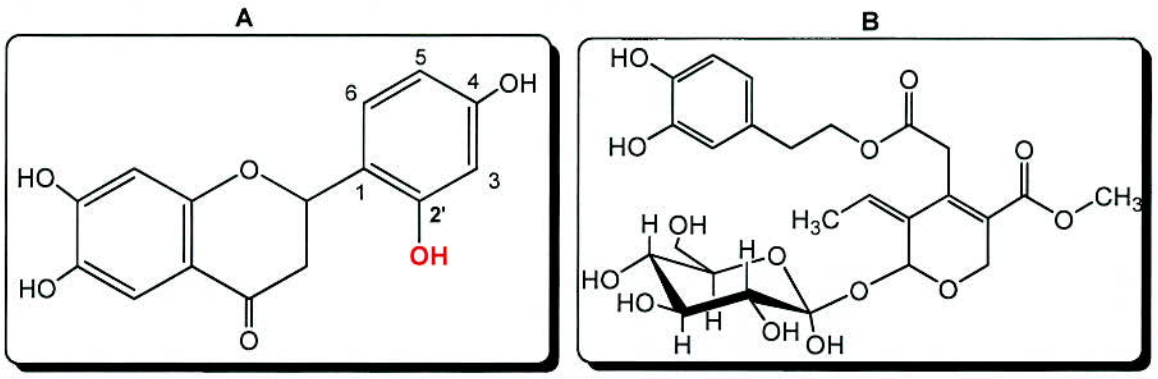

Figure 1.8: Chemical structures of flavanone (A) supposed to show anti MRSA activity because of the hydroxyl group in position 2' compared to oleuropein (B)

1.5.3 Small Colony Variants

S. aureus that are deficient in oxidative metabolism have been associated with a spectrum of persistent, recurrent and antibiotic resistant infections. This deficiency in growth can lead to mutations in genes of bacteria. The appearance of mutations in genes of *S. aureus* led to bacteria known as small colony variants (SCVs). These bacteria have characteristic and distinct features such as slow growth rate, the absence of pigmentation, a reduced range of carbohydrate utilisation and a general lack of virulence factor production. Defects in electron transport lead to a decrease in capacity to produce ATP that is used for cell-wall biosynthesis leading to a slower growth rate and, therefore smaller colonies, which are approximately one tenth of the size of wild type strains. Reduced membrane potential results in decreased uptake of cationic compounds and decreased pigment formation (McNamara & Proctor, 2000; Proctor *et al.*, 2006).

SCVs have been described for a wide range of bacterial genera and species including *S. aureus*, methicillin resistant *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *S.* serovars, *E. coli, V. cholerae*, and *Shigella* spp. (Proctor *et al.,* 2006). Unlike wild type strains, SCVs survive within cultured endothelial cells shielding the bacteria from host defences and from antibiotics that cannot cross the host cell's plasma membrane. SCVs often trigger long lasting and recurrent infections and their colony morphology frequently leads to insufficient or false identification in laboratory diagnosis (Roggenkamp *et al.,* 2004). Electron microscopy of SCV has shown that these cells had incomplete, branched and multiple cross walls, without regular cell separation (Proctor *et al.,* 2006). It has been speculated that the emergence of SCVs may significantly contribute to the chronic persistent course of an infection (Roggenkamp *et al.,* 2004). The occurrence of SCV is not well understood and needs more research attention. SCVs could be part of the normal life cycle of *S. aureus* and might be one of the survival strategies that *S. aureus* uses for internalisation and survival in the host. Natural compounds, such as oleuropein might be able to eliminate SCVs or provide evidence of the development of SCV from *S. aureus* wild type strains.

1.5.4 Quorum sensing

The view of bacteria as single-celled organisms acting individually changed with the discovery of the quorum sensing mechanism. Microorganisms interact with one another by the production of secondary metabolites. In recent years, particular groups of secondary metabolites have been characterised for their role in the regulation of gene expression in a cell-density-dependent manner, and this behaviour has been collectively referred to as quorum sensing or cell–cell

communication (Keller & Surette, 2006). It has been established that bacterial cells can communicate with each other through diffusible signal molecules. These signal molecules, also referred to as autoinducers when exceeding a threshold concentration, bind to receptors and change gene expression of the bacterial cell (Keller & Surette, 2006).

A number of pathogenic bacteria co-ordinate the expression of virulence factors through such mechanisms. Most of the well-described systems for Gram-negative bacteria act through the accumulation of diffusible molecules such as the *N*-acyl homoserine lactones whereas Gram-positive bacteria employ small post translationally processed peptide molecules (Ermolaeva *et al.,* 1999). In *S. aureus,* the *agr* quorum sensing system, believed to play a central role in pathogenesis, decreases the expression of several cell surface proteins and increases the expression of many secreted virulence factors in the transition from late-exponential to stationary phase growth *in vitro* (Yarwood & Schlievert, 2003). Keller & Surette (2006) suggested that cell to cell signalling in *S. aureus* was mediated by oligopeptides and that the signalling was highly specific and had a role in intraspecific competition, as well as in coordinated, cooperative behaviour.

The influence of natural products on quorum sensing has not been investigated. Oleuropein might prevent the accumulation of autoinducers by denaturing the oligopeptide chain and therefore avoiding the host virulence expression in *S. aureus*. However this is speculative and needs to be addressed in further studies.

1.5.5 Biofilms

Many staphylococcal infections are not caused by the free-living organism but rather by groups of interacting cells termed biofilms. Bacterial biofilms are defined as a community of cells attached to either an abiotic or a biotic surface, are encased in a self-produced matrix, and generally exhibit an altered growth and gene expression profile compared with that of planktonic, or free-living bacteria (Yarwood & Schlievert, 2003). Yarwood *et al* (2004) described two main stages of staphylococcal biofilm formation The first stage involves attachment of cells to a surface which is likely to be mediated by cell-wall associated adhesion. The second stage involves cell multiplication and formation of a mature structure consisting of many cell layers associated with the production of extracellular factors including polysaccharides of the extracellular matrix.

Intracellular signalling, encoded by the agr-locus in *Staphylococcus*, is assumed to be involved in biofilm development. Detachment of cells expressing *agr* from the biofilm structure may contribute to the spread of additional infection sites in the host. If the *agr*-locus is not expressed, cells remain in the biofilm and may contribute to persistent low-level infections as is seen with biofilm forming *S. epidermidis* (Yarwood & Schlievert, 2003). However, loss of agr function may lead to the enhanced survival of staphylococci in the host and may contribute to persistent chronic infections (often biofilm associated). Biofilm associated infections are generally resistant to antibiotic therapy and staphylococcal infections include endocarditis, osteomyelitis, and implanted device related infections (Yarwood & Schlievert, 2003). In Figure 1.9, the biofilm process is shown from the initial stage to the final development.

1. Colonization by individual cells;
2. Micro colonies reach sufficient cell density for agr-dependent gene expression;
3. Possible signaling between micro colonies;
4. Portions of the biofilm detach through as yet unknown mechanisms and parts of the biofilm population become metabolically inactive and lose membrane integrity;
5. Following new growth into the voids left by the detached cells and the cycle is repeated;
6. The micro colony finally reaches a relatively quiescent state where any growth is slow and agr expression is undetectable

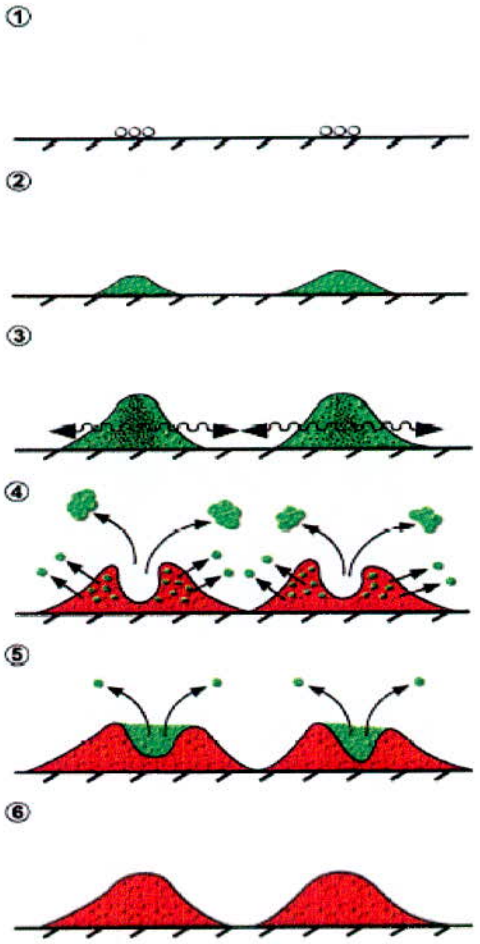

Figure 1.9: Model of agr expression in *S. aureus* biofilms

Adapted from Yarwood *et al.*, 2004

The formation of biofilms on surfaces can be regarded as a universal bacterial strategy for survival (Heipieper *et al.*, 1991; Otto, 2004). There is much interest in the development of agents, which can kill and/or remove biofilm bacteria. Phenolic compounds, such as oleuropein may depress biofilm formation. In this study, biofilms were not investigated but some bacteria used in this study are known biofilm formers (*S. epidermidis and P. aeruginosa*).

1.6 Aims and objectives of the study

The aim of this study was to develop an improved method for the extraction and purification of oleuropein from olive leaves and characterise its activity against bacteria by exploring the mode of action.

The first objective was to extract and purify oleuropein from crude material. A high performance liquid chromatography method was designed to determine the quantity of oleuropein in olive fruits and leaves. Further work focussed on the extraction method and the purity of the crude extract and a two phase system necessary for a countercurrent chromatography (CCC) process was developed. Flash chromatography was introduced as an additional purification step.

The antimicrobial properties of oleuropein were investigated. Initially broth methods and spectrophotometric assays were used, followed by the disc diffusion test, agar plate assay and the microtitre assay against a range of bacteria. Further work concentrated on methicillin resistant and methicillin sensitive *Staphylococcus aureus*. Time kill studies were designed and different concentrations of oleuropein and inoculum were tested.

The mode of action of oleuropein on bacteria including clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) was investigated. Transmission electron microscopy (TEM) was used to observe cell wall damage. Leakage of cell constituents was explored using the Bradford assay and the ninhydrin test on cell-free supernatant. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to determine protein denaturation in oleuropein treated cells. A method to test glycoproteins via a lectin reporter was also investigated.

Chapter Two

2 Material and Methods

All materials for the purification of oleuropein were from Fisher Scientific, Loughborough, UK unless otherwise indicated. In the microbiological work, all chemicals were purchased from Sigma-Aldrich and Fisher Scientific. Mueller Hinton Agar and Broth (MHA and MHB), Trytone Soya Agar and Broth (TSA and TSB) were purchased from Oxoid (Basingstoke, UK). Phosphate Buffered Saline (PBS, 0.1 M, pH 7.4) was used as diluent throughout the study unless otherwise stated.

Oleuropein and hydroxytyrosol (purity not declared) were purchased from Extrasynthese, Lyon, France. Caffeic acid and p-coumaric acid were supplied by Sigma-Aldrich and were labelled as at least 95% pure. All phenolic compounds were powders except hydroxytyrosol, which was a viscous liquid. Olive extracts were prepared from the cultivar *Olea europaea* L. (Trilye type). Olive fruits and leaves were collected from the Trilye region (Mudanya-Turkey) in October 2005 and kept at -12°C for approximately three months prior to use.

The purity of oleuropein in the sample was determined by mass balance. By using the oleuropein standard calibration curve the HPLC peak area was used to quantify the amount of oleuropein in the sample. The ratio of amount of oleuropein in the sample and the total amount of oleuropein in the crude extract expressed as a percentage was equivalent to the purity of oleuropein.

2.1 Extraction and purification of oleuropein

A review of the literature suggested that absolute methanol as well as methanol mixed with water in different proportions may be used to extract oleuropein from olive leaves and fruits. Approximately 1 g of olives was extracted with 25 mL methanol-water (50:50 v/v). Similarly, 1 g of olive leaves was extracted with 25 mL

absolute methanol. A mortar and pestle were used to grind the materials for approximately 5-10 minutes. Larger amounts of extract were prepared by grinding approximately 20 g of olive leaves with 1 litre methanol. The extract was concentrated by rotary evaporation to 100 mL. In another experiment, a different batch of leaves was stored in methanol for up to fourteen days at 4°C prior to sonication at 60°C (Sonomatic, Jencons, England). The sonication of crude material was introduced to compare the extraction to that afforded by the manual method with mortar and pestle.

The composition of all extracts was determined using a Waters 2695 High Performance Liquid Chromatograph equipped with an autosampler, a temperature monitoring system set to 40°C, and a Waters 2996 photodiode array detector. The column was a 250 mm x 4.6 mm ID, 5 μm bead diameter, YMC-Pack Pro C18 column (12 nm pore size). All solvents were of HPLC grade. The mobile phase contained (A) Water with 0.05% Trifluoroacetic acid (TFA) and (B) Acetonitrile with 0.05% TFA, which were degassed by vacuum prior to use. The solvent gradient started with 95% A and 5% B, reaching 50% A at 25 min, 5% A at 25.5 min, and 95% A at 27 min, followed by an isocratic plateau for 3 min and return to initial conditions. The total run time excluding equilibration was 30 min. Data acquisition and remote control of the HPLC system was monitored with Millenium[32] Empower 2 software.

Oleuropein calibration was done in methanol with a total run time of 10 min. For quantitative determination of oleuropein in extracts from crude materials, samples were run for 30 min. Oleuropein standards were prepared in duplicate using 10 mg oleuropein from Extrasynthese dissolved in 100 mL deionised water at 20°C.

Aliquots of 2, 5, 10, 15, 20, 25, and 30 µL were used to prepare standard curves of peak area versus concentration. Caffeic acid, hydroxytyrosol, and p-coumaric acid calibrations were also undertaken with a total run time of 33 min. All olive extracts were dried down by a concentrator (Eppendorf 5301), dissolved in methanol and filtered (Gelman, Acrodisc 13, 0.45 µm) prior to injection of 10 µL into the HPLC.

2.2 Solvent selection for countercurrent chromatography

In a successful CCC run, it is important that the compound of interest is equally dispersed in a two-phase system. The solvents in the two-phase system for olive leaf extracts were selected using a Liquid Handling Robot shown in Figure 2.1 (PerkinElmer Multiprobe II Robot). The liquid handler was programmed to produce all solvent mixtures shown in Table 1.5 (heptane, ethyl acetate, methanol, butanol and water) in a total volume of 4 mL. The crude olive extract was added at a final concentration of 72.5 mg mL^{-1} in methanol. A total of 56 extracts consisting of 28 samples for lower and 28 for upper layer were automatically prepared. Aliquots of 1 mL of each layer were removed, dried in a centrifugal concentrator (Eppendorf 5301) (at 30, 45 and 60°C) and redissolved in 1 mL methanol before analysis on HPLC. This experiment was done ones only.

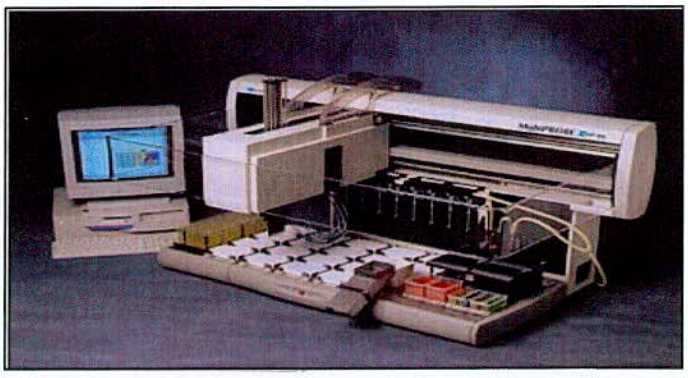

Figure 2.1: Liquid handling robot used for selecting solvent system ratios based on heptane, ethyl acetate, methanol, butanol and water

In order to test the stability of oleuropein in methanol and the selected solvent mixture no 7 from Table 1.5 (consisting of ethyl acetate, heptane, methanol and water) concentrations of 5 and 10 mg mL^{-1} oleuropein were prepared, stored at room temperature for seven and eleven days, respectively, and tested using HPLC. Oleuropein stability experiments were carried out ones only.

2.3 Countercurrent chromatography

The separation of oleuropein from its crude mixture was accomplished by two countercurrent chromatograph machines as illustrated in Figure 2.2. The separation was tested initially on the Mini CCC before scale up to the Midi machine. The Midi CCC was a "J" type coil planet centrifuge and the control system used was with a rotor radius of 110 mm. Two stainless steel coils were used (total volume of 840 mL) total length of tubing 944 cm, bore 3.6 mm. Normal phase operation was applied: the machine was filled with stationary aqueous phase (lower) and the mobile phase was the organic (upper) phase. Run conditions were 1100 rpm with flow rates of 25 and 50 mL min^{-1} for the Midi and 1 mL min^{-1} for the Mini apparatus with the UV detector adjusted to 230 nm. After optimisation the Midi process was done ones whereas the Midi process was done twice.

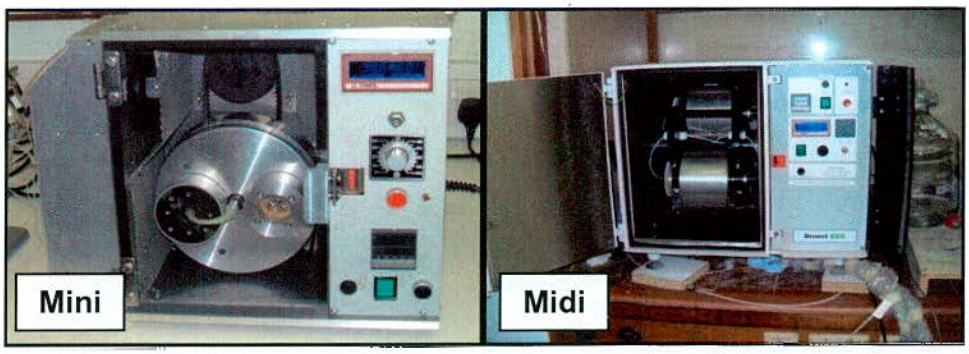

Figure 2.2: Countercurrent chromatography apparatus with maximum operating volumes of 100 µL (Mini) and 50 mL (Midi). Machines manufactured by Brunel Institute for Bioengineering, Brunel University.

2.4 Flash chromatography

Flash or column chromatography enables the elimination of compounds that can interfere in CCC separation. Hence, a higher purity of the compound can be achieved. For flash chromatography, a gradient column was prepared in a 250 mL burette filled with silica particles (Silica 60A, Particle size 35-70 µm, Davisil) suspended in hexane. Hexane was used as column solvent (300 mL). At the end of the procedure fractions were collected and analysed using HPLC as described previously.

Hexane (100%) was run through the column followed by the mixtures of hexane (75%) and ethyl acetate (25%) before changing the solvent proportions of hexane:ethyl acetate to 50:50, 25:75 and 0:100 before switching to ethyl acetate:methanol 50:50 and 0:100. Experiment was done ones only.

A crude olive leaf extract of around 10 mL was run through the burette (190 mL Silica dissolved in hexane). In a second experiment the gradient was with hexane and ethyl acetate (0, 25, 50, 75, 100% ethyl acetate) as mobile phase followed by ethyl acetate with methanol (10, 20, 50, 100% methanol) instead of using hexane to elute polar components. Experiment was done ones only.

Figure 2.3: Flash chromatography column filled with silica particles 35-70 µm dispersed in hexane

2.5 Microorganisms and their cultivation

All microbiological media were dissolved in distilled water and autoclaved at 121ºC for 20 minutes. Microorganisms (Table 2.1) were obtained from National Culture Collections and from the microbiology laboratory of St. Mary's Hospital London. The clinical strains originated from blood specimens of infected patients. Stock cultures of microorganisms were prepared by growing microorganisms in Tryptone Soya Agar (TSA) overnight. The colonies were transferred using a sterile loop into a suspension of porous ceramic beads in a cryopreservative vial before frozen storage at -80°C. Stock cultures were renewed every 6 months. The routine procedure for bacterial growth comprised overnight growth in Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) at 37°C. In MHB, the culture was grown in 20 mL universals in a shaking incubator at 200 rev/min velocity (Stuart Orbital Shaking Incubator S 150).

2.6 Determination of antimicrobial activity

Antimicrobial activity was determined using four methods: disc diffusion, agar supplementation, Minimum Inhibitory Concentration (MIC) and time-kill assays in buffer. All microorganisms were grown overnight at 37°C in MHA except *Streptococcus* sp, which were grown in Sheep Blood Agar (Oxoid).

2.6.1 Disc diffusion method

The method from the British Society for Antimicrobial Chemotherapy (BSAC) was used (Andrews, 2001). A loopful of bacteria (5-10 colonies) from an overnight culture on MHA was inoculated into 10 mL MHB and incubated at 37°C in a shaking incubator until the turbidity was equal to or greater than 0.5 McFarland standard (Oxoid, UK). An aliquot of 100 µL was transferred into 900 µL distilled water. Depending on the bacteria, further dilutions were prepared (1:10 for *Enterococci*,

Streptococci, *Enterobacteriaceae*, *Pseudomonas* spp., 1:100 for *Staphylococci* and *Streptococcus pneumoniae*). A sterile cotton swab was used to spread the bacteria onto MHA. Sterile paper discs of 6 mm diameter (Fisher Scientific, UK) were placed onto the agar surface. Aliquots of 10 µL oleuropein (1, 2, 5, 10%) in deionised water were pipetted onto the paper discs. Plates were incubated at 37°C for 24 h in duplicate and examined for zones of inhibition. The diameter of the zones of inhibition was measured around each disc using a ruler. The disc diffusion test was done in duplicate and the means were determined.

2.6.2 Oleuropein supplemented agar

Sensitivity to oleuropein was determined by streaking bacteria onto MHA supplemented with 0.25, 0.5, 1.0, 1.5, and 3.0% oleuropein prior to autoclaving. Plates were incubated at 37°C for 24 h and assessed visually by categorising susceptibility into no growth and confluent growth. Experiment was done in duplicate.

2.6.3 Determination of minimum inhibitory concentration (MIC)

Oleuropein solutions (0.2, 0.5, 1, 2%) were prepared in 5 mL MHB and inoculated with 10 µL overnight bacterial suspension in MHB (macrobroth assay). After incubation at 37°C in a shaking incubator for 24 h, absorbance was read at 595 nm (WPA UV1101 Biotech Photometer). In addition, experiments were conducted in 96 well microtitre plates (microbroth assay) and the absorbance was measured at 540 nm in 150 µL volumes in each well with a microtitre plate reader (MWGt -Maxline Microplate Reader, Molecular Devices). MICs were defined as the minimum concentration which displayed no growth by absorbance at 37°C after 24 h. The macrobroth and microbroth assay were done ones only.

Table 2.1: Microorganisms used in this study

Name of microorganism	Source
Gram-positive bacteria	
Bacillus subtilis	NCIMB 2591[1]
Enterococcus faecalis	ATCC 29212[2]
Micrococcus luteus	NCIMB 196[1]
Staphylococcus aureus (MRSA)	ME-30IVUK[6]
Staphylococcus aureus (MRSA)	ME-80[6]
Staphylococcus aureus (MSSA)	ME-5S[6]
Staphylococcus aureus (MSSA)	ATCC 29213[2]
Staphylococcus aureus (MSSA)	NCIMB 6571[1]
Staphylococcus epidermidis	ATCC 35984[2]
Streptococcus pneumoniae	SPN-009[3,6]
Streptococcus pyogenes	GAS010[4,6]
Streptococcus pyogenes	GAS104[4,6]
Gram-negative bacteria	
Escherichia coli	ATCC 25922[2]
Escherichia coli	NCIMB 8020[1]
Pseudomonas aeruginosa	NCIMB 950[1]
Yeast	
Candida albicans	NCYC 597[5]

[1]NCIMB – National Collection of Industrial and Marine Bacteria; [2]ATCC - American Type Culture Collection; [3]SPN- *Streptococcus pneumoniae*; [3]GAS – Group A *Streptococci*; [5]NCYC – National Collection of Yeast Cultures; [6]St. Mary's Hospital London.

2.6.4 Bacterial time-kill studies

Bacterial killing assays were conducted to examine the biocidal action of oleuropein. *MSSA*-ATCC 29213 and MRSA-ME-30, ME-80 and ME-5S were grown overnight at 37°C on MHA. Five to ten colonies were transferred with a loop into 10 mL MHB. After 24 h at 37°C in a shaking incubator, absorbance was read at 595 nm. The culture was diluted to 10^6 cfu mL^{-1} indicated by an optical density of 1.8-2.0 at 595 nm. Cells were collected by centrifugation (4000 g for 10 min) followed by two wash cycles in sterile Phosphate Buffered Saline (PBS). The time-kill studies were undertaken with 1:1000 dilutions of the centrifuged bacterial suspension by transferring 500 μL into 4.5 mL PBS. Oleuropein was added from a 20% stock solution in deionised water to obtain final concentrations of 0.5, 1 and 2% in a total volume of 5 mL. Viable counts were determined by serial ten-fold dilution in PBS followed by inoculation of 0.1 mL onto MHA containing four to six sterilised glass beads (4 mm in diameter, Merck, Germany). All plates were prepared in duplicate before spreading the bacterial suspension by gently shaking the glass beads on the agar surface. The glass beads were decanted into a disinfectant container and the plates were incubated overnight at 37°C. Colonies were counted using a colony counter. Experiment was conducted in triplicate for MSSA 29213, MRSA-ME30 and in duplicate for MSSA-ME5S and MRSA-ME80. Standard errors were based on standard deviation values.

2.7 Mode of antimicrobial action studies of oleuropein

The effect of oleuropein on bacterial cell walls and membranes was investigated using transmission electron microscopy, measurements of nucleic acid and protein leakage, fluorescence microscopy and sodium dodecyl sulphate polyacrylamide gel electrophoresis.

2.7.1 Transmission Electron Microscopy

The transmission electron microscope used was a JEOL 200FX with an acceleration of 80kV. Colonies of five to ten MSSA-ATCC 29213 and MRSA-ME30 cells were picked from MHA plates and grown overnight at 37°C in 10 mL MHB in a 20 mL universal under shaking conditions. Aliquots of 1 mL were transferred into 1.5 mL eppendorf microtubes, centrifuged at 14000 g for 10 min and washed twice in Phosphate Buffer (PB – 0.1M pH 7). Absorbance was recorded at 595 nm before cells were treated with 2% oleuropein for two, four and six hours. Cells were fixed for two hours in 2.5% glutaraldehyde in PB and further processed as described by Johnston *et al* (2003) using PB instead of cacodylate buffer. Cells were embedded in resin and polymerised at 65°C for 12 h. Ultrathin sections were cut using a diamond knife (Ultracut, Reicher-Jung, Austria) and collected on mesh copper grids before staining with lead nitrate mixed with sodium citrate in deionised water (Ellis, 2007). The experiment was done in triplicate.

2.7.2 Leakage of cell constituents

Leakage of low molecular weight compounds, such as nucleotide components (purines, pyrimidines, pentoses and inorganic phosphate) as well as amino acids from cells can be measured at 260 nm (Johnston *et al.,* 2003). Freshly prepared

bacterial suspensions of 5 mL in MHB were centrifuged and washed in duplicate in PBS prior to treatment with 1% oleuropein. At defined time points (0, 1, 2, 4, 6 and 24 h), suspensions were filtered through a 0.45 μm filter (Millipore) and a Sephadex gel column in a pasteur pipette filled with approximately 3 mL Sephadex G-25 (bead size of 50-150 μm) in PBS. Aliquots of 100 μL were diluted in 2.9 mL PBS and absorbance was measured in UV glass cuvettes in a spectrophotometer (WPA UV1101 Biotech Photometer) at 260 nm. In addition, a 0.2% charcoal solution was used to attempt to eliminate absorbance interferences by phenolic compounds. Experiment was done ones only.

2.7.3 Leakage of proteins

Leakage of proteins from cells treated with oleuropein was determined using three separate methods as described below.

In the Bradford assay, Coomassie dye binds to proteins causing a shift in the absorbance maxima from 465 to 595 nm (Bradford, 1976). The Bradford assay was used to determine the amount of protein leaking from oleuropein treated bacterial cell cultures. The 2 mL micro assay kit from Sigma-Aldrich was used. Bovine Serum Albumin standards (BSA) of 2, 5, 7.5, 10, 20, 40 and 80 μg mL^{-1} were prepared from a stock solution (0.1 g mL^{-1}) in PBS and a standard curve was constructed (Appendix: 7). Overnight cultures of MRSA-ME30 and MSSA 29213 were grown in 10 mL MHB and washed twice in PBS. Cells were then treated with 1% oleuropein for 0, 1, 2, 4, 6 and 24 h, centrifuged (4000 g) and filtered through a 0.45 μm microfilter (Millipore) prior to adding 1 mL Bradford reagent into cell free supernatant. This was incubated at room temperature for at least 5 minutes before absorbance was measured at 595 nm using a spectrophotometer (WPA UV1101

Biotech Photometer). Since the colour of oleuropein interfered with the absorbance reading in the Bradford assay, an additional step was introduced to remove the interfering substances. Cell free supernatants were treated with Sephadex as described in section 2.7.2 prior to mixing with the Bradford reagent. After optimisation the experiment was done ones only.

The ninhydrin test detects amino groups in proteins. In this test ninhydrin binds with α-amino acids, free amino and carboxylic acid groups on proteins and peptides. The ninhydrin reaction is explained as follows: First an oxidative deamination reaction occurs that removes two hydrogens from the alpha-amino acid to yield an alpha-imino acid. Ninhydrin is then reduced and loses an oxygen atom with the formation of a water molecule. The NH group in the alpha-imino acid is rapidly hydrolyzed to form an alpha-keto acid with the production of an ammonia molecule. This alpha-keto acid further undergoes decarboxylation when heated to form an aldehyde that has one less carbon atom than the original amino acid releasing a carbon dioxide molecule. Ninhydrin with amino groups attached forms an intensely blue-purple product that can be measured at 570 nm. This was considered as an alternative technique to the Bradford assay. Standard curves with 2% ninhydrin reagent were prepared with stock solutions of 400 mM aspartic acid dissolved in 0.05% glacial acetic acid (Appendix: 8, 9). Bacterial cultures were prepared in the same way as described in the bacterial killing assay. Oleuropein at 1% was added to cells in a total volume of 5 mL following incubation for four hours with shaking (200 rev min^{-1}) at 37°C. Suspensions were then centrifuged and cell free supernatant was placed in a boiling water bath for 10 min followed by cooling at room temperature. Ethanol (5 mL 95%) was added to the mixture, vortexed and absorbance was read in

disposable cuvettes at 570 nm in a Lambda 25, UV-Vis Spectrometer (Perkin Elmer). The ninhydrin test was done in duplicate. Standard errors were based on standard deviation values.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Hames, 1990; Shi & Jackowski, 1998) was used to determine the size of proteins leaking out of cells following oleuropein treatment. The bacterial cells were prepared as for the bacterial time kill studies and were exposed to 1% oleuropein for 4 h and diluted 1:10 in PBS. Cells were centrifuged and the pellet was dissolved in 500 μL 0.9% NaCl solution. A 30 μL solution of 1 mg mL^{-1} of lysostaphin in deionised water (from *Staphylococcus staphylolyticus*) was added and cells were incubated at 37°C. The sample buffer was prepared (using 2% SDS, 20% glycerol, 0.1 M Tris-HCl pH 6.8 and 1% mercaptoethanol, Bromophenol Blue) and 100 μL was added into the cell suspension before placing in boiling water for 3-5 min. After cooling and vortexing, 20 μL was pipetted into the wells and SDS-PAGE was run at 180 V for approximately 1 hour. The gel was then stained in Brilliant Blue-G250 (40% methanol, 10% acetic acid, 0.25% Brilliant Blue in water) for at least 45 min and destained for 30-60 minutes with a solution containing 10% methanol and 5% acetic acid in water. Pictures were taken with a digital camera (Pentax Optio M20). Experiment was conducted in triplicate.

2.7.3.1 Determination of glycoproteins

Glycoproteins were determined using Wheat Germ Agglutinin (WGA) lectin covalently conjugated with a fluorescent compound. It was hypothesised that oleuropein treated cells would have lower fluorescence than untreated cells if glycoproteins become denatured by the action of oleuropein. The method of Roth *et*

al (1978) for the conjugation of fluorescein 5, (6)- isothiocyanate (FITC) with Wheat Germ Agglutinin (WGA) was used. However, instead of using dimethyl sulphoxide, 500 μL acetonitrile was used to dissolve 2 mg of FITC. FITC conjugate shows affinity to alpha and epsilon amino groups of lysine found in bacterial cells. WGA-Lectin (10 mg) was dissolved in 2 mL deionised water and brought to pH 9.0 with carbonate buffer (0.1 M, pH 11.0). An aliquot of 100 μL FITC stock solution was mixed with the lectin solution for 20 min with stirring. The mixture was left overnight at 4ºC and then passed through a 10 mL Sephadex column (2% of column volume) in PBS to separate unbound FITC. Overnight cultures of MRSA-ME30 and MSSA 29213 grown in 5 mL MHB in 20 mL universals were centrifuged and resuspended in 5 mL PBS. Aliquots of 50 μL of FITC conjugate were added to 1 mL washed cells. The suspension was incubated in a shaking incubator at 200 rev min^{-1} for one hour at 37°C. The mixture was then centrifuged (4000 g for 10 min at 4°C), resuspended in PBS followed by fixation in PBS containing 2% paraformaldehyde. For microscopic examination, a Magnum-T Epi-fluorescence Microscope with a mercury bulb HP200v/HBO 100W illumination attached to a Nikon Coolpix 4500 Digital Camera from CETI Medline Scientific Limited was used. The experiment was carried out in triplicate.

For the FITC amino group binding without WGA, the same concentrations and preparation of FITC as well as bacteria were used. Cells were treated with 1% oleuropein in PBS prior to centrifugation and addition of FITC. In addition to MRSA-ME30, MRSA-ME80 and MSSA 29213, *S. epidermidis* a biofilm former was included because of its enhanced glycocalyx surface. The experiment was carried out in triplicate.

Chapter Three

3 Extraction and purification of oleuropein from raw olive plant material

3.1 Introduction

The oleuropein content in the olive plant varies from 1-14 mg g^{-1} in fruits and 10-140 mg g^{-1} in leaves. Current extraction processes are both lengthy and expensive. Therefore, studies were initiated with the aim of extracting oleuropein by Countercurrent Chromatography (CCC), a dynamic purification method that is potentially more efficient than conventional batch-based solvent purification. Equipment and supervision for this work was kindly provided by Dr Ian Garrard at the Bioengineering Institute at Brunel University. The work has included development of a method detecting oleuropein using high pressure liquid chromatography, selection of an appropriate solvent system for extracting oleuropein from olive leaves and several purification experiments using the 17 mL Mini- and 840 mL Midi-scale CCC apparatus at Brunel University. Since the surfactant properties of the leaf extract adversely affected the purification by CCC, an additional purification step based on flash chromatography was also investigated.

3.2 Detection of oleuropein by HPLC

A typical HPLC chromatogram of a commercial oleuropein sample is shown in Figure 3.1.The purity of the commercial oleuropein was 83% calculated on the basis of peak area. Compared to the commercially-available oleuropein, crude olive plant extracts contained many compounds and the 10 min run time was too short to allow all components to elute completely. Therefore, the run time was extended to 30 min to resolve all components. The retention time of oleuropein in crude extracts was between 15.3 and 15.4 min. A regression line of peak areas of purchased standards

of oleuropein, caffeic acid, p-coumaric acid, and hydroxytyrosol was used for the quantification of these compounds in olive extracts (Appendix: 1, 2, 3). Peaks were identified on the basis of their retention time and the UV spectrum (Figure 3.2).

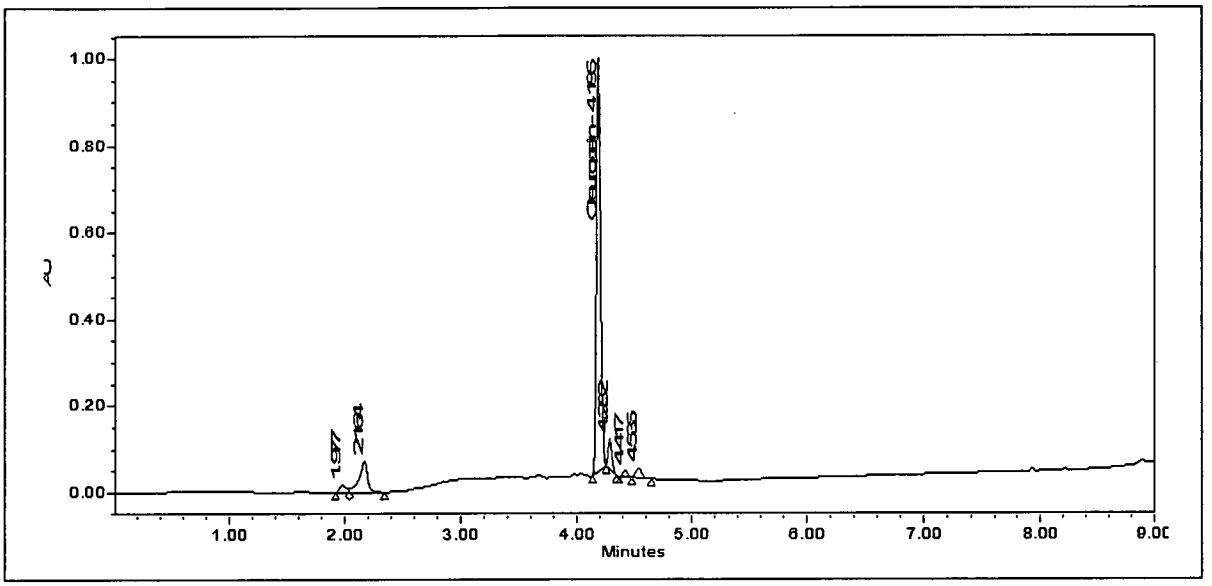

Figure 3.1: HPLC chromatogram of 20 μL injection of commercially-available oleuropein (Extrasynthese, France) with a 10 min run time. The oleuropein peak is shown at 4.2 min achieved with a YMC-Pack Pro C18 column.

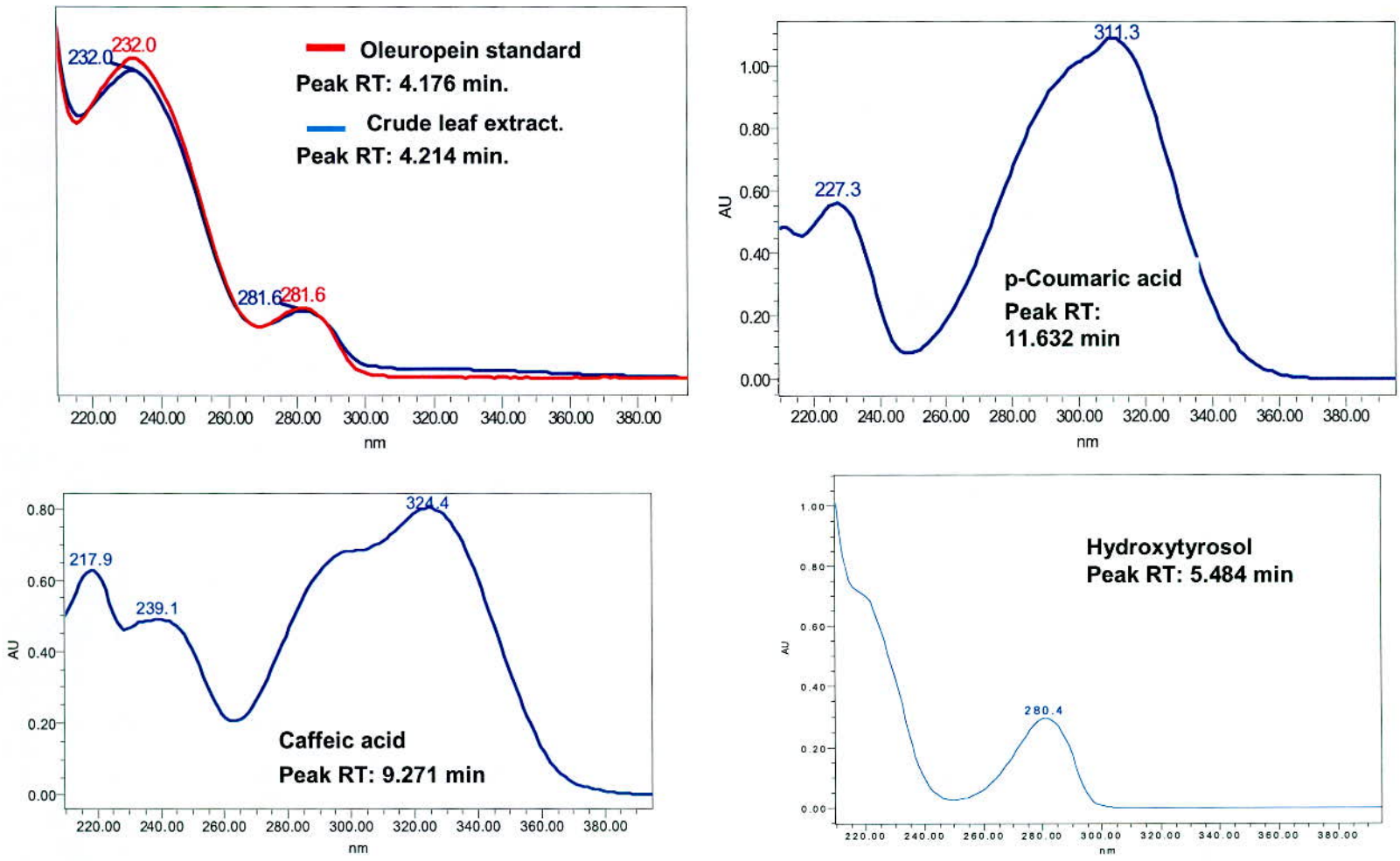

Figure 3.2: Absorbance spectra of oleuropein, p-coumaric acid, caffeic acid and hydroxytyrosol compared to crude olive leaf extract

3.3 Extraction of oleuropein from plant material

Initially, the work focused on finding a suitable solvent for the extraction of oleuropein from its plant source. The factors which were considered important for solvent selection were: i) Maintaining the integrity of the compound of interest without inflicting any damage to the structure, such as degradation and irreversible bond breakage; ii) Separation of substantial yields of oleuropein from the crude mixture; iii) Selection of an economic solvent which does not interfere with the identification process.

Three different concentrations of methanol (50, 80, 100%) in deionised water were used to extract oleuropein manually with mortar and pestle from approximately 1.0 g of olives and olive leaves. The yields of oleuropein were quantified using HPLC and the results are shown in Figure 3.3. Pure methanol resulted in the highest yield of oleuropein of 7.6 mg g^{-1} from olive leaves and 1:1 proportions of methanol:water resulted in oleuropein of 2.8 mg g^{-1} from olive fruit.

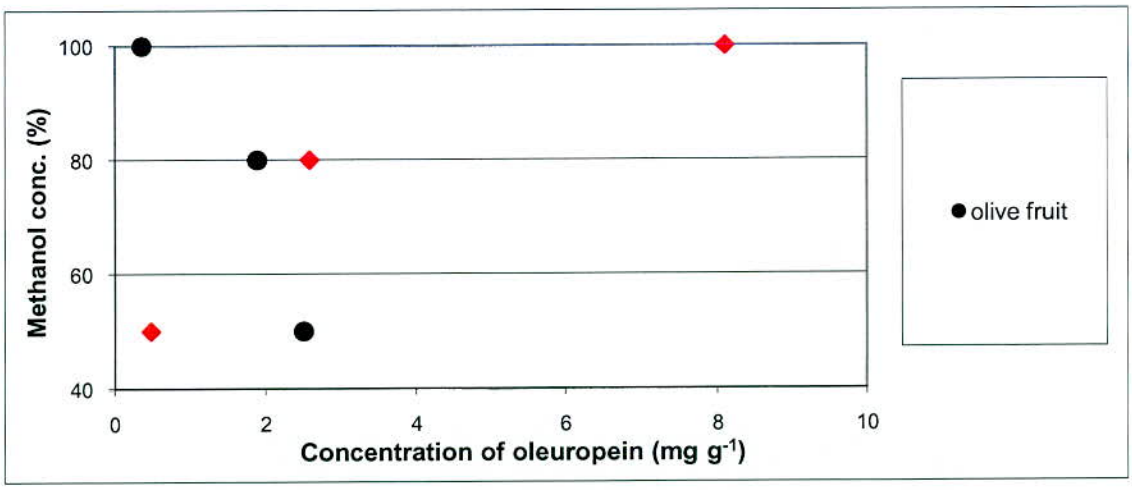

Figure 3.3: Extraction of oleuropein from olive leaves and untreated olive fruit using 50, 80 and 100% methanol in deionised water

Experiment was done ones only

In some circumstances, the crude plant extracts were stored refrigerated and processed at a later date. Therefore, it was essential to investigate the stability of the extracts in the solvent used. A solution of commercial oleuropein (10 mg mL^{-1}) was stored in methanol at room temperature and tested for changes in HPLC peak area at various intervals of time over a 7 day period. As shown in Figure 3.4, oleuropein was stable in methanol for up to three days. By day 7, oleuropein concentration was reduced by 6.3%, indicating that some degradation took place over this time period.

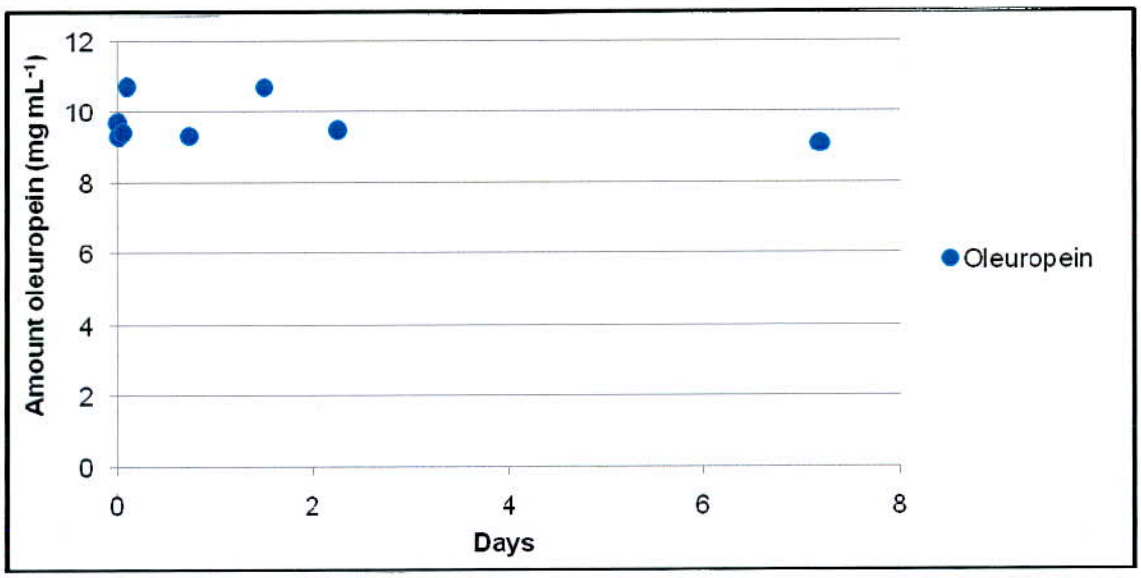

Figure 3.4: Stability of oleuropein stored in 100 mL methanol at room temperature for 7 days determined by HPLC

Experiment was done ones only

Oleuropein was stable for 11 days in solvent system No 7 (heptane, ethyl acetate, methanol, water, 1:19:1:19). This was the system selected for CCC as detailed in the section below. The results showed some fluctuation in oleuropein content but remained steady overall (Figure 3.5).

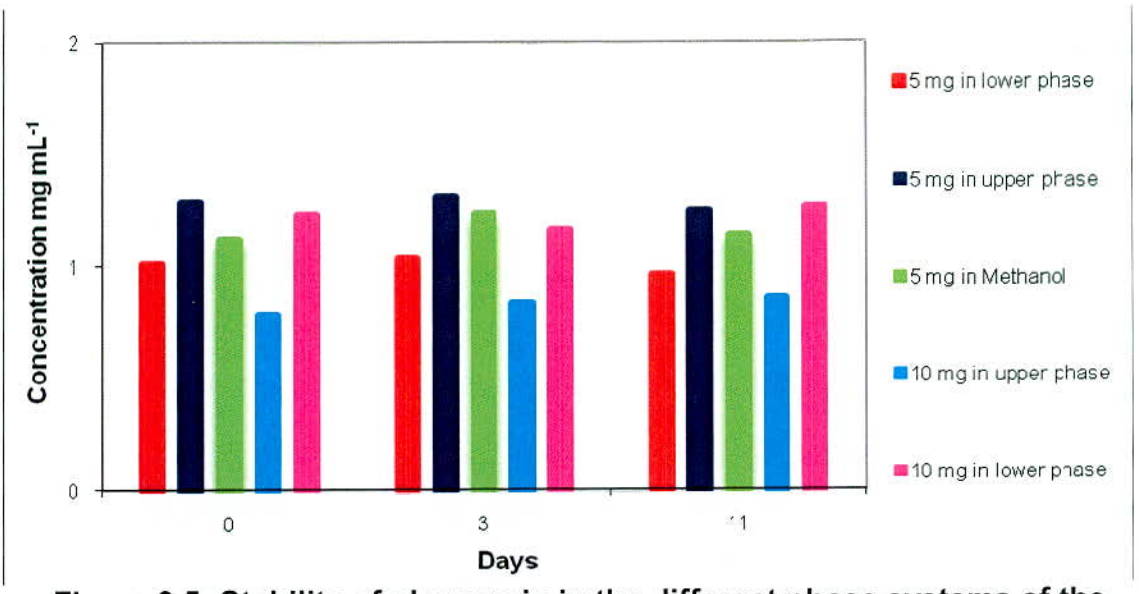

Figure 3.5: Stability of oleuropein in the different phase systems of the selected solvent system (lower phase: ethyl acetate & butanol; upper phase: methanol & deionised water and only methanol) for a period of 11 days determined by HPLC

Experiment was done ones only

3.4 Purification of oleuropein by Countercurrent Chromatography

The initial work comprised the selection of an appropriate two-phase solvent system for CCC processing. The solvent system must consist of two immiscible phases (lower and upper layer formed in a mixture). The compound of interest should ideally settle equally well in both phases. If the compound significantly favours the mobile phase, separation would be impeded as the compound elutes rapidly with the mobile solvent front. On the other hand if the stationary phase is significantly favoured the length of the CCC run is increased and major peak band broadening occurs, possibly to the extent that the eluted peak is not recognised above the baseline. Either phase in CCC can be used as stationary phase. In this case, the lower phase (higher density) was selected to be the stationary phase, so that the volatile organic phase was mobile. This would facilitate the subsequent

drying of fractions. To determine the optimum solvent system for the CCC purification, a liquid handling robot mixed proportions of heptane, ethyl acetate, methanol, butanol and water with the crude extract as shown in Table 3.1. The mixtures decreased in polarity from top to bottom of the table. The distribution ratio D is the parameter to describe the concentration of a solute between the upper and lower phase of a phase system. The solutes to be separated on CCC should ideally fall within the range D = 0.2 to 5. In normal operation, retention of a solute depends upon the phase volume ratio and the distribution ratio of the solute (Garrard, 2005). Unlike HPLC, there is no adsorption of solutes in CCC. If D is less than 0.2, peak resolution is poor as the solute is not retained sufficiently in the CCC column. However, if D is much greater than 5, it takes a long time to elute from the column and thus significant peak broadening occurs (Garrard, 2005).

$$D = \frac{C_S}{C_m} = \frac{peak\ area\ oleuropein\ \ lower\ phase}{peak\ area\ oleuropein\ \ upper\ phase};$$

C_s is the concentration of the sample component in the stationary phase and C_m is the concentration of the component in the mobile phase

Equation 2: Definition of the distribution ratio for the solvent selection in countercurrent chromatography in normal phase operation

As shown in Table 3.1, the distribution ratio of oleuropein in solvent system No 7 was D = 1.6. Sutherland *et al* (2000) have determined that when the mobile phase is lighter (upper phase), better stationary phase retention occurs with the mobile phase pumped from tail periphery to head centre of a multi-layer coil. Solvent systems No 17 to 28 were not appropriate as oleuropein was only found in the lower phase.

In addition to oleuropein, the phenolic compounds caffeic acid, p-coumaric acid and hydroxytyrosol were tested in olive leaf extracted with methanol. The peak area determination showed that caffeic acid was present in trace amounts and p-coumaric acid was present up to 2.0%. However, p-coumaric acid content decreased after CCC application to around 0.70%. Therefore, all further applications focused on oleuropein content.

Table 3.1: Solvent system selection and distribution ratio calculation for oleuropein in solvent mixtures made of heptane, ethyl acetate, methanol, butanol and water using the liquid handling robot

System No	Oleuropein determined by HPLC peak area							
	Solvent proportions by ratio					Peak Area (uV.s)		Distribution Ratio
	Heptane	*Ethyl acetate*	*Methanol*	*Butanol*	*H_2O*	Upper phase	Lower phase	Normal Phase
1-5	Oleuropein was not detected							
6	0	1	0	0	1	252628	254877	1.0
7	1	19	1	0	19	12476	20462	1.6
8	1	9	1	0	9	17028	44482	2.6
9	1	6	1	0	6	18169	75193	4.1
10	1	5	1	0	5	19401	88913	4.6
11	1	4	1	0	4	17393	105489	6.1
12	1	3	1	0	3	11719	146642	12.5
13	2	5	2	0	5	10441	174615	16.7
14	1	2	1	0	2	5212	201635	38.7
15	2	3	2	0	3	2021	82236	40.7
16	5	6	5	0	6	973	49535	50.9
17-28	Oleuropein was found only in the lower phase							

Experiment was done ones only

3.4.1 Mini-CCC

The crude leaf extract (100 µL, 72.5 mg mL^{-1}) was processed with the Mini-CCC and solvent system no 7 with the following conditions: 17.2 mL coil, 1.0 mL min^{-1} flow rate pumping from tail to head (centre of the column), upper organic phase as mobile, 2100 rpm spin at 30°C with a UV detection at 230 nm, 100 µL sample loop, crude sample in methanol. The CCC chromatogram shown in Figure 3.6 (A) was obtained. The noisy CCC chromatogram is due to the presence of ethyl acetate in the mobile phase, which saturates the detector at the wavelength used. Fractions were collected every minute, dried in a concentrator and analysed by HPLC.

The crude olive leaf extract was analysed prior to CCC processing on HPLC and the purity was determined to be 5.9% (Figure 3.7). As shown in Figure 3.6 (A) the fraction collected at 22nd min run time from the CCC chromatogram resulted in the highest purity at 57.6% determined by HPLC peak area (Figure 3.8). The injection volume of 100 µL with a sample concentration of 72.5 mg mL^{-1} resulted in 7.25 mg being loaded onto the CCC column. In 7.25 mg sample there is 7.4% oleuropein, therefore the content of oleuropein in the sample is 0.5365 mg. The overall recovery was 76.2% thus 23.8% was unaccounted for. This may be due to inconsistencies in measuring small samples or to variations in the initial batch of crude material. Nevertheless, the purity of oleuropein increased from the crude value of 5.9% to 57.6% as determined by HPLC peak area (Figure 3.8).

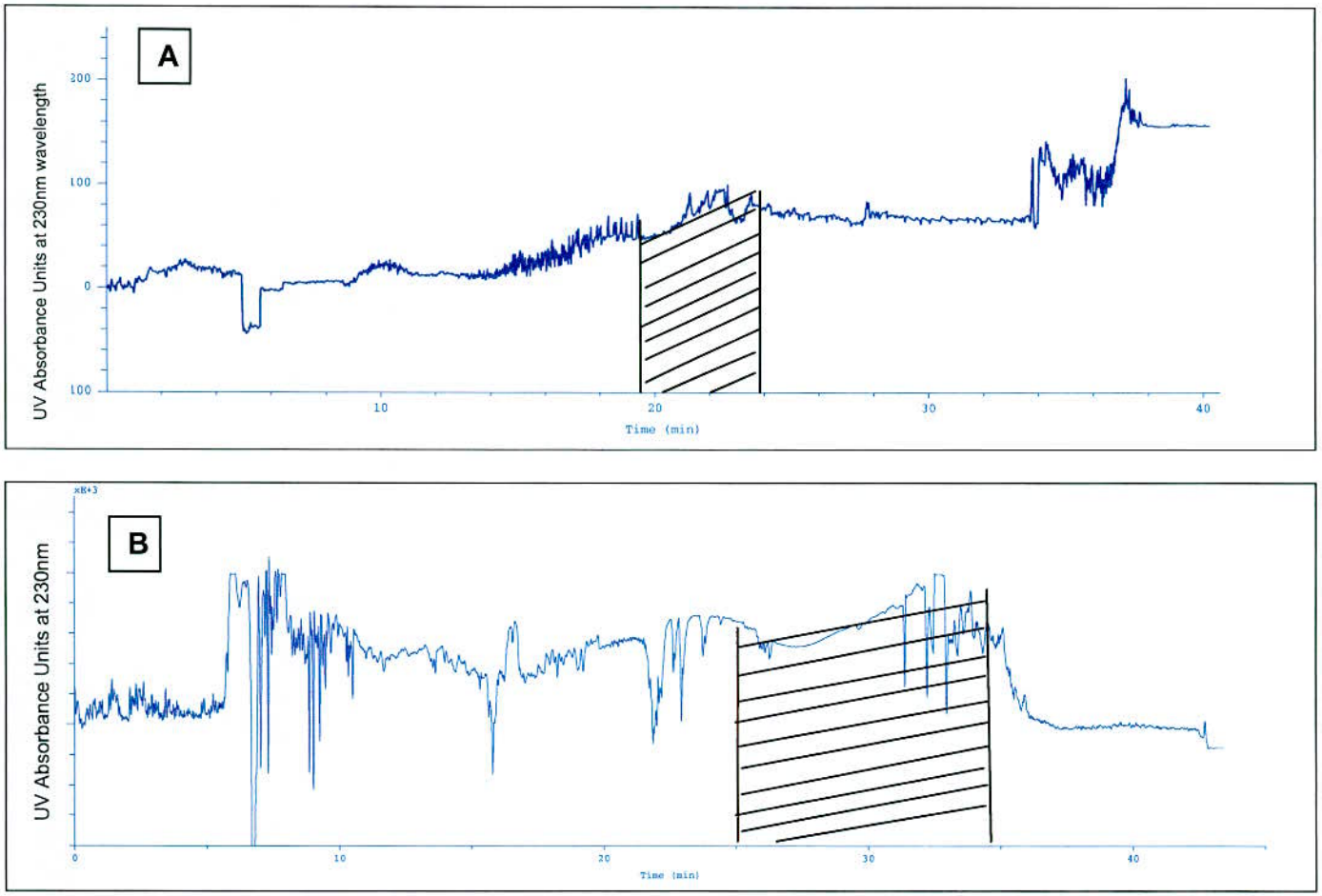

Figure 3.6: Countercurrent chromatography of crude olive leaf extracts (100 µL, 72.5 mg mL^{-1}) in methanol with solvent system ethyl acetate, butanol, methanol and water on the Mini-CCC (A) and Midi-CCC (B) (50 mL, 32.5 mg mL^{-1})

Experiments were done ones only (hash lines represent fractions with oleuropein)

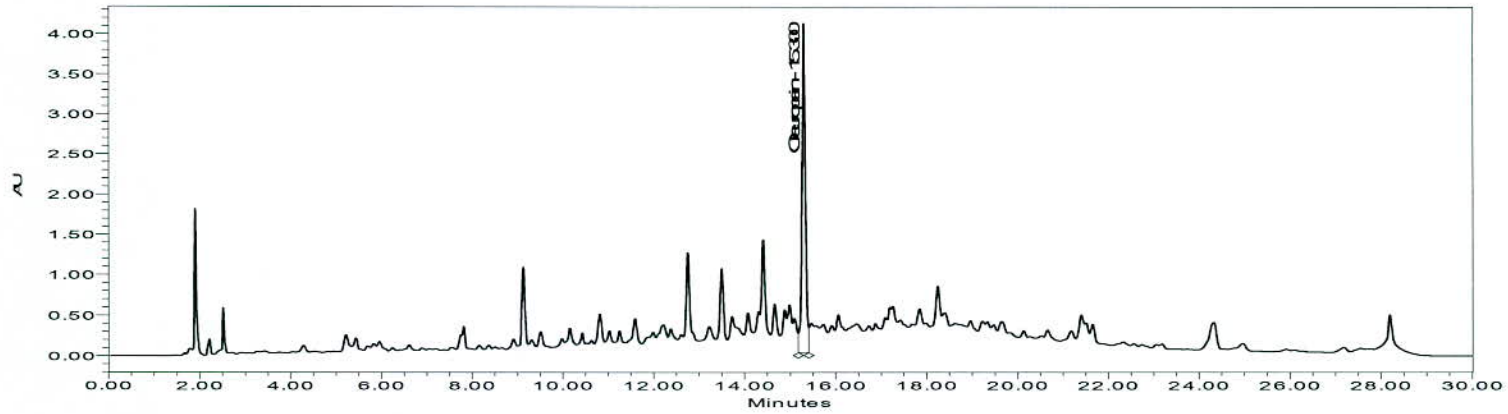

Figure 3.7: HPLC chromatogram of crude olive leaf extract in methanol prior to countercurrent chromatography

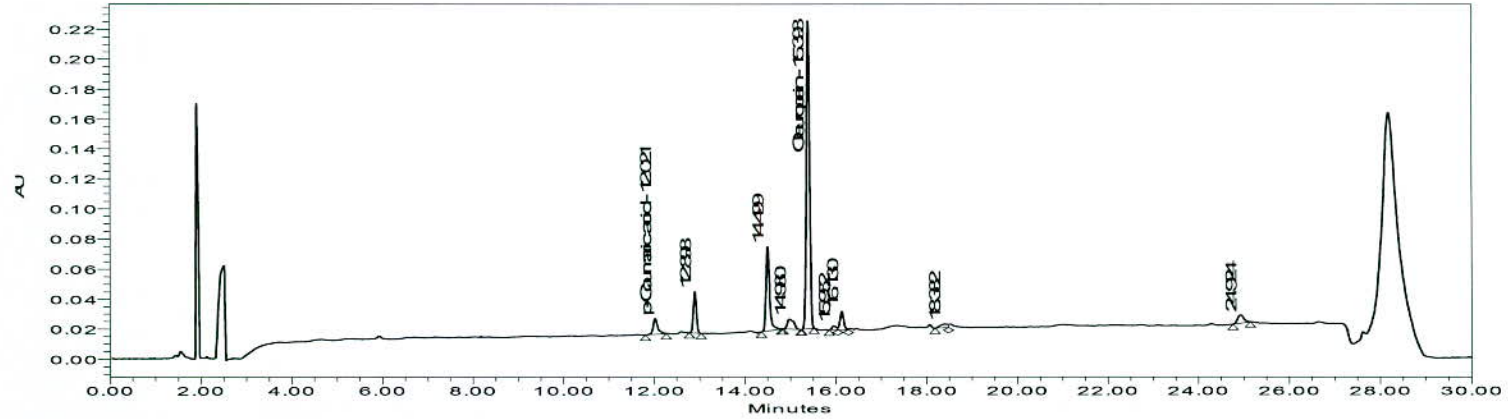

Figure 3.8: HPLC chromatogram of the fraction with highest oleuropein content from olive leaf extract subjected to countercurrent chromatography using the Mini-CCC

3.4.2 Midi-CCC

The results obtained using the Mini-CCC suggested that scale-up of the method would be worthwhile. The Midi-CCC centrifuge was used with the same solvent system and the following conditions: Two coils were arranged in series with a total coil volume of 839 mL (409 mL + 430 mL), 50.0 mL min^{-1} flow rate pumping from tail to head, upper organic phase as mobile, 1100 rpm spin at 30°C with UV detection at 230 nm, 50 mL sample loop. The concentration of the crude olive leaf extract was 32.5 mg mL^{-1} in methanol. The CCC chromatogram shown in Figure 3.6 (B) was obtained. A reconstructed chromatogram of the CCC chromatogram from the HPLC peak area in each fraction is shown in Figure 3.9, where the purity (%) of the collected fractions is displayed on the y-axis and the CCC signal (V) on the right y-axis. Fractions were collected every minute, dried in a concentrator and analysed by HPLC. Fractions with the highest oleuropein content in lower and upper phase are displayed in Figure 3.10.

As shown in Figure 3.9, oleuropein was found in fractions 25-35 in both the upper and lower phase. Throughout the CCC process, stripping of the stationary phase occurred from the column. However, in this experiment, both the upper and lower phase of all fractions were analysed separately. The highest purity obtained was 64% with an oleuropein content of 4.5 mg in the lower phase of the fraction. Total amount of crude olive leaf extract injected was 1625 mg calculated by multiplying the concentration with the sample loop. The sum of the obtained amount of oleuropein in upper with lower phase of all fractions was 35.4 mg. Having used a 2.7% purity of oleuropein from a different extracted batch suggested a total oleuropein content of 43.9 mg and a purification yield of 80.6%. Due to different

amounts of oleuropein in each leaf extract, the purity of the crude extract varied.

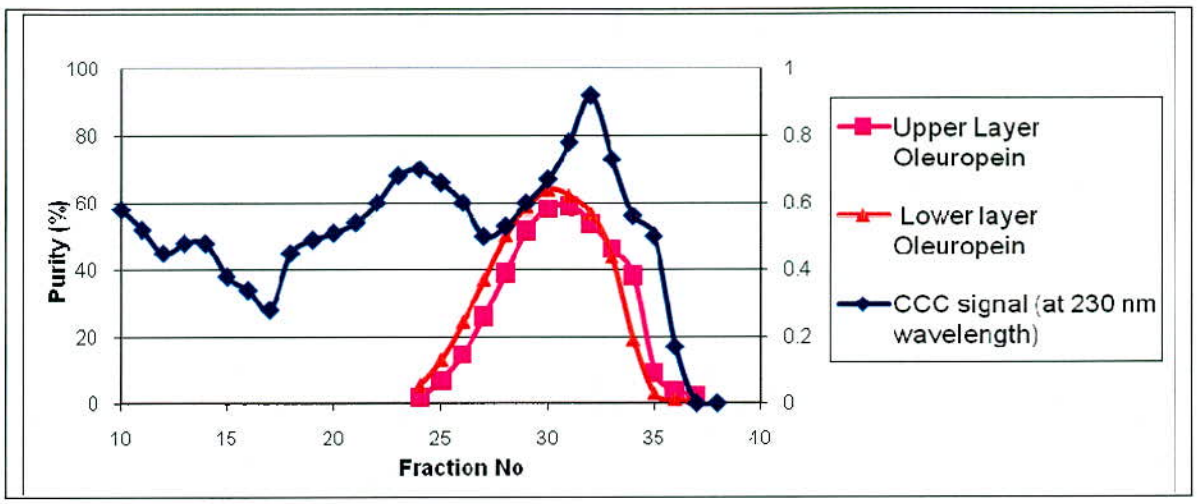

Figure 3.9: Oleuropein purity in lower and upper phase fractions collected during the Midi-CCC process after transformation of the CCC signal

The purity of the oleuropein in the leaf extract increased from 2.7 to up to 64%. Oleuropein eluted within 25-35 min in a 40 min run time. The amount of oleuropein distributed in both layers was similar as would be expected from its distribution ratio of D=1.6.

Fractions containing oleuropein from the Midi-CCC process were combined, dried in a rotary evaporator (Büchi Rotavapor R-205) and tested for biocidal activity against Methicillin-resistant *Staphylococcus aureus* in PBS at 37°C. As shown in Figure 3.11 (A) there was a reduction in viable counts of nearly 3 log cfu mL^{-1} within 2 h of exposure to 2 % CCC extract, whereas 2% oleuropein from Extrasynthese showed a reduction of 5 log cfu mL^{-1} within 3 h exposure (Figure 3.11 (B)).

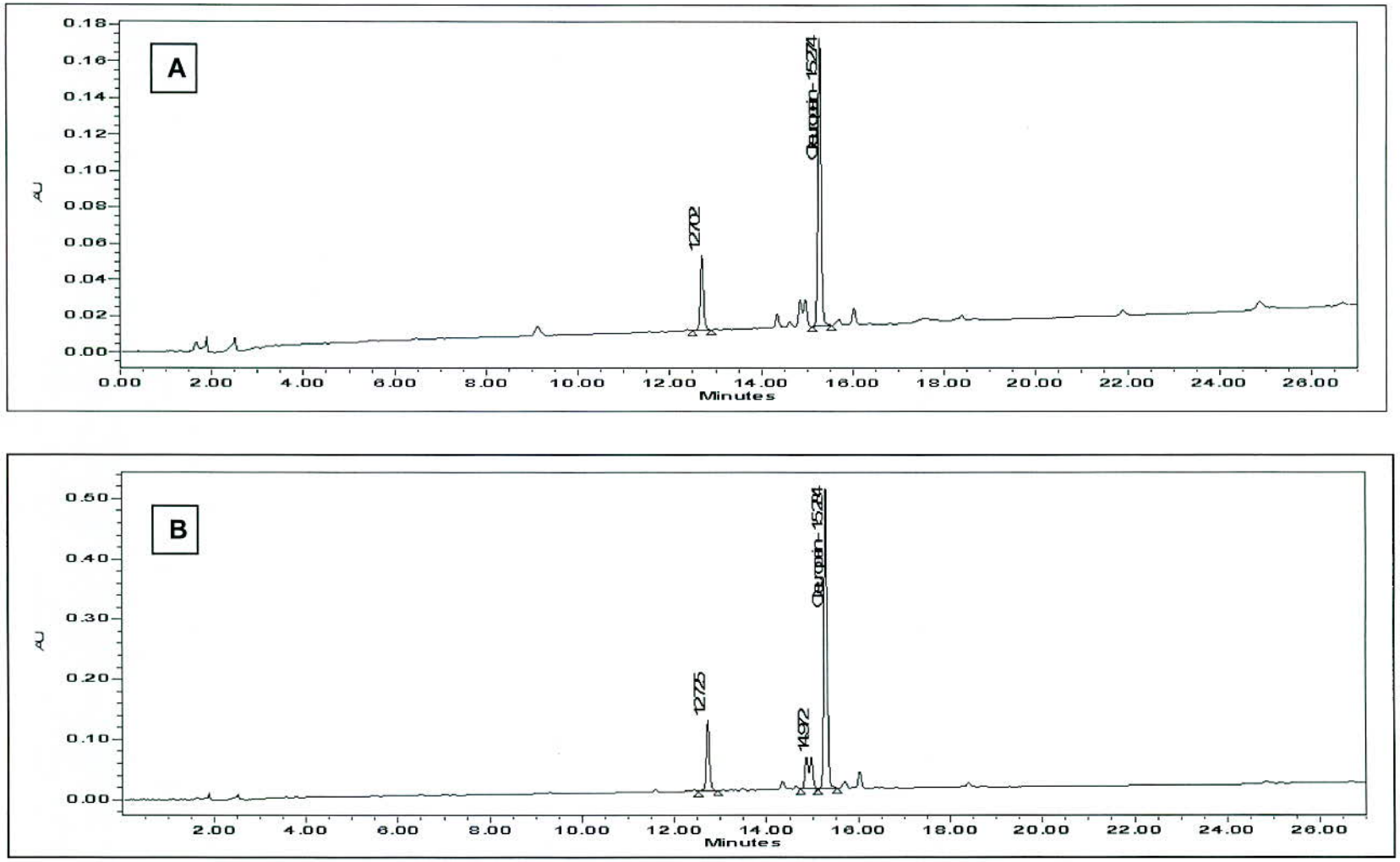

Figure 3.10: HPLC chromatogram of upper (A) and lower phase (B) 10 µL of collected fraction after 32 min of the CCC run with a 10 µL injection with a 30 min run time. The oleuropein peak is shown at 15.4 min achieved with a YMC-Pack Pro C18 column

In the presence of the CCC extract at 1%, there was a reduction of 2 log cfu mL^{-1} within 3.5 h, whereas at 0.5% there was a 1 log cfu mL^{-1} reduction after 4 h of exposure. The reduction in numbers was used to calculate killing rates as shown in Figure 3.11(A). A reduction of 50% in viable numbers occurred in the presence of 2% extract within 2.7 h, 1% extract within 5.4 h and 0.5% extract within 14.7 h, respectively. Compared to the bacterial killing assays from section 4.4, only the CCC-2% extract resembled the killing time of commercial oleuropein. At lower concentrations, the olive leaf extract was less biocidal than the commercial preparation. However, the olive leaf extract was 60% pure, whereas oleuropein from Extrasynthese had a purity of 83%. Nevertheless, the CCC extract with 60% purity demonstrated bactericidal activity against MRSA.

The results shown in Figure 3.11 demonstrate the biocidal activity of oleuropein against MRSA. The killing time for half of the bacterial population (Figure 3.11) was approximately 7 and 16 h at 1 and 0.5% olive leaf extract, respectively. If the purity of oleuropein in the extract could be increased to that of the commercial product from the supplier Extrasynthese, the killing rate for MRSA-ME30 would probably be improved.

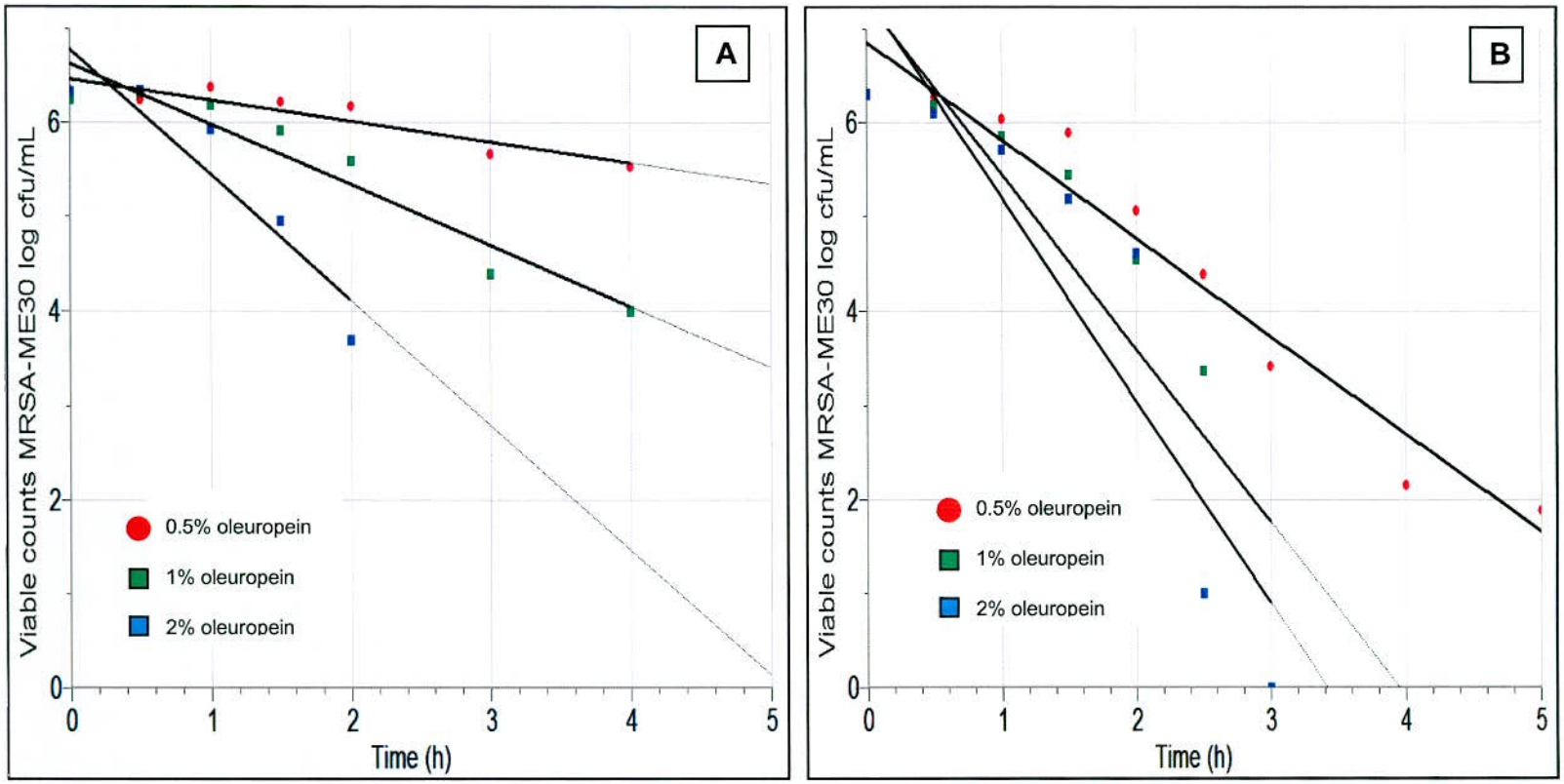

Figure 3.11: Viable bacterial count reduction of CCC extracts oleuropein with a purity of 60% against MRSA (A) and oleuropein from Extrasynthese with a purity of 83% (B) against MRSA in PBS at 37°C

Sensitivity limit was 3 log cfu mL^{-1} for (A) and 1 log for (B); experiment was done ones only

3.4.3 Improved Midi CCC processing

During the Midi-CCC experiment described in 3.4.2, stripping of the stationary phase occurred. This can take place when the injection concentration is too high or due to the presence of surfactant molecules in the crude mixture. Stripping can adversely affect the purification by reducing the amount of stationary phase in the column and thus the resolution of the compounds. Furthermore, the presence of the stationary phase in the fractions can add contaminants back into the purified components as the stationary phase often contains slow or non-eluting components. Finally, stripping of the stationary phase is undesirable as each fraction has to be separated into two phases which is a labour-intensive process. Generally, stationary phase stripping is alleviated by reducing the injection loading. For a better separation of oleuropein from the mixture, a reduced concentration was chosen and also a reduced injection volume into the coil. The flow rate was also halved to improve resolution. The same Midi-CCC centrifuge was used with modified conditions; 839 mL coil volume (409 mL + 430 mL), 25.0 mL min^{-1} flow rate pumping from tail to head, upper organic phase as mobile, 1100 rpm spin at 30°C with UV detection at 230 nm, 50 mL sample loop with 20 mL partially filled injection of crude sample with 13.5 mg mL^{-1} in lower phase resulted in 270 mg crude injected. With a purity of 5.9% the total amount of oleuropein in the crude olive leaf extract was 15.93 mg. Fractions were collected every minute with the exact volumes shown in Appendix: 4 & 5.

However, in spite of adaptations to the conditions, stationary phase stripping occurred in almost all fractions. Oleuropein was found in fractions 35-57. The lower phases of those fractions contained 2.08 mg oleuropein, whereas the upper

phases contained 13.46 mg oleuropein (Figure 3.12) resulting in 15.54 mg oleuropein in total. The error of 2.5% from the calculated amount was most likely due to inaccurate calculations of peak areas. Purity (%) increased from 5.9% in the crude extract to up to 92% in 4 mL of the lower phase of fraction 43. The upper phase (21 ml of the same fraction) had a purity of 83.5% with the highest oleuropein content of 1 mg. An increase in purity was achieved from 5.9% to around 90% in the combined fractions. The distribution of oleuropein in fractions 34-53 reduced the efficiency of the separation. The injection volume was reduced about 10 fold but stationary phase stripping still occurred. It was assumed that the presence of surfactants in the crude extract was causing the stripping. Therefore, an additional separation step prior to the CCC run needed to be considered. In the next section, attempts to achieve further improvements in separation using flash chromatography are described.

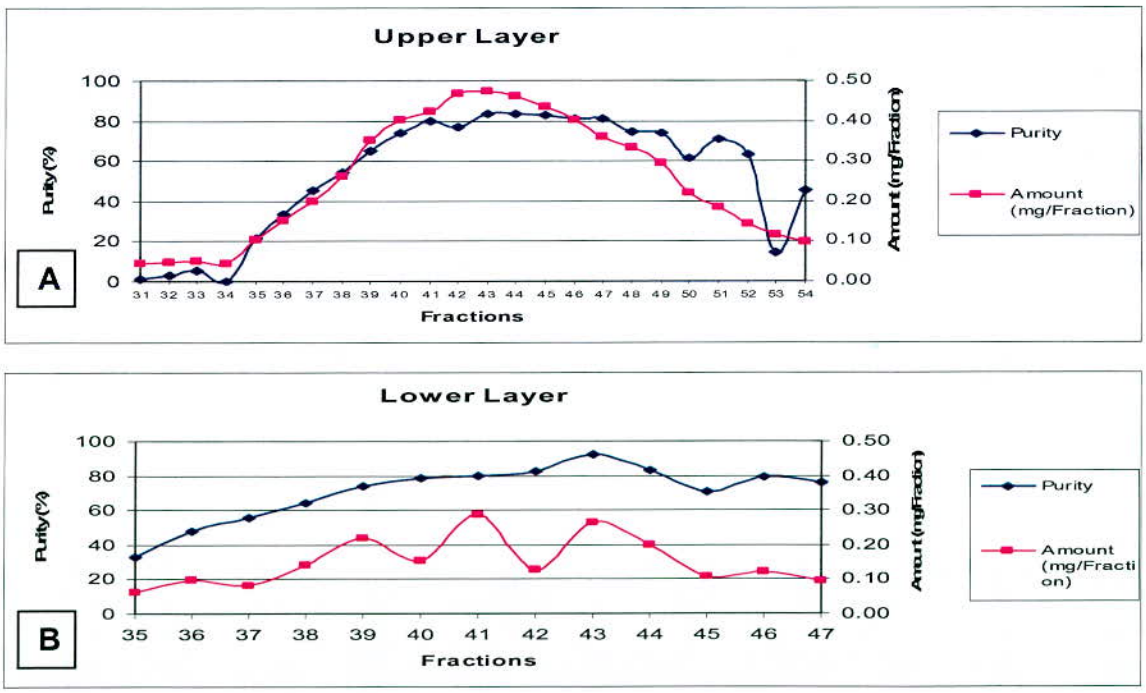

Figure 3.12: Oleuropein purity in (A) upper phase and (B) lower phase of fractions collected during Midi-CCC processing of crude olive leaf extract

Flash chromatography, often used as additional separation process in natural compound extraction, was incorporated. The eluting compounds were driven out of the column by the mobile phase, whereas solvents were changed with coloured fractions eluting (Figure 3.13). Figure 3.13A shows the elution of olive leave extract with 100% ethyl acetate whereas Figure 3.13B shows the elution of the oleuropein fraction with 100% methanol.

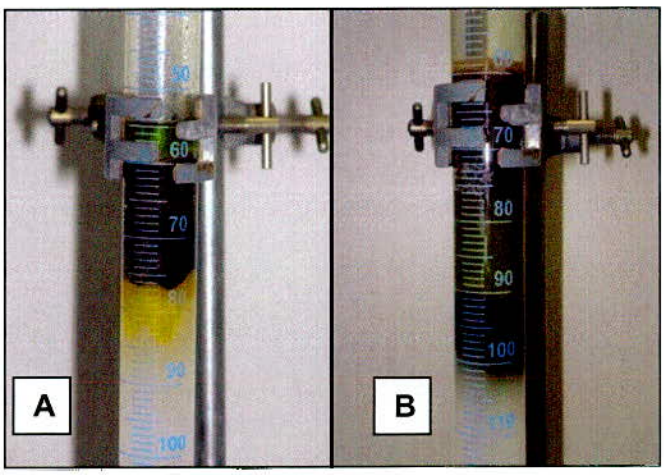

Figure 3.13: Treatment of olive leaf extract using flash chromatography prior to CCC (A) yellow fraction eluting with 100% ethyl acetate and (B) dark oleuropein fraction eluting with 100% methanol
Experiment was done ones only

In a second experiment, different solvent proportions were tested. Methanol was used instead of hexane and the gradient interval was reduced to 0, 10, 20, 30, 50, 75, 100% methanol. The solvents and volumes used were 100 mL hexane, 300 mL ethyl acetate followed by 200 mL 10, 20 and 30% methanol and 100 mL 50, 100 % methanol. Moreover, the extract was dissolved in chloroform, which precipitated the extract and also removed some oleuropein from the mixture (oleuropein was found in the sticky particles and the liquid phase analysed by HPLC). The solvent chloroform is a common solvent for extracting compounds from plants. However, with the olive leaf extract, it formed sticky residues after the rotary evaporation of

the crude olive extract. By adding small amounts of methanol (1 to 2 mL) the precipitate became liquid again and 11 mL were used for the flash chromatography.

The oleuropein content of the olive leaf extract prior to treatment with flash chromatography was 41.10 mg (Figure 3.14 A). The HPLC fraction with the highest oleuropein content was subjected to flash chromatography and was found to contain 24.30 mg oleuropein. Some impurities from the crude extract were eliminated but the purity increased only slightly from 5.9% to 6.5% (Figure 3.14 B). The total amount of oleuropein collected from the fractions was 31.27 mg giving a yield of 75.7%. The total content of oleuropein in the extract was 50.23 mg. Fractions collected with pure methanol contained the highest oleuropein amounts with 33.07 and 14.56 mg (total collected of 48.76 mg) resulting in a yield of 97.1%. The purity was calculated to be 24.5% in the fraction with the highest oleuropein content giving an increase in purity of almost five fold (Figure 3.14 C).

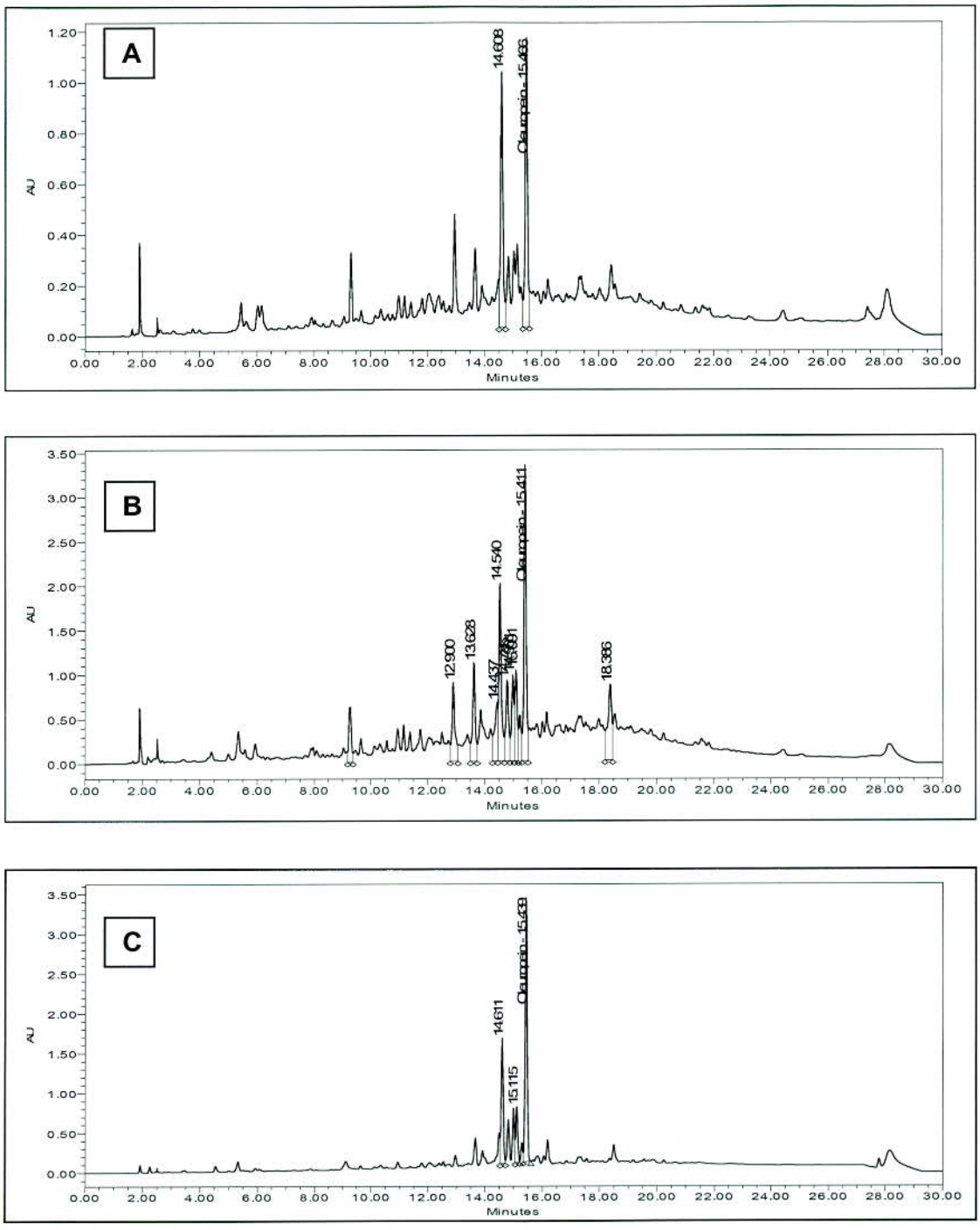

Figure 3.14: HPLC chromatogram of crude olive leaf extract in methanol

(A) before treatment with flash chromatography

(B) after flash chromatography with hexane, ethyl acetate and methanol

(C) after flash chromatography with ethyl acetate and methanol

The results show that a wide range of contaminants were successfully eliminated using flash chromatography. Further purification with the CCC may result in almost 100% pure oleuropein as greater loadings and less stripping of the stationary phase would occur (Figure 3.15). Unfortunately, this second purification was not performed as the Midi-CCC machine was not available at the time and time constraints on the project prevented further work from being performed.

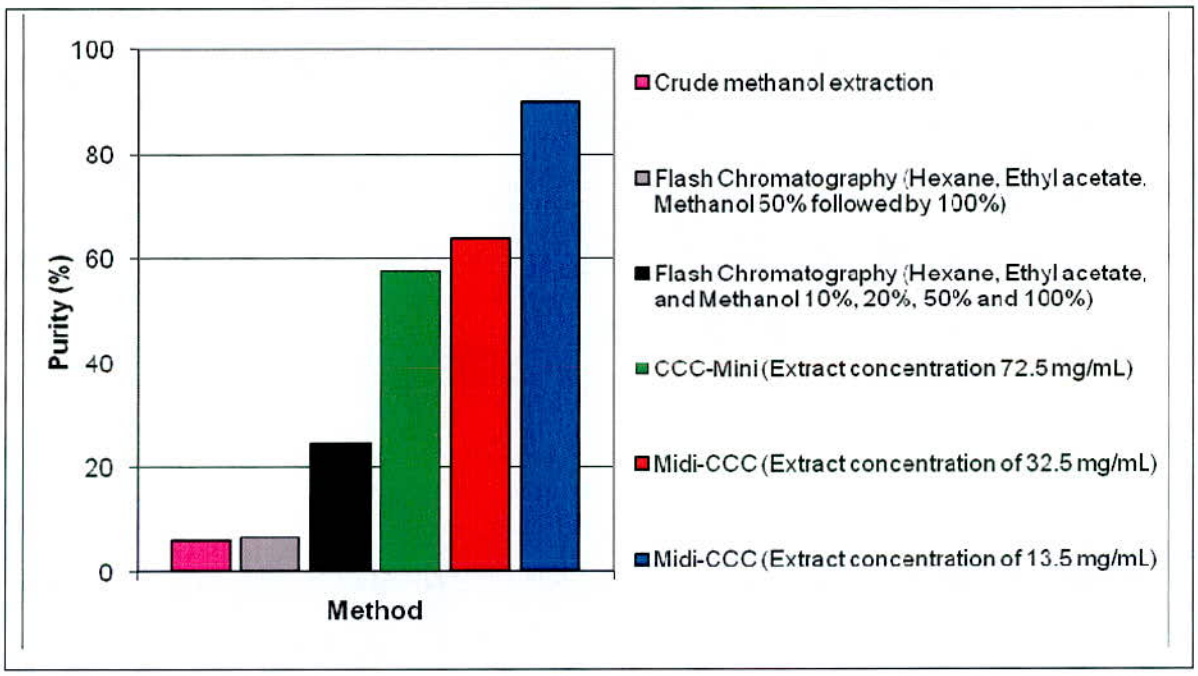

Figure 3.15: Purity of oleuropein extracted from olive leaves and subjected to methanol extraction using flash chromatography and countercurrent chromatography

3.5 Discussion

The objectives in this chapter were to extract, separate and purify oleuropein from its crude material. Olive leaves were preferred to untreated olive fruits as the oleuropein content in leaves was greater as shown in Figure 3.3. A randomly chosen selection of olive leaves was extracted with methanol and subjected to countercurrent chromatography. The problem that emerged with the CCC process was the stripping of the liquid stationary phase effect, caused most likely by the presence of surfactant molecules in the crude mixture. These were most likely removed with the additional purification step of flash chromatography. The lipids in the olive leaf extract may be the origin for the stripping effect but it is unclear whether oleuropein or other components in the crude mixture were the cause.

The separation from the crude extract resulted in the distribution of oleuropein in several time-defined fractions each of which consisted of two phases. Flash chromatography was applied as an additional purification step to remove surfactant contaminants from the crude mixture. Although purity increased with flash chromatography, the successive CCC process was not applied because of unavailability of the CCC machine at that time. Nevertheless, flash chromatography is a relatively cost-effective technique for the initial removal of contaminants.

The reason for the lower content of oleuropein of the first flash chromatography process was that the jump in polarity from the solvent ethyl acetate to pure methanol at the end of the purification was too fast. A number of the non-polar contaminants were most likely removed. However, polar components seemed to be in greater proportion and remained with the oleuropein fraction when used with

the solvent system described in section 1.3.2. The second run with the flash chromatography improved the purity of the compound from 5.9% up to 24.5%.

Overall, the novel CCC technique applied to the purification of oleuropein from olive leaves was established as a viable method although there were difficulties working with a glycosidic natural compound. Stationary phase stripping occurred probably because of the polar and unpolar properties of the target molecule combined with the presence of other surfactant molecules. However, oleuropein demonstrated good stability in the solvents used for both the initial extraction and the CCC purification.

In respect to the oleuropein content described in Table 1.2, the amount of oleuropein obtained from the leaf extract was approximately 20% of the content published by Malik & Bradford (2006) who achieved 36 mg g^{-1} and only 5% of the content published by Savournin *et al* (2001) who achieved 140 mg g^{-1}. The difference in oleuropein content probably reflects the original source of the material. However, the standard curve used to calculate oleuropein contents by Savournin *et al* (2001) was obtained by using 100% pure oleuropein, which resulted most likely in increased oleuropein levels.

Ranalli *et al* (2006) investigated olive cultivars from Italy in different periods of the year with oleuropein values of 8.5 mg g^{-1} in March and 7.7 mg g^{-1} in October. In this study, 1.0 g of crude olive fruits and 1.0 g of leaves were used to select the solvent for the extraction. Furthermore extractions with 21.0, 21.4 and 24.0 g olive leaves resulted in oleuropein values of around 7.12, 7.26 and 9.12 mg g^{-1}, respectively. The purity of oleuropein varied in each extracted batch ranging from

2.7, 7.4 and 5.9%, respectively. The oleuropein content in the black olive fruit used in this study was 2.8 mg g^{-1} compared to 10 mg g^{-1} in a study of the phenolic profile of categorised maturity in olives by Vinha *et al* (2005).

Comparison of results from different studies is difficult because of different extraction methods applied as well as the amount and the cultivar of crude material selected. Although the extraction results from this study showed lower oleuropein contents when compared to those published in the literature, they are of the same order of magnitude expected when variations in extraction method and type of olive plant are taken into account.

Preparative work for CCC consisted of the selection of an appropriate two phase solvent system. The semi-purified material of the first CCC technique resulted in an increase in purity of oleuropein from 2.7% to around 60%. The purified extract from the CCC run was used in the antimicrobial assay against MRSA-ME30 cells and it demonstrated good bactericidal activity.

There is a need to optimise CCC run parameters such as sample concentration, injection volume and flow rate because of stationary phase stripping. As a result of this, a second CCC run was performed and this resulted in an increased purity up to 92%. However, the amount of oleuropein extracted was only 15.5 mg and the content was spread over several fractions with stripping of the stationary phase in most of the samples.

Kubo *et al* (1985) used liquid chromatography techniques (rotation locular countercurrent chromatography & droplet countercurrent chromatography) to purify oleuropein but the process was lengthy and the raw material was olive fruits.

Walter *et al* (1973) used countercurrent distribution (CCD) to extract and purify oleuropein from olive fruits. In this method the extract was mixed in a tube containing a two phase liquid system and transferred to a next tube (up to 40 tubes in the instrument) resulting in a separation of compounds. The CCD method is a time consuming and labour intensive process without recovery of solvents.

The range of extraction and purification methods for phenolic compounds in general and oleuropein in particular illustrates the interest in finding a reliable method. Escribano-Bailón & Santos-Buelga (2003) published an overview of polyphenol extraction and purification procedures from foods. The most recent methods in extraction of olive plant polyphenols are reported below.

Japón-Luján & Luque de Castro (2006a) extracted the phenolic matrix from 1 g olive leaves and suggested the use of superheated liquid extraction (SHLE), using the solvents ethanol:H_20: 70:30 at a 6 bar pressure and temperature of around 140°C. Conventional extraction procedures at temperatures of 30-40°C were compared to the proposed method using the same solvents. The oleuropein content was 23 mg g^{-1} of leaves obtained with SHLE after 13 min extraction time. Similar contents were recorded with the conventional extraction method but the extraction time was 48 h. The HPLC chromatogram identified oleuropein, verbascoside, apigenin-7-glucoside and luteolin-7-glucoside. Many other peaks were shown but not named and this demonstrated the difficulty of separating oleuropein from its crude material. However, the SLHE method had the advantage of using non toxic solvents and it was fast.

The main feature of many extraction methods is that they are individually unsuitable for purifying compounds and an additional purification step usually needs to be incorporated. Moreover, the use of high pressures and temperatures might be a safety concern in some laboratories. Priego *et al* (2004) researched the separation and determination of phenolic compounds by capillary electrophoresis (CE) after ultrasonic-assisted extraction of alperujo. Similar to the HPLC, CE is a method to detect individual components in a mixture by running small sample volumes e.g. 1 μL through a capillary coated with fused silica (silanol groups) particles on the edges into a buffer reservoir. An electroosmotic flow is forced to carry charged particles through the capillary centre (Holme & Peck, 1998). However, the ultrasonic-assisted extraction combined with the capillary electrophoresis was applied as an analytical tool to separate and determine phenolic compounds rather than the purification of individual phenolic components.

Another study by Japón-Luján *et al* (2006a) described the same process but with HPLC detection and showed a decrease in the amount of phenolic compounds after solid phase extraction (column chromatography) and liquid-liquid extraction. Direct injection of the sample after ultrasonic treatment resulted in greater peaks areas of the phenolic matrix.

The microwave assisted technique suggested by Japón-Luján *et al* (2006b) resembled the ultrasonic extraction in which the phenolic matrix was isolated from olive leaves and later detected. One gram of olive leaves was dispersed in the solvent (ethanol-H_20: 80:20) and microwave energy was applied for 8 min with 200W. The extract was centrifuged and injected into the HPLC equipment which

resulted in around 25 μg g^{-1} oleuropein. The yield was therefore considerably lower than the extraction procedures that were applied elsewhere. However, if quantities can be disregarded and the detection of phenolic groups is paramount, then the microwave assisted extraction process saves time and manual strain.

Kefalas (2007) used ground olive leaves, which have undergone several leaching stages by column chromatography and reported a purity of around 95%. Several extraction methods are listed in the European patent registry (Quintanilla *et al.*, 2001; Kefalas, 2007).

Extraction of oleuropein followed by purification from crude olive leaves with the CCC equipment requires more work to ensure the complete elimination of impurities. Although stability was demonstrated, the possible degradation of the compound needs to be considered. In the past successive leaching stages in various solvents over a certain period of time have been used to extract compounds from crude material. The disadvantage was the loss of quantity due to excessive processing and the possible degradation of the compound. Whether CCC is the right choice for the purification of oleuropein needs to be further investigated. However, a suitable extraction method is paramount for the separation of components from the crude material. Flash chromatography is without doubt an effective technique that could be introduced as an intermediate purification step.

3.6 Conclusions

Using countercurrent chromatography, the purity of oleuropein in olive leaf extract increased from 5.9% to over 90%. It is possible that a combined CCC purification with flash chromatography could produce improved purity compared with the current commercial standard from Extrasynthese in France. Moreover, higher sample loadings on the CCC machine would be possible after flash chromatography resulting in greater content of oleuropein. Extrasynthese do not declare if their oleuropein is chemically synthesised or extracted from olive leaves but the purity was found to be around 80% as determined by HPLC. The fact that a commercially available product has such a low purity demonstrates the difficulties in purifying this compound.

The objective to extract and purify oleuropein was achieved by using countercurrent chromatography. The use of CCC in the purification of oleuropein showed that a crude natural component can be isolated by using a predesigned solvent system. The time period for extracting and purifying oleuropein from crude olive leaves was 2-3 days whereas the actual CCC process took only around 40 min. Hence, the CCC technique is a powerful and very attractive approach for separating components from a mixture and could potentially be used for industrial applications where vast solvent volumes could be recovered and re-used.

Chapter Four

4 The antimicrobial properties of oleuropein

4.1 Introduction

The published literature on the antimicrobial activity of oleuropein against antibiotic resistant strains such as methicillin resistant *Staphylococcus aureus* (MRSA) is limited. Moreover, the methods used in the past precluded comparison because of different conditions used such as growth media and target strains. In this study, the antimicrobial activity of oleuropein against twelve Gram-positive and five Gram-negative bacteria and one yeast was investigated in both agar and broth-based growth media. MRSA strains were tested as well as their methicillin sensitive counterparts to establish if methicillin resistance conferred greater resistance to natural antimicrobials such as oleuropein.

A range of microbiological techniques including the broth assay, disc diffusion test, agar plate assay and bacterial killing studies with kinetic rate determination was applied to determine the oleuropein concentration with measurable antimicrobial activity. Strains sensitive to oleuropein were considered for further investigations of the antimicrobial mode of action of oleuropein.

4.2 Determination of antimicrobial activity of oleuropein using spectrophotometric methods

In previous studies the antimicrobial effect of oleuropein was measured visually or by measuring turbidity changes in the microbial suspension (Furneri *et al.*, 2002). Microbial growth in laboratory environments is dependent on the presence of nutritive medium. However, the constituents of a growth medium might interfere in the reaction between microorganism and antimicrobial agent by binding to the antimicrobial agent. This possibility was tested in Tryptone Soya Broth (TSB) and Mueller Hinton Broth (MHB) in the presence of different concentrations of oleuropein (0, 0.5, 1 and 2%).

As shown in Figure 4.1A, absorbance values of sterile TSB supplemented with 1.5% and 2% oleuropein increased up to 0.15 units when incubated at 37°C for six hours. Similarly, absorbance increased by 0.05 units in the presence of 0.5 and 1% oleuropein under the same conditions. The colour of TSB changed after oleuropein was added from yellow to light green and turned brownish over the course of the experiment. This indicated that components in the TSB medium were reacting with oleuropein at 37°C and resulted in an increase of absorbance. Therefore, growth measurements by means of spectrophotometry needed to incorporate adjustments to control for colour changes of oleuropein during incubation. Absorbance measurements at 660 nm with 0.25, 0.5 and 1% oleuropein in MHB showed similar increases over 24 h (Figure 4.1C). When measured at 595 nm in MHB, the absorbance of the medium almost doubled in the presence of oleuropein over 24 h (Figure 4.1B).

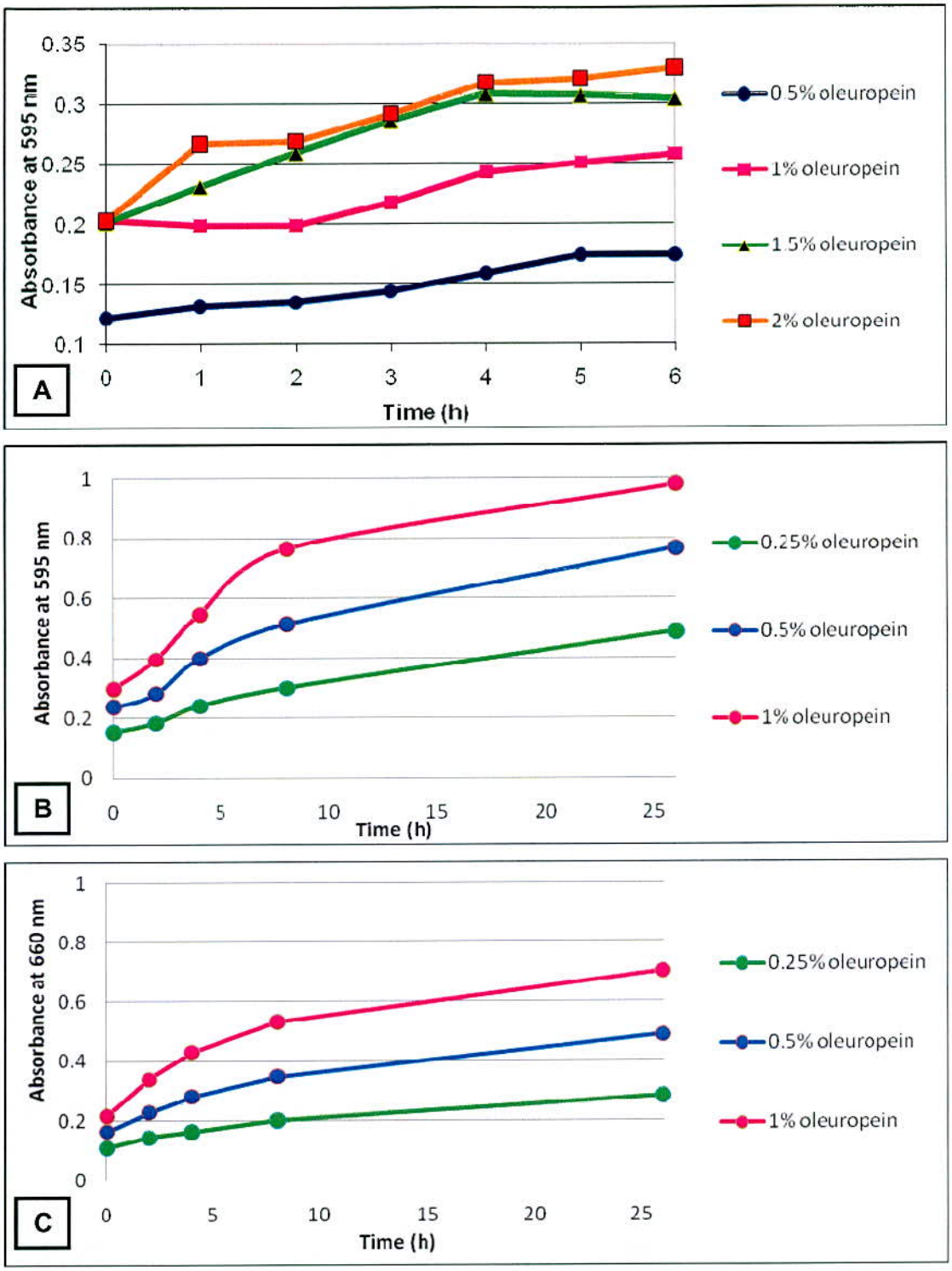

Figure 4.1: Absorbance of sterile Tryptone Soya Broth for 6 h (A) and Mueller Hinton Broth (B+C) supplemented with oleuropein at 0.5, 1.0, 1.5 and 2% incubated at 37°C for 26 h

Experiment was done ones only

It is possible that oleuropein changed the colour of the medium when incubated at 37°C due to oxidation. The absorbance increase at 660 nm was less pronounced than at 595 nm in the presence of oleuropein. However, using 660 nm instead of 595 nm as the absorbance wavelength did not entirely eliminate interference by coloured compounds. Of the two media tested, TSB showed a lower increase of absorbance with oleuropein than MHB. Consequently, TSB was used in subsequent experiments.

In the 96 microtitre plate assay, the amount of sample and oleuropein was 75 µL giving a total volume of 150 µL. Oleuropein concentrations of 0.2 to 5% were added into MRSA-ME30, MRSA-ME80, MSSA 29213 and MSSA-ME5S suspensions prior to absorbance reading at 540 nm (Figure 4.2).

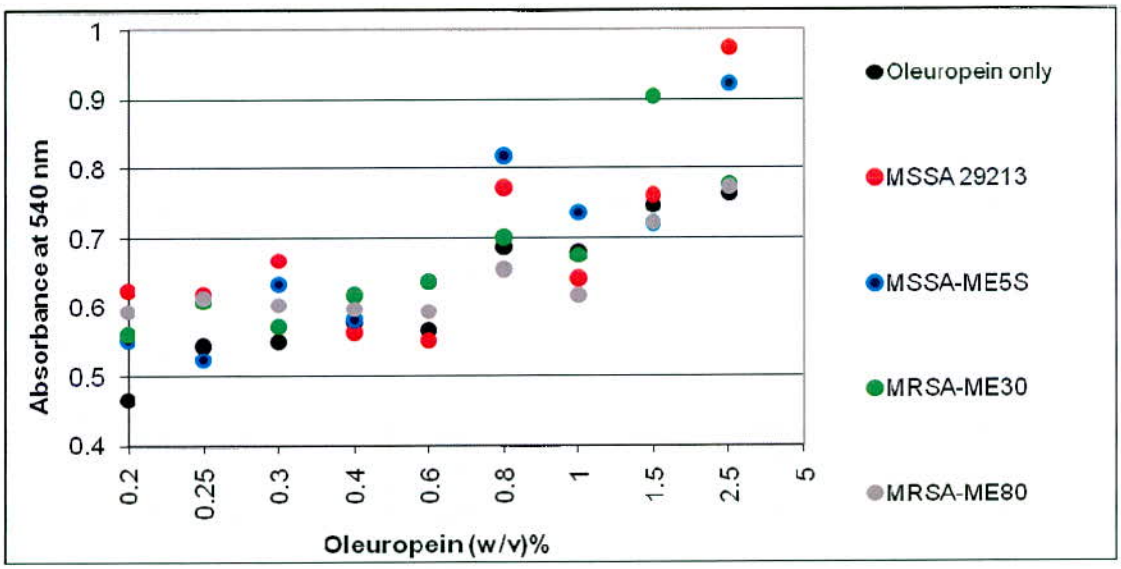

Figure 4.2: Absorbance of five target bacteria in TSB supplemented with 0.2 to 5% oleuropein and incubated at 37°C for 24 h

Experiment was done ones only

As shown in Figure 4.2, the absorbance values for four bacteria increased with an increase in oleuropein concentration at 37°C after 24 h. Oleuropein and all strains

showed the same trend in growth. At 1% oleuropein, absorbance was 0.6 to 0.7 in the presence of microorganisms whilst the uninoculated oleuropein had an absorbance of 0.68 units. The absorbance readings for the controls were similar to those obtained for inoculated bacterial samples at all oleuropein concentrations (0.2-5%) tested. Colour formation in the oleuropein-supplemented broths obstructed the spectrophotometric determination of bacterial growth.

The findings from the broth-based experiments indicated that oleuropein changed the colour of the medium with increased concentration, time and temperature probably due to oxidation. Therefore, broth-based spectrophotometric methods for the determination of antimicrobial activity were unsuitable for studies with oleuropein. At the end of the microtitre assay, a sterile loop was dipped into each well and streaked onto Tryptone Soya Agar prior to incubation at 37°C for 24 h. The plates were then categorised into growth (+) and no growth (-). The results in Table 4.1 show the inhibition of MRSA and MSSA after the microtitre plate assay at oleuropein concentrations ranging from 0.8 to 5%. The lowest oleuropein concentration that indicated no growth was 0.8% with MSSA-ME5S, 1% with MRSA-ME30, and 2.5% with MRSA-ME80 and MSSA 29213.

Table 4.1: Oleuropein-treated bacteria from the microtitre plate incubated at 37°C for 24 h in Tryptone Soya Agar

Bacteria	Oleuropein concentration				
	5%	*2.5%*	*1.5%*	*1%*	*0.8%*
MSSA-29213	-	-	+	+	+
MSSA-ME5S	-	-	-	-	-
MRSA-ME30	-	-	+	+	+
MRSA-ME80	-	-	+	+	+

- No Growth; + Growth
Experiment was done ones only

4.3 Determination of antimicrobial activity of oleuropein in solid media

Antimicrobial properties can be measured using paper discs. The discs containing the antimicrobial agents are applied on the inoculated surface of a solid test medium and the clear zone of inhibition around the discs is measured following incubation at appropriate temperatures. Several events take place during the process: i) discs absorb water from the agar medium which dilute the working concentrations of the antimicrobial agent ii) the antimicrobial diffuses through the adjacent agar medium iii) the zone of inhibition around the disc indicates the extent of inhibition of the bacteria (Barry & Thornsberry, 1991).

Paper discs are often used to categorise bacteria into resistant, moderately susceptible and susceptible strains. The diameter of the zone of inhibition is indirectly proportional to the Minimum Inhibitory Concentration (MIC) (Barry & Thornsberry, 1991). Because different compounds diffuse at different rates, zones observed with different agents cannot be compared with each other (Barry & Thornsberry, 1991). The MIC is defined as the lowest concentration of antimicrobial that inhibits the visible growth of an organism (Sahm & Washington, 1991).

As shown in Figure 4.3, oleuropein at 10% inhibited the growth of MSSA-ME5S and MRSA-ME30. Table 4.1 shows that none of the sixteen target organisms used in this study were inhibited by 1 and 2% oleuropein. At 5% oleuropein, *C. albicans* and one of each MRSA and MSSA were inhibited. The results shown in table 4.1 are giving the average of two separate experiments. The highest zone of inhibition

at 10% oleuropein was measured against MSSA-ME5S (26.5 mm in diameter) followed by MRSA-ME30 (24 mm) and *C. albicans* (22.5 mm). MSSA-29213 exhibited an inhibition zone of 17 mm and MSSA 6571 a zone of 9 mm in the presence of 10% oleuropein. The Gram-negative *E. coli* strains averaged at 9.5 mm whilst the zones for *Micrococcus luteus* were about 13.5 mm in diameter.

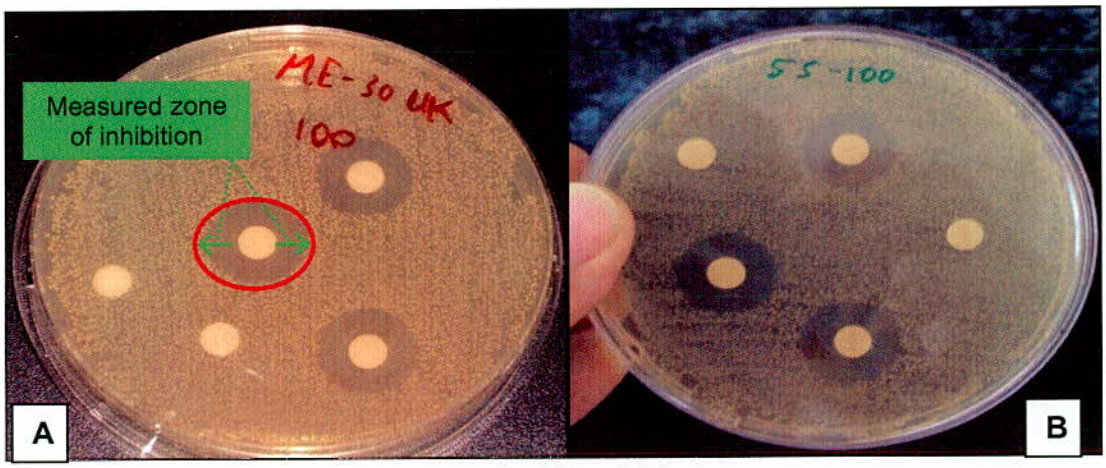

Figure 4.3: Disc diffusion test with 10% oleuropein showing inhibition zones against (A) MRSA-ME30, and (B) MSSA-ME5S in TSA incubated at 37°C for 24 h

Experiment was done in duplicate (n = 2)

All strains were also tested in oleuropein supplemented agar (Table 4.3). Having the antimicrobial agent mixed with the molten agar before it solidifies ensures even distribution throughout the agar and increases the exposure of the bacterial cell to the antimicrobial. In contrast to the disc diffusion method, a range of microorganisms may be inoculated on a single plate but only one antimicrobial concentration can be tested per plate. Moreover, additional growth supplements such as calcium, magnesium or sodium chloride can be added into the molten agar (Figure 4.4).

Table 4.2: Inhibition zones (in mm) of microorganisms in the presence of 1, 2, 5 and 10% oleuropein in TSA at 37°C for 24 h as determined by the disc diffusion method

	Oleuropein concentration in %			
Microorganisms	**1**	**2**	**5**	**10**
Bacillus subtilis NCIMB 2591	0	0	0	0
Candida albicans NCYC 597	0	0	13.5	22.5
Enterococcus faecalis ATCC 29212	0	0	0	ND
Escherichia coli ATCC 25922	0	0	0	8.5
Escherichia coli NTCC 8020	0	0	0	10.5
Micrococcus luteus NCIMB 196	0	0	0	13.5
Pseudomonas aeruginosa NCIMB 950	0	0	0	ND
Staphylococcus aureus MRSA (ME-30)	0	0	16	24
Staphylococcus aureus MRSA (ME-80)	0	0	0	ND
Staphylococcus aureus MSSA (ME-5S)	0	0	18	26.5
Staphylococcus aureus NTCC 6571 (MSSA)	0	0	0	9
Staphylococcus aureus ATCC 29213 (MSSA)	0	0	0	17
Staphylococcus epidermidis ATCC 35984	0	0	0	15.5
Group A *Streptococcus* 10	0	0	0	ND
Group A *Streptococcus* 104	0	0	0	ND
Streptococcus pyogenes	0	0	0	ND
Inhibition zone in diameter (mm) ; ND not determined; Results represent means of two experiments (n = 2)				

Table 4.3: Inhibition of microbial growth on Mueller Hinton Agar supplemented with 0.25, 0.5, 1.0, 1.5 and 3% oleuropein and incubated at 37°C for 24 h

	Oleuropein concentration in %				
Microorganisms	**0.25**	**0.5**	**1**	**1.5**	**3**
Bacillus subtilis NCIMB 2591	+	+	+	+	+
Candida albicans NCYC 597	+	+	+	+	+
Enterococcus faecalis ATCC 29212	-	-	-	-	-
Escherichia coli ATCC 25922	+	+	+	+	+
Escherichia coli NTCC 8020	+	+	+	+	+
Micrococcus luteus NCIMB 196	+	+	+	+	ND
Pseudomonas aeruginosa NCIMB 950	+	+	+	+	ND
Staphylococcus aureus MRSA (ME-30)	+	+	+	-	-
Staphylococcus aureus MRSA (ME-80)	+	ND	+	ND	-
Staphylococcus aureus MSSA (ME-5S)	+	-	-	-	-
Staphylococcus aureus NTCC 6571 (MSSA)	+	-	-	-	-
Staphylococcus aureus ATCC 29213 (MSSA)	+	+	+	-	-
Staphylococcus epidermidis ATCC 35984 (MSSA)	+	-	-	-	-
Group A *Streptococcus* 10	-	-	-	-	-
Group A *Streptococcus* 104	+	+	+	ND	-
Streptococcus pyogenes	+	-	ND	ND	-
+ Growth; - No Growth; ND not determined; Results represent two experiments (n = 2)					

As shown in Table 4.3, oleuropein was active on bacteria at concentrations of 0.25-3.0%. MICs were 0.25% for *Enterococcus faecalis* and Group A *Streptococci* 10. The MICs for all MSSA strains except MSSA-29213 (MIC 1.5%) as well as *S. pyogenes* were 0.5% whereas MRSA-ME30 showed a MIC of 1.5%. In 3% oleuropein supplemented agar, *E. faecalis,* Group A *Streptococci, S. pyogenes, S. aureus*, and *S. epidermidis* were inhibited. The most sensitive organisms were *E. faecalis* and Group A *Streptococcus* 10 at 0.25% oleuropein. Among the five *S. aureus* isolates tested, variability in the level of susceptibility was noted. Two MSSA (NCTC 6571, ME-5S) were inhibited by 0.5% oleuropein. The remaining three isolates (two MRSA, one MSSA) required at least 1.5% oleuropein to inhibit growth (Figure 4.4).

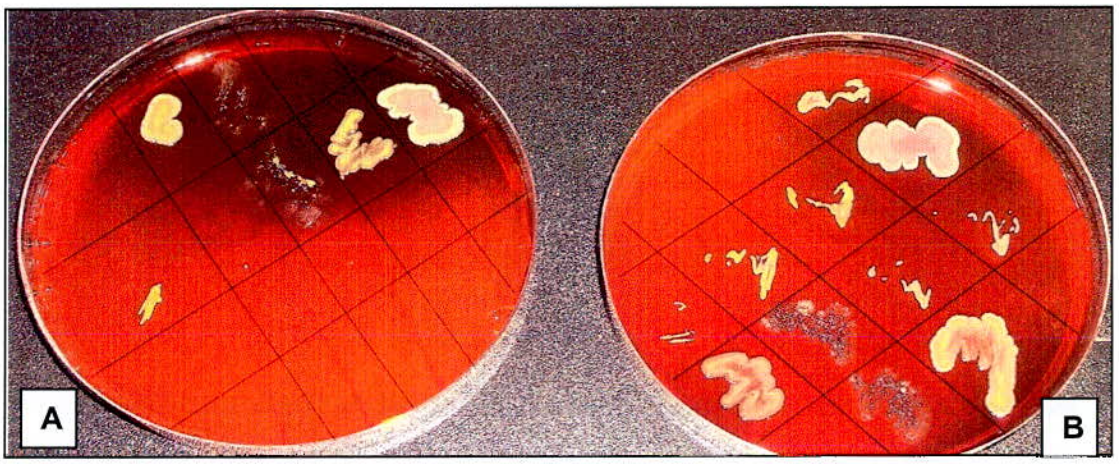

Figure 4.4: Inhibition of growth of 16 bacteria on MHA supplemented with (A) 3%, and (B) 0.5% oleuropein incubated at 37°C for 24 h

Experiment was done in duplicate (n = 2)

The results in Table 4.2 differ from the results in Table 4.3. The lower concentrations of oleuropein required to inhibit microorganisms in the oleuropein agar plate indicated that antimicrobial activity was lost in the disc diffusion test.

4.4 Bacterial killing studies

Time kill studies have the advantage of quantifying cell survival after antimicrobial treatment. Moreover, they determine living cells only, whereas in the spectrophotometric techniques dead cells are detected as well. Time kill studies also offered the opportunity to measure antimicrobial activity without growth as a buffer solution was used and replication of cells was avoided. Oleuropein concentrations of 0.5, 1 and 2% were used and the bacteria exposed for 0, 1, 2, 4, 6 and 24 h. Bacterial time kill studies were undertaken on MRSA strains ME-30, ME-80, MSSA strains ME-5S and MSSA 29213 in duplicate and the average of three individual experiments for MRSA-ME30 and MSSA 29213 and two individual experiments for MRSA-ME80 and MSSA-ME5S was used in Figure 4.5.

As shown in Figure 4.5A, numbers of MSSA 29213 were reduced by up to 6 log cfu mL^{-1} within 6 h of exposure to 2% oleuropein whereas 1% and 0.5% resulted in a 4 and 3 log cfu mL^{-1} reduction after 8 h, respectively. Viable numbers of MRSA-ME30 were reduced by up to 6 log cfu mL^{-1} within 4 h of exposure to 2% oleuropein. After 6 h in the presence of 1 and 0.5% oleuropein, numbers were reduced by 5 and 4 log cfu mL^{-1}, respectively (Figure 4.5C). For MSSA-ME5S, a reduction of over 5 log cfu mL^{-1} occurred within 4-5 h of exposure to 2% and 1% oleuropein whereas a reduction of over 4 log occurred after 6 h exposure with 0.5% oleuropein (Figure 4.5B). MRSA-ME80 showed a reduction in viable counts of 5 log cfu mL^{-1} after 6 h at 2 and 1% oleuropein and 4 log cfu mL^{-1} with 0.5% oleuropein (Figure 4.5D). The most susceptible strain to oleuropein was MSSA-ME5S and the most resistant was MSSA 29213.

In all bacterial killing assays, pin-prick colonies were observed after 2-4 hours at 37°C. These colonies were characterised by their very small size compared to colonies in the control plate and were ultimately killed at a later time point. These colonies were isolated and streaked on a fresh MHA plate to reveal any differences in visible growth or colony colour. However, the second generation colonies were similar in size and shape as well as colour to the original colony. Further studies on these colonies were considered and described in Chapter 5.

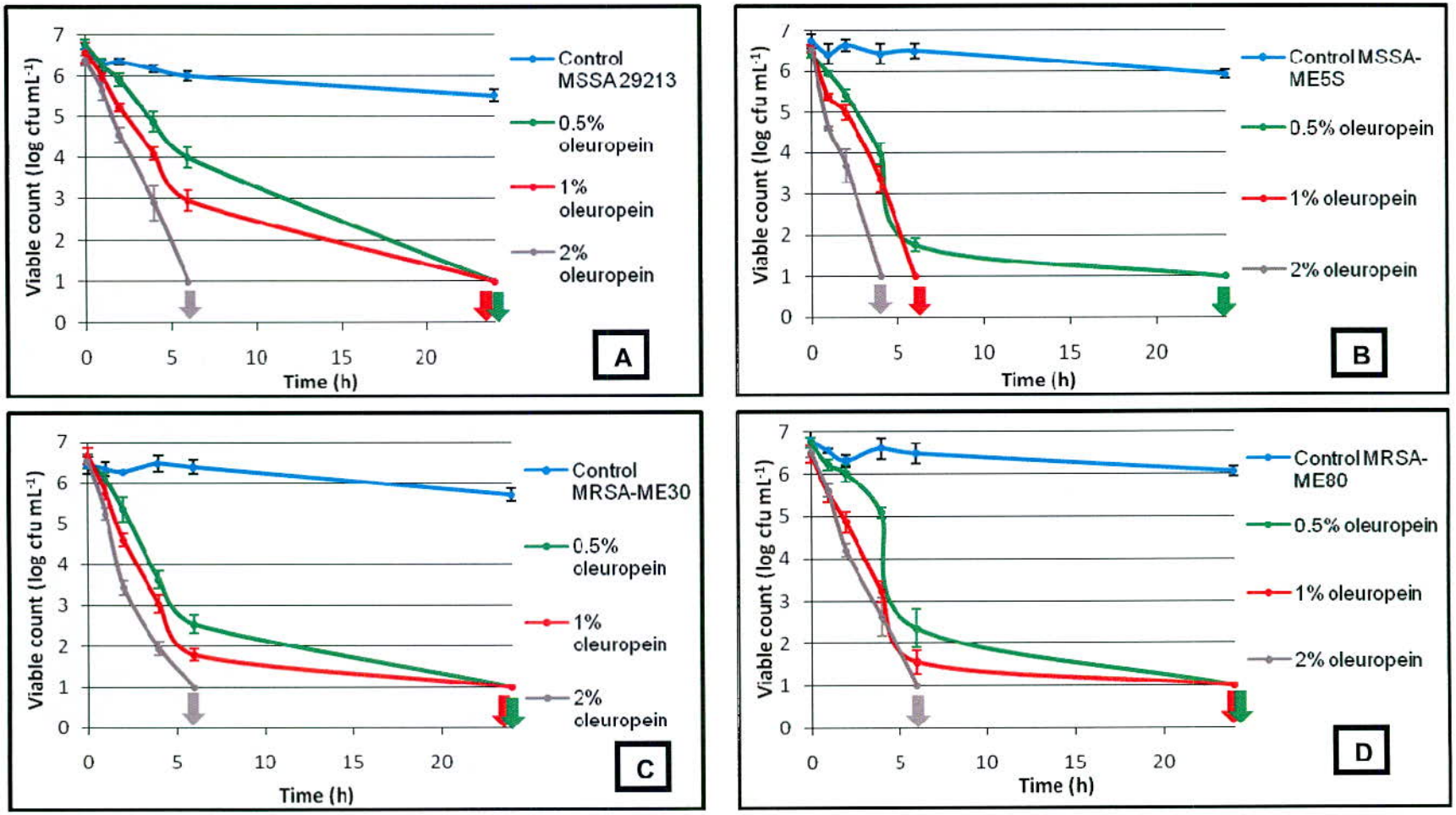

Figure 4.5: Inactivation of MSSA 29213 (A), MRSA-ME5S (B), MRSA-ME-30 (C) and MRSA-ME80 (D) in PBS (0.1M pH 7.4) at 37°C in the presence of 0.5, 1 and 2% standard oleuropein

Sensitivity limit was 1 log cfu mL^{-1}; standard error bars are based on standard deviation values (error bars = ±SD) and represent three experiments with MRSA-ME30 and MSSA 29213 (n = 3) and two experiments with MRSA-ME80 and MSSA-ME5S (n = 2)

The rates of bacterial reduction were calculated using Logger Pro 3.3 software. A linear trend line was drawn through the data points as shown in Figure 4.6 and the equation shown below was used to calculate the time in which half of the bacterial population was killed.

Equation 3: Formula for calculating time taken for 50% reduction of the bacterial population in the presence of 0.5, 1 and 2% oleuropein

$$x = \frac{(\frac{D}{2}) - b}{m},$$

where D is the starting number of cells (cfu mL^{-1}) at the time point zero, b is the y-axis interception point, m is the slope and x (h) is the time for killing 50% of bacteria.

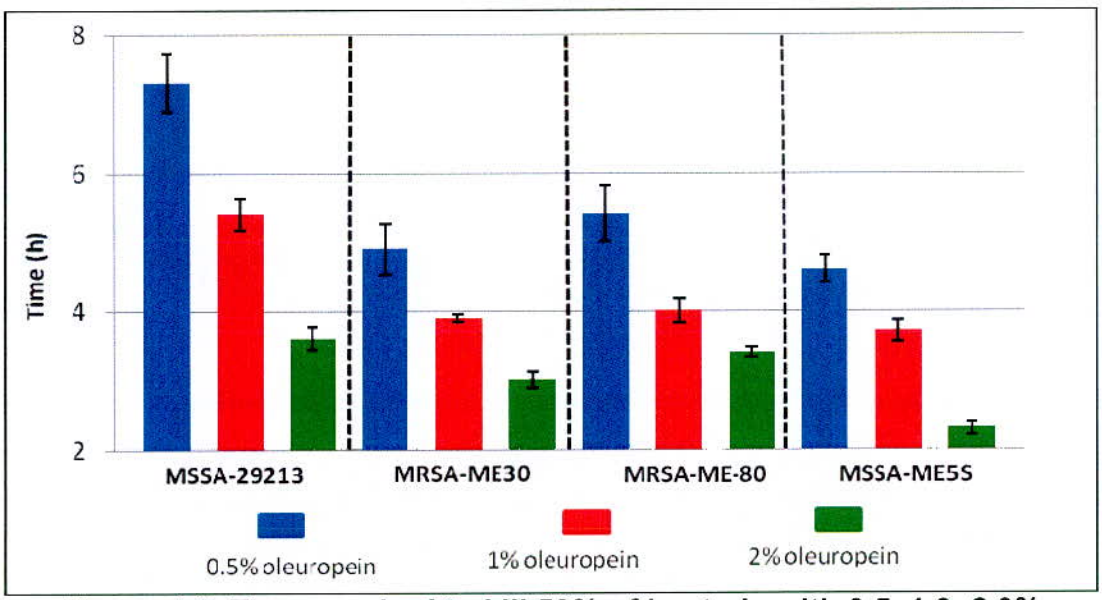

Figure 4.6: Time required to kill 50% of bacteria with 0.5, 1.0, 2.0% oleuropein at 37°C

Standard error bars are based on standard deviation values (error bars = ±SD) and represent three experiments for MRSA-ME30 and MSSA 29213 (n = 3) and two experiments for MRSA-ME80 and MSSA-ME5S (n = 2)

As shown in Figure 4.6, the time to kill 50% of MSSA and MRSA with 2% oleuropein ranged between 2.3 and 3.6 h. The use of lower concentrations of

oleuropein (1 and 0.5%) extended the time to 3.7-5.4 h and 4.6-7.3 h, respectively. MSSA 29213 was the most resistant and MSSA-ME5S was the most susceptible strain to oleuropein.

Growth of MRSA in MHA and MHB is often supplemented with 2% NaCl. Therefore, the bacterial killing assay was undertaken in the presence of 2% NaCl. The bacteria were grown overnight in MHB supplemented with 2% NaCl and viable counts were determined on MHA containing the same percentage of salt. The pH of a control containing salt and oleuropein but no bacteria was monitored at 37°C.

The pH of oleuropein suspended in PBS diminished with time. A 2% oleuropein solution at a pH of 7.2 and 6.9 with 2% NaCl in the buffer resulted in pH 6.2 and 6.1 after 24 h incubation at 37°C, respectively (Figure 4.7 A & B). This indicated a drop of one pH unit without salt and 0.8 pH units with salt at 37°C within 24 h. The pH of 0.5 and 1% oleuropein solutions was reduced from between 0.4 and 0.7 pH units both with and without salt. Whilst the drop in pH was substantial, the extent of reduction in pH was not greatly affected by the presence of salt.

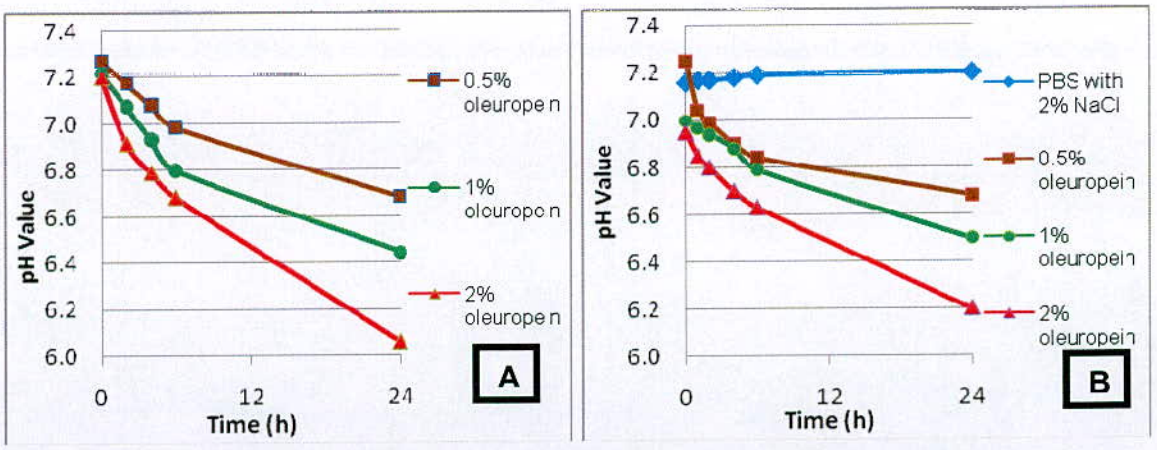

Figure 4.7: pH changes in oleuropein from Extrasynthese (France) in 0.1 M phosphate buffered saline (pH 7.4) in (A) and supplemented with 2% NaCl (B) at 37°C

Experiment was done ones only

The use of 2% NaCl in the growth medium resulted in almost equal time kill curves to those without salt (Figure 4.8). A reduction of 50% in viable counts was calculated using the Logger Pro 3.3 software (Table 4.4). The time to kill half of the bacterial population of MRSA and MSSA with 2% oleuropein was 2.90-3.5 h and 3.7-5.0 h with 1% oleuropein. A 0.5% concentration of oleuropein needed 4.6-8.0 h to kill 50% of the microorganisms. As in section 4.1.5, the most susceptible strain was MRSA-ME5S and the most resistant was MSSA 29213.

Table 4.4: Time required to kill 50% of bacteria with 0.5, 1.0, 2.0% oleuropein at 37°C (A) with 2% NaCl in MHA and MHB (B) without NaCl using the linear equation

	Oleuropein concentrations		
A ***Bacteria***	***0.5%***	***1%***	***2%***
MSSA 29213	8.0 h	5.0 h	3.0 h
MRSA ME30	5.1h	4.1 h	2.9 h
MRSA ME80	4.8 h	4.3 h	3.5 h
MSSA ME5S	4.6 h	3.7 h	2.9 h
B ***Bacteria***	***0.5%***	***1%***	***2%***
MSSA 29213	7.3 h	5.4 h	3.6 h
MRSA ME30	4.9 h	3.9 h	3.0 h
MRSA ME80	5.4 h	4.0 h	3.4 h
MSSA ME5S	4.6 h	3.7 h	2.3 h

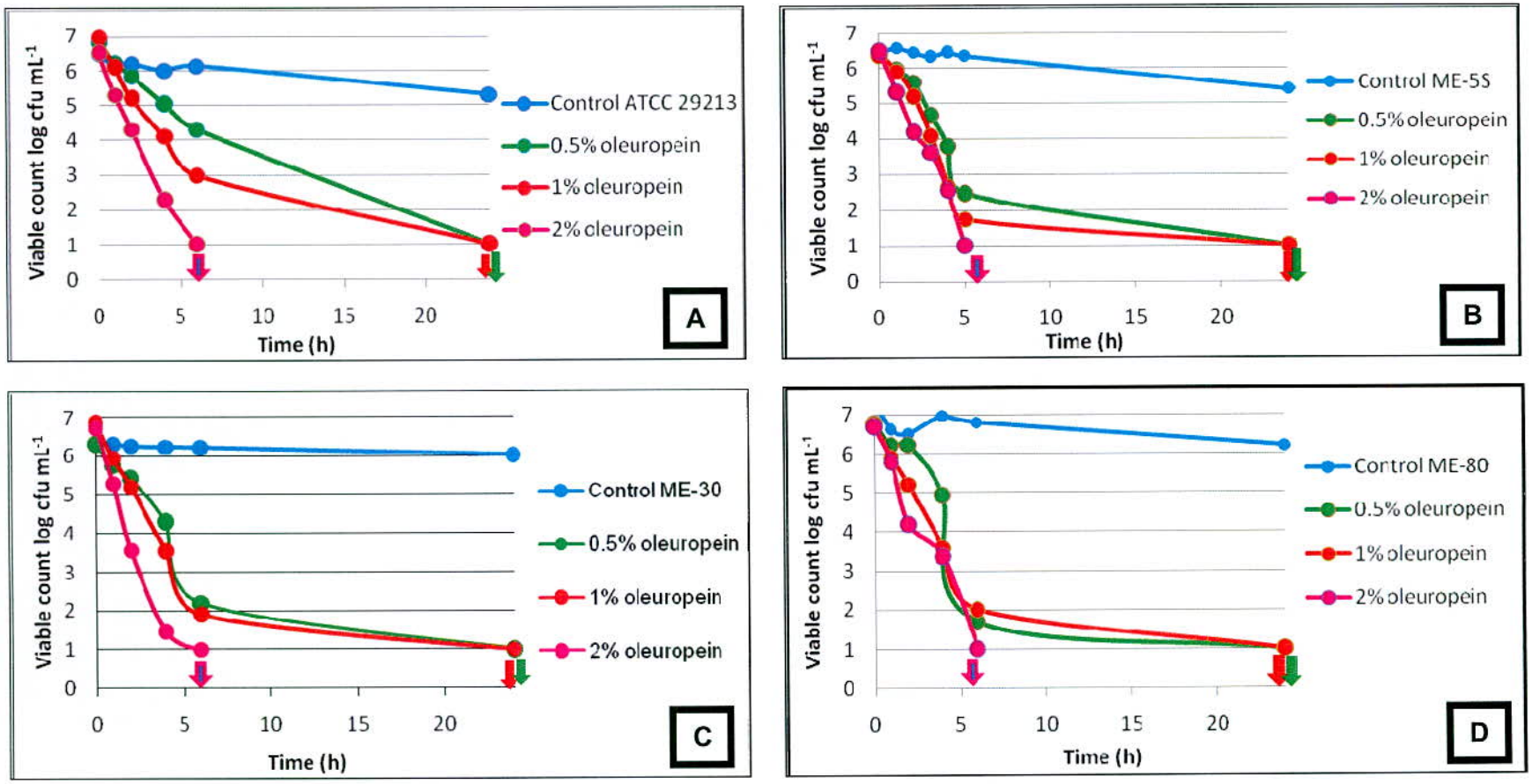

Figure 4.8: Inactivation of MSSA 29213 (A), MSSA-ME5S (B), MRSA-ME30 (C) and MRSA-ME80 (D) in PBS (0.1M pH 7.4) at 37°C in the presence of 0.5, 1 and 2% oleuropein and 2% NaCl in the growth medium MHA and MHB

Sensitivity limit was 1 log cfu mL^{-1}; experiment was done ones only

4.5 Discussion

In this work the antimicrobial potential of commercial oleuropein was investigated against a wide range of microorganisms including methicillin resistant *Staphylococcus aureus.* The effect of oleuropein on antibiotic resistant strains has not been investigated extensively in the past.

The initial work focused on the growth inhibitory properties of oleuropein using spectrophotometric methods but the results were inconclusive due to interference by coloured byproducts of oleuropein oxidation, splitting the compound into elonic acid and hydroxytyrosol. Therefore, a range of other techniques was considered to circumvent the colour formation caused by oleuropein. Previous works with oleuropein may have taken into account oleuropein interference with absorbance readings (Furneri *et al.,* 2002; Pereira *et al.,* 2007). The use of 660 nm as the absorbance wavelength decreased the interference of oleuropein and might be incorporated in rapid absorbance methods depending on the study purpose.

The disc diffusion test was chosen to distinguish between strains susceptible and resistant to oleuropein. Although the disc diffusion test showed an inaccurate picture of the inhibition of bacterial growth by oleuropein, it supported the initial selection of resistant and susceptible microorganisms. The diffusion of liquid oleuropein into the agar was probably restricted because of the absorption of the compound into the paper discs thereby reducing the antimicrobial activity. Furthermore, the incubation temperature of 37°C might have desiccated oleuropein on the disc and consequently diminished the diffusion rate into the agar.

The results of the disc diffusion test have shown that 10% oleuropein inhibited the growth of mainly Gram-positive strains. The results shown in Table 4.3 illustrated the antimicrobial activity on Gram-positive (MSSA and MRSA) strains with the agar plate method. In accordance with Hugo & Bloomfield (1971a), the antimicrobial effect on Gram-negative strains was less, possibly because of adsorption of the antimicrobial agent in the cell wall, which contains larger amounts of lipids than in Gram-positives.

The results represented in Table 4.2 & Table 4.3 show discrepancies in the inhibitory concentration of oleuropein. In the disc diffusion test, inhibition of *C. albicans*, MRSA-ME30 and MSSA-ME5S was observed with an oleuropein concentration of 5% but this might have occurred also with a concentration lower than 5%. In the agar plate method, MRSA-ME30 was inhibited with 1.5% and MSSA-ME5S with 0.5% oleuropein. The results from the microtitre plate assay shown in Table 4.1 indicate that a concentration of 0.8% oleuropein inhibited the growth of MRSA-ME5S whereas MRSA-ME30, ME80 and MSSA 29213 needed 2.5% oleuropein. The composition of the growth medium TSA used in the disc diffusion test and the microtitre plate assay might have affected the results. These results demonstrate the variation possible when different methods for determining antimicrobial activity are used.

Bisignano *et al* (1999) tested the antimicrobial effect of oleuropein using paper discs and reported MICs ranging from 31.25-125 μg mL^{-1} (0.003-0.01%) oleuropein against penicillin resistant *S. aureus* strains. The low MIC values by Bisignano *et al* (1999) may have been achieved because of the use of dimethylsulphoxide

(DMSO) as a solvent, which probably contributed to the antimicrobial action. In this work the disc diffusion test was used to identify sensitive strains and quantify the inhibition zones.

The oleuropein agar plate assay was a satisfactory method to determine MICs of oleuropein. By dissolving oleuropein in the agar medium, the contact between antimicrobial and microorganism was instant. The MIC results ranged between 2.5 for *E. faecalis* and 15 mg mL^{-1} for MRSA-ME30, respectively. Sudjana *et al* (2009) reported MICs achieved with olive leaf extract using the microdilution assay of 250 mg mL^{-1} for *E. faecalis* and 8 to 125 mg mL^{-1} for MRSA. However, the oleuropein MIC values were high compared with MICs for antibiotics that are often in lower figures (µg mL^{-1}). Oleuropein might be adversely affected by constituents in the growth medium and constrained in its activity. Both MRSA and MSSA strains showed susceptibility to oleuropein and further studies were undertaken using these microorganisms.

Selected MRSA and MSSA strains were subjected to time kill studies. The results show that 2% oleuropein needed 2.3-3.6 h to kill 50% of these microorganisms. Lower concentrations of oleuropein (1 and 0.5%) required between 3.7-5.4 h and 4.6-7.3 h, respectively. Similar half-times were reported for tea tree oil, where the antimicrobial activity against *staphylococci* showed a log 5 and a log 6 reduction in viable cells after 2 h with 0.5% tea tree oil (Carson *et al.*, 2002; Cox *et al.*, 2000).

The time kill curves presented in Figure 4.5 exhibit a prolonged shoulder in the first 6 h of exposure to oleuropein. The shoulder may represent the time needed to break into the cell barrier. With the decline in cell size at certain time points, it is

possible that microorganisms switch to minimal cell activities but this theory needs to be confirmed. The assumption that oleuropein leads to the formation of small colony variants was questioned in the following chapter.

Supplementation of salt in the growth medium has not improved the antimicrobial activity of oleuropein. Kloos & Lambe (1991) used salt in proportions of 5 and 6.5% against a range of *S. aureus* strains without inhibiting growth. Overall, similar time kill rates were obtained without or with 2% NaCl in the growth medium. *S. aureus* strains are salt tolerant and at pH values above 5 showed no change in viable count (Bergdoll, 1989). The possible pH range of *S. aureus* has been tested from pH 4.5 to 9.3 and an optimum pH of 7 to 7.5 was demonstrated (Bergdoll, 1989). However, the organism's sensitivity to acids increased with an increase in temperature (Bergdoll, 1989). Therefore, the reduction in pH to 6 at 37°C for 24 h shown in Figure 4.7 can be neglected when pondering possible synergy mechanisms with NaCl. Tassou & Nychas (1995) investigated synergy mechanisms of oleuropein with NaCl with conductance measurements and reported that no synergy occurred by combining NaCl (0.5% w/v) and oleuropein (0.2%).

Unlike hydroxytyrosol, eugenol and thymol, oleuropein is soluble in water. This has the advantage of allowing measurement of the antimicrobial activity without interference from a solvent, such as ethanol. Solubility in water is an economical advantage in the possible use of oleuropein as an additive in detergents and cleaning agents. However, the rather long contact time required would hamper the usage of oleuropein in such a product. The activity of oleuropein may be improved

with an additional agent used in synergy, such as ethylene-diamine-tetraacetic-acid (EDTA). EDTA is a well known chelating agent binding to calcium and magnesium groups present in the bacterial cell wall. Russell & Chopra (1996) reported that the use of EDTA led to the binding of cations and released 50% of the lipopolysaccharide molecules causing non-polar phospholipids associated with the inner membrane to be exposed at the cell surface so that hydrophobic molecules can enter the cell. EDTA potentiated the antimicrobial activity of eugenol and thymol against *S. aureus*, *E. coli* and *P. aeruginosa* as illustrated with a delay in the lag phase (Walsh *et al.,* 2003). However, the delay in growth appeared to be concentration dependent and subsequent growth using eugenol and thymol occurred.

With respect to oleuropein, the hydrophilic and hydrophobic properties of the molecule may have the potential to kill the bacterial cell and eventually reduce the contact time when applied in synergy with EDTA. Bacterial time kill studies have the advantage of calculating viable cells rather than dead cells. Overall the plate assay is the most effective and reliable way to obtain results.

Oleuropein was effective against a range of microorganisms including MRSA and MSSA. The response of oleuropein against these bacteria was dose and time dependent ranging from 0.5 to 5%. Compared with the literature, oleuropein had higher MIC values in this study. However, the antimicrobial activity of oleuropein against these bacteria has been confirmed and further substantiated in chapter 5.

4.6 Conclusions

The work in this chapter on the antimicrobial effect of oleuropein against 15 bacteria and one yeast including antibiotic resistant bacteria such as MRSA has shown that the natural compound oleuropein from the olive plant can be used to kill a wide range of bacteria. Oleuropein was bactericidal against methicillin sensitive *Staphylococcus aureus* (MSSA) and methicillin resistant *Staphylococcus aureus* (MRSA). The viable numbers of MSSA 29213, MRSA-ME30, MSSA-ME5S and MRSA-ME80 were reduced by 5 log cfu mL^{-1} in the presence of 2% oleuropein after 4-6 hours of exposure.

If the diffusion time of oleuropein through the cell wall of Gram-positive and Gram-negative bacteria could be reduced, for instance by a synergy mechanism, the application of oleuropein in cleaning agents for medicinal and cosmetic use might be considered. However, this requires further research work in model systems and in real product formulations. For improving the effectiveness of oleuropein against bacteria, more information on the mode of action is necessary.

Chapter Five

5 Mode of antimicrobial action of oleuropein

5.1 Introduction

The mode of action of oleuropein against bacteria was described in the literature as affecting the cell membrane leading to leakage of cell constituents (Juven *et al.*, 1972). However, information is lacking on destruction of particular cell wall constituents that are sensitive to oleuropein. In this chapter, a wide range of cell wall assays were undertaken to research the mode of action of oleuropein against Gram-positive bacteria.

The anticipated antimicrobial mechanism of oleuropein can be explained in following steps: i) Contact of oleuropein with microorganism; ii) adherence and adsorption into the lipid bilayer; iii) disruption of essential cell wall conjunctions; iv) disintegration of cell wall blocks v) Leakage of intracellular constituents due to the fall in osmotic pressure; vi) cell death.

Leakage of low molecular weight compounds was measured in this chapter. Another element measured was protein leakage. Proteins are cell wall components and are important determinants of the surface properties of cells (Hammond *et al.*, 1984). Furthermore, lectins were used to detect oleuropein damage of glycoproteins, which are located on the exterior of the cell membrane. Transmission electron microscopy (TEM) of treated MSSA and MRSA cells was also undertaken.

5.2 Leakage of low molecular weight compounds

Low molecular weight metabolites include nucleotide components such as purines, pyrimidines, pentoses and inorganic phosphate as well as amino acids and inorganic ions (Johnston *et al.,* 2003). By measuring these components, the antimicrobial action of oleuropein would be linked to cell wall damage. The presence of purines and pyrimidines can be detected by ultraviolet absorbance at 260 nm (Johnston *et al.,* 2003). However, most phenolic compounds including oleuropein also absorb at 260 nm wavelength. As shown in Figure 3.2, absorbance maxima of oleuropein occur at 232 and 280 nm. Therefore, it was anticipated that the measurement of leakage of low molecular weight compounds using spectrophotometric techniques may be subject to some interference.

Charcoal is an adsorbent agent used in various fields to separate interfering substances from a suspension. In order to reduce interference, 0.2% charcoal was added to the cell free supernantant of oleuropein treated cells. After a short time (3-5 min) of gentle mixing the suspension was centrifuged and the charcoal pellet was removed. In addition, the cell-free supernatant was passed through a sephadex gel column before UV measurement.

Figure 5.1 shows the UV absorbance of cell-free supernatant of MRSA-ME30 before and after oleuropein exposure. The results demonstrate that the use of charcoal and sephadex treatment to remove absorbance interference caused by oleuropein was insufficient. As expected, the control without oleuropein indicated that MRSA-ME30 cells showed minor leakage between 0 and 6 h but enhanced leakage after 24 h. Since it was not possible to eliminate or control for absorbance interference by oleuropein, UV spectrophotometric methods were abandoned.

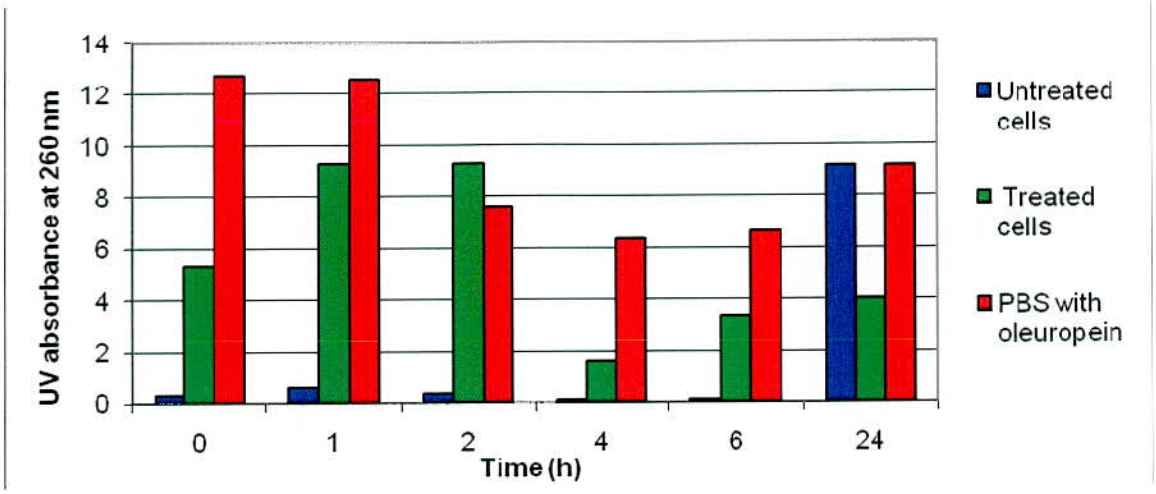

Figure 5.1: UV absorbance of cell free supernatant from MRSA-ME-30 exposed to 1% oleuropein in PBS at 37°C for 24 h
Experiment was done ones only

5.3 Leakage of proteins and amino acids

In order to measure the leakage of proteins from cells, two different methods were considered: the Bradford assay and the ninhydrin test.

The Bradford assay is a colourimetric assay and one of the most widely used methods for the quantification of proteins (Thomas & McNamee, 1990). With a shift of the absorbance maximum from 465 to 595 nm caused by the Bradford dye complex, the protein content was quantified as the amount of absorbance proportional to the protein present. Using bovine serum albumin (BSA), a standard curve for the Bradford assay was prepared.

Figure 5.2 shows the leakage of proteins from MRSA-ME30 when left untreated and when treated with 1% oleuropein for up to 24 h. Absorbance readings of the cell-free supernantant were corrected using absorbances from the PBS controls. The amount of protein leaking out of MRSA-ME30 treated with oleuropein after 24 h was around 40 µg compared to 20 µg in the control. The extent of protein

leakage after six hours was similar in the treated and untreated cells. The increase in absorbance of treated cells showed that oleuropein caused leakage of proteins. Proteins leaked slowly but steadily from untreated cells whereas in treated cells the protein leakage commenced after two hours with a rapid increase.

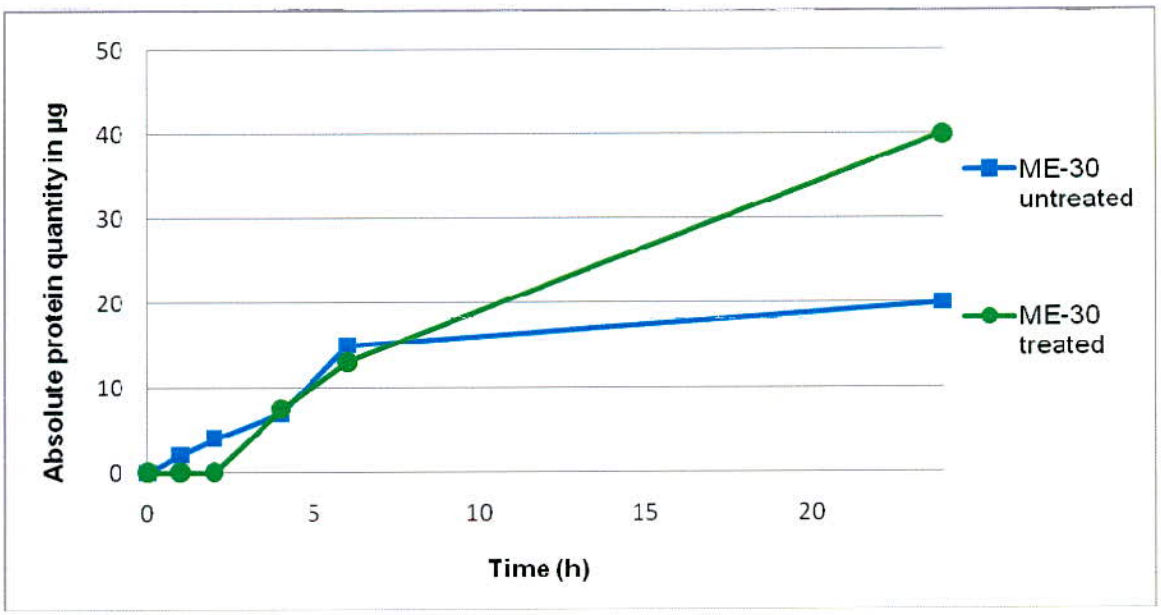

Figure 5.2: Release of protein from MRSA-ME30 exposed to 1% oleuropein in PBS (0.1 M, pH 7.4) at 37°C for up to 24 h

Experiment was done ones only

In the ninhydrin test, proteins are subjected to acid hydrolysis and the total amino acids released are measured using the ninhydrin reagent (Marks *et al.,* 1985). Experiments with a range of proteins have demonstrated that the release of total amino acids was linearly related to the original amount of protein present (Marks *et al.,* 1985; Starcher, 2001).

Starcher (2001) showed that the ninhydrin test was several times more sensitive than the Bradford assay by comparing different proteins including haemoglobin, myoglobin, lysozyme, tyrosine, albumin and gelatine. The individual amino acids of a protein can be liberated by hydrolysing the peptide bonds with an acid or heat.

The reaction formed between a primary amino group and the ninhydrin reagent forms the coloured product called Ruhemann's purple (Prochazkova *et al.*, 1999). The reaction is well known and has been studied for years and is extensively used for amino acid analysis. In this work, the ninhydrin reagent was added to the cell-free supernatant of cells treated with 1% oleuropein. Amino acid contents were compared with a standard curve prepared with aspartic acid.

As shown in Figures 5.3 and 5.4, the amino acid content of cell-free supernatant of the untreated MRSA-ME30, MRSA-ME80, MSSA 29213 and MSSA-ME5S were 175.8, 164.1, 197.3 and 263.8 μg mL^{-1}, respectively. Oleuropein-treated (1%) cell-free supernatants had an average amino acid content of 154.7 (MRSA-ME30), 132.8 (MRSA-ME80), 143.9 (MSSA 29213) and 163.0 (MSSA-ME5S) μg mL^{-1} after 4 h, respectively. In summary, a reduction of 12 to 38% of amino acid leakage in all treated *S. aureus* occurred (12% MRSA-ME30; 19% MRSA-ME30; 27% MSSA 29213; 38% MSSA-ME5S). For comparison, the amino acid contents of cell-free supernatant of *E. coli* ATCC 29212 and *P. aeruginosa* were also determined. The amino acid values for *E. coli* were 82 and 100 μg mL^{-1} for treated cells. The controls of *E. coli* and *P. aeruginosa* showed amino acid contents of 82 and 114 μg mL^{-1} (Figure 5.4).

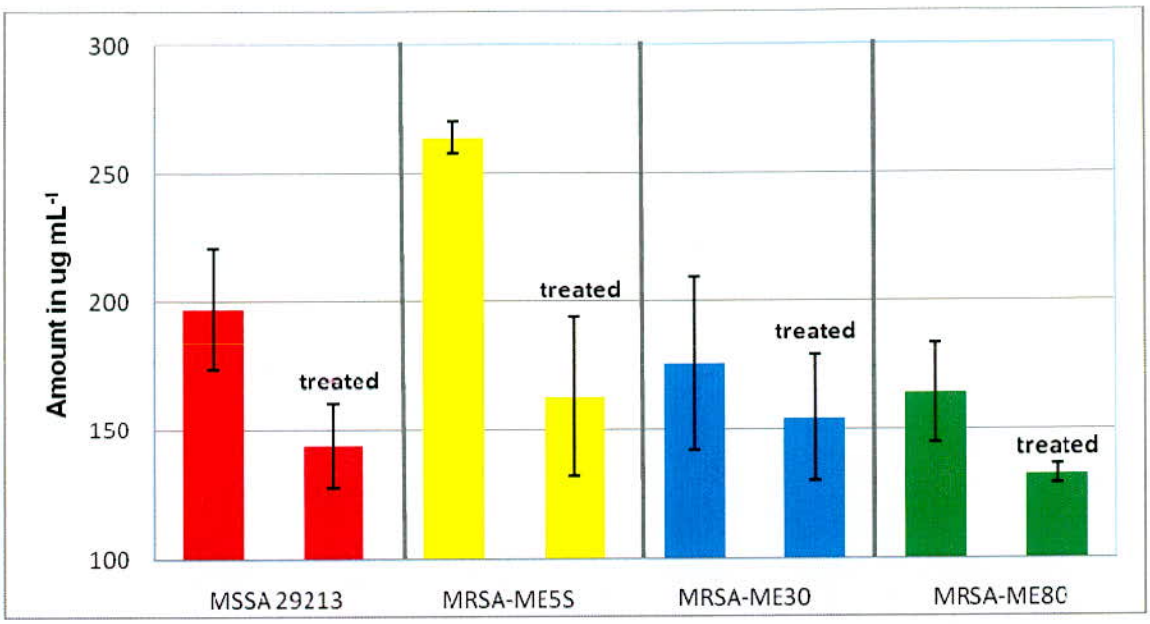

Figure 5.3: Amino acid contents of cell-free supernatant of MRSA and MSSA cells treated with 1% oleuropein at 37°C for 4 h using the ninhydrin test

Standard error bars are based on standard deviation values and represent the means of two experiments (error bars = ±SD; n = 2)

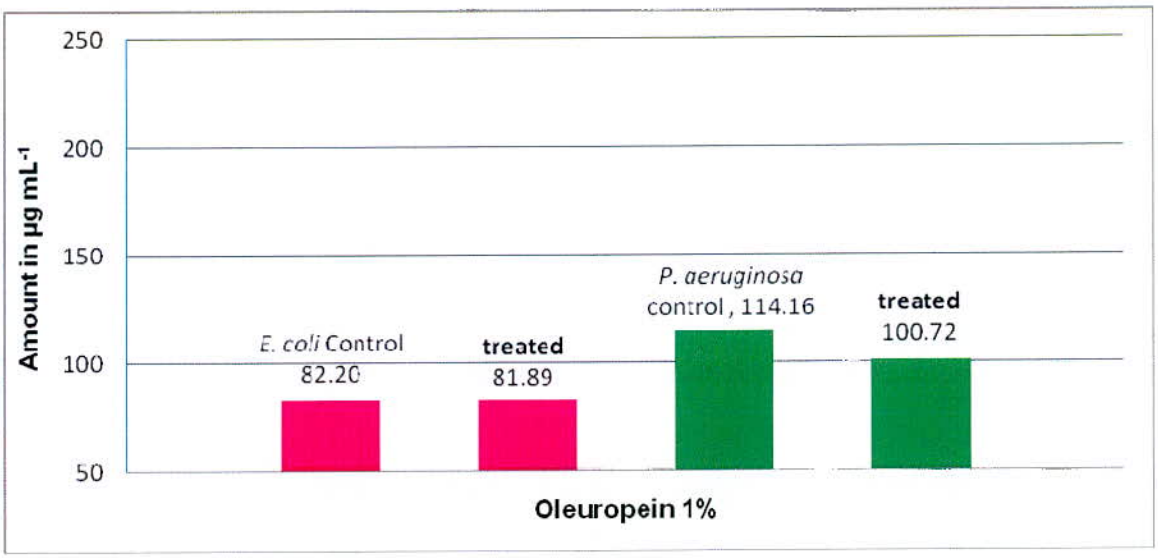

Figure 5.4: Amino acid content of cell free supernatant of *E. coli* and *P. aeruginosa* cells treated with 1% oleuropein at 37°C for 4 h using the ninhydrin test

Experiment was done ones only

In this study, MSSA 29213, MSSA-ME5S, MRSA-ME30, and MRSA-ME80 were treated with 1% oleuropein and cell-free supernatant was assessed for particular band patterns using SDS-PAGE.

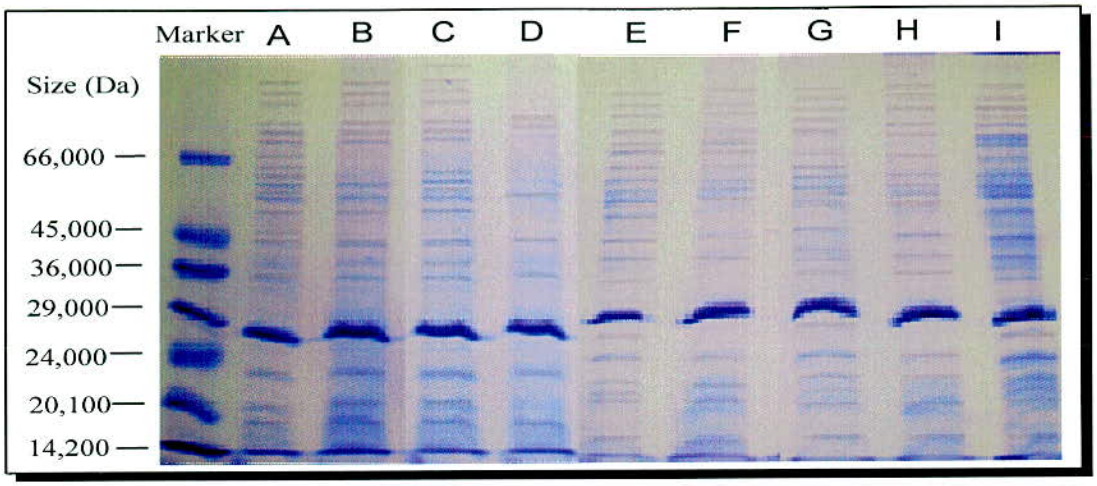

Figure 5.5: SDS PAGE of 25 µL volume for MRSA and MSSA cells treated at 37°C for 4 h with 1% oleuropein in PBS (0.1 M pH 7.4)

Gel was stained with Brilliant Blue G-250
Experiment was done in triplicate

Well Position	Microorganism and treatment
A	Control MRSA-ME80
B	MRSA-ME80 with 1% oleuropein
C	Control MSSA-ME5S
D	MSSA-ME5S with 1% oleuropein
E	Control MSSA 29213
F	MSSA 29213
G	Control MRSA-ME30
H	MRSA-ME30 with 1% oleuropein
I	MRSA-ME30 with 0.5% oleuropein

Marker : Protein	Molecular size in Da
Bovine serum albumin	66,000
Ovalbumin	45,000
Glyceraldehyde-3-phosphate Dehydrogenase	36,000
Carbonic Anhydrase	29,000
Trypsinogen	24,000
Trypsin Inhibitor	20,000
α-Lactalbumin	14,200

As shown in Figure 5.5 starting with MRSA-ME80, the untreated control showed three bands below the 66,000 Da mark and another band at around 45,000 Da. The oleuropein treated cell-free supernatant of MRSA-ME80 was lacking these bands. The MSSA-ME5S control showed four extra bands above the 66,000 Da mark and at least three additional bands above 45,000 Da which were absent in the treated sample. Another band at around 36,000 Da was absent from the

treated MSSA-ME5S sample but present in the control. MSSA 29213 showed bands around 50,000 and 30,000 Da in the control which were lacking in the oleuropein-treated sample. In the case of MRSA-ME30, the proteins found in the control were also found in the treated samples except at 28,000 Da and above 20,000 Da. In addition, protein bands in the 1% oleuropein-treated sample were more faded than in the 0.5% oleuropein sample.

The SDS-PAGE showed that bands from MRSA and MSSA were eliminated within 4 h exposure to 1% oleuropein. In particular, bands in the range of 45.000 and above were absent in the oleuropein-treated sample. Thus heavier proteins seem to be more prone to oleuropein treatment. This result demonstrates the mode of action of oleuropein against MRSA and MSSA. It is proposed that the elimination of certain proteins leads to further disruption of cell wall activity and leakage of cell constituents. Cell wall disruption of these cells was further investigated using transmission electron microscopy described in section 5.5.

5.4 The effect of oleuropein on glycoproteins

Bacteria contain glycoproteins anchored to the exterior of the cell wall. These glycoproteins show affinity towards lectins, which are proteins derived from plants, animals or microorganisms. The lectins attached to glycoproteins can be visualised with the use of a fluorescent reporter via fluorescence microscopy.

The work on glycoproteins was designed to examine if oleuropein caused glycoprotein degradation. It was postulated that the bond between protein within the cell wall and oligosaccharides might be a target for the mode of action of oleuropein. Wheat Germ Agglutinin (WGA) lectin from *Triticum vulgaris* was used

as it was reported that this lectin showed affinity with *S. aureus* (Lotan *et al.,* 1975; Morioka *et al.,* 1987; Slifkin & Doyle, 1990). WGA lectin was initially conjugated with Fluorescein (5) – isocyanate (FITC). An aliquot of conjugated FITC was then inoculated with MSSA 29213, MRSA-ME30 and MRSA-ME5S. After incubation and centrifugation, the cells were examined for glycoproteins using a fluorescence microscope. No fluorescence was observed with any of the strains indicating that the WGA lectin failed to bind with the cell surface glycoproteins.

An alternative method was introduced to investigate amino acid binding by FITC. Maeda *et al* (1969) demonstrated that FITC reacted with alpha amino groups (below pH 9.5) and epsilon amino groups (above pH 9) of neocarzinostatin (protein of an antibiotic) and insulin forming non covalent bonds. This allows the observation of FITC conjugated cells using fluorescence microscopy. MSSA 29213 and MRSA-ME80 were treated with 1% oleuropein followed by FITC conjugation before inspection by fluorescence microscopy.

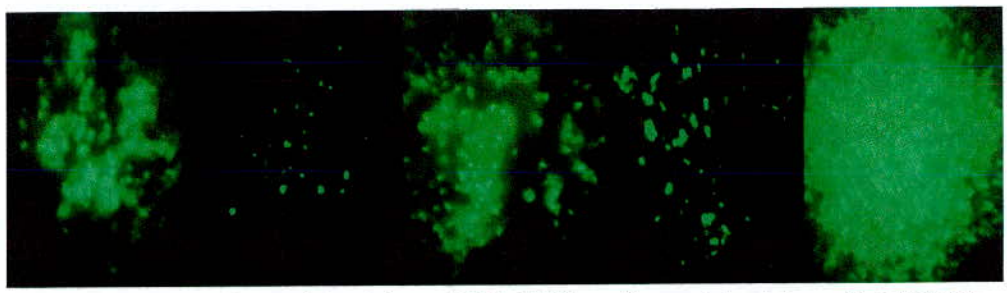

Figure 5.6: Fluorescence microscopy images of fluorescein (5)- isocyanate conjugation of alpha & epsilon amino groups in MSSA 29213, 1% oleuropein treated MSSA 29213, MRSA-ME80 and *S. epidermidis*

Experiment was done in triplicate

As shown in Figure 5.6, the brightness of 1% oleuropein-treated MSSA 29213 was lower compared to the control strain and the distance between fluorescence particles seemed to be wider in the treated sample. MRSA-ME80 cells without treatment displayed similar fluorescence images to MSSA 29213 but showed a higher intensity of fluorescence in the oleuropein-treated sample compared to oleuropein-treated MSSA 29213 cells. *S. epidermidis* ATCC 35984 is a biofilm former and was included as positive control because of its enhanced glycocalyx layer, which displayed extended fluorescence. The intensity of fluorescence reflected the binding of FITC particles with amino groups. The lower intensity of fluorescence of oleuropein-treated samples indicates a disruption of these molecules.

5.5 Transmission electron microscopy of oleuropein treated MSSA and MRSA

Transmission Electron Microscopy (TEM) was used to investigate morphological damage to cells of MRSA-ME30 and MSSA 29213 exposed to oleuropein. Cells were treated with 2% oleuropein and examined after 0, 2, 4 and 6 h.

The results in Figures 5.7 & 5.8 indicate that partial cell lysis occurred within two hours of exposure to 2% oleuropein for MRSA-ME30 and four hours for MSSA 29213. Figure 5.7 shows TEM images of MSSA 29213 cells incubated at 37°C in phosphate buffer for up to 6 h with and without the presence of 2% oleuropein.

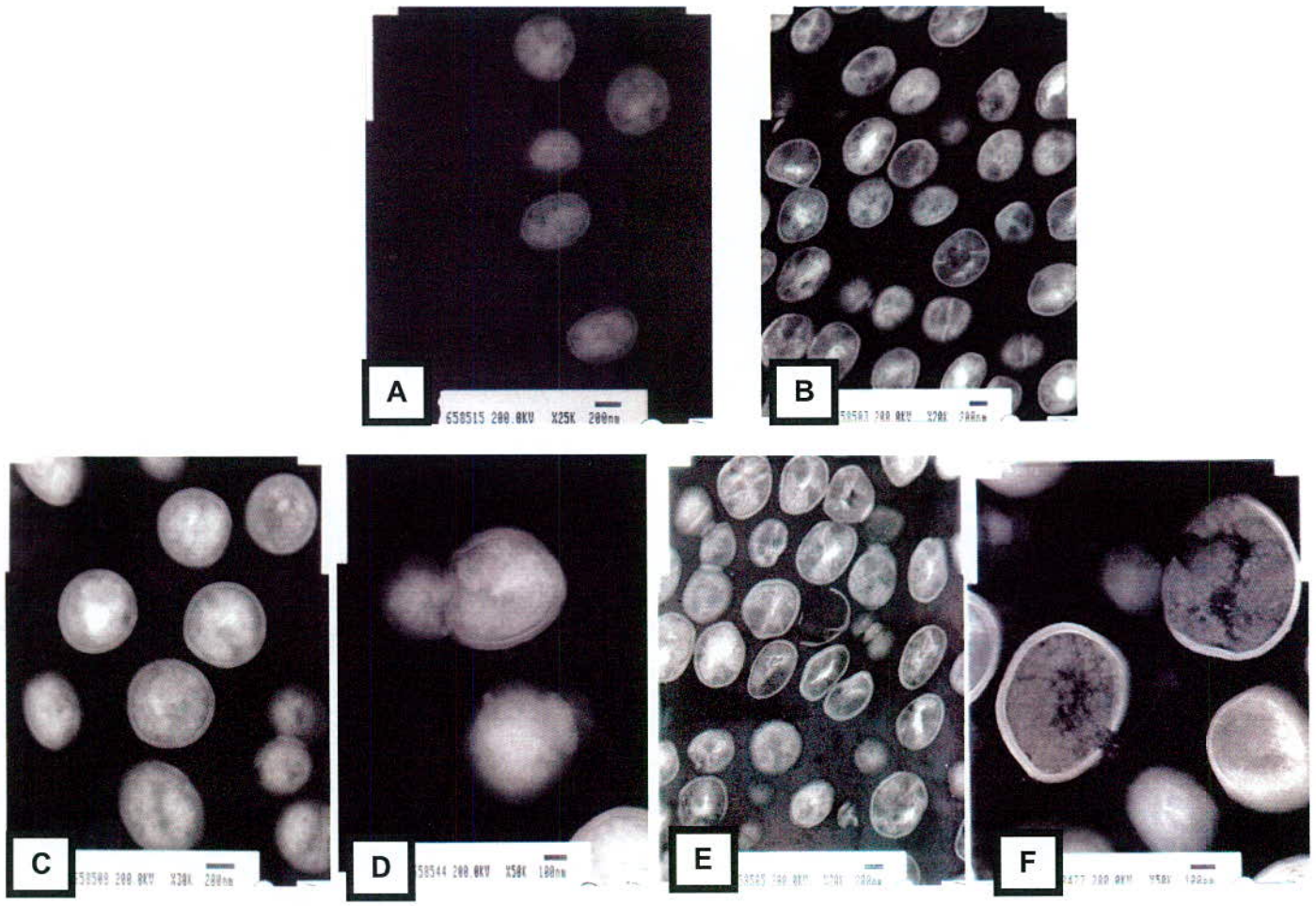

Figure 5.7: Transmission Electron Micrographs of MSSA 29213 in phosphate buffer (0.1 M pH 7); A: Control 4 h x25,000; B: Control 6 h x20,000; C: 2% oleuropein treated 2 h x30,000; D: 2% oleuropein treated 4 h x50,000; F: 2% oleuropein treated 6 h x20,000; G: 2% oleuropein treated 6 h x50,000; Scale bar = 200 nm for A, B, C & E; Scale bar for D and F = 100 nm ▬

Experiment was done in triplicate

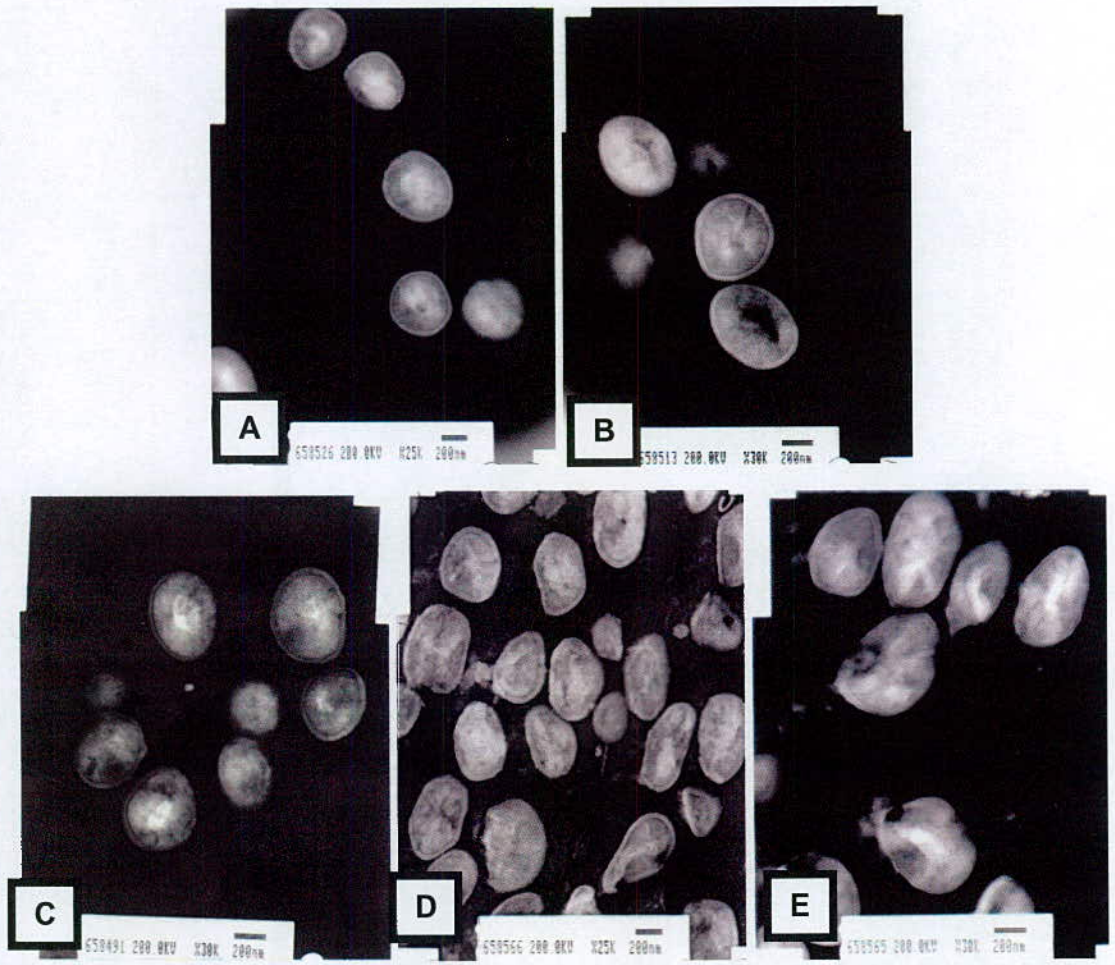

Figure 5.8: Transmission Electron Micrographs of MRSA-ME30 in phosphate buffer (0.1 M pH 7); A: Control 2 h x25,000; B: Control 6 h x30,000; C: 2% oleuropein treated 0 h x30,000; D: 2% oleuropein treated 4 h x25,000; E: 2% oleuropein treated 4 h x30,000; Scale bar = 200 nm ▬

Experiment was done in triplicate

Time kill studies were also undertaken with MRSA-ME30 and MSSA 29213 treated with 2% oleuropein. As shown in Figure 5.9, a 7 log reduction of viable colonies was achieved with 2% oleuropein after 24 h for MRSA-ME30 and MSSA 29213. The time to kill 50% of viable numbers was calculated using the Logger Pro software.

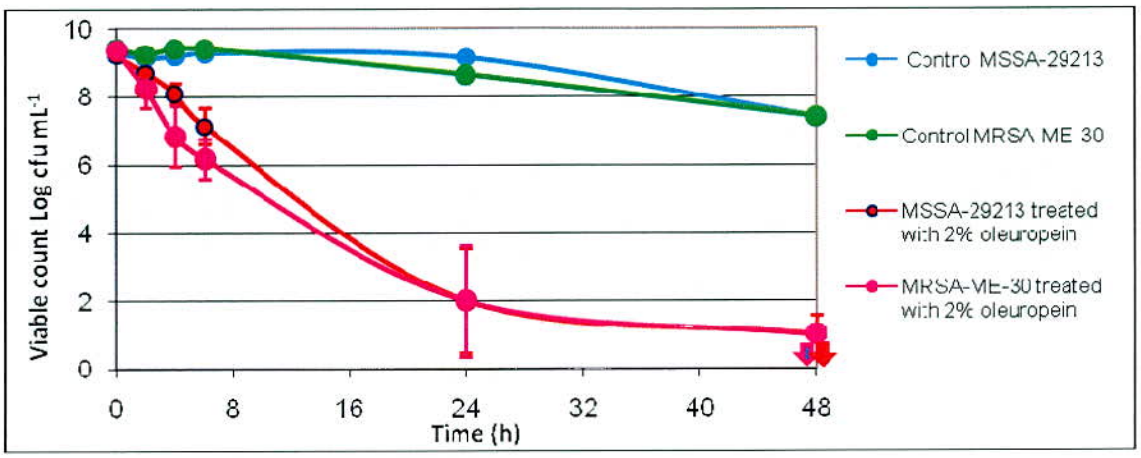

Figure 5.9: Inactivation of MRSA-ME30 and MSSA 29213 using 2% oleuropein in phosphate buffer (0.1 M pH 7.0) for 48 h

Sensitivity limit was 1 log cfu mL^{-1}; error bars are based on standard deviation values and represent three individual experiments (error bars = ±SD; n = 3)

The time to kill 50% of MRSA-ME30 with 2% oleuropein with a starting inoculum of 9.4 cfu mL^{-1} was 9.7 h and 13.2 h for MSSA 29213 (9.2 cfu mL^{-1}). In section 4.4, a reduction of half of the inoculum was achieved after 3.0 and 3.8 hours for MRSA-ME30 and MSSA 29213, respectively. The results showed that if the inoculum was increased by > 2 log cfu mL^{-1} compared to the bacterial time kill studies from section 4.4, the time to kill 50% of the cell mass more than tripled.

5.6 Discussion

The results in section 5.2 have shown that oleuropein interfered with the spectrophotometric assay for detecting leakage of low molecular weight compounds. Attempts to reduce oleuropein interference were made using Sephadex gel and charcoal but both initiatives failed. Because oleuropein and its by-products absorbed at wavelengths between 230-300 nm, the compounds needed to be completely removed. However, additional steps to remove oleuropein might have the disadvantage of removing low molecular constituents from the cell free-supernatant. Moreover, Sephadex gel chromatography could be improved by running lower sample volumes (2% of column volume) and if necessary multiple runs (Gegenheimer, 1990). In this work the sample volume was 16% of the column volume and this has most likely reduced the separation efficiency. A list of suggested polyphenol binding agents and procedures is given by Pierpoint (1996). Polyvinylpolypyrrolidone or Amberlite XAD-4, both polyphenol binding agents, might have solved the problem of oleuropein interference but these were not tested due to time constraints.

Bacterial cells contain proteins throughout the cell wall. Protein leakage in response to oleuropein treatment was examined using the Bradford assay. The Bradford assay was reported as four times more sensitive in protein detection than the Lowry assay (Bradford, 1976; Marks *et al.,* 1985). The Lowry assay was unsuitable for measuring cells treated with polyphenols because polyphenols reduce the Folin-Ciocalteu reagent (Bensadoun & Weinstein, 1976; Matoo *et al.,*

1987). Absorbance interferences were noted with the Bradford assasy but it was possible to construct adequate controls for these.

As shown in Figure 5.2, the quantity of leaked protein was 40 μg mL^{-1} in the treated and 20 μg mL^{-1} in the control after 24 h. These values were higher than those given by Kwon *et al* (2003). In the latter study protein leakage from cinnamic aldehyde treated *S. aureus* cells reached 9 μg mL^{-1} after 2 h exposure compared with 3 μg mL^{-1} in the control (Kwon *et al.*, 2003).

The ninhydrin test has been reported to eliminate phenolic interferences but there are no published reports on the use of this test in combination with oleuropein (Marks *et al.*, 1985). The ninhydrin test is a method to detect amino acids and proteins with a free NH_2 group forming a purple product with an absorbance maximum at 570 nm. The results in this study showed that oleuropein-treated cells had lower amino acid contents in the cell-free supernatant than untreated cells. This might be the result of protein denaturation caused by oleuropein. Pierpoint (1996) proposed that phenolic compounds form irreversible covalent linkages and modifications of amino acid residues and proteins mainly occured as a consequence of the oxidation of their vicinal dihydroxy groups to quinones and semiquinones, which are catalysed by polyphenoloxidase in alkaline conditions and in the presence of metal cations. Therefore, it is hypothesised that oleuropein caused denaturation of proteins resulting in lower amino acid contents of oleuropein-treated cells. This might be answered by using high performance liquid chromatography, gas chromatography or ion exchange chromatography on cell-free supernatants (Tristram & Rattenbury, 1981).

Overall the ninhydrin test was successful in eliminating oleuropein absorbance interference but the results did not support the hypothesis that oleuropein caused increased protein leakage from cells. Instead, the amino acid leakage in cell-free supernatant of treated cells was reduced by 38% for MSSA-ME5S and 12% for MRSA-ME30, respectively (Figure 5.3). The Gram-negatives *E. coli* and *P. aeruginosa* have not shown any difference of amino acid leakage following oleuropein exposure. It should be noted that the standard curve was prepared with aspartic acid and this may not represent all the different amino acids present in the sample. The standard curve could be prepared using a range of different amino acids.

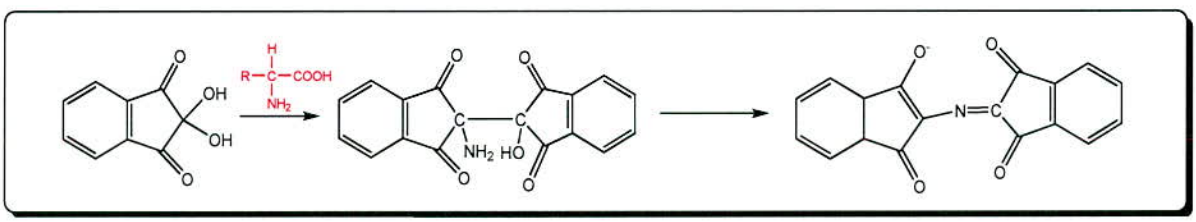

Figure 5.10: Ninhydrin reaction with the amino group forming a purple coloured product

these proteins. Fischetti (2006) reported that up to 25 or more surface proteins were found on most Gram-positive organisms, which display multifunctional properties. Glycoproteins are attached to the exterior of the cell wall and they support the cell wall structure and transport certain nutrients across the membrane. The attempt to measure glycoproteins using the lectin binding assay was unsuccessful as Wheat Germ Agglutinin (WGA) lectin was not binding to the bacterial cells. WGA specifically binds to *N*-acetyl-glucosamines located in the peptidoglycan layer of *S. aureus.* Previous studies on WGA binding to *S. aureus* were done with a different strain of *S. aureus* (Lotan *et al.,* 1975).

Another lectin which was suggested was Concanavalin but this lectin needs to be tested on MSSA 29213 and MRSA cells (Muñoz-Crego *et al.,* 1999). It is also possible that a longer incubation time in the presence of FITC conjugated lectin was needed. Moreover, when FITC was used without the lectin, attachment to amino groups in particular with lysine in MSSA 29213 and MRSA-ME80 occurred. The fluorescence microscope images obtained with FITC indicated the presence of amino groups in *S. aureus*. It was observed that the intensity of fluorescence was reduced with oleuropein-treated samples. However, the results are qualitative rather than quantitative and therefore the method was unsuitable for the detection of cell surface activity of oleuropein. A longer period of oleuropein treatment might have shown less fluorescence intensity but the drawback of the method is that it does not reveal the location of these amino groups.

Konno *et al* (1999) reported that leaves containing oleuropein have protein-denaturing, protein-crosslinking, and lysine-decreasing properties that could be explained in terms of alkylating activities. When a protein was mixed with the leaf extract, protein denatured and formed a high-molecular-mass complex. At the same time, the lysine content of the protein decreased to one-third to one-fifth of the original, although other amino acids were not affected (Konno *et al.,* 1999). This could be further investigated using a fluorescence spectrophotometer to quantify the intensity of fluorescence or using an amino acid analyser.

SDS-PAGE analysis showed that some proteins were missing from oleuropein-treated MSSA and MRSA cells (Figure 5.6). The results suggest that oleuropein acts on cell surface proteins and eliminates them from the SDS-PAGE band

spectrum. Overall, a range of bands were deleted in MRSA and MSSA cells treated with oleuropein. Further information is required about the size of these proteins which could be achieved using Western blotting technique or using Matrix-assisted laser desorption-ionization (MALDI-TOF) a soft ionisation technique used in mass spectrometry of biological molecules.

An alternative method to SDS-PAGE is two-dimensional SDS-PAGE in which an additional process prior to SDS-PAGE is inserted (Garrels, 1983). The first-dimension is isoelectric focusing, where immobilised pH gradient strips are dehydrated in a cassette containing urea buffer to solubilise proteins in the sample. Proteins migrate in the strip until their charge becomes neutral and then stop their migration. In principle, proteins separate according to their isoelectric points. The strip is then placed on the top of the resolving gel for the second-dimension in which the proteins are separated according to their molecular weights. Mixtures of different proteins can be resolved and the relative amount of each protein can be determined by means of the spot size.

A study by Konno *et al (*1999) showed that oleuropein (0.6%) denatured ovalbumin when mixed with tropolone, a polyphenol oxidase inhibitor extracted from privet leaves. Tropolone (2 mM) was reported to be effective in eliminating the brownish colour formation caused by oxidation and polymerisation of oleuropein (Konno *et al.,* 1999). However, when extracted oleuropein from privet leaves was added without the enzyme fraction, the protein denaturing activity was absent. This suggests that oleuropein is enzymatically activated in spite of the presence of a polyphenol oxidase inhibitor, which eliminates the colour formation. Oleuropein

from Extrasynthese used in this study seemed to work in the same way. Oxidation of oleuropein might be an indicator of a protein denaturing reaction.

The present work showed that oleuropein has the potential to be utilised as effective antimicrobial against MSSA and MRSA. The mechanism of bacterial inactivation by oleuropein and most likely other polyphenols is by partial cell wall degradation. It is suggested that cell surface proteins become denatured during the oleuropein treatment. It is proposed that the cell wall synthesis in MRSA and MSSA is interrupted leading to a partial disruption of the cell wall resulting, in turn, to leakage of cell constituents and ultimately cell lysis.

Chapter Six

6 General Discussions

The phenolic compound oleuropein was extracted from its plant source and its purification was investigated using a novel Countercurrent Chromatography (CCC) purification method. The purification with CCC was successful but needs further adjustment to optimise the process. The antimicrobial activity of CCC-extracted oleuropein against Methicillin-resistant *Staphylococcus aureus* (MRSA) was similar to that of commercial oleuropein.

CCC is a process which has the potential for use in the purification of natural compounds. However, the operator for a CCC run needs to be familiar with certain chemical and physical properties of the compound and the liquid-liquid two-phase system. Depending on the compound and its commercial application, CCC has the advantage of potentially producing a purer end-product. A pure compound might be desired for medicinal and pharmacological reasons but sometimes the purity of the compound needs to be balanced against the demands of economics and time. An increase of 5% in purity might not improve antimicrobial activity but may add substantially to the cost of the process.

The diversity of plant compounds is often linked with antimicrobial properties. It is common to test natural compounds for their antimicrobial activity. There are several reasons why these compounds are all tested in similar ways: i) lack of knowledge about antimicrobial properties, ii) development of further studies if compounds indicate bactericidal activity, iii) hope in finding the ultimate medicine, iv) marketing reasons. Hence, most compounds of natural origin undergo similar research procedures to pre-select novel compounds. The research on oleuropein has progressed over the years and studies on its antimicrobial activity has open

the way for further utilisation. Oleuropein showed antimicrobial properties against two species of MRSA and two species of MSSA bacteria. The antimicrobial action against Gram-negatives was restricted because of the additional layer in the cell wall. Bisignano *et al (*1999) suggested that the lower activity of oleuropein against Gram-negative organisms compared to Gram-positives was due to the glycosidic group, which renders the compound incapable of penetrating the cell wall. This work suggests that oleuropein seems to be absorbed by cell wall constituents of which some become degraded. The Gram-negative cell wall seems to be more resistant to the absorption by oleuropein which eventually becomes oxidised and loses its antimicrobial activity.

Oleuropein resulted in a reasonable reduction (5 to 6 log cfu mL^{-1}) of MRSA and MSSA within 3-7 hours. The formation of pin prick colonies at certain time points led to the theory that small colony variants (SCV) were formed as a defence mechanism of cells. This was investigated by transmission electron microscopy (TEM) and results of recultured colonies revealed that these colonies were in fact the same as the wild type. A typical characteristic of SCV is that cells are heterogeneous in a single culture and include empty cells surrounded by a cell wall (Seaman *et al.,* 2007). Pin prick colonies of oleuropein-treated samples were recultured in agar medium and prepared for TEM. However, there was no indication that SCV were formed as result of oleuropein treatment. In contrast, the cells seemed to have regained their original metabolic strength and looked similar to the original strain. The lack of growth of oleuropein-treated cells indicated that cells became smaller in size. The defence mechanism of cells subjected to adverse environmental conditions lead to depressed cell activitites (Gilbert *et al.,*

1990). This process could be the result of quorum sensing involvement. The disturbance in cell wall integrity might switch the cell to a reduced life cycle by either activating or deactivating intracellular cell activities such as electron transport regulators and virulence expression.

The mode of action of oleuropein and other polyphenols has not been elucidated in detail in previous studies. By investigating the cell wall interactions between oleuropein and bacteria via transmission electron microscopy and cell wall leakage, partial breakage of the barrier was observed in this study (Figure 5.7). SDS-PAGE of oleuropein-treated supernatant showed that band patterns were absent suggesting denaturation of proteins. As a result of this, amino acid contents were reduced shown with the ninhydrin test (Figure 5.3). Amino groups were analysed by FITC bonding and fluorescence intensity. The results suggest that α- and ε-amino groups of lysyl groups were targeted by oleuropein. Whether this occurs in the cell wall or within the cell needs to be continued by further work. Cellular contents such as nucleic acids and denatured proteins were released and eventually cells were killed. Whether oleuropein targets particular surface proteins or extracellular glycoproteins needs to be further researched.

As with most natural compounds, their use in disinfectants and detergents is limited because of relatively low content of the active compound in a mixture as well as the high cost of purifying the compounds. There are much more effective agents available to eliminate bacterial growth, such as alcohols, chlorhexidine, formaldehyde, hydrogen peroxide, phenol, triclosan, strong acids or bases. However, some of these agents are harmful for humans. Moreover, they could

result in toxic reactive by-products, damaged surfaces, colour removal and metal corrosion.

In summary, oleuropein has the potential to kill bacteria but its usage as a disinfectant is debatable because of economical considerations. Oleuropein and other polyphenols are already utilised as food supplements to complement a healthy diet. For medicinal products, such as ointments and cosmetics the antibacterial effect of oleuropein needs more research to determine and extend the multiple target sites for cell inactivation. Whether biofilm formation can be prevented by oleuropein and whether the quorum sensing mechanism of certain bacteria is activated in the presence of polyphenols is unknown.

From the chemistry prospect, natural product research has facilitated the development of assays for the purification and characterization of penicillins, cephalosporins, aminoglycosides, tetracyclines, erythromycin, related macrolides, vancomycin and teicoplanin (Walsh, 2003). The future objective for the extraction of compounds will always be the improvement of purity of compounds of interest and a rapid hassle-free application suitable for scale-up within the framework of a green process. Hence, research in this field offers much more than the discovery of new antibacterial products it also confronts researchers with new structures and the innovation of required analysis methods. The increasing demand for natural products in consumer goods will maintain the level of interest in research in this field.

Chapter Seven

7 Conclusions and Future Work

In conclusion, the novel CCC method of extracting oleuropein seems to be a reliable technique for separating crude mixtures from natural sources into their single components. Oleuropein was successfully separated within 40 minutes and this could be further improved by incorporating flash chromatography. Whether CCC is an appropriate method for oleuropein depends on the solvent system used and the surfactant properties of the compound. However, CCC is an economical and environmentally friendly approach as the solvents could be reused.

Oleuropein was effective against common pathogens such as MRSA and MSSA prevalent in the hospital environment. This study suggests that the usage of oleuropein in combination with other agents in detergents and cleaning products could help to kill a wide range of bacteria. Moreover, oleuropein is a product from a natural source and hence there is no limitation on its availability. With many synthetic detergents the degradation of the ingredients could cause environmental concerns, which would not be an issue with oleuropein.

This study has shown that oleuropein acts on the cell wall. This includes the breakdown of cell wall material and the release of internal contents. There is evidence of protein degradation and this suggests that the antimicrobial activity is orchestrated by the affinity of oleuropein for certain amino acid groups within the cell wall barrier. A similar mode of action may be used by a wide range of natural compounds but would need further research.

The antimicrobial studies and the possible mechanism of the bactericidal effect of oleuropein against selected microorganisms were investigated. Selected methicillin resistant *Staphylococcus aureus* were killed by oleuropein. The antimicrobial potential of oleuropein against bacteria resistant to antibiotics such as penicillin needs further investigations.

The affinity of oleuropein to lysine groups was shown by fluorescence microscopy observations. Fluorescence spectrophotometry could quantitatively validate these findings. In addition, the amino acid contents in the supernatant of treated cells could be analysed by using high performance liquid chromatography (HPLC), gas chromatography or an amino acid analyser. In the latter method the samples are subjected to four different processes including hydrolysis, derivation, HPLC separation and data interpretation. By using these methods an accurate picture of the affinity of oleuropein with certain amino acids could be presented.

Peptide mass fingerprinting a method to analyse proteins could be applied to sequence migrated proteins from the SDS-PAGE process. Using a mass spectrometer the absolute mass of these proteins could be determined and potential proteins in bacteria targeted by oleuropein identified. Moreover, it would indicate whether these proteins are present in the cell wall or within the cell. Subsequently, mutagenesis of bacteria lacking the proteins targeted by oleuropein could substantiate the mode of action of oleuropein and perhaps other phenolic compounds found in the plant kingdom. The defence mechanism in bacteria needs further recognition. Oleuropein and other natural compounds need to be further investigated on its strategy to target biofilm forming bacteria and the quorum sensing mechanism.

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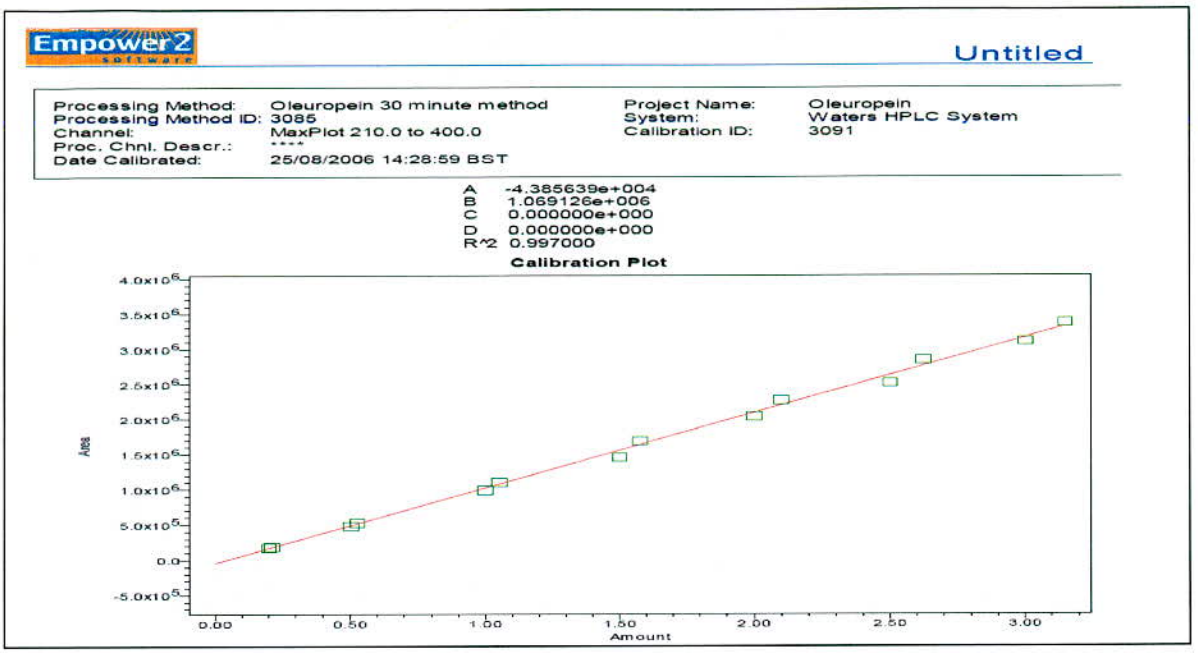
Appendices

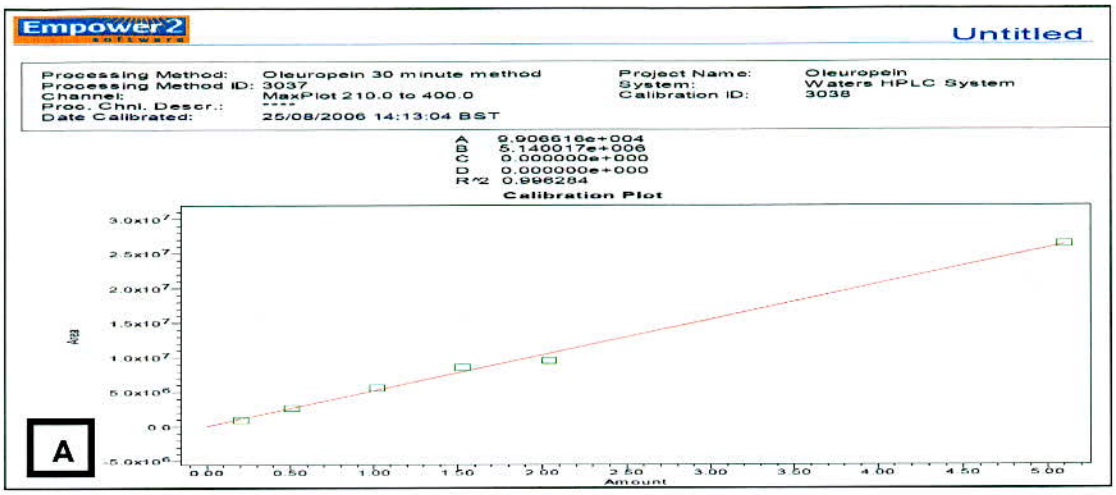


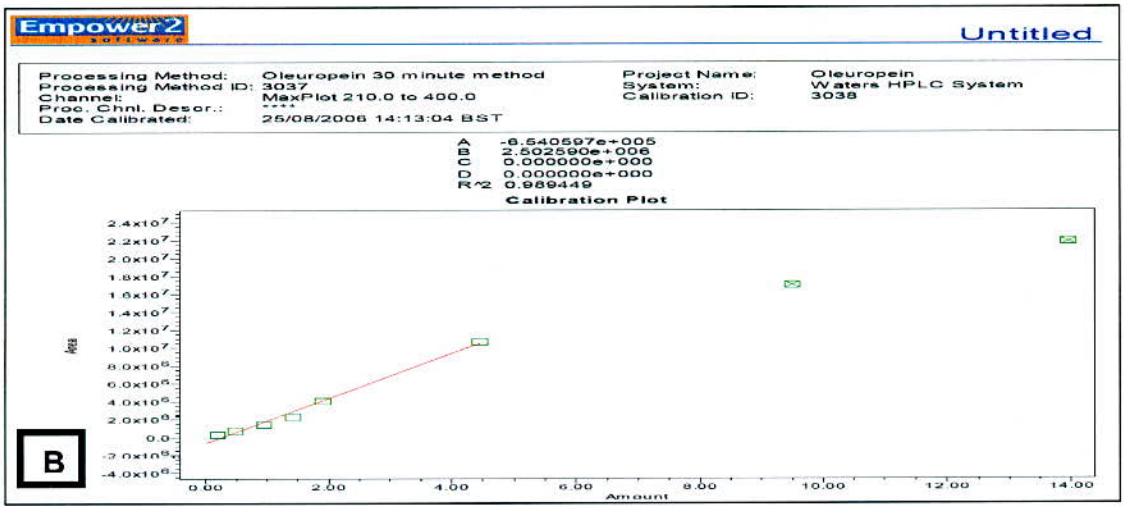
Appendix 1: Calibration curve of oleuropein verified by HPLC

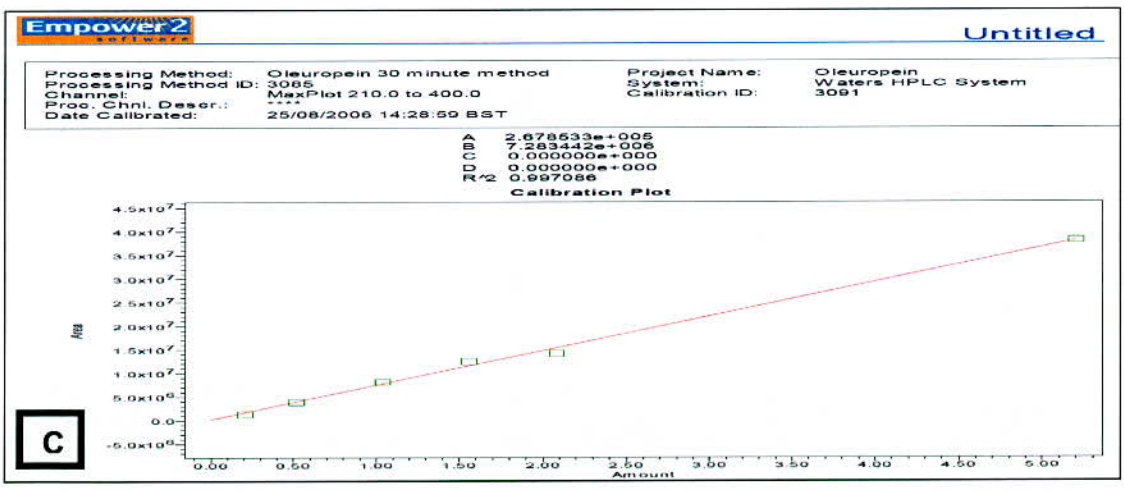
The absolute amounts of oleuropein were calculated by using the formula given below, whereby A and B are the variables calculated from the calibration curve, with A = -43856 and B = 1069126 (values are given in Microvolt x seconds; µV.s). The calibration curve is linear over a range from 0-3 µg injected amount with $R^2 = 0.997$.

$$Amount = \frac{Peak\ Area - A}{B}$$

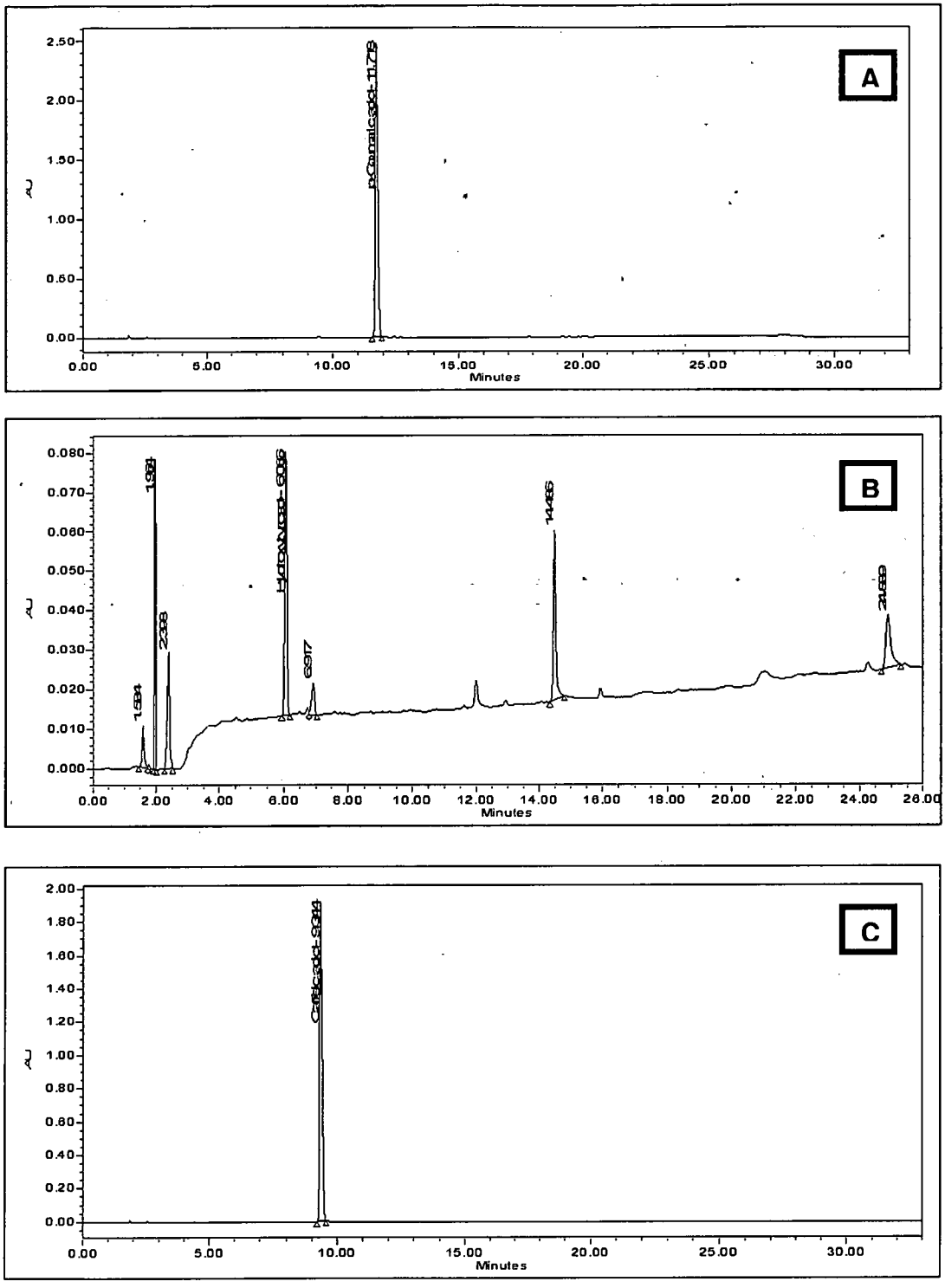
Equation 4: Formula for oleuropein quantification







Appendix 2: Calibration curve of standard p-coumaric acid (A), hydroxytyrosol (B) and caffeic acid (C)



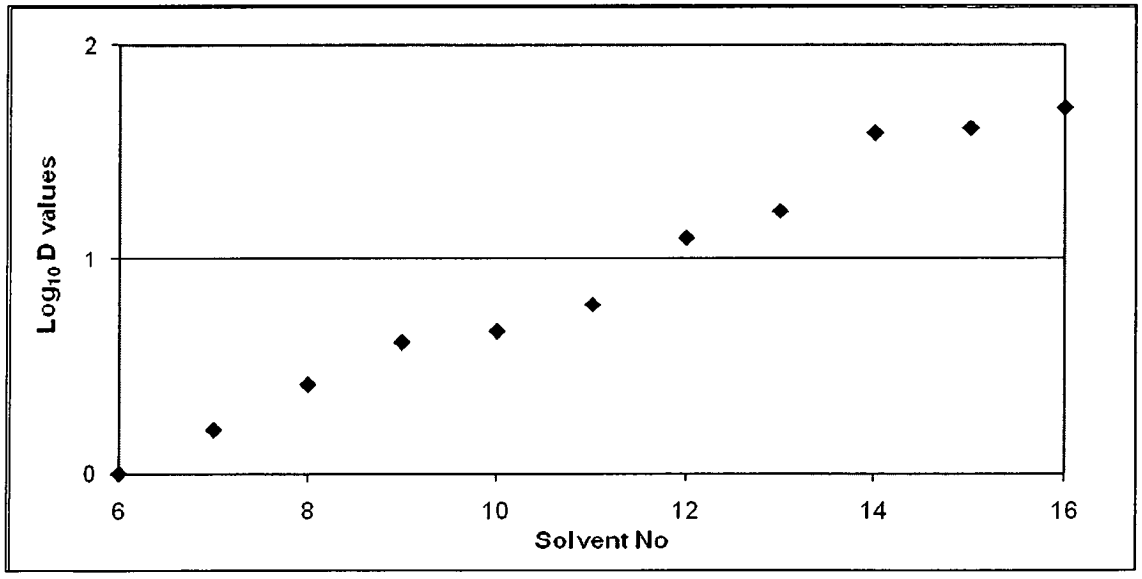
Appendix 3: HPLC chromatograms of standard p-coumaric acid (A), hydroxytyrosol (B), and caffeic acid (C) on a 33 min HPLC run

Appendix 4: Upper phase data analysis of Midi-CCC process

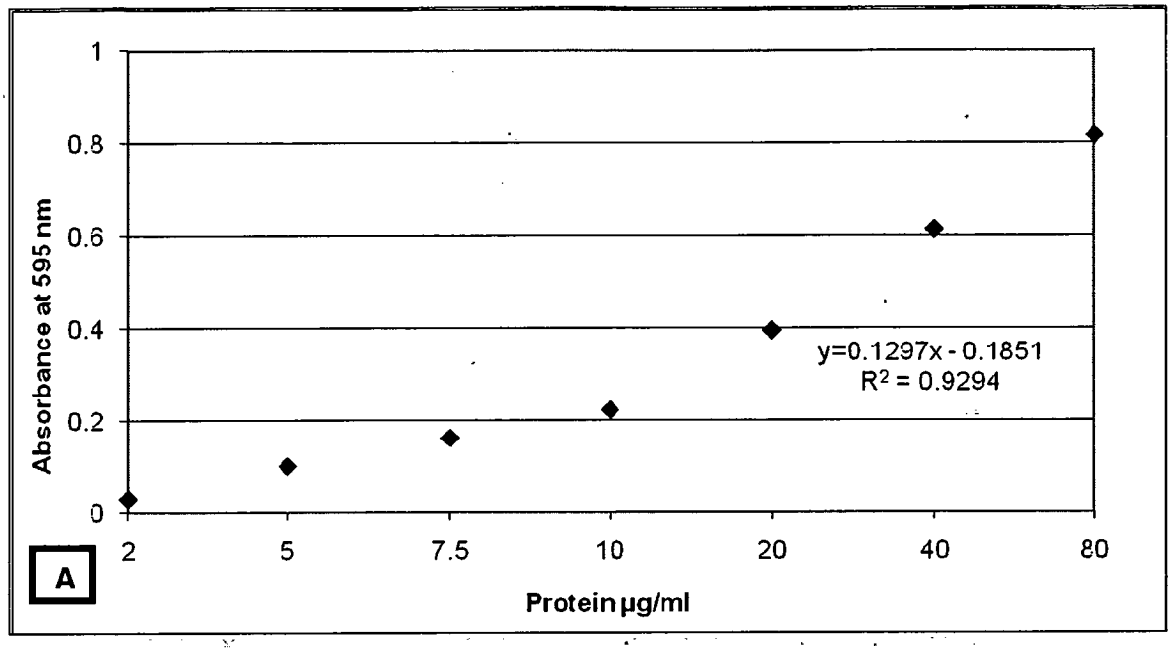
Fraction No	Area (uV.s)	in 10uL	Amount in Fraction (mg)	Conc. (mg/mL)	Volume of Fraction (mL)	Purity (%) Peak Area
CCC crude sample in LP	7838482	7.37	14.75	0.74	20	
31	2522	0.04	0.10	0.00	24	1.29
32	7244	0.05	0.11	0.00	23	3.09
33	11704	0.05	0.11	0.01	21	5.2
34	5147	0.05	0.10	0.00	21	0.28
35	66319	0.10	0.41	0.01	40	21.33
36	116864	0.15	0.30	0.02	20	33.26
37	168761	0.20	0.42	0.02	21	44.96
38	234834	0.26	0.52	0.03	20	54.19
39	332564	0.35	0.70	0.04	20	65.1
40	386189	0.40	0.88	0.04	22	73.66
41	406715	0.42	0.84	0.04	20	79.66
42	454361	0.47	0.93	0.05	20	76.84
43	462771	0.47	1.00	0.05	21	83.58
44	448677	0.46	0.97	0.05	21	83.59
45	422082	0.44	0.87	0.04	20	82.56
46	384593	0.40	0.88	0.04	22	80.77
47	341397	0.36	0.79	0.04	22	80.97
48	313300	0.33	0.80	0.03	24	74.57
49	270334	0.29	0.68	0.03	23	74.01
50	190618	0.22	0.50	0.02	23	61.31
51	152507	0.18	0.42	0.02	23	70.9
52	110275	0.14	0.33	0.01	23	62.86
53	80093	0.12	0.29	0.01	25	14.4
54	59987	0.10	0.22	0.01	23	45.2
55	x	x	x	x	x	x
56	24809	0.06	0.15	0.01	23	30.72
57	14373	0.05	0.12	0.01	22	20.46
Amount in Upper Phase 13.46 mg						

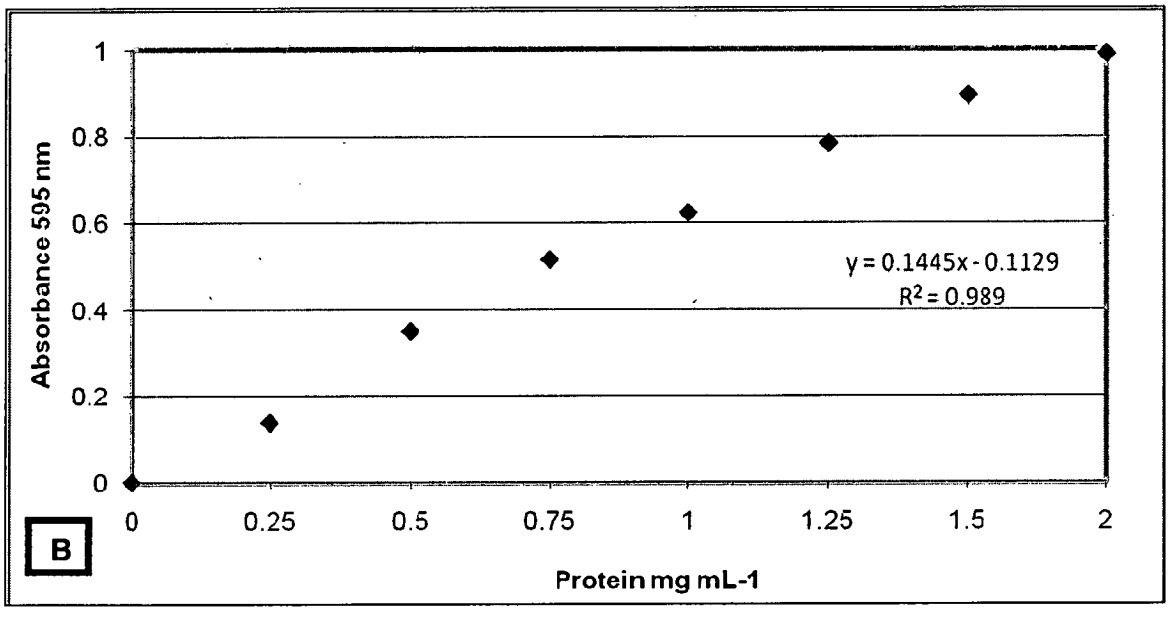
Appendix 5: Lower phase data analysis of Midi-CCC process

35	91407	0.13	0.06	0.01	5	32.48
36	169040	0.20	0.10	0.02	5	47.57
37	241892	0.27	0.08	0.03	3	55.24
38	335713	0.36	0.14	0.04	4	64.08
39	421712	0.44	0.22	0.04	5	73.89
40	508668	0.52	0.16	0.05	3	78.23
41	569376	0.57	0.29	0.06	5	79.87
42	631650	0.63	0.13	0.06	2	82.56
43	664329	0.66	0.26	0.07	4	92.12
44	668936	0.67	0.20	0.07	3	82.95
45	339513	0.36	0.11	0.04	3	70.69
46	171278	0.40	0.12	0.04	3	79.36
47	467896	0.48	0.10	0.05	2	75.85
48	x	x	x	x	0	x
49	x	x	x	x	0	x
50	111618	0.21	0.04	0.02	2	x
51	x	x	x	x	0	x
52	179957	0.21	0.04	0.02	2	31.95
53	x	x	x	x	0	x
54	x	x	x	x	0	x
55	19450	0.08	0.02	0.01	2	39.02
56	x	x	x	x	0	x
57	21972	0.09	0.02	0.01	2	14.8
	Amount in LP		**2.08 mg**			
	Total		**15.54 mg**			

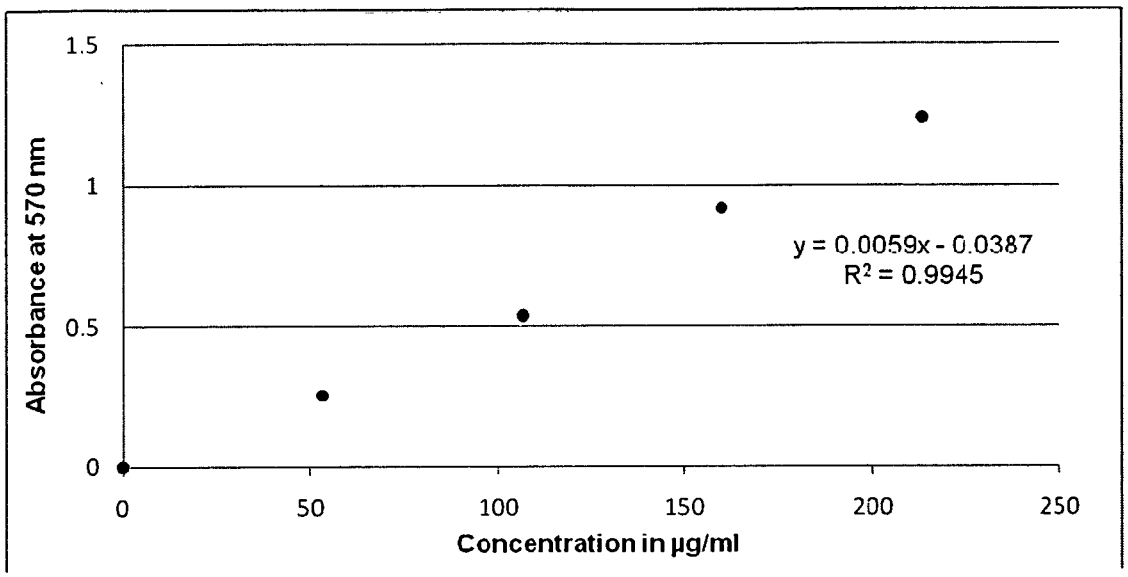


Appendix 6: Standard curve for the solvent selection





Appendix 7: Bradford agent (2%) standard curve in μg mL^{-1} (A) and mg mL^{-1} (B) protein achieved with bovine serum albumin



Appendix 8: Ninhydrin standard curve achieved with the amino acid aspartic acid

Appendix 9: Sample preparation for the ninhydrin test

	Volumes in mL				
Tube No	1	2	3	4	5
Sample	0.0	0.5	1.0	1.5	2
PBS	2.0	1.5	1.0	0.5	0
Ninhydrin	1.0	1.0	1.0	1.0	1

Verbal presentations as result of this research project

1. PhD Research Forum at Thames Valley University in April 2008 and Brunel University Research Meeting in June 2008

Title: Investigation of cell membrane damage in *Staphylococcus aureus*

Abstract: Many natural compounds have a diverse range of antimicrobial properties but their mode of action is not fully understood. In this study, oleuropein, a polyphenol found throughout the olive tree, was investigated. The purpose was to elucidate the mechanism of antimicrobial action of oleuropein on the cell walls of methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus*. The mode of action of oleuropein on methicillin-resistant and methicillin-sensitive *S. aureus* cell envelope has been investigated systematically. Previous work with bacterial time kill studies and TEM had revealed that oleuropein eradicated the methicillin-resistant strain faster than the methicillin-sensitive *S. aureus*.

2. Annual MPhil/PhD Conference at Thames Valley University in September 2006 and Brunel University Research Meeting in August 2006

Title: A natural compound from olive leaves is able to kill bacteria

Abstract: As more and more publications appear reporting bacteria to be resistant to killing by antibiotics and other chemical agents, attention is turning to natural compounds in the fight against bacterial disease. Olive leaves contain substantial amounts of phenolic substances, including the polyphenol oleuropein. The purpose of this study was to develop a method for extraction of oleuropein from olive leaves and characterise the activity of this compound against a range of bacteria. In collaboration with the Brunel Institute for Bioengineering at Brunel University, phenolic compounds from olive leaves were extracted in solvent and oleuropein separated from this mixture by countercurrent chromatography.

3. PhD Research Forum at Thames Valley University in February 2006

Title: Phenolic components in olives - Antimicrobial activity of a natural substance

Abstract: In microbiological studies, twelve strains of bacteria (including species commonly associated with hospital infections) were tested for their sensitivity to oleuropein by disc diffusion on agar media. All of the bacteria were killed by oleuropein, but the amount of oleuropein required to achieve this inhibition varied from 0.25 to 3.0%. Gram-negative species and *Bacillus subtilis* required a larger amount of oleuropein to show inhibition compared to species of Gram-positive cocci (including *Staphylococcus*), suggesting that the structure of the bacterial cell wall is an important determinant in susceptibility to oleuropein.

Posters presented as result of this research project

1. Annual MPhil/PhD Conference at Thames Valley Conference in February 2008

Title: The polyphenol oleuropein disrupts components of the cell wall of bacteria

Abstract: Plant products differ in the presence, type and content of phenolic compounds, which have natural antibacterial activity. Phenolic compounds, in contrast to antibiotics, show a multitude of actions against pathogens. In the olive plant the main polyphenol is oleuropein, which is found throughout but chiefly in the olive leaf. It is believed that the site of action of oleuropein is the bacterial cell wall and its chemical constituents. In this study oleuropein was utilised to demonstrate its antibacterial effect on the cell wall of Methicillin sensitive *Staphylococcus aureus* (MSSA) and Methicillin-resistant *S. aureus* (MRSA). Transmission Electron Microscope (TEM) images of bacteria treated with oleuropein indicated partial cell lysis within two hours for MRSA and four hours for MSSA. Nucleic acid studies on MRSA demonstrated the leakage of internal material from the cell.

2. Countercurrent Chromatography Conference at Brunel University April 2006;
   West Focus: Modernisations of Traditional Chinese Medicines a UK perspective Brunel University Conference in April 2006

Title: The antimicrobial action of olive phenolics against bacteria

Abstract: The antimicrobial properties of oleuropein, a major phenolic constituent of olives, were investigated with a view to developing novel cleaning and sanitising agents for use in hospital environments. Twelve strains of bacteria, including clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), were tested for their sensitivity to oleuropein (0.25-3.0%) in agar media. Growth of some Gram-positive bacteria was inhibited by concentrations as low as 0.25% oleuropein. In phosphate buffered saline, the action of 0.5% oleuropein was biocidal against several strains of *S. aureus*.

3. Annual MPhil/PhD Conference at Thames Valley University in September 2006

Title: Interactions between natural antimicrobials and bacterial cells

Abstract: Table olives and olive oil contain the compounds oleuropein and hydroxytyrosol. These natural phenolic substances have been shown to have both antioxidant and antimicrobial properties. The aim of this project is to investigate the mode of action of those compounds towards clinical microorganisms so that they could be better exploited in controlling the spread of diseases.