An investigation into the chemical nature and mechanism of action of antiviral compounds of natural origin

Michael B. Maelzer

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Abstract

The research described in this thesis concerns the antiviral activity of constituents found in a pomegranate rind extract, PRE, from the husk of the fruit of *Punica granatum* L., as has been described by Stewart et al., (1998). To investigate the mode of action, to identify the active molecule responsible for the activity and to establish a bioassay that can be used to test for antiviral activity of plant extracts were the main points of concern.

A partially purified pomegranate rind extract (pPRE) was obtained by a multi step purification process with the main points being extraction of the freeze dried rind followed by column chromatography using an ionexchange resin. It was confirmed by mass spectrometric analysis that the hydrolysable tannin punicalagin was present but its isolation was not achieved.

Phage particles are bacterial viruses and have been used as model systems for the study of viruses. Analysis of bioassays for monitoring the antiviral activity were carried out and an improved bioassay has been established, modified and applied successfully using *Acenitobacter* HER1401 and its corresponding phage. It was found that both extracts, PRE and pPRE, have phagicidal activity that was increased if added ferrous ions were present. Ferrous ions on their own showed no activity. This modified bioassay decreased the assay time considerably, was not susceptible to regrowth and showed good reliability.

The increased antiviral activity in the presence of ferrous ions suggested that free radicals might be involved in the mechanism of action. Therefore free radical detection assays to monitor the superoxide and hydroxyl radicals were investigated. It was found that superoxide radicals were not generated or involved in the generation of peroxide. The assay testing for hydroxyl radicals showed that hydroxyl radicals were generated but only in the presence of atmospheric oxygen. An additive effect was observed when the contact time of the hydroxyl radical detection assay was extended suggesting that ferrous ions get recycled by pPRE.

The mechanism of action of the antiviral activity involves the generation of hydroxyl radicals but as pPRE on its own showed antiviral activity it is likely that there is a secondary mechanism of action involved.

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Declaration

I declare that the research in this thesis, unless otherwise formally indicated within the text is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed:

Dated: 14/6/2010

Chapter one

Introduction

1. Introduction

1.1 General review of *Punica granatum* L

1.1.1 Botanical description

Punica granatum L., commonly known as pomegranate, is so named because of its abundance of seeds (In latin: poma is fruit and granatum is grains). It shares its botanical family, *Punicacae,* only with the *Punica protopunica* which has only been found on the island of Socotra, lying just off the Yemeni coast. More than 1000 cultivars of *Punica granatum* L. are known (Levin, 1994), originating from the Middle East and extending throughout the Mediterranean in the west, to India and China in the east and to the American south west of California and Mexico. The pomegranate plant can be considered a small tree or a big shrub. It usually grows to no more than 15 feet with a pale brownish bark. The buds and young shoots are red in contrast to the leaves which are thick, glossy and almost evergreen. The flowers are large and solitary, the crimson alternating with the lobes of the calyx. The fruit is the size of an orange and has a thick reddish-yellow rind. This thick and leathery pericarp contains numerous arils, a seed within a translucent juice containing sac. Thin membranes extend into the interior of the fruit providing a framework form which the arils are suspended (Lansky, 2007). The fruit is made up of 4 main parts: the seeds about 3%, the pericarp about 13%, the oil about 20% and the juice about 30% of the weight of the fruit. The pericarp (peel) also includes the interior network of membranes (Lansky, 2006).

1.1.2 Historical and cultural uses

There are three different kinds of pomegranate; one very sour, one moderately sweet and one very sweet. The juice of the first can be used as a substitute for unripe grape juice and the very sweet one is served as a popular desert in Syria (Grieve, 1982). *Punica granatum* L differs from most other plants as it has influenced various cultures. In Greek mythology Persephone (daughter of Zeus) was tempted by Hades (King of the underworld) to eat the fruit of the

pomegranate and was forced in return to spend one third of the year in the underworld. It is still used in some Jewish ceremonies and has also been used as decoration in temples and needlework for a very long time. It is one of the holy plants mentioned in the Koran and also features prominently in Christianity, Budhism and Zoroastrianism (Langley, 2000). The pomegranate is a symbol of life, longevity, femininity, knowledge, morality, immorality, spirituality (Madihassan, 1984). The plant has found a variety of applications over the years. The bark is used for tanning and dyeing, giving Moroccan leather its typical yellow colour. It has also found wide applications in folk medicine. The first record has been found on the Ebers papyrus where the bark has been used as a cure for tapeworm by the Egyptians in 1550 BC (Grieve, 1982). As far as medical applications go in ayurvedic medicine this plant is referred to as a pharmacy in itself, the bark and roots are believed to have antihelmintic and vermifuge properties (Naovi, et al., 1991). The rind is considered a powerful astringent and cure for diarrhoea and oral aphthae and the fruit a refrigerant (Arseculeratne et al., 1985) and blood tonic (Frawley, 1986). Dried pomegranate rind decocted in water is used both internally and externally for a variety of problems demanding astringents and/or germicides, especially for aphthae, diarrhoea and ulcers in India (Nagaruja, 1990), Tunisia (Boukef *et al.*, 1982) and Guatemala (Cacers *et al.*, 1987). The flower has been used as a remedy for diabetes mellitus in Unani medicine, traditional Middle Eastern medical system (Saxema, 2004).

1.1.3 General research on *Punica granatum* L.

In recent years the pomegranate has been the subject of research all over the world. In 2008 there were 40 publications compared to 19 from 1953 to 2000 (Science Direct). These publications can generally be divided into three main groups: food science, plant extract purification and medical research of extracts and fractions. The first is concerned with the storage, health benefits and cultivation of the pomegranate fruit. The most recent involved a study of various different factors such as temperature, pressure and viscosity on pomegranate juice extract (Magerramov, 2007). A major part of the publications has been

concerned with increase of storage time of the fruits after they have been harvested. The main problems with long term storage are scolding of the rind and decay which is often caused by the presence of fungal inoculum present in the blossom end of the fruit. The latest technique to increase the shelf life involves pre-treatment using polyamines combined with low temperature storage (S.H. Mirdehghan, 2007). Another interesting approach was to use a common food additive potassium sorbate instead of the fungicide Fludioxonil, the fungicide was more effective but when the former was combined with chilled storage ($7.2^{0}C$) the same results were observed (Palou, 2007). However the best results using controlled storage only were achieved when a combination of O_2 and CO_2, 5 kPa of O_2 and 15 kPa of CO_2, was used at $7.2^{o}C$ and 90-95% relative humidity, this resulted in extension of the storage period by up to 6 months (Defelippi, 2003). The health benefits will be discussed in greater detail as this is the area of concern of this piece of work.

1.2 Secondary metabolites

All organisms need to change, interconvert and transform a vast amount of organic molecules to enable them to live, grow and reproduce. They need to produce energy, in the form of ATP, and building blocks to make their own tissues. An integrated and complex network of chemical reactions, regulated and mediated by enzymes, is used for this purpose, and these reactions are referred to as the metabolic pathways. Most of the crucially important molecules like carbohydrates, proteins and nucleic acids are polymeric materials. Different organisms vary widely in their ability to synthesize and transform chemical compounds. Plants for example are very efficient at synthesizing organic compounds using photosynthesis and inorganic material present in the environment whilst animals and micro organisms rely on their dietary intake to provide them with these raw materials, for example eating plants. In most species producing energy from organic compounds via oxidative reactions, these reactions are generally referred to as primary metabolism. The degradation of

carbohydrates and sugars, for example, generally proceeds via the same well known route called glycolysis and the Krebs/citric acid cycle. Secondary metabolism is concerned with compounds that are not so readily available and have a more limited distribution in nature. These reactions do not occur in all conditions and in the vast majority of cases it is not understood what their benefits are. Some are produced for their toxicity in order to repel predators, as well as a protection against viruses, bacteria and fungi, or as colouring agents to attract or warn other species, but it is thought that they are all of some benefit of their producer (Winks, 1999). It is this area of secondary metabolites that is mainly responsible for most pharmacologically active natural products. Secondary metabolites are derived from primary metabolites generated during processes such as glycolysis, photosynthesis and the Krebs cycle. So called building blocks employed in biosynthesis are divided into four main groups: acetate, shikimate, mevalonate and deoxyxylulose phosphate pathways. In addition to these pathways amino acid derived products also play an essential part in natural product synthesis. For the synthesis of poly phenolics the acetate and shikimate pathways are mainly used. To appreciate how a natural product is elaborated it is useful to divide the structure into its basic building blocks to then deduce how they are joined together and how they have been synthesized. In the case of punicalagin the molecule can be divided into three building blocks, ellagic acid, a glucose moiety and a polyphenolic subunit. Ellagic acid is made of two gallic acid units that are combined by lactonisation, gallic acid is formed by dehydrogenation and enolisation of 3-dehydroxyshikimic acid which is part of the shikimic acid pathway (Knaggs, 2000).

1.2.1 Natural products and their biosynthesis

Natural products go through a wide variety of transformations as they get metabolised into more complex molecules. Therefore there is a wide variety of often structurally similar molecules present within the plant. Because of this a lot of these compounds have some properties in common. For example a lot of the compounds are derived from phenolic compounds. They therefore have phenolic

functional groups, which gives the simple starting molecules and the more complex metabolites antioxidant properties. It is important to recognise that even though there are a variety of different compounds present within the plant they do have a lot in common. Structural similarities are there due to chemical reactions that are often aided by enzymes present but they all start of with glycolysis that yields the basic building block pyruvate.

The starting point for flavonoids, phenylpropenes, lignans, coumarins, hydrolysable tannins as well as some alkaloids such as the opium alkaloids is shikimic acid. Shikimic acid, a metabolite of pyruvate, is a precursor of gallic acid, benzaldehyde, tyrosine and phenylalanine. The base for all of them is an aromatic C-6 ring and a 3-carbon side chain attached to the aromatic ring. These relatively simple molecules and slightly more complex ones such as dimers are classed as hydroxybenzoic acids. The amino acid phenylalinine is used to form many natural product groups including phenylpropenes, lignans, coumarins and flavanoids.

There are two types of tannins, hydrolysable and condensed tannins. In *Punica granatum* L. only hydrolysable tannins are present. Punigaligan, present in *Punica granatum* L., is a hydrolysable tannin, or gallotannin, derived by the metabolism of gallic acid into the dimer ellagic acid which, in turn, undergoes further metabolism to produce punigaligan. Polymerisation of flavanoids via condensation reactions give non-hydrolysable tannins also called condensed tannins (Dewick, 2002).

1.3 Phytochemical review of *Punica granatum* L.

1.3.1 About *Punica granatum* L.

The pomegranate plant can be divided into different parts that can be used as sources for medicinal purposes. From the fruit; husk, seed and juice and from the plant; flower and leaf, bark and root. In the case of *Punica granatum* L. the main natural product classes are flavonoids, alkaloids and tannins.

The fruit husk is a rich source of flavonoids, alkaloids and tannins as well as some complex polysaccharides (Jahfar, 2003). The tannins present are all hydrolysable tannins and include punicalagin, punicin and punicalin. The flavonoids include the phytoestrogenic compounds luteloin, quercetin and kaempferol, which can be also be found in the husk (van Elswijk, 2004). The only alkaloid present in the fruit husk is pelletierine. Pomegranate seed oil consists to 80% of conjugated fatty acids like punicinic acid, minor components present are sterols and steroids such as campesterol and testosterone (Abd El Wahab, 1998). In the juice anthocyanins are present, from the group of flavanoids, that are also responsible for the bright red colour for example cyanidin 3-O-glucoside (Hernandez, 1999). The root and bark are rich sources of alkaloids especially the pelletierines like pseudo, N-methyl-, Norpseudo- pelletierine. (Neuhofer, 1993) The flower of the plant contains triterpenoids such as ursolic acid and olenaolic acid (Huang, 2005). The leaves contain some ellagotannins that are present in the husk like punicalagin but also punicafolin which only occurs in this part of the plant and also the flavone apigenin (Nawwar, 1994).

1.3.2 Flavanoids

This group of natural products are widely spread throughout the plant kingdom and have been attributed with various medicinal properties. They are derived from combining parts of the shikimate and the acetate pathways. Cinammic acid and malonyl units coupled to coenzyme A react in a ratio of one to three to give a polyketide which is then folded with the help of the enzyme to allow Claisen reactions yielding chalcones followed by a Michael type nucleophilic attack to give a flavonone. Chalcones act as precursors for a wide range of flavonoid derivatives throughout the botanic world (Dewick, 2002). Flavanoids can be oxidised further to give anthocyanins and due to their extended chromophores they absorb light in the visible spectrum. This ability causes anthocyanins to act in a variety of plants and berries as colour attractants to insects and birds as an aid in pollination and dispersal, for example blackberries (Wang, 2006). Compounds present in husk and flesh of the fruit are flavones such as luteloin,

quercetin and kaempferol that can be used to treat hormonally dependent cancers such as breast and prostate cancer due to their mammalian oestrogen like properties (van Elswijk, 2004) (Tsimogiannins, 2004) .

1.3.2(1) Glycosides

Because of the phenolic and acidic groups present in flavanoids and, for example, gallic acid they are often bound to sugar molecules forming glycosides. Glycosides enhance the diversity of other natural product classes. The term glycoside is used for natural products that are bound to a sugar entity. Therefore they are composed of two parts, sugar moiety and aglycone. The aglycone can be a wide variety of natural products such as flavanoids, coumarin and terpene. They can be divided into four classes C-, O-, N- and S- glycosides depending on the linkage between the sugar and the aglycone. They get their name due to a carbon-carbon, a carbon-oxygen bond, carbon-nitrogen and carbon-sulphur bond that connects aglycone and the sugar moiety. Glycosides are usually more polar than the aglycone and therefore increase the water solubility. They are usually linked via acetal functions which can be hydrolysed in the stomach, acid hydrolysis, to release the aglycone (Dewick, 2002). There are two glycosides of interest and they can both be found in the husk. They are glycosides of the flavones, Luteolin and Kaempferol (van Elswijk, 2004).

1.3.3 Alkaloids

The second important group are the alkaloids. Alkaloids are metabolised from amino acids that contain a primary, secondary, or tertiary amine group or an amide group, therefore giving rise to a diverse group of chemical structures. This is the group of natural products that has contributed more to medicines and pharmaceutical products than any other. They include nicotine, caffeine, morphine, cocaine to just name a few. The name alkaloid was first used by a german pharmacist called Karl Friedrich Wilhelm Meissner due to their alkaline properties. These properties stem from the primary, secondary or tertiary amine groups present in their structure. However exceptions such as quaternary amine,

amide and phenolic groups present in some alkaloids give them either neutral or acidic properties (Dewick, 2002).

The main alkaloids present in the pomegranate plant are pelletierine (1-(2-piperidyl)propan-2-one), isopeletierine (2-acetonylpiperidine)and punicine, with the empirical formula of $C_8H_{15}ON$ and a molecular weight of 144.22, they are all present in the root and bark only pelletierine is also present in the husk. The alkaloid pelletierine has been mentioned in pharmaceutical and medical literature for about 130 years. Initially there were thought to be pelletierine and three derivatives, pseudo-pelletierine, iso-pelletierine and methylisopelletierine, which were separated by paper chromatography and tested for their antihelminthic properties using liver fluke (Wilbaut, 1956). It was found that the active isomer with the empirical formula of iso-pelletierine was the most active and was most likely to be responsible for anthelmintic activity of the bark extract of *Punica granatum* L.

Pelletierine tannate is a composition of structurally related alkaloids from pomegranate, pelletierine, pseudo, iso and methylpelletierine hydrochloride salts. It occurs as a lightly yellow or greyish amorphous, odourless powder having an astringent taste and a weak acidic reaction. Pelletierine tannate has the same properties as pelletierine but it is preferred to the pure alkaloid and its sulphates on account of its greater insolubility in the stomach, with a consequent decreased tendency to absorption, the occurrence of which may give rise to symptoms of intoxication, and is used to treat tape worm (British pharmaceutical codex, 1907).

1.3.4 Hydroxybenzoic acids and their more complex metabolites

Various hydroxybenzoic acids have been reported to have medicinal properties. The monomer gallic acid, the predecessor of galo- and ellago-tannins, can be found in many natural tannins and is widely distributed in many plants. It is formed by branchpoint reactions from shikimic acid, and is used widely as an antioxidant in the food industry. It was also found to have antiproliferative effects on cancer cells by generating hydrogen peroxide. Phenolic compounds in the presence of transition metal ions produce reactive oxygen species such as H_2O_2

via autoxidation at physiological pH. However this was not due to the direct reaction between the metal ions and the phenolics but could occur due to the presence of a metal ion chelator (Lapidot, 2002). It was reported that the presence of a chelator like EDTA is necessary to form redox cycle interactions between flavanoids and iron ions in aqueous solution at physiological pH (Hodnik, 1988). A dimer formed from gallic acid units is called ellagic acid which has been studied more intensely over the last few years and has been the subject of various publications. Ellagic acid has been reported to cause apoptosis in cancer cells of cervical carcinoma cells as well as Bladder T24, leukaemia MOLT-4, breast MCF-7 and HS578T and prostate DU 145 cells (Narayanan, 1998; Lin, 2006; Losso, 2004; Mertens-Talcott, 2005). The two more complex hydroxybenzoic acids 3,3'-Di-O-methylellagic acid and 3,3',4'-Tri-O-methylellagic acid have both shown considerable anti oxidant activity when tested *in vitro* on rat brains (Zhang, 2004). This is consistent with the findings of strong anti oxidant activity of pomegranate seed oil (Schubert, 1999). Interestingly it was reported that ellagotannins, in this case punicalagin, present in food cannot be absorbed *in vivo*. However when they reach the colon they hydrolyse and release ellagic acid (EA) that is absorbed by the human microflora and is responsible for the apoptosis of cancer cells, colon cancer Caco-2 cells, via the mitochondrial pathway. EA is metabolised by the colonic microflora to give bioavailable hydroxyl-dibenzo-pyran-6-one derivatives. (urololithin derivatives) (Larorosa, 2006) This suggests that most of the antioxidant activity observed and reported for various plant ellagotannins is mainly due to the fact that EA is released and is therefore responsible for their activity.

Quinic acid is also present in the husk and juice and, even though it has no proven medical activity itself, its derivatives have been found to have virucidal and anti tumour properties. They have been found to possess potent hepatoprotective activity and derivatives synthesised from quinic acid have been found to be active against certain lung cancer lines, A549 and H460 (Kim, 2007 and Arthurs, 2008). Brevifolin carboxylic acid 10-monopotassium sulphate was the first reported natural occurrence of an ellagotannin bearing a potassium

sulphate residue, however potassium sulphate derivatives have been found with flavanoids (Hussein, 1997 and Harbone, 1982). Hydroxycinnamic acids have been found to lower cancer cell metastasis by down regulating of the metalloproteinase expression. Especially caffeic acid and caffeic acid phenyl ethyl ester have shown activity against various enzymes such as lipoxygenases, glutathione S-transferase and xanthine oxidase. It has been found to have antitumor and anti-inflammatory activity. It was also reported to have apoptosis inducing functions as well as inhibitory effects of viral replication, HIV (Hwang, 2001).

1.3.5 Tannins

The term tannin was first used by Seguin in 1796 to describe a substance present in plant extracts that had the ability to combine with proteins of animal hides, prevent their putrefaction and turn them into leather. Most true tannins have a molecular weight of 1000-5000 and can be detected quantitatively making use of their ability to adsorb onto standard hide powder. To be an effective tannin the molecule must be small enough to enter the interstices between the collagen fibrils of animal skin but also big enough to crosslink between protein molecules of adjacent fibrils at several points. Tannins are widely distributed throughout the plant kingdom. Most plant families embody species that contain tannins. When tannins occur they are usually localised in specific parts of the plant such as leaves, fruits, barks and stems. Chemically, tannins are complex substances. They usually occur as a mixture of polyphenols that are difficult to separate because they do not crystallize. That is why they are often referred as tannin extracts rather than tannins. Due to the application of chromatographic techniques the constituents of these tannin extracts have been analysed and as well as the complex mixtures of constituents present small polyphenols have been found. Complex tannins are generally considered to have arisen from the polymerisation of these polyphenols. In some plants the role of tannins is to provide resistance against verticillium wilt. An increase in tannin concentration of

oak leaves is also responsible for the desertation of the leaves by the larvae of the winter moth as the leaves mature in June (Bell, 1981).

Recently authors have distanced themselves from the old definition of tannins as a phytochemical group but as examples of polyphenols illustrating particular aspects of gallic acid and flavan-3-ol phytochemistry (Okuda, 1995). The characteristics of tannins are largely due to the presence of a substantial number of phenolic groups, (1-2 per 100 mol. wt.) within a moderately sized molecule. Tannis are divided into two main groups; hydrolysable tannins and condensed tannins (proanthocyanidins). Both classes of tannins frequently occur in the same plant but usually one predominates in certain plant parts.

Hydrolysable tannins are formed from several molecules of phenolic acids such as ellagic acid and gallic acid that that are linked to a central glucose molecule by ester bonds. Two examples of hydrolysable tannins are gallitannins and ellagitannins which are composed of gallic acid and hexahydroxy-diphenic acid. Ellagic acid can be produced by the lactonisation of hexahydroxy-diphenic acid during chemical hydrolysis of the tannin (Luck, 1994).

Condensed tannins are also known as non-hydrolysable tannins or as proanthocyanidins. Proanthocyanidins are polymers that when treated with hot acid some of the carbon-carbon bonds are broken, yielding anthocyanidin monomers. Basically these tannins contain only phenolic nuclei but are frequently linked to carbohydrates or proteins. Most of these tannins result from the condensation of two or more flavan-3-ols such as catechin (Foo, 1996).

Hydrolysable tannins are also present in a wide variety of plants and plant products like raspberries, strawberries and pomegranate. They can also be found in red wine and have been reported, (Frankel, 1993), to play a part in the French paradox. This suggests that despite the intake of a high fat diet, there is a strikingly low incidence of coronary heart disease in France compared to the other western countries (Sun, 2002). This has been partly attributed to the consumption of red wine which is higher in natural polyphenols then other alcoholic beverages such as white wine or beer. It has been demonstrated that

these compounds inhibit platelet aggregation and protect low density lipoproteins from oxidation (Sun, 2002). In the plant *Punica granatum* L. hydrolysable tannins are predominantly present and it is these which seem to be responsible for the antioxidant, anticancer and probably the antiviral activity (Lansky, 2006).

1.3.5.1 Hydrolysable tannins from Punica granatum L.

The molecule of concern in this project is called punicalagin, see fig 1.3, from the plant *Punica granatum* L., a polyphenol that belongs to the family of the hydrolysable tannins. It contains three molecular subunits; ellagic acid, glucose and a polyphenolic group. The glucose molecule forms the linking part to the other two molecules attached to it via two sets of ester bonds each. The 16 phenol groups and one hydroxyl group present make the molecule very polar and water soluble. Ellagitannins are present in all parts of the pomegranate plant. They are hydrolysable tannins where the individual units are bound together by ester linkages. Punicalagin is the one that has received most attention in recent years due to its broad spectrum of applications and because of its high abundance in the plant. It is present in all parts of the plant, husk, leafs, root and bark.

Punicalin is a precursor of punicalagin, it was shown to have the ability to block HIV rgpl20 from binding to CD4 (Weaver, 1992). It has also been found to inhibit carbonic anhydrase, although to a lesser extent than other ellagitannins such as punicalagin, granatin A and B. Inhibition of carbonic anhydrase can be therapeutically useful for the treatment of glaucoma and diuresis by reducing the amount of water present by lowering sodium reabsorption (Satomi, 1993).

It is usually conjugated with a glycoside moiety and even more commonly as part of a polymeric molecule called ellagotannins. These ellagotannins are part of the hydrolysable tannins family that can be hydrolysed to free ellagic acid via the spontaneous lactonization of hexahydroxydiphenic acid as has been reported by Mar Larrosa 2005. However these studies did not take the important issue of bioavailability of ellagic acid or ellagotannins into account. Ellagotannins are hydrolysed to yield EA that is then metabolised by the human colonic microflora

into 2,8-dihydroxy-6H-dibenzo(b,d)Pyran-6-one (urolithin A) derivatives (Cerda, 2004).

Punicacortein which exists in four different isomers A, B, C and D, is another ellagotannin that has been reported to have anti HIV properties by inhibiting HIV-reverse transcriptase and HIV replication in T9 lymphocytes. (Nonaka, 1990) All the ellagitannins have ellagic acid moieties that are connected via ester linkages that can be hydrolysed. They have antioxidant properties due to their polyphenolic nature and also show the same properties as ellagic acid due to its release after hydrolysis.

In recent years it has been suggested that polyphenols with anti oxidant properties might offer similar beneficial effects to other parts of the body and research is ongoing

Fig 1.3 Structure of punicalagin

1.3.6 Other secondary metabolites with medicinal relevance

Also present in the husk are flavones and flavone derivatives. Of high interest are the so called phytoestrogens such as quercetin, kaempferol and luteolin. This interest is due to their possible role in preventing a range of diseases such as

hormonally dependent cancers like breast and bladder cancer (Kim, 2002; van Elswijk, 2004). The activity is due to their structural similarities to mammalian estrogens and they therefore show weak estrogenic behaviour (Kuiper, 1998). Possible chemopreventive effects have been suggested as in cultures where more foods rich in phytoestrogens are consumed there are fewer cases of hormonally dependent cancers (Setchel and Cassidy, 1999). However in the plant most of these phytoestrogenic compounds occur predominantly as 0-glycosidic conjugates which are more polar but reduces their activity dramatically. Using simple acid hydrolysis releases and activates the phytoestrogens explaining the benefits of diets rich in these compounds (van Elswijk, 2004). This is similar to the way ellagic acid is believed to be released when ellagotannins are hydrolysed in the colon.

Apigenin is a flavone that can be found in the leaves of *Punica granatum* L. Its various medicinal properties have made it a very popular in recent years. It is unique because it has got various effects on the central nervous system such as anxiolytic, sedative and anti-depressive properties (Avalone, 2000, Yi, 2008). It was reported that it has antidepressive effects too when tested on rats against blanks and Fluoxetine when given in oral form. However the treatment has to be chronic to be effective as acute oral administration did not give positive results. Treatment resulted in normalisation of changes in central monoaminergic neurotransmitter, the HPA axis and adenylyl cyclase activity systems in chronic mild stress (CMS) rats (Yi, 2008). Apigenin also has sedative effects and can induce apoptosis in cancer cells. It has been used to induce apoptosis in 22Rv1, cancer cells *in vivo*. This is accompanied by an increase in reactive oxygen species suggesting that free radicals are involved in the mechanism of action (Shukla, 2008). Although sedative effects have been attributed to apigenin the mechanism of action is still unknown (Avallone, 2000).

Luteolin is another flavanoid that has been attributed with anxiolytic effects. It seems to have similar properties to apigenin. It also has effects on the CNS. Luteolin and apigenin seem to have similar properties to classic

benzodiazepines, and $GABA_2$ as well as 5HT have been suggested as possible target sites (Coleta, 2008).

Anthocyanidins are present in a variety of foods especially in red wine and fruits including the pomegranate. They have got very strong antioxidant activity. Recently anthocyanidins have been used in combination with Doxorubicin which has side effects that could be minimised by the presence of an antioxidant. Cyanidin and delphinidin showed the most potent protection against cytotoxicity as well as lipid peroxidation when tested on H9c2 cardiomyocytes. As with most of these active compounds metabolic products exist that have extra glycoside moieties, but these adducts show significantly reduced activity although they blocked intracellular reactive oxygen species (ROS) (Choi, 2006).

Table 1.3 The molecular structure of active biochemical constituents and their location within *Punica granatum L.*

(H=husk, B=bark, R=root, F=Fruit, S=Seeds)

Type of molecule	Compound name	Chemical structure	Extracted from	References
Piperidine alkaloids	Pelletierine		H, B, R	Neuhofer 1993
Piperidine alkaloids	N-Methylpelletierine		B, R	Neuhofer 1993
Piperidine alkaloids	Pseudopelletierine		B, R	Neuhofer 1993
Piperidine alkaloids	Norpseudopelletieine		R	Neuhofer 1993
Hydroxybenzoic acids	Gallic acid		H, F	Amakura 2000 Huang,T. 2005b

Hydroxybenzoic acids	Ellagic acid		F, H, S	Amakura 2000 Huang, T. 2005
Hydroxybenzoic acids	3,3'-Di-O-methylellagic acid		S	Wang 2004
Hydroxybenzoic acids	3,3',4'-Tri-O-methylellagic acid		S	Wang 2004
Hydroxycinnamic acids (phenylpropanoids)	Caffeic acid		F, H	Artik 1998, Amakura 2000
Hydroxycinnamic acids (phenylpropanoids)	Chlorogenic acid		F, H	Artik 1998, Amakura 2000

Hydroxycinnamic acids (phenylpropanoids)	p-coumaric acid		F, H	Artik 1998, Amakura 2000
Cyclitol carboxylic acids and their salts	Quinic acid		F, H	Artik 1998, Amakura 2000
Cyclitol carboxylic acids and their salts	Brevifolin carboxylic acid 10-monopotassium sulphate		L	Hussein 1997
Ellagitannins	Punicalin		B, H, L, R	Tanaka 1985

Ellagitannins	Punicalagin		B, H, L, R	Tanaka 1985
Ellagitannins	Granatin A		H	Tanaka 1990

Ellagitannins	Granatin B		H	Tanaka 1990
Ellagitannins	Punicacortein A		B, R	Tanaka 1985

Ellagitannins	Punicacortein B		B, R	Tanaka 1985
Ellagitannins	Punicacortein C		B, R	Tanaka 1985

Ellagitannins	Punicacortein D		B, R	Tanaka 1985
Flavanols	Quercitin		F, H	Tsimogiannis, 2004, van Elswijk 2004
Flavanols	Kaempferol		H	Van Elswijk 2004

Flavanol glucosides	Kaempferol 3-0-glucoside		H	Van Elswijk 2004
Flavanol glucosides	Kaempferol 3-0-rhamnoglycoside		H	Van Elswijk 2004
Flavones	Luteolin		H	Van Elswijk 2004
Flavones	Apigenin		L	Nawwar 1994

Flavone glycosides	Luteolin 7-0-glucoside		H	Van Elswijk 2004
Flavone glycosides	Apigenin 4'-0-β-glucopyranoside		L	Nawwar 1994
Anthocyanidins	Delphinidin		H	Choi 2006
Anthocyanidins	Cyanidin		H	Choi 2006

Anthocyanidins	Pelargonidin	HO O+ OH OH OH	H	Choi 2006

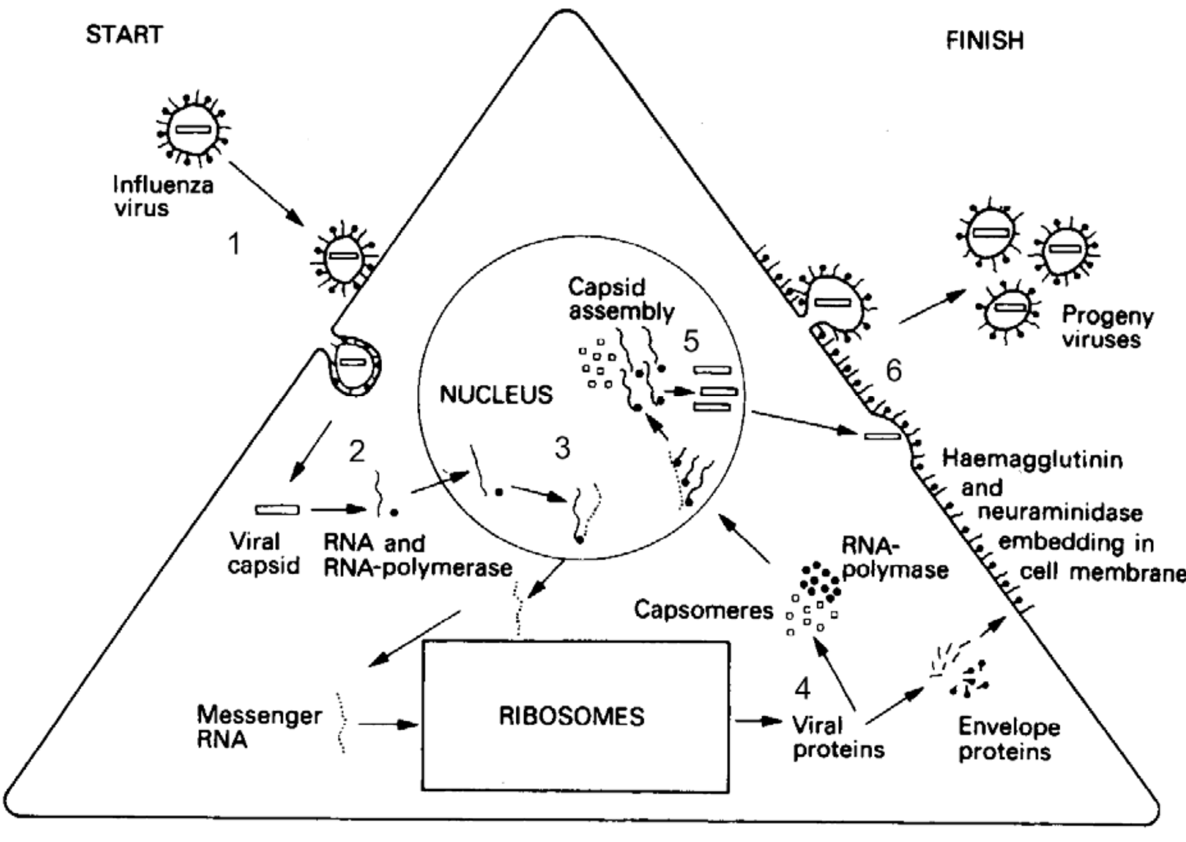
1.4 Viruses

All species of life animal, plant and bacteria are susceptible to infection by viruses. Viruses are small particles that are composed of a core of genetic material, either RNA or DNA, surrounded by a protein coating called the capsid. The capsid protects the viral genes from inactivation by various enzymes, for example tissue nuclease enzymes, that would otherwise digest the naked viral chromosome. For many of them the protein coating plays an important role in the attachment of the virus to receptors on susceptible cells (Hugo, 2004). Furthermore in some bacterial viruses the capsid is further modified into an insertion mechanism for the viral genome to overcome the barrier of the cell wall of their hosts. It is composed of a variety of subunits called capsomers. Each capsomer is made of the same amino acid sequence. The capsid is formed from repeating units of capsomers. Therefore only 300 nucleosides are required to specify the capsid, leaving genetic capacity for other functions. This subunit structure allows the construction of virus particles by self assembly involving processes such as crystallisation. The two main structural groups are icosahedral and helical symmetry. There is also a third more complex structure that is comprised of a number of different structural components and occurs mainly in bacterial viruses. Therefore no coat synthesising enzymes are required. In addition to the protein coat some virus particles are surrounded by a protein envelope that has been derived from the cytoplasmic membrane from their previous host. Due to the simple structure they are very small varying from 28-250 nm (poliovirus-poxvirus) in humans. Viral particles can not synthesise their own proteins and can only grow and replicate in other cells which they infect by injecting their DNA/RNA, this is called the viral replication cycle. There are six essential steps in the multiplication of human viruses each of which can be used as a target for antiviral drugs.

1. Attachment or adsorption of the virus to the cell via specific receptors
2. Penetration of the virus into the cell

3. Uncoating and release of the viral nucleic acid from the capsid
4. Biosynthesis: viral genes are expressed resulting in the production of viral nucleic acid and proteins
5. Assembly of virus pieces to yield complete virions
6. Release of virions from the cell

See figure 1.4 for diagram of viral replication cycle of influenza virus (Wagner, 2003)



There are four scenarios of virus/host-cell interactions that can occur (Wagner, 2003).

1. Expression of the encoded proteins generating new viral particles. This usually results in the deaths of the host cells which explodes (lysis) and releases the viral particles that then infiltrate new cells within a tissue.

2. Elimination of the virus from the cell. The infection is aborted without recognisable effect on the host cell

3. Survival of the infected cell unchanged, except that it now carries the virus in a latent state

4. Survival of the infected cell in a dramatically altered state, for example the cell is transformed and might have cancerous properties.

There is a subfamily of viruses called retroviruses that establish and maintain a close association with their host cells following the primary infection to gain a great survival advantage. By associating itself closely with the host the virus only needs to breach the environmental barrier between individual host cells. In addition, once infected the host serves as a continuous source of infectious viral particles (Wagner, 2003).

To achieve this long lasting relationship the retro viruses integrate their genome into that of the host cell and therefore turn them into cellular genes. The retro viruses owe their uniqueness to the important ability to convert viral genomic RNA into cellular DNA. The key to this lies in the activity of one enzyme called reverse transcriptase.

A typical retrovirus consists of a single viral protein, the envelope, which is important for the receptor recognition. The capsid is made up of a second viral protein called group specific antigen. Inside there is the viral genome and also a few copies of three important enzymes reverse transcriptase, protease and integrase. These are important for the early stages of viral infection (Wagner, 2003).

1.4.1 Impact on human health

Viral infections pose serious risks to humans as they can cause severe illness and in many cases there is only limited medical help available. One of the problems with viral infections is that once the virus has entered a cell it is much harder to be identified and treated. A viral disease that is wide-spread is measles. The World Health Organisation estimated that 20 million people are affected each year and that 600 children die daily as a result of measles infection. The most obvious symptom that can be seen is a red rash that starts on the face and arms and spreads over the body (Strebel, 2003). While prophylaxis for measles is available there is no current medication once the patient is infected. Some viral infections do not break out straight away but are carried by the host often unknowingly. For example the herpes virus has a significant feature as it strikes a life long partnership with the host and undergoes periodic reactivation. There are several different herpes viruses, the most common ones are HSV-1 and -2. It is thought that virtually 100% of adults carry the HSV-1 virus, although in many cases the infection never breaks out. HSV-2 is genital herpes, a sexually transmitted disease that is wide spread and the infection can become chronic. Currently the seroprevalence, number of people who test positive, of the population of north America is 20% and in developing countries such as sub-saharan Africa is 30-80% among women (Nazli, 2009). These viruses can establish a latent infection of the ganglia of nerves that supply the site of primary infection and the latent disease is reactivated by a number of stress factors (Lemke, 2007). The problem when treating herpes infections is that current treatments can control the reactivation of the virus but can not eliminate the latent virus. Another viral disease that strikes annually is caused by the influenza virus that is commonly known as cold and flu which are respiratory illnesses. The World Health Organisation estimates that 5-15% of the people in the northern hemisphere are affected each year and for 3-5 million of these infections result in hospitalisation. In 250000-500000 of those hospitalised, death is the outcome. There are three types of influenza A, B and C where the A strain is responsible for the majority of influenza epidemics. Prophylaxis is available but

constant genetic mutations often cause changes in the structure of the virus resulting in limited success of this treatment method and making this virus extremely difficult to control (Smith, 2008).

The reason why viral diseases are so widely spread is due to the fact that they are easily transferred from one human to another by droplet, airborne or contact transmission, sexually or intravenously. They are very adaptable and in some cases have been transferred from one species to another as was the case with avian bird flu. These examples show some of the diversity and trickery that viruses posses and the impact they have all over the world and emphasise the need for new approaches in the search for drugs that can tackle viruses.

1.5 Antiviral agents and the search for new treatments

1.5.1 Antiviral agents

There are two classes of antiviral agents depending on their specific needs. Non-specific agents such as interferons are active against a number of different viruses and are produced as an immune response by the human body (Stewart, 1974). The second class are specific antiviral agents such as acyclovir that specifically target cells that are infected with the herpes virus (Sudo, 2005). A different approach for non-specific antiviral agents is to use aggressive compounds such as disinfectants, such as bleach, to kill the viruses before they can enter the patient. Topically applied antiviral agents for the treatment of verucas, warts and anogenital warts, caused by the papilloma virus, can be treated more aggressively and non-specifically as they will not be ingested but applied as cream or a tincture. A drug commonly used to treat this condition is podophyllum with the active ingredient being podophyllotoxin. Podophyllum is a resin obtained from six species of the Berberidaceae family and can be used to treat warts. Genital warts can only be treated at low concentrations due to the compound's aggressive nature (BNF, 2003, Drugs.com). Podophyllotoxin acts as a mitotic spindle inhibitor and is thus cytotoxic (Dewick, 2002). It is too toxic for systemic use.

Specific antiviral agents are required when viral infection has taken place in the body and the following problems have been encountered finding such agents. There are several compounds known that have inhibitory effects on mammalian viruses in tissue culture, but only few can be used in the treatment of human viral infections. The main problem is the selective toxicity of these drugs as they need to be specific for reactants that are only present in infected cells. Many effective inhibitors of metabolic processes, even if more or less specific for the pathogen, have unwanted side effects. The therapeutic index is the ratio of benefit of a drug compared to unwanted side effects (Rang, 2003).

To determine the therapeutic index extensive animal testing is required which is time consuming and expensive. Consequently only very few inhibitors can be considered as being safe anti viral drugs (Wagner, 2003). The sites for antiviral drugs to attack include prevention of adsorption of the viral particle, prevention of intracellular penetration of the adsorbed virus, inhibition of protein or nucleic synthesis and prevention of the release of viral particles from host cell, see fig1.4. The problem with the first two sites is that drugs using these targets are most likely to be given as a prophylaxis. Therefore the preferred strategy has been to identify viral functions that differ significantly from or are not found in the host and are therefore unique. Therefore a lot of research has gone into understanding the viruses' life cycles and attempting to specifically block these sites. Amantadine hydrochloride is a prophylactic used against influenza A, it does not prevent adsorption of the virus but inhibits the viral penetration and acts at the surface protein-receptor interaction of the cycle. It is a primary amine that stops the acidification of the virus infected vesicles that is essential for the completion of the viral entry. Most antiviral drugs work at different stages of the replication cycle inhibiting enzymes that are needed for replication (Balzarani, 1998). The most common ones are the nucleoside-related reverse transcriptase inhibitors (NRTIs) for example Zidovudine, which inhibit nucleic acid synthesis, viral protease inhibitors (PIs) eg. Saquinavir which inhibit the cytochrome P450 system and Non-nucleoside reverse transcriptase inhibitors (NNRTIs) for example Neviraparin. These analogues act at various points of the replication

cycle. For example thymidine analogues are mistaken by a reproducing enzyme (reverse transcriptase) for thymidine but cause premature termination of viral DNA (Hugo, 2003). Other possible sites of attack for antiviral agents include the prevention of adsorption of viral particles to the host cell, prevention of intracellular penetration of the adsorbed virus and inhibition of protein or nucleic synthesis, these types of drugs are called non nucleoside analogues. However the problem is that these drugs are not very selective and therefore also act on healthy mammalian cells. More recent types of nucleoside analogues (Aciclovir) have been developed which only become activated in infected host cells that express specific enzymes (thymidine kinase) (herpes specific enzyme). The enzyme initiates the conversion of the antiviral to a monophosphate and then to the tri-phosphate derivative which inhibits viral DNA polymerase (Sudo, 2005). The host cell polymerase is not inhibited to the same extent and the antiviral tri-phosphate is not produced in uninfected cells. Different conditions require the drugs to act at different stages of the replication cycle. For example reverse transcriptase inhibitors prevent the production of DNA in newly infected cells. However they do not prevent reactivation from previously infected cells (HIV). This is because this enzyme is not involved in this process. Therefore new analogues are being developed for preventing reactivation like protease inhibitors that act later on in the replication cycle. The majority of the nucleoside drugs are formulated as pro drugs because it decreases the toxicity and ensures that a higher proportion of the drug can cross the cell membrane. This is achieved by forming enzymatically hydrolysable functional groups to the hydrophilic group at the 5-O position such as esters, carbonates and carbamates (Anastasi, 2003). A new approach for the treatment of viral infections, mainly influenza strains, uses neuraminidase as a target. These neuraminidase inhibitors such as Tamiflu stop the release of the viral particles from the host cell. This results in a reduction of new viral infection of healthy cells and shortens the time that the patient suffers from the infection. Because they act at the last stage of the viral replication cycle they are less likely to cause mutation of viruses that can lead to resistance. (medilexicon.com)

1.5.2 Biotechnological products

Interferons are low molecular weight particles that are produced by virus infected cells, they are small proteins that inhibit the transcription of viral RNA. There are three types IFNά, IFNβ and IFNγ. They are the body`s response to viral infections and tumours and belong to the cytokine family. They are divided into two separate groups alpha and beta are type 1 and gamma is type two. This is due to the different receptors they bind to. Leukocytes produce IFNά, fibroblasts produce IFNβ and activated T-cells produce IFNγ (Balkwill, 1989). Receptor type 1 stimulation results in antiproliferative and antiviral activity, whereas type 2 stimulation results in weaker antiviral activity, increased immunomodulator properties and also a wider range of immune functions such as macrophage activation. IFNά has been approved and used successfully for the treatment of Hepatitis B and genital warts. IFNγ has been used in the treatment of multiple sclerosis and granulomatous disease. With technologies such as gene splicing and other genetic engineering techniques being developed it has been possible to make therapeutic quantities of these proteins. In the 80s when interferons were first licensed for the treatment of genital warts the yields of interferons obtained were poor. Only through genetic engineering was it possible to produce viable amounts of interferons. This is achieved by introducing a gene into the fast growing bacteria *Escherichia choli* to produce interferons (Hugo, 2003).

Antisense oligonucleotides are single-stranded deoxyribonucleotide oligomers, (about 20 nucleotides) with a nucleotide sequence designed to be complementary to a target mRNA transcript (Pakunlu, 2006). This class of drugs is specific for RNA viruses such as Influenza A and Ebola (Kabanov, 2002). Antisense drugs are the generation of short oligonucleotide polymers that are complementary to specific portions of viral mRNA molecules. These antisense oligonucleotides can be designed to specifically inhibit the translation of an important viral gene product with little or no toxicity to other proteins. However a problem encountered with these compounds as unmodified oligonucleotides are highly unstable *in vivo* in circulation and within cells due to rapid nuclease digestion. Therefore modifications of the compounds have to be made to make

them more stable and site specific. Despite a lot of research that has been done on these compounds site specific delivery is still a major hurdle. So far there is only one licensed oligonucleotide drug, Formivirsen, which is administered locally into the confined space of the eye but due to significant side effects its application is limited (Spurges, 2007).

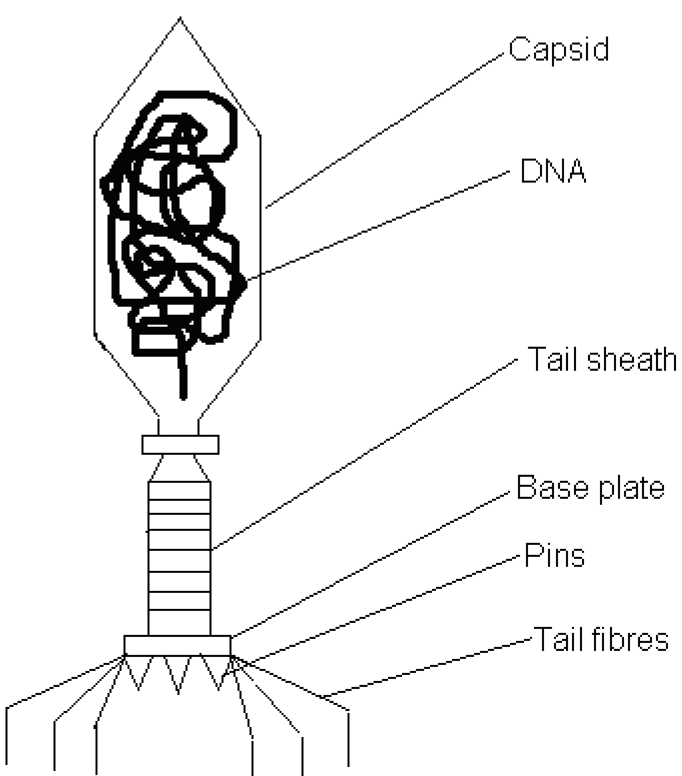
1.6 Bacteriophages

1.6.1 Morphology and properties

Viruses that use Bacteria as their hosts are called bacteriophages or are more simply termed phages. Phages posses a protein tail, tail fibre, base plate, pin and a capsid (head) that contains the genetic material, the tail is used to attach to the host cell, see fig 1.6. The common structures are icosahedral, helical and symmetrical cylinder. Each different phage is very specific and only attacks bacteria of a single species. The dimensions of the phage heads vary from 60x90 nm for Escherichia coli phage to 30x30 nm for certain Bacillus species phage. The tails can vary in length from 15-200 nm and can be rather complex structures. The majority of phages have double stranded DNA, however the small types tend to have single stranded DNA or RNA. After infection of the host bacterium one of two responses can occur either lytic or lysogenic. A lytic response is when the phage infects the bacterium, replication of the viral genetic material takes place, followed by lysis of the cell and release of the new phage particles. A lysogenic response is when the invading genome does not change the direction of cellular activity but incorporates its viral nucleic acid into the bacterial chromosomes. What results is called a prophage. When experiments are performed plating out phage mixed with sensitive indicator bacteria it is common that a combination of lytic and lysogenic responses occur. This is called lysogeny. The plaques, partial areas of clearing on the bacterial lawn, that can be observed after incubation are only in lytic cells (Wagner, 2003). Within a few minutes of infection the viral genome produces early mRNA expressing early proteins. These are responsible for interfering with the host cell macromolecular synthesis and start to make the first viral DNA. Once the build up of its

components has started the late RNA is produced. The RNA strands are transcribed from genes that specify the proteins of the phage coat forming the capsid. An enzyme to digest the cell wall, lysozyme, is also expressed in the cell at this stage and it eventually causes the lysis of the cell thereby releasing the viral particles. Phages have been found to kill bacteria that cause cholera, scarlet fever and anthrax *in vitro* but were found to have no activity *in vivo*. Since the development of antibiotics phage therapy has almost been redundant (Hugo, 2003). However due to the ease of handling of phage, compared to human viruses in the laboratory, and their rapid multiplication cycles they have been extensively used as experimental models.

Fig 1.6 shows basic structure of bacteriophage



1.6. 2 Bacteriophage as diagnostic agents

In 1998 Stewart and Denyer reported and patented a novel approach called the phage amplification assay for the rapid identification of specific bacterial

infections using appropriate phage particles. The method is based on the phage lytic cycle with plaque formation as the assay end point. This method has been reported to be sensitive and to give quantitative results within 4 hours. This was a significant reduction in assay time in comparison to previous assay done for the identification of bacterial strains as they required enrichment of the bacteria which often took several days.

The phage amplification assay comprises of four stages,

1) Phage infection of target bacterium,
2) Destruction of exogenous phage particles
3) Amplification of phage within host bacteria
4) Plaque formation of infected host with the aid of helper bacteria.

It was reported to work for a variety of different bacteria such as *Pseudomonas aruginosa, Salmonella typhimurium* and *Staphylococcus aureus* (Stewart et al, 1998).

1.6.3 Phage therapy in the treatment of bacterial infections

Prior to the discovery of antibiotics and their widespread application phage therapy was an alternative to treat infections. Phage preparations were used as early as 1919 by Felix D’Herelle, a French Canadian Microbiologist, to treat dysentery with clinical success. He also used phage therapy to treat patients suffering from bubonic plague, cholera and various other illnesses yielding good clinical results (D’Herelle, 1927). D’Herelle’s laboratory in Paris and the Eli Lilly Company (Indianapolis, Ind) produced phage preparations for commercial use but the efficacy of these preparations was controversial due to a lack of understanding of the spectrum of phage activity and the phage cycle.

Following the discovery of antibiotics phage therapy was abandoned in the western world after World War 2, but they continued to be used solely or in combination with antibiotics in the Soviet Union and Eastern Europe to treat infections. The Eliava institute in Georgia was and is the only institution whose

only focus of research was phage research and production. It produced various phage preparations for all the Soviet Union to be used in various fields of medicine surgery, urology and ophthalmology. Bacterial strains were isolated and collected from all over the Soviet Union and the socialist countries resulting in a vast collection of different bacterial strains and their corresponding phage.(Sulakvelidze, 2001), (Miedzybrodzki, 2005). This collection was and still is constantly updated with new phage particles isolated from new bacterial strains resulting in specific treatment of bacterial infections. (Kutadeladze, 2008) A recent application of phage therapy is a combination of a polymer and various phage strains called bioderm. Bioderm acts as a slow releasing system for phage which is applied to wounds. It has been shown to give good results for the treatment of burns wounds and ulcers that had been infected by an antibiotic resistant *S. aureus* strain (Markoishvili, 2002). Nowadays where antibiotic resistant bacterial strains are a more common occurrence phage therapy seems like a very reasonable alternative treatment that has the benefit of being highly specific.

1.6.4 Bacteriophage as model systems for studying mammalian viruses

In 1996 Maillard reported that bacteriophage can be used as a model system for human viruses in a variety of ways. The idea being that they have several advantages over human viruses. They only infect bacterial cells and are therefore not pathogenic to humans, their infection cycle is much more rapid than that of human viruses and complex and expensive media are not needed for propagation. The lytic infection cycle ends with lysis of the bacterial host, subsequently forming plaques, which are easy to assess, bacteriophages are wide spread in the environment and extremely diverse in their structure. They can therefore be used to study a variety of viruses of higher organisms.

Chapter two

Isolation and purification

2. Isolation and purification of plant extract

2.1 Introduction

In pharmaceutical research it is always of interest to find novel lead molecules and finding bioactive compounds from natural origin has become the subject of increased attention in recent years. Lead molecules have got "drug like" structures, which means that they are either similar to existing drugs and/or likely to fit the required receptor. These molecules can be optimised by synthesising additional functional groups that improve the pharmacokinetic properties of the product. The process is called lead optimisation. This is an important process as it helps to get the drug absorbed to ensure enough arrives at the target receptor to cause the required response. However it is always favourable to find a molecule that is "Intrinsically drug like" thereby reducing the time spent modifying it (Lednicer, 2006). In order to help finding molecules with drug like activity a quick screening method was required. A theory called Lipinski's rule of five has been applied in many cases. It states that the molecule in question should comply with four basic rules. It should not have more then 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, the molecular weight should not exceed 500 and the octanol, -water partition coefficient log P should be less then 5. There are only four rules but due to the fact that they all include multiples of 5 is where the name originates from. (Lipinsky, 1997) If a molecule passes all four parameters then it is likely to have good permability and oral bioavailability. In order for a molecule to pass the quick screening test it is required to pass at least two of the four parameters. It is important to get these molecules of interest in as pure a form as possible and if possible isolate them at a high enough rate compared to the starting material in order to make this approach feasible.

Taking this into consideration there are usually two steps involved. First a quick screening method is undertaken that has a high sample throughput but usually lacks accuracy. This screening method checks for activity. When interesting hits are found it is essential for the second step to be fast and effective. It involves the isolation and purification of the active lead molecule from the extracts

identified by the previous screening. Therefore it is important to have efficient and rapid preparative chromatographic separation techniques at hand. It is important to get purified natural products to allow for complete spectroscopic identification and characterisation as well as for biological testing, for the supply of pharmaceuticals, standards and as a starting material for synthetic work. However obtaining pure products from plant extracts can be a long, labour intensive and expensive undertaking involving many steps and the amount of the desired product may be minute. Therefore it is important to have an open mind and try to make use of the many different methods available. Trial and error will always be involved to ensure the right method or technique is found. The nature of the molecule of interest can range from highly polar to very lipophilic. The molecule determines which techniques can be used for isolation and purification. Because of the bioactivity of these compounds it is important to have gentle separation conditions in order to not lose activity. This usually limits the temperatures used during the extraction and the solvents that can be used. (Hostettman, 1998)

Isolation and purification of hydrolysable tannins, in this case ellagitannins, has been labour intensive in the past due to the polyphenolic nature of the molecule and the presence of predecessor molecules such as ellagic acid and gallic acid that have similar structures and are therefore difficult to separate. Early methods made use of cellulose and polyamide as solid supports in column chromatography (Mayer, 1977; Spencer, 1988). In addition to the columns these methods involved various other techniques to get a small amount of the desired product. The first different approach using an ion-exchange resin called Amberlite XAD-16 to isolate total pomegranate tannins (TPT) from an aqueous extract which can then be separated using analytical HPLC and tandem HPLC-ES/MS to give punicalagin at a high yield, 80-85%w/w (Seeram, 2004). The latest method for the isolation of punicalagin is by using high-speed countercurrent chromatography. This method has a 30% yield of punicalagin with a purity of 92% in a relatively short amount of time due to the very little sample preparation required. When the crude sample was analysed it was found to

contain about 41% of punicalagin which makes this method very effective. (Lu, 2007)

2.1.1 Sample preparation

Chromatographic separation techniques are used very widely in natural product research and isolation. The plant extract needs pre-chromatographic sample preparation in order for it to be suitable for use in chromatographic separation columns as their efficiency can be compromised by the excessive presence of impurities. Sample preparation followed by preparative chromatographic separation techniques are the most commonly used. A suitable sample pre-treatment can save a lot of time and effort in subsequent steps and make isolation considerably easier. Simple preliminary steps are often useful to remove most of the undesired material. Because of often costly and sensitive solid support in chromatography columns it is important to pre-treat samples to lower the risk of permanent damage to equipment, especially when working with HPLC. Classical pre-treatment techniques include selective extraction, filtration precipitation and centrifugation as well as simple open column chromatography. More modern approaches that give rapid results and good recovery include supercritical fluid extraction and solid phase extraction. (Hostettmann, 1997) The preparation of biomolecules that have properties different to the normal classes of secondary metabolites such as biopolymers, for example, require special handling. Sample pre-purification can be achieved by special techniques such as dialysis, ultracentrifugation combined with more commonly used techniques such as centrifugation, precipitation ion exchange and gel filtration. (Wehr, 1990)

2.1.2 Preparative chromatography

To separate and purify the molecule of interest chromatography can be used as it is one of the most powerful and most well established separation techniques available. Chromatographic techniques usually consist of a mobile phase and a stationary phase and make use of the different solubility or attributes of molecules within both of these phases. The stationary phase is usually of a solid nature like gel or resin and the mobile phase is usually liquid or gas in which the

sample under investigation is dissolved and then pumped through the solid support packed in a column. Due to different adsorptive properties of the different constituents in the sample they have different retention times and therefore get flushed out separately. The simplest technique is gravity column liquid chromatography (LC) that uses gravity to move the mobile phase past the solid support. A slurry of silica gel is poured into a glass separating column. More advanced methods are high pressure liquid chromatography (HPLC) where the system is pressurised to quicken the process and to increase the separation. The columns used contain a solid phase of smaller particle size resulting in increased backpressure. HPLC makes use of liquid mobile phase and a solid stationary phase. This method quickens the process and increases the sample throughput achievable compared to gravity column liquid chromatography.

More recently a lot of research has gone into counter current chromatography. This method was first introduced by Y. Ito in 1981, a pioneer in preparative chromatography. It uses a liquid-liquid system with two immiscible solvents, one acts as the stationary phase and the other as the mobile phase, a centrifuge is incorporated in order to get the two phases moving. This concept has got several advantages over the commonly used solid stationary phases. There is no irreversible adsorption of the sample on the solid support therefore reducing sample loss. This is combined with lower solvent consumption and the ability for quantitative recovery of the introduced sample. There is also a much lower risk of sample denaturation. On the downside there are some new problems that need to be solved. The most obvious being how to retain the stationary phase against the flow of the mobile phase and how to reduce mass transfer resistance i. e.: how to mix the phases and how to increase interfacial contact. Another concern is how to minimise laminar flow spreading the sample bands (Ito, 1981). Another disadvantage is the time it takes for a single run which can be a day or even longer. For natural products this is a particularly interesting method as there is no column packing material required and research in recent years has made full use of its potential. In China high speed counter current chromatography has been used successfully to separate plant extracts. In comparison to counter

current chromatography the centrifuge spins faster reducing the time of a single run dramatically to hours rather then days. In the eight months of 2009 there have been twenty three publications on high speed counter current chromatography on the data base of science direct alone. A lot of them have been on isolation and purification of components from plant extracts. It was reported by H. Yin, 2008 that high speed counter current chromatography has been used for the preparative isolation of two benzoxazinoid glucosides from *Acanthus ilicifolius L*. It has also been successfully used for the separation of five diterpenoids from *Tryperygium wilfordii* (Zhou, 2008). This shows the wide variety of application of this technique; isolation, purification and separation all using the same basic method. There are slightly different methods of high speed counter current chromatography and they can be used for separations.

2.1.3 Sample analysis

Analysis is an important part when purifying natural products or during organic synthesis as this is how the progress of purification, isolation or synthesis is determined and checked. There is a variety of techniques available and depending on the purpose, different techniques are chosen in order to maximise their potential. The most commonly used methods are:

- Colour tests or spot tests
- Thin layer chromatography (TLC)
- UV-Spectroscopy
- Fluorescent spectroscopy
- Infrared spectroscopy
- High pressure liquid chromatography (HPLC)
- Mass spectrometry (MS)
- Nuclear magnetic resonance spectroscopy (NMR)

The methods are in order of complexity and cost of equipment. If only confirmation of the presence of a certain functional group is required then a colour test or spot test using a specific reagent such as ferric chloride for the presence of phenolic groups is sufficient. Many such tests for a wide variety of functional groups are known (Kemp, 1986; Criddle, 1967). In order to check for different molecules of the same type TLC can be useful as it is possible to separate these using this cheap and basic technique.

UV spectroscopy can be used to monitor reactions in solution. The appearance or disappearance of absorbances at specific wavelengths within the spectrum will be indicative of the presence or absence of relevant functional groups (Greenwald, 1985). For example, hydroxylation of aromatic groups will result in a change in the lambda max for the aromatic ring system. Fluorescent spectroscopy can only be used if usually the resulting product of a reaction gives off fluorescent light and can therefore be detected using this method. Its big advantage over UV-vis spectroscopy is that if all reagents were previously checked interference is almost impossible. It works by having a monitoring molecule that reacts with the molecule of interest and results in fluorescent species. However this unique property also limits its applications but it has been used by research groups successfully. One approach to solve this problem is to add fluorescent markers that allow this method to be more widely used (Hoshina, 2007).

Infrared spectroscopy is a very useful tool for the determination of functional groups present as different functional groups give different IR spectral absorbances. Absorbances from different groups present on a molecule appear in the overall spectrum at characteristic wavenumbers. Quantitative analysis of substances within a sample using IR spectroscopy is difficult and is not commonly used restricting its use in chemical research to structure elucidation. It is routinely used to provide supporting data for results obtained from other instrumental techniques.

High pressure liquid chromatography (HPLC) is a separation technique that allows for the identification of specific, known substances. This is an extremely versatile technique and identification of analytes can be optimised by varying column chemistries, eluting solvent conditions and detection systems and setup. Several different detection systems are available. The most commonly used one being UV-Vis spectrophotometry. Setting the detector to the maximum wavelength of absorption of a particular analyte yields both qualitative and quantitative data. In recent years this method has increasingly been combined with Mass spectrometry (MS) where HPLC separates the different constituents which are then analysed using MS. These HPLC-MS machines are an integral part for most research groups nowadays as they have been proved highly efficient.

Mass spectrometric (MS) analysis is commonly used to determine the mass of molecules. There is a variety of methods available in mass spectrometry. The methods analyse ionised molecules but they differ in the way they ionise the molecule or atom of interest. There are 4 steps which lead to the analysis of the molecule and its mass/charge ratio.

1) Ionisation of the sample: The sample is ionised by one of a variety of ionisation techniques and gets the sample into a gas ion state.
2) Acceleration: Once the ions are formed they are immediately extracted and focused and accelerated using a series of electronic lenses to increase their kinetic energy.
3) Separation: The ions are then filtered by the analyser according to their mass/charge (m/z) ratio. Analysers can be combined in series to get better separation.
4) Detection: Ions terminate their flight by striking the sensor of the detector which measures electrical charge.

The mass is determined by the mass/charge ratio which is measured by the change of the ionised molecule when passing a magnetic field. The bigger the

molecule and or the smaller the charge the less it will be influenced by the magnetic or electric field (Kellner, 2003). The nature and the size of the molecule as well as its sensitivity determine what ionisation technique should be used. In this instance two suitable methods were chosen electron spray-time of flight MS and matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF) MS. They are both powerful analysing tools for determining the relative molecular mass of macro-molecules and were both introduced in the 1980s (Sørensen, 2007).

There are two different methods for the ionisation of the sample molecule and they are hard and soft ionisation. In hard ionisation a considerable amount of energy is transferred to the analyte resulting in unimolecular dissociation reactions. The most common one used is called electron ionisation. Most other techniques are soft ionisation techniques that result in little fragmentation and thus provide molecular mass information. The following ionisation techniques are all soft ionisation techniques.

The MALDI method makes use of easily ionisable matrixes that transfer their charge onto the analyte and the resulting ions are extracted before their mass/charge (m/z) ratios are determined by time of flight (TOF) analysis. This method can be difficult to master and it can be hard to get good reproducibility however used to its full potential this can be a very powerful and accurate technique.

Electrospray ionisation is the most commonly used method to ionise samples. This method combines nebulization and ionisation performed at atmospheric pressure. The ionisation mechanism is not clearly understood but it is thought that highly charged droplets are generated during nebulization and subsequent evaporation of the neutral solvent molecule resulting in a very mild ionisation method. ESI-TOF is a modern approach trying to achieve a MS that is reduced in size, benchtop format, with the analytical potential of a larger machine. It combines ESI which is one of the most commonly used methods for ionising samples with a TOF mass analyser. This results in a wide range of molecules

that can be analysed with high accuracy. This method can be used for large molecules and is therefore ideal for the analysis of the tannin punicalagin.

Both methods make use of the TOF analyser to determine the mass of the ion. This method is suitable for large molecules and uses the time the ion travelled a given distance under vacuum to determine its mass. The mass, shown as the m/z ratio, is saved and then combined with the relative abundance for presentation in the format of the m/z spectra. Software is used to calculate empirical formula according to the different m/z ratios (Irungu, 2008).

Recently a lot of research has gone into ion traps, this is a component that is added to the system and is situated after the ionisation setup and the acceleration of the molecule. Ion traps can be incorporated into different setups and will increase the clarity of the spectrum. An electrostatic ion gate pulses open and closed to inject ions into the trap. The ion trap is typically filled with helium gas. Collisions with helium atoms lowers the kinetic energy of the ions resulting in their being directed to centre of the trap. The ions are thus trapped. The trapped ions are further focused before being ejected sequentially from low to high m/z-ratio. Ions are ejected through holes in the endcap electrode and detected using an electron multiplier but TOF detection is also possible (Jonscher, 1997).

Nuclear magnetic resonance (NMR) spectroscopy has been widely applied for the determination of molecular structures. This method works by applying a strong magnetic field to the sample. The two most commonly used methods are proton and carbon-13 NMR. This is due to the wide distribution of these two elements. Other methods such as fluoride-19 or phosphorous-31 are also used when investigating molecules where these elements are present. The protons have got a spin state of + and – 0.5 and when a strong external magnetic field is applied any atomic nuclei with a nuclear spin now have different energy levels. The sample is now irradiated with a short pulse of electromagnetic radiation in the radio frequency. Nuclei that absorb the radiation are promoted to the higher energy level and the difference in energy is only very small but very specific to

where structurally it is positioned within the molecule. Once the nuclei fall back down into the lower energy level the energy given out is measured and analysed. The difference between the energy levels is proportional to the strength of the external magnetic field applied. (Clayden, 2001) Due to the fact that the difference in energy levels is very small the magnetic field applied has to be very strong. The sample, which is mostly in solution, is also spiked with a small quantity of, for example, tetramethylsilane which is added to define zero on the scale. This is important for the interpretation of the spectra. There are two basic parameters to look out for on NMR spectra, where the peaks are in relation to the zero marker, this is referred to as chemical shift, and whether or not there is splitting which is caused by closely related protons, this is referred to as proton-proton coupling. Splitting frequently results in multiple peaks that with the use of integration of the area under the peak can be used to determine protons in close proximity. What separates this technique from others is that it identifies protons which can be used to determine the location of functional groups present within the molecule (Kellner, 2003). In combination with MS it is highly useful in determining structures and it is therefore an essential tool in chemical research. It has, for example, helped significantly to elucidate the complex structure of the steroid saponin isolated from *Yucca filamentosa* (Plock, 2001). However, a problem when using NMR in combination with plant extracts is that due to their often extensive size and the presence of impurities the interpretation of the spectra can become increasingly complex.

2.2. Materials and Methods

2.2.1 Materials used

164 pomegranates of the type Valencia were obtained from a local supermarket (J. Sainsburys plc).

Syringe filter Centricon ^{tm} 0.45 µm, Amicon, Danvers, MA01923, USA

Soxhlet extractor fitted with 250 ml round bottomed flask

Thimble: Whatmans cellulose 19x90 mm

Centrifuge: Sorvell RC-5B centrifuge

HPLC: Pump: LDC Analytical Spectra Series P200

Column: C-18 reverse phase Phenomenex 250 x4.60 mm, 5 microns

Detector: LDC Analytical Spectra Monitor 3100 X/ variable wavelength monitor.

Chart recorder: BBC Coerz Metrawatt SE-120

Mass spectrometers:

Kratos MALDI-III, time of flight MS

Matrixes: Alpha-cyano-4-hydroxycinnamic acid (97%),

3-5-Dimethoxy-4-Hydroxycinnamic Acid, predom trans isomer 98%

4-Hydroxy-3-Methoxycinnamic Acid 99%

2-(4-Hydroxyphenylazo)-Benzoic Acid 97%

All obtained from Aldrich Chemicals Gillingham

Bruker Daltonics MicrOTOF

Bruker HCT plus ion trap

Thin Layer Chromatography (TLC): DC-Fertigplatten, Cellulose 20x20cm, 0.1mm thickness, (Merck Darmstadt)

Alugram Sil gel/uv$_{254}$, (Machery-Nagel Düren)

Solid supports for column chromatography: Cellulose powder (Aldrich chemicals, Gillingham)

Polyamide 6S (Riedel de Haën, Seelze Germany)

Amberlite XAD-8 (Supelco, Bellefonte, USA)

Silica Gel, 0.060-0.2mm, pore size 6nm

(Arcos Organics, Loughborough)

Water purifier: ELGA Purelab UHQ PS MK3

Solvents were all obtained from Fisher Scientific, Loughborough and of laboratory grade, except trifluoroacetic acid (TFA) (redistilled from glass) and the HPLC solvents were bought of HPLC grade, water used was obtained from a reverse osmosis water purifier of the type mentioned above.

2.2.2 Preparation and storage of pomegranate rind

Pomegranates were cut up and the rind was separated from the flesh. The rinds were rinsed with water and frozen in freezer bags at -20°C, prior to freeze drying. The freeze dryer cooling coil was set at -80° C. About 250 g of the frozen rind quarters were put into three separate 1 l beakers with as little contact area as possible between the individual rind segments. A typical drying cycle lasted 30 hours. The water content was about 55% of the wet weight. The dried rind was stored in freezer bags at -20°C.

2.2.3 Extraction methods

2.2.3 (I) Cold extraction

Fifty g of the dried rind were placed in a beaker and 500 ml of solvent (water and methanol) were added and allowed to stand for 18 hours. Solids were removed by filtration and the extract stored at <5°C.

2.2.3 (II) Soxhlet extraction

A soxhlet apparatus was used with extraction chamber volume 200ml, 250 ml round bottomed flask, cellulose thimble, the total volume of solvent was 200 ml,

weight of dry rind was 1.9 g and extraction time was 8 hours. Once the extraction was complete the solvent was evaporated to dryness under reduced pressure. Solvents used were methanol, dichloromethane, ethyl acetate, 60-80°C petroleum ether and isopropanol.

2.2.3 (III) Standard method for preparing PRE

The following method was developed by Stewart *et al.* (1998) at Nottingham University. Pomegranate (*Punica granatum*) rind was blended and boiled in distilled water, 25% w/v, for 10 minutes. After centrifugation (12,400 rpm, 4°C, 30 mins) the supernatant was autoclaved (121°C, 15 min), cooled and stored at -20°C. A further purification of the pomegranate rind extract was achieved by membrane filtration using a Centricon ^{tm} 0.45 μm filter and the filtrate was stored at 4°C.

2.2.4 Preparative scale purification methods

2.2.4 (I) column chromatography using cellulose

Ten grams of Celite was added to 50 grams of Cellulose powder and packed using 1M AcOH into a 12x300 mm glass column. 0.5 grams from soxhlet methanol extraction was dissolved in a minimum amount of de-ionised water and applied to the column. The column was eluted using 1M AcOH with 10% w/v of DMF as determined by cellulose TLC to give the best separation of fractions.

2.2.4 (II) column chromatography using polyamide-6S

Ten grams of polyamide-6S were pre-swollen in water and put into 50 ml glass funnel fitted with a glass wool plug. 0.5 grams from hot methanol extraction were dissolved in minimum amount of water and applied to the column by means of a funnel. A hundred and fifty ml of methanol was used to elute impurities followed by a gradient of methanol with 5, 10, 15 and 20% of DMF content used to elute the tannins from the solid support.

2.2.4 (III) column chromatography using Amberlite XAD-8

Ten grams of the resin were weighed out and prepared by wetting it in 200 ml of methanol for 30 minutes, followed by washing it with water and filling it into a glass column 12x300mm. 0.5 grams of sample from the hot extraction were diluted in the minimum amount of de-ionised water and were put onto the column and washed with 2-3 l of water. Once the water that was eluted was clear the column was attached to a vacuum line to dry the resin before using 200 ml of methanol to take the phenolic compounds off. The solvent was then taken off under reduced pressure.

The resin was recycled by washing it with 1.5 l of 0.1M NaOH followed by elution with water until neutral pH was reached. The same procedure was applied but 1.5 l of 0.1 M HCl was used instead.

2.2.4 (IV) Calcium precipitation

The product from soxhlet extraction using methanol was suspended in 50 ml of water, 0.5 g of $Ca(OH)_2$ was dissolved in 200 ml of deionised water. The mixture was placed in a sonicator bath for 15 minutes and then left to stand for 30 minutes. The solution was then centrifuged and the precipitate washed 3 times with de-ionised water. The precipitate (ppt) was acidified using dilute HCl to bring the polyphenols into solution.

2.2.4 (V) Final method for purification of pomegranate rind extract

A soxhlet extraction using petroleum spirit to remove waxy-non polar constituents was set up and run for 60 minutes. This was followed by the slightly more polar solvent ethyl acetate for another 60 minutes to remove more polar constituents. The rind was then extracted using isopropanol for 8 hours. The resulting isopropanol was filtered using filter paper to remove any rind residues and was removed under reduced pressure. The resulting product was then dissolved in the smallest amount of warm water and applied to a previously prepared Amberlite XAD-8 ion exchange column, see method 2.3.3 (I). The column was then washed using 4 litres of water. The column was then attached to a vacuum

line to remove any residual water for 30 minutes. The dry column was then washed with 200 ml of methanol to remove the polyphenols. The first 15-20 ml of eluted methanol was discarded and the rest of the eluted methanol was collected and transferred into a 250 ml round bottomed flask. The methanol was taken off under reduced pressure. The product was called purified pomegranate rind extract (pPRE).

2.2.5 Analysis of products

2.2.5 (I) Colour test for phenols

Two drops of ferric chloride, Fe^{3+}, $Fe(Cl)_3$ dissolved in water 1% w/v were added to 1 ml of test solution in a test tube.

2.2.5 (II) Thin layer chromatography analysis and silica gel column chromatography

A silica gel TLC plate was used and a variety of solvent systems were investigated for use as analytical tools for evaluating the acetylated product. The solvent system used successfully was composed of 70/30=ethyl acetate/toluene. This solvent system was then used for a silica gel column using 50 g of silica in toluene.

2.2.5 (III) HPLC analysis

All fractions were dissolved in 1 ml of HPLC grade water and filtered using a 0.22 µm membrane filter (Milipore). The solvent system was water and methanol with 0.1% trifluoroacetic acid (TFA) each. The flow rate was 1ml/min and the injection volume was 200 µl. The column used was a milipore 250x4.60 mm, 5 microns, C-18 column and the wave length used for the detection was 286nm. See table 2.2.5 for solvent gradient composition.

Table 2.2.5 gradient for HPLC analysis

Time (minutes)	% solvent A water + 0.1%TFA	%solvent B Methanol + 0.1% TFA
1	100	0
20	75	25
30	5	95
35	5	95
40	95	5

2.2.5 (IV) Mass spectrometric analysis

2.2.5 *(IV)a Matrix assisted laser desorption- time of flight Mass spectrometry (MALDI-TOF MS)*

Samples were prepared and applied in a sandwich method. The sample was dissolved in chloroform and the matrix was dissolved in methanol and applied individually to the target. It was also tried to mix the matrix and the sample prior to application and to add multiple layers of matrix to increase the chance of the sample being ionised. Positive and negative ion mode was used.

2.2.5 (IV)b Electron spray ionisation time of flight (ESI-TOF/MicroTOF) mass spectrometric analysis

A sample of 1 mg/ml of pPRE was prepared and diluted 1/100 before analysis.

2.2.5 (V) Analysis of derivatives

In order to facilitate the analysis by TLC and MALDI-TOF MS, acetylated derivatives of the extract were prepared. Using a small amount of pPRE, approximately 2mg and treated it with 2 drops of acetic anhydride and pyridine was left for four hours (Mayer, 1977).

2.2.5 (VI) Method for the analysis of the product

To check for the presence of phenolics three different methods were applied: the $FeCl_3$ colour test, HPLC and Mass spectrometry.

The colour test for phenols, $FeCl_3$ test, was used to check whether or not polyphenolic components were present in a fraction. It forms a coloured complex with phenols, dark green-blue giving a quick indication of their presence.

HPLC was used to test purified fractions and to separate the constituents and to check the amount of impurities present.

The final product, pPRE, was then tested using an ESI-TOF mass spectrometer.

2.3 Results

2.3.1 Extractions

Table 2.3.1 Results from various extraction methods

Solvent	Method	Results (as judged by intensity of $FeCl_3$ colour test)	Results as judged by HPLC analysis)
Water	Cold	+++	Very complex
Water	soxhlet	+++	Very complex
Methanol	Cold	++	very complex
Methanol	soxhlet	+++	Very complex
60-80^0 petroleum ether	soxhlet	Little polyphenolic evidence	N/D poor extraction efficiency
Ethyl acetate	soxhlet	Little polyphenolic evidence	N/D poor extraction efficiency
Iso-propanol	soxhlet	++	complex

2.3.1.1 Soxhlet extraction

The standard thimble used was filled with 1.9 g of freeze dried rind and extracted using propan-2-ol. After eight hours the extracted solvent was dried under reduced pressure giving 0.5 g of a yellow powder at a rate of 27% w/w. It was found that the solvent of choice had to be of a polar nature to extract the polyphenolics. Propan-2-ol was preferred to methanol as it seemed to extract less impurities and gave a cleaner chromatogram when analysed using HPLC.

2.3.1.2 Preparative chromatography

An approach using column chromatography was investigated. The most commonly used solid support in column chromatography is silica gel but because of the high polarity of the molecule, due to the presence of 16 phenol groups present, streaking is caused which made this method unsuitable.

2.3.1.2(I) Cellulose solid support

It was reported that cellulose chromatography can be used to separate punicalagin from the methanol extract (Mayer, 1977). Cellulose TLC plates were prepared and various different fractions were tested. After a suitable mobile phase composition was established using 1 M AcOH and 10% v/v DMF a column was set up. The bands observed were very broad and difficult to differentiate. Celite was added to increase the powder flow and therefore the packing properties but the result was not sufficient to get separation.

2.3.1.2(II) Polyamide solid support

Polyamide was chosen as the stationary phase as that has been reported to purify punicalagin using Methanol only to elute the polyphenol (Haslam, 1989). However, methanol on its own did not result in purified product, therefore a gradient using up to 20% DMF was used to get the polyphenols of the solid support. Fractions were tested for polyphenolic constituents using ferric chloride. Positive samples were evaporated to dryness under reduced pressure to give an orange solid. Different solvent systems were used to obtain separation but due to the strong interaction of polar compounds and the polyamide this method was only successful on a small scale giving a poor yield. K. Hostettmann reported that molecules with up to 4 phenol groups can be eluted using methanol but as the molecule of interest has 16 phenol groups it bound too strongly to the stationary phase (Hostettmann, 1998). Various different solvent systems had to be used making this approach very labour intensive and the final product still showed a complex chromatogram when analysed using HPLC.

2.3.1.2(III) Amberlite XAD-8 ion exchange resin

In order to achieve good separation of the polyphenolic entities a stationary phase had to be found that did not interact too strongly with phenolic groups. In order to achieve this ion exchange resins were investigated. It was reported that a macroreticular resin called Amberlite XAD-8, polar acrylic ester resin, was successfully used to separate tannins and other polyphenolic molecules from cassava root (Lalaguna, 1993). The resin is made of methylmethacrylate and has a high surface area. The mechanism of interaction between the resin and the phenolics is not fully understood but hydrogen bonding is probably involved. This resin did not bind as strongly to the phenolic compounds as polyamide. Because of this water was used to elute the impurities from the column and methanol was used to take off the large polyphenols rapidly. A yield of 15% w/w was achieved using the methanol and propan-2-ol soxhlet extraction product. This was a quick and simple way to purify plant extracts without the need of solvent gradients to get the products required.

2.3.1.3 Calcium precipitation

Polyvalent cations such as calcium are known to form precipitates with a number of product substances including polyphenolics and alkaloids. An additional method used involved the formation of a calcium salt of the phenolic compounds from the crude extract (Mayer, 1989). This method was unsuccessful as the resulting product was difficult to separate. The final product was put on the polyamide column and eluted with 500 ml of methanol followed by methanol with 0.1%TFA and a small amount of purified sample was obtained. The resulting product was analysed using HPLC and a less complex chromatogram was obtained. However this approach was highly labour intensive and only small amounts of product were obtained.

2.3.2 Analysis of products

2.3.2 (I) Colour test for phenols

If polyphenols were present an intense green blue colour could be seen due to a complex formed between the phenols and the ferric ion, see figure 2.3.2 for possible structure of complex. This method was fast and used throughout to monitor the presence of polyphenolic substances in extracts and fractions.

$3Ar(OH)+Fe(Cl)_3 \rightarrow Fe(OAr)_3+3HCl$

Fig 2.3.2 shows possible structure of ferric complex

2.3.2 (II) Thin layer chromatography analysis and silica gel column chromatography

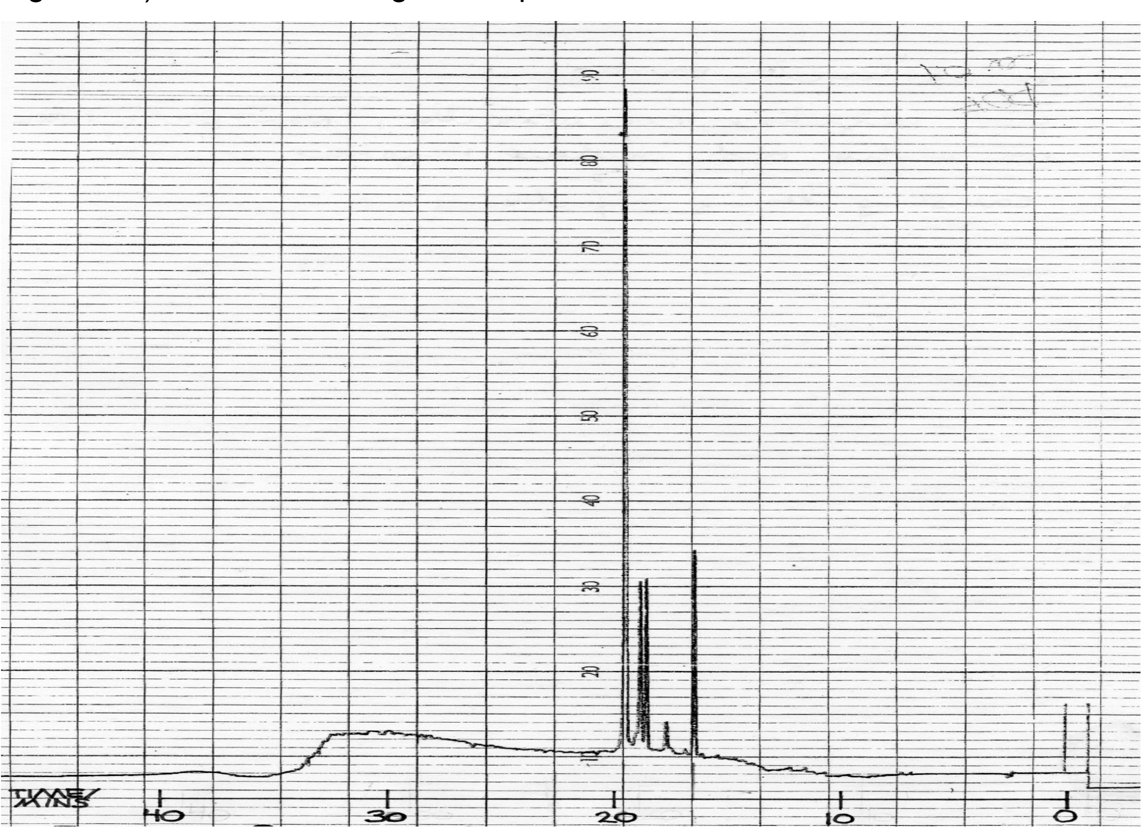
This method was unsuitable for the analysis of pPRE due to the polar nature of polyphenols which causes severe streaking. It was used for the monitoring of the acetylation reaction. The acetylated product was less polar than the original molecule and therefore silica gel could be used as solid support for TLC and column chromatography. A suitable mobile phase, 30/70 toluene/ethyl acetate, was found after the products were tested using silica TLC. The same mobile phase was then applied to a silica column and three different fractions were obtained to aid structural analysis.

2.3.2 (III) HPLC analysis

HPLC was used to assess the purity of purified samples. It makes use of the different partitioning of the molecule in question between, a C-18 column which is

highly non-polar and the mobile phase which is more polar. The mobile phase was pumped continuously through the column and depending on the nature of the molecule it was retained for different lengths of time by the column before being eluted and detected. The polarity of the mobile phase can be altered as it is composed of two components, methanol and water spiked with an organic modifier. In this case trifluoroacetic acid (TFA). The strength of this technique is that the mobile phase composition can be altered to maximise the separation of the sample under investigation to give better separation of peaks. The chromatogram obtained by the final method of purification, 2.2.6, is shown in fig 2.3.2.

Fig 2.3.2a) HPLC chromatogram for pPRE at 286 nm



2.3.2 (III) Mass spectrometric analysis

2.3.2(III) a MALDI-TOF mass spectrometric analysis

The power of the MALDI-TOF mass spectrometer for the analysis of polyphenolic components from plant origins was first demonstrated when the degree of polymerisation of proanthocyanidin in apples was determined by Omnishi-Kamayama, 1997. This allowed them to identify up to pentadecamers (15) rather than only pentamers (5) which were the biggest polymers analysable when fast atom bombardment (FAB) MS was used previously.

The determination of polyphenols using various different matrices was investigated to determine the purity of the samples. The resulting spectra showed few signals indicating that this approach was unlikely to give extract and fraction profiles that could be regarded as being qualitatively reliable. To increase the chances of getting results using this technique some of the sample was acetylated. The MALDI-TOF mass spectrometer was set up but the resulting spectra showed no improvement of quality compared to the non-acetylated product. The same method and matrix used to analyse this molecule were successfully used and published by Kulkarni (2004) 4 months later.

2.3.2(iIi)Electro-spray ionisation time of flight (ESI-TOF/MicroTOF) mass spectrometric analysis

The mass found: 1107.0466, $C_{48}H_{28}O_{30}Na$

Required mass of analyte: 1107.05578

When a sample of pPRE was analysed a peak at 1107 was observed which is the molecular mass of punicalagin, mr=1084, with a positively charged sodium ion, mr=23, also present, see fig 2.3.1a. This shows that punicalagin is present in pPRE and confirms that the isolation and purification process was successful. In 2.3.1b the molecular formula generator results are shown but the molecular mass of punicalagin is not shown. This is probably due to the fact the tolerance was set at 5ppm and the difference in masses of found and required mass is 8ppm.

Fig 2.3.2b) Electro-spray time of flight mass spectrometric analysis results for pPRE, Na ion of punicalagin 1107 can be seen

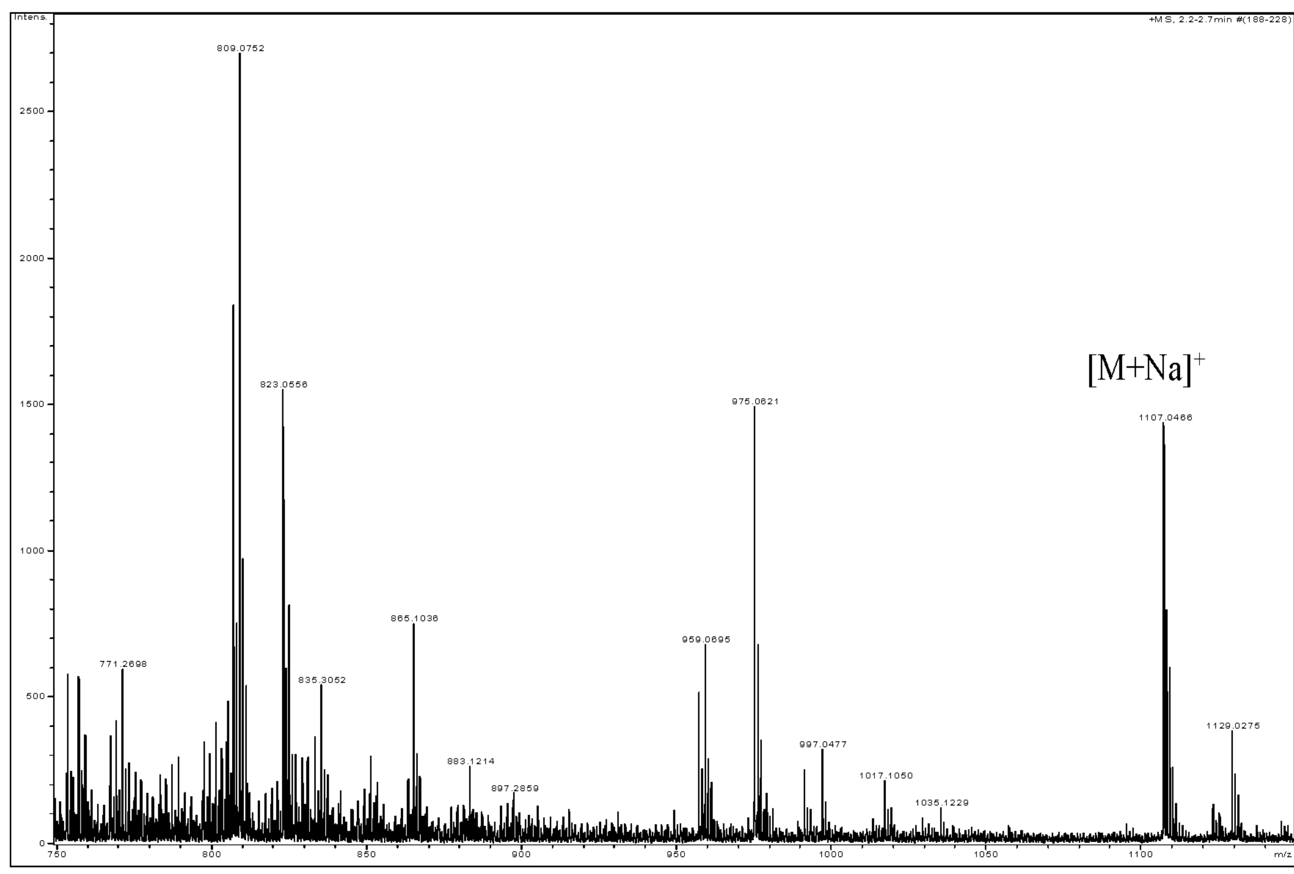
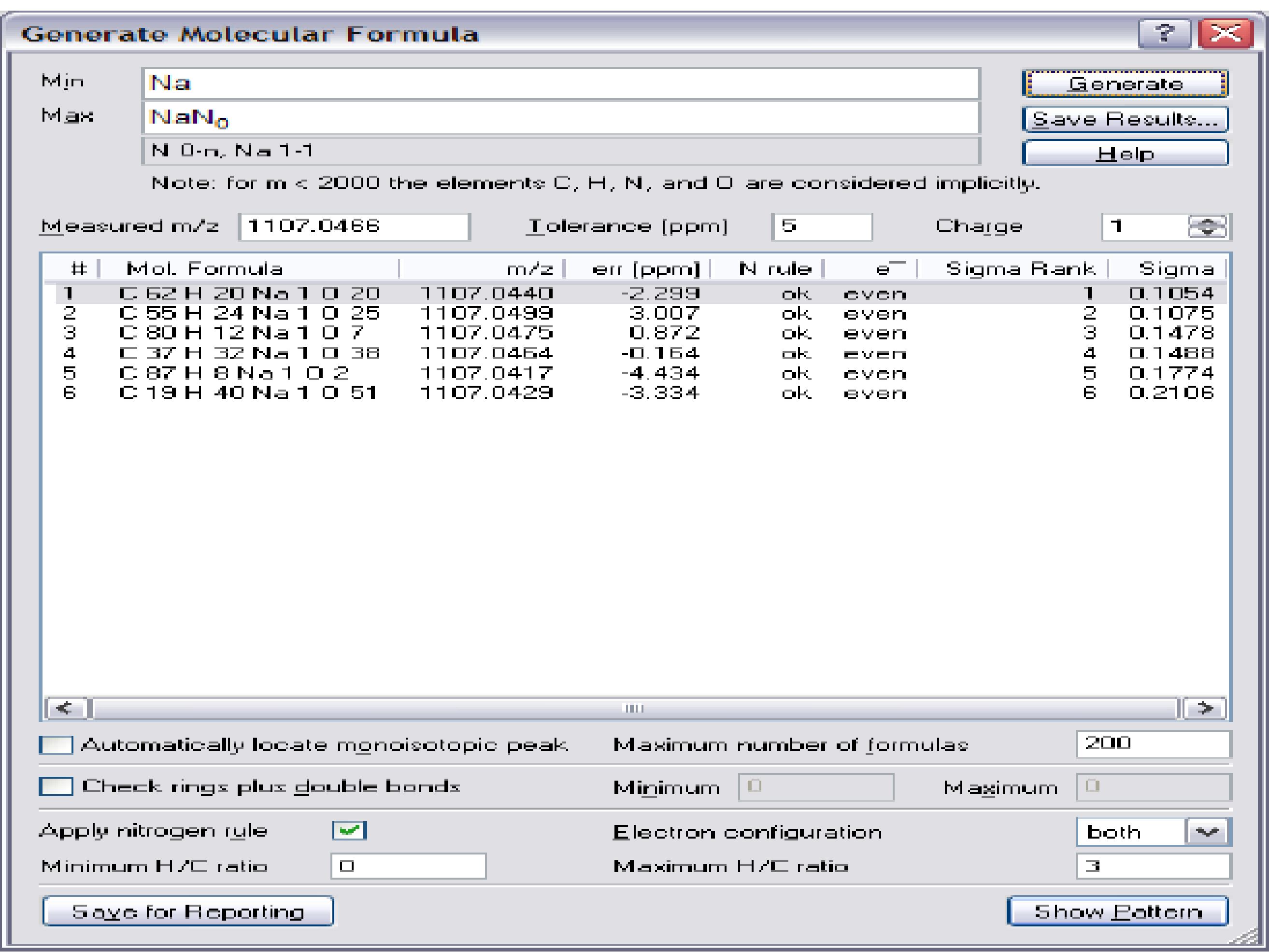


Fig 2.3.2c) General molecular formula results of Na ion of punicalagin



2.4 Discussion

Prior to extraction sample preparation was carried out to remove for example waxes and chlorophyll that are usually present in natural products. A commonly used process is a gradient of solvents with different polarity using a variety of stationary phases such as ion exchange resins or silica gel. A problem with plant extracts is that a variety of different molecules are extracted and therefore it is difficult to know which one(s) are responsible for the activity. The aim of this part of the project was to obtain the hydrolysable tannin, punicalagin, which is thought to be responsible for the pomegranate`s anti viral properties, in as pure a form as possible. Another obstacle lies in the nature of the molecule, a polyphenol, a very polar molecule that is difficult to separate due to a limited number of stationary phases available for chromatography, as well as a wide variety of plant constituents with the same characteristics present that make it more difficult to separate.

The molecule of interest is polar and therefore polar solvents were used. There are many other constituents in the plant material that are also extracted leaving a complex mixture to be separated.

Extractions using the polar solvents methanol (soxhlet and cold) and water (cold) were carried out as it was reported by Stewart (1998) and Schilling (1985) that these are rich in punicalagin. To test for their presence of phenolic compounds a $FeCl_3$ colour test was used. The problem with these two solvents was that they also extracted a wide variety of non-phenolic compounds.

In an attempt to obtain a more pure extract and eliminate polar contaminants, such as sugars, sample preparation was done using soxhlet extractions with a range of organic solvents. Using solvents like petroleum ether 60°-80^oC, dichloromethane and ethyl acetate in a soxhlet extraction were explored, see table 2.4.1. It was hoped that fewer highly polar substances would be extracted and therefore the efficiency of the soxhlet extraction would result in punicalagin being isolated in a purer form. This would be a very cheap and efficient way to

obtain the desired product . The polyphenol concentration tested using the colour test was very low making this method unsatisfactory.

Other solvents were explored such as finding azeotropic mixtures that would lower the boiling point of the solvent making the extraction milder and therefore putting less stress on the extracted products. Another advantage of using azeotropic mixtures was that different polarities could be achieved by combining for example solvents such as methanol and acetone. Various different azeotropic mixtures were tested but the results were not satisfactory.

When samples were analysed and compared using HPLC the propan-2-ol extract showed the cleanest chromatogram and it was also less complex then the methanol one. It was reported by Kulkarni, 2004 and others before that the solvent of choice was methanol but by using propan-2-ol a purer starting material could be achieved prior to chromatography. To further reduce the amount of impurities that are present in the final product, a series of extractions were carried out as sample preparation. Firstly a very non-polar solvent in a soxhlet extraction, petroleum ether 60°-80° C, followed by a more polar one, ethyl acetate, before extracting with the actual solvent, propan-2-ol. This lowered the amount of non-polar impurities present in the extract prior to chromatography. The resulting extract was then dried under reduced pressure leaving a yellowish orange crystalline solid behind. This was then re-suspended in the minimum amount of water before being put on an LC column packed with Amberlite XAD-8 ion-exchange resin. A brownish yellow colour could be seen where the tannins had been bound by the resin. The resin was washed with water until the eluting liquid was colourless. The column was then dried using a vacuum line for 30 minutes followed by the removal of the product with methanol. A yellow plug could be seen. The initial 10 ml of elutant was brown and was decanted. The rest of the methanol extract was combined and dried under reduced pressure to give a yellowish orange crystalline solid which was referred to as pPRE. A sample of pPRE was then analysed using ESI-TOF mass spectrometric analysis. Ions of the combined mass of punicalagin and sodium appeared at a mass of 1107, could be seen. This is the sum of the mass of a sodium ion and punicalagin,

23+1084. This showed that punicalagin was successfully purified and present in pPRE. A problem when purifying and analysing natural extracts is that there are a lot of precursor molecules present that have similar or the same functional groups as the target compound making them difficult to separate and cause more complex spectra when analysed using HPLC and ESI-TOF. To improve the separation and isolation high speed counter current chromatography could be applied as recent publications suggest that it has successfully been used to separate natural products and that difficulties associated with this method previously have been overcome. (Peng, 2008, Huang, 2008) This method could also be scaled up to give reasonable amounts of product and there is a possibility that it could be automated and combined with separating and analysing tools. In mass spectrometry ion traps have found their way into many systems as they increase the abundance of target molecules by concentrating them prior to release. There is now an ESI-TOF fitted with an ion trap which increases the potential of this method.

2.5 Conclusion

This chapter was concerned with the isolation, purification and analysis of the hydrolysable tannin punicalagin from the rind of the pomegranate. For the isolation and purification a combination of a soxhlet extraction and liquid chromatography using an ion exchange resin called Amberlite XAD-8 were used. This method was simpler and less time and material consuming then previous methods applied for this purpose. The resulting orange-yellow coloured crystalline powder was purified but isolation of punicalagin was not achieved. The sample was analysed using HPLC and ESI-TOF mass spectrometry and the presence of punicalagin was clearly shown.

Chapter three

Establishment and development of bio-assays for the determination of virucidal activity

3. Establishment and development of bio-assays for the determination of virucidal activity

3.1 Introduction

The aim of this experiment was to devise a reliable, cheap and quick method for the testing of antiviral activity of a compound. As has been mentioned in the introduction on antivirals one of the problems is that such methods have to be very specific and due to the high cost of working with human viruses and the length of the replication cycle it seems like a very feasible idea to use a species of bacteria and its corresponding phage as a primary model. By using phages rather then viruses the replication cycles are shortened and the safety requirements in the laboratory and therefore the cost of the tests are significantly reduced. Bacteriophages have been suggested as model systems for studying human viruses by Maillard (1996).

3.1.1 The phage amplification assay

Stewart *et al* (1998) used phage in a patented method which can be used to detect specific bacteria at low concentrations. This assay is based on the lytic cycle of phage and is used to detect specific bacteria from contaminated sources at very low concentrations. The principle is that the added bacteriophage interacts and infects the specific host bacterium during contact time. After this step the surplus phage particles are killed using a virucide that does not interact with the bacterium. The phage particles that have infected the bacterial cells are safe and are now ready for the subsequent release of new and viable bacteriophage. These can then be amplified in number by added helper bacteria. The number of phage can then be determined by comparing the number of plaques produced on a lawn of helper bacteria after incubation with the number of plaques produced by equivalent controls in the absence of the original target bacteria. Until this assay was introduced testing for trace bacteria took several days because there was need for enrichment of the bacteria. This method can detect trace amounts of bacteria in 4 hours which is a significant improvement

and due to the high specificity of phage particles it is also highly selective. The antiviral used by Stewart *et al* was pomegranate rind extract (PRE). It was used successfully with a variety of different bacteria and their corresponding phage such as *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Staphylococcus aureus* with assay times being 4 hours for all of them.

3.1.2 Bacteriophage assay as model system for studying viruses

Bacteriophages have been used as model systems for human viruses for several reasons:

- They are bacteria specific and therefore pose minimal risk to the handler making it a low cost method as safety measures are not as high.
- Their infection cycle is more rapid than that of human viruses and expensive culture media are not needed for their propagation.
- The life cycle ends in lysis of the bacterial host, subsequently forming plaque forming units (pfu), these can be measured by counting.
- Bacteriophages are widespread in the environment and are highly diversified in their structure and can therefore be used to study a wide spectrum of viruses of higher organisms (Maillard, 1996).

Gracia Mendez de Corao tried to make use of the bacteria/phage system and the natural phagocide used by Stewart et al to develop an assay to test for antiviral properties of plant extracts. A quick screening method was derived using a 96-well plate microtitre assay in her Ph.D. thesis, 2001. The compound in question was added to a previously titred amount of bacteriophage for long enough to ensure the killing of the phage. In Mendez de Corao`s work the virucide was also PRE and ferrous ions and the contact time was 3 minutes, same parameters as Stewart (1998). This was followed by the addition of the appropriate host bacteria. Two different bacterial strains and their corresponding phage were used in the tests for phagicidal activity of plant extracts. They were *Pseudomonas aeruginosa* NCIMB 10548 and *Escherichia coli* ATCC13706. The mixture was

then placed into a 96-well microtitre plate and incubated in an appropriate growth medium. Antiviral activity was then deduced by the presence of cloudiness of sample wells compared to control wells that did not contain any virucide. If the well was cloudy it indicated that the virucide had destroyed the bacteriophage and therefore the bacteria could divide and grow. However, if the bacteriophages were not destroyed, they infected the bacteria causing lysis (cell death) and therefore the well remained clear. A problem with this way of detection is that in the reaction of the natural polyphenols with ferrous ions a complex is formed that can be seen as dark blue/black precipitate which could lead to misinterpretation of results. This method was relatively quick to do but, as the results were deduced by observation only, the time required for the assay procedure was longer as it could not be automated as was initially anticipated. Results obtained by Mendez de Corao`s work showed that this method could be used for measuring phagicidal and bacteriocidal activity as she tested a variety of plant extracts. This method was used to check for the antiviral activity of potential virucides. Many different ones were tested but extracts from *Chamaecrista cf. flexuosa*, *Mimosa tomentosa* and *Byrsonima chrysophilla* showed the most potent antiviral activity. However due to cloudiness being used to monitor the outcomes the reliability of these results is questionable. Another limiting factor in this assay is that regrowth can occur and there is no way of checking for this. Regrowth occurs when there are contaminating bacteria strains present. Due to the size of each individual well of a 96-well plate it is impossible to spot such contamination. This can again lead to misinterpretation of results making this assay semi-quantitative at best. It was shown that pomegranate rind extract (PRE) in the presence of ferrous ions showed phagicidal activity and confirmed the results reported by Stewart *et al.*, 1998. Electron microscopy of the phage treated with PRE and ferrous ions showed clearly that they were deformed, lacking tails and were static compared to the intact untreated phages. Such damage was similar to the free radical damage seen with treatment by bleach. This method showed that bacteria/bacteriophage systems can be used to test for

antiviral activity of plant extracts and with modifications in terms of monitoring the outcome, this could be made into a more reliable assay.

Following on from Mendez de Corao's work the following methods were designed to monitor phagocidal activity of plant extracts from *Punica granatum* L. in a dose dependant matter. For this to be achieved a different way to monitor the outcome of the assay had to be derived. One of the issues when designing such a method is to get the right balance between time taken to complete the assay, throughput, and accuracy required. Precision is an important factor as bad reproducibility can cause a sharp increase in time spent trying to get continuously precise results. For example Mendez de Corao`s method was quick but the accuracy and precision was low. Therefore it can primarily be used as an indicator test to give an idea of antiviral activity present. In a perfect world a very precise, accurate and high throughput method would be designed that can give instant results. However as this is difficult to achieve the goal was to increase the accuracy and precision of the assay but at the same time not to lower the sample throughput too much. When working with living organisms it is difficult to achieve good precision as there are many factors influencing the growth of phage/bacteria systems. For example different temperatures in the laboratory, age of bacteria since being defrosted, continuous thickness of growth medium on agar plates and the time elapsed since they were prepared to just name a few.

The following method tried to establish a model system that showed the anti-viral properties of different extracts from pomegranate rind as well as to elucidate the mode of action of activity of this substance employing bacteriophage and the natural host bacterium for the phage.

3.2. Materials

The bacterial host used was *Acinetobacter baumanii* HER 1401 and the corresponding phage was HER 1401 BS46. They were both obtained from Lavalle University Canada and were suspended in TSB and glycerol. The phage is a double stranded DNA phage, *A. baumanii* is non fermentative, gram negative, non motile and oxidase negative and is known to cause lung infections. This bacterial strain and its corresponding phage were successfully used in a variety of assays by a fellow research student in the department and was therefore chosen as a model system.

Dried media and media components, agar and broth, were obtained from Oxoid Ltd. (Basingstoke, GB) and subsequently from Fisher Scientific (Loughborough, GB).

Tryptone Soya Agar (TSA)

Tryptone Soya Broth (TSB)

Technical Agar Nr 3

Chemicals used:

Gelatine, Fisher Scientific (Loughborough GB)

$MgSO_4 \cdot 7H_2O$, Acros Organics (Loughborough GB)

$FeSO_4 \cdot 7H_2O$, Acros Organics (Loughborough GB)

Tris(hydroxymethyl)aminomethane (Trizma), Acros Chemicals (Loughborough GB)

Water purifier: ELGA Purelab UHQ PS MK3

The water used in any of the reagents was purified water of laboratory or HPLC grade was obtained by reverse osmosis using the purifier listed above

Syringe filters: Centricon tm 0.45 µm filter (Amicon, Danvers, MA 01923, USA)

0.22µm Acrodisc (Gelman Sciences)

3.3 Preparation of reagents

3.3.1 Tryptone soya broth (TSB)

A 10 ml volume of TSB was prepared at the standard strength of 30 g per litre of deionised water followed by autoclaving at 121°C for 15 minutes.

3.3.2 Tryptone soya agar (TSA) plates

The agar plates used TSA as the growing medium at concentration of 40 g per litre of deionised water which was autoclaved at121^{o}C for 15 minutes before the plates were poured.

3.3.3 Lambda buffer

One molar Trizma base was prepared by adding 12.11g of Trizma base to 100 ml of deionised water. The pH was adjusted to 7.2 using dilute HCl and autoclaved at 121°C for 15 minutes.

For Lambda buffer 6ml of the previously prepared solution was added to 2.5 ml of 2% w/v of Gelatine and 2.5 g of $MgSO_4 \cdot 7H_2O$ in a 1 l volumetric flask and made up with deionised water. The buffer was then dispensed into 100 ml capped glass containers and autoclaved at 121°C for 15 minutes.

3.3.4 Pomegranate rind extract (PRE)

The crude PRE extract was prepared after the method of Stewart *et al.* (1998). The only difference being that freeze dried rind was used instead of the fresh rind in the protocol. The loss of weight during drying was taken into account.

Pomegranate (*Punica granatum* L.) rind was blended in purified water (25% w/v) and boiled for 10 minutes. After centrifugation (12 400 g, 4°C, 30 mins) the supernatant was autoclaved (121°C, 15 mins), cooled and stored at -20°C. A further purification of the extract was achieved by syringe filtration filtration through a 0.45 μm filter and the filtrate stored at 4°C.

3.3.5 Purified pomegranate rind extract (pPRE)

The pPRE was obtained, using the methods described in section 2.2.6. A stock solution was prepared by dissolving 0.001g of pPRE in 10 ml of HPLC grade water, this was followed by filter sterilisation using a 0.2 μm cellulose acetate filter.

3.3.6 Sloppy Agar

Sloppy agar was prepared by boiling 300 ml of purified water and dissolving 2.4 g of technical agar No 3 at a concentration of 4% g/l of agar. To that 18 g of TSB dissolved in 300 ml of hot but not boiling purified water was added. Aliquots of 5 ml of this mixture were pipetted into 15 ml Bijoux bottles followed by autoclaving at 121°C for 15 mins.

3.3.7 Ferrous sulphate

Ferrous sulphate stock solution was prepared by dissolving 0.0533 g of ferrous sulphate hepta hydrate in 10ml previously degassed HPLC grade water. The solution was prepared just before use to minimise oxidation. The solution was then sterilised by filtration using a syringe filter, 0.22μm, and used straight away to minimise oxidation.

3.3.8 Bacterial suspension

Bacteria were taken from -80°C freezer, defrosted and once in suspension 1-4 colonies were spread onto a TSA plate and incubated for 16-18 hours at 37°C. From this plate one colony was suspended in 10 ml of TSB and again incubated for 16-18 hours. The optical density of the resulting suspension was measured at 600 nm and adjusted to 0.460-0.470 using TSB, which corresponds to 1.4x10^{8}-1.5.x10^{8} cfus/ml of *Acenitobacter* her 1401.

3.3.9 Phage suspension

The concentrated phage suspension was prepared and titrated using 1in 10 dilutions to find the concentration that results in countable plaques. Typical

phage titres were $1x10^{10}$ plaque forming units (pfu) for the stock solution, phage stock was maintained in Lambda buffer at 4^oC.

3.4. Assay of phagicidal activity by plaque counting

3.4.1 Method

Sloppy agar was chosen as the medium to mix the antiviral with the bacteria and appropriate phage particles.

a. Sloppy agars aliquots (5ml) were melted in a boiling water bath for 5 minutes and then left to cool down in a 50^oC water bath for 90 minutes.

b. The Eppendorf rack was kept in a 37^oC water bath for 30 minutes. The tubes contained all the reagents used in the assay. All the reactions were done in the tubes to reduce clumping of phage particles and therefore assure reproducibility.

c. Autoclaved Eppendorf tubes (2ml) were used to mix 100 µl of 10^3 pfu/ml of phage particles suspension with 100 µl of antiviral suspension plus 100 µl of dilute ferrous sulphate solution if required in the experiment or 100 µl of sterilised water to keep the volume consistent.

d. Three minutes contact time was allowed before the mixture was added to the sloppy agar and vortexed for 5 seconds.

e. A volume of 100 µl of the bacterial suspension with an optical density of about 0.460 at 600nm was added to the sloppy agar and vortexed for 5 seconds before being poured onto TSA plates.

f. All experiments were done in 5 replicates and controls were done for each component added individually on the day to assure reproducibility. The concentration of phage and bacteria was constant throughout the experiment and only the concentrations of the plant extracts and ferrous sulphate were adjusted.

g. The plates were left to settle for at least 60 minutes before incubation at 37^oC for 16-18 hours.

Plaques are counted and are commonly referred to as plaque forming units (pfu) and activity of the anti viral was deduced by the reduction in pfus.

3.4.2 Results and discussion

3.4.2.1 Optimisation of reagents

3.4.2.1 (I) Phage titre

The phage stock suspension was of a concentration of 10^{10} pfu/ml so to deduce the right concentration to give countable plaques a phage titre was carried out. This was achieved by doing serial dilutions. First two agar plates were prepared for each concentration and the phage was mixed with the inoculum and incubated for 18 hours. It was found that at concentrations above 10^4 there were too many pfu to count and below 10^3 not enough pfu were observed. Therefore 10^3 and 10^4 pfu/ml were the concentrations of choice and 5 replicates of each of these concentrations were done, see table 3.4.2.1(I) The data shows that the best results were achieved at a concentration of $1x10^4$ pfu/ml. This was confirmed by a lower relative standard deviation.

Table 3.4.2.1(I) Determination of the optimum concentration of phage particles to yield countable and reproducible pfus, phage concentration against pfus

Phage conc in pfu/ml	replicates					average	stdev	RSD%
	I	II	III	IV	V			
$1x10^3$	36	27	25	22	22	26.4	5.77	0.219
$1x10^4$	135	93	115	124	143	122	19.39	0.159

3.4.2.1 (II) Ferrous sulphate titre

It is important to find the most suitable concentration of ferrous ions required to get complete kill of the phage particles. The reason for that being that the ferrous ion is highly reactive and therefore if present in excess side reactions could occur that might cause interference. To find the optimum concentration of ferrous

sulphate in HPLC grade water a serial dilution was done using two replicates. PRE stock solution, 10^4 pfu phage suspension and standardised inoculum were used in the experiment. A stock solution with a concentration of 5 mM was prepared and serial dilutions were done. Only two replicates were done initially to get an idea of the concentration ranges. It was found that the cut off point was between $5x10^{-2}$ mM and $5x10^{-3}$ mM, which is equivalent to no plaques seen at concentrations $5x10^{-2}$mM/l or higher. Therefore six serial dilutions with five replicates each were done in this concentration range, see table 3.4.2.1(II)b. Taking into account that temperature can influence the number of phage particles killed and the variability between 3.4.2.1(II)a where all phage particles were killed at $5x10^{-2}$ and at the same concentration in table 3.4.2.1(II)b where some phage particles survived it was decided that for this assay the concentration of ferrous sulphate should be $5x10^{-1}$ to ensure complete killing of all the phage particles. By choosing this concentration it was assured that there would be enough ferrous ions present to react with any antiviral compounds present in the purified plant extract or in the crude PRE at lower concentrations.

Table 3.4.2.1(II)a Determination of critical ferrous sulphate concentration in relation to phagicidal activity

Conc of $FeSO_4$ in mM	Replicates in pfu		Average pfu
	I	II	
blank	87	74	80.5
5×10^{-4}	73	87	80
5×10^{-3}	73	60	66.5
5×10^{-2}	0	0	0
5×10^{-1}	0	0	0
5	0	0	0

Table 3.4.2.1(II)b Determination of critical ferrous sulphate concentration in relation to phagicidal activity

$FeSO_4$ in mM	Replicates in pfu					Average pfu	stdev	RSD%
	I	II	III	IV	V			
blank	214	203	223	220	210	214	7.97	3.72
5×10^{-3}	212	213	203	208	196	206	7.02	3.4
1×10^{-2}	147	144	138	121	140	138	10.12	7.33
2×10^{-2}	126	128	154	141	126	135	12.32	9.13
3×10^{-2}	105	108	113	114	102	108	5.12	4.74
4×10^{-2}	50	56	37	46	44	46	7.06	15.35
5×10^{-2}	5	20	16	10	6	11	6.47	58.82

3.4.2.1 (III) Optimisation of the temperature

Due to the difficulties encountered in getting good reproducibility when using this method there was a need to improve on the original assay. Lower temperatures could cause clumping of the phage particles and as the phage stock solution is diluted to the required strength on the day of the experiment it was important to have an evenly dispersed stock suspension. In the original method there was a measure to minimise the impact of clumping of the phage particles in the stock suspension as it was placed on a rotary shaker in an incubator kept at 37^0C prior to dilution. However after the dilutions were done the resulting diluted phage suspensions were kept at room temperature which was likely to be the reason for the inconsistency observed with the results. Therefore a modification to the method in order to ensure a homogenous phage solution for the experiment was reassured. This was achieved by preparing a water bath that was equipped with a shaker and that had an Eppendorf test tube rack containing all the reactants in the corresponding Eppendorf tubes. When comparing the relative standard deviations of the blank samples shown in table 3.4.2.1(III) it can be seen that there is a reduction in the values from 17.3 to 8.7 which equals an improvement in reproducibility. This modification to the original method gave results obtained when using active samples that were more consistent and more reproducible.

Table 3.4.2.1(III) Comparison of preparation methods on the reproducibility of plaque counts at fixed concentrations of all reagents

Type of method	I(pfu)	II(pfu)	III(pfu)	IV(pfu)	V(pfu)	Average	stdev	RSD%
original	71	34	63	32	52	50	17.3	0.343
modified	115	109	121	107	128	116	8.7	0.075

3.4.2.2 Assay of phagicidal activity by plaque counting

A phage titre was performed to find out the optimum concentration of phage suspension. This concentration varied between 10^3 and 10^4 which gave between 110 and 220 pfu. A difficulty found with this method was reproducibility due to temperature sensitivity of the system. The phage particles need to be kept at constant temperature as clumping can occur and can cause lower plaque counts and that can lead to misinterpretation of data. Previous experiments gave very poor precision as temperatures throughout the day and on different days caused high diversity in the results obtained. This problem was overcome by keeping the Eppendorf rack containing all the reagents in Eppendorf tubes in a water bath at 37°C to reduce clumping of the phage particles and therefore increase the reproducibility of the method. The phage suspension and the lambda buffer were placed in the water bath prior to dilution to make sure that no clumping occurred. Once up to temperature the dilutions were done. At this time the pPRE was also put into the water bath and left in there for 15 mins. The last reactant to be placed in there was the ferrous sulphate solution as that was prepared at the last possible moment to ensure minimum oxidation. This ensured that at least some of the factors that caused poor reproducibility in the first place were regulated. The problem with this assay is the labour intensity combined with the varied reproducibility caused by factors that could not be influenced such as a non-air-conditioned laboratory. Small changes in temperature can cause big changes in plaque forming unit formation and the way the bacteria divide. The increased accuracy achieved with this method and the possibilities to do a variety of interesting experiments were prohibited by reproducibility issues. However the trends could be seen clearly and good results were obtained. The varying concentrations of the virucide against pfus can be seen in table 3.4.2.2(I) and 3.4.2.2(II) show a clear indication of the power of the method. The problem with this sensitive method was that small changes in the behaviour of the bacteria and the resulting pfu made it very difficult to deduce the exact activity of virucidal concentrations. For example one day a gradient was done and the concentration that causes 100% kill of phage particles was determined and the next day when

the same experiment was repeated some phage still survived and plaques could be seen.

Table 3.4.2.2(I) Determination of phagicidal activity of varying concentrations of pPRE and fixed concentrations of Fe^{2+} at 0.5 mM by monitoring pfu formation

Conc pPRE	Fe^{2+}	I(pfu)	II(pfu)	III(pfu)	VI(pfu)	V(pfu)	average	stdev	RSD%
blank		214	203	223	220	210	214	7.07	0.033
0.001 mg/ml	✓	212	213	203	208	198	207	6.3	0.030
0.002 mg/ml	✓	170	142	162	157	152	157	10.52	0.067
0.004 mg/ml	✓	126	128	154	141	126	135	12.32	0.091
0.006 mg/ml	✓	105	108	113	114	102	108	5.13	0.048
0.008 mg/ml	✓	50	56	37	46	44	47	7.05	0.15
0.01 mg/ml	✓	5	20	16	10	6	11	6.47	0.59

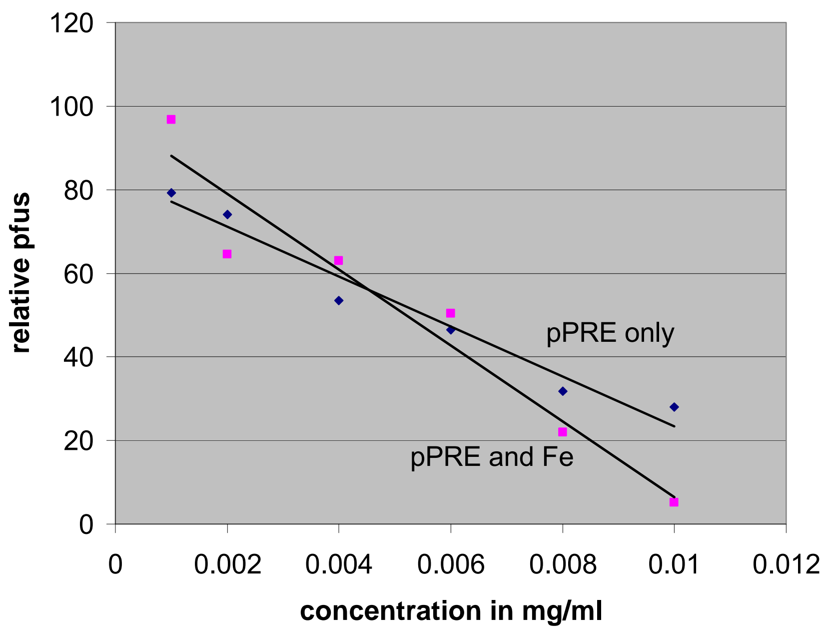
Table 3.4.2.2(II) Determination of phagicidal activity of varying concentrations of pPRE in the absence of Fe^{2+} by monitoring pfu formation

Conc pPRE	Fe^{2+}	I(pfu)	II(pfu)	III(pfu)	VI(pfu)	V(pfu)	average	stdev	RSD%
blank		115	109	121	107	128	116	8.66	0.075
0.001 mg/ml		115	84	93	87	80	92	13.81	0.150
0.002 mg/ml		106	92	101	63	69	86	19.23	0.223
0.004 mg/ml		90	66	46	46	64	62	18.13	0.292
0.006 mg/ml		65	52	46	49	56	54	7.36	0.136
0.008 mg/ml		56	41	28	37	23	37	12.73	0.587
0.01 mg/ml		47	34	22	29	35	33	9.18	0.278

Table 3.4.2.2(III) Normalised reduction in plaque forming units with increasing concentrations of pPRE, Fe^{2+} at fixed concentration of 0.5mM, statistical analysis showed no significant difference, r^2=0.930221

Conc in mg/ml	PPRE only	PPRE + Fe^{2+}
blank	100	100
0.001mg/ml	79.3	96.7
0.002mg/ml	74.1	73.4
0.004mg/ml	53.4	63.1
0.006mg/ml	46.6	50.5
0.008mg/ml	31.9	22.0
0.01mg/ml	28.5	5.1

Fig 3.4.2.2 Graph of normalised pfu as a function of concentration of antivirals



This experiment used the partially purified extract (pPRE) obtained from the purification process described in chapter 2 section 2.2.4(V). A general decrease in plaque counts with increasing concentration of pPRE was observed see fig 3.4.2.2(III). This indicated that there was a dose dependent relationship between the concentration of pPRE and the number of phage particles that are killed. This was interesting as it shows that the antiviral component present in pPRE was of a high enough concentration and therefore confirmed that the purification process was successful in relation to the active constituent. It also proved that the method and its modification were suitable for monitoring dose dependent activity of antiviral compounds. The methods used by Stewart et al., 1998, made use of the phage/bacteria model to determine whether any traces of bacteria were present acting as a sensitive limit test. In Mendez de Corao's 2002 method the idea was to make a high throughput method that was able to give quick indications for plant extracts to determine whether antiviral activity was present. This method also enabled the analyst to monitor the difference in phage killing potency of two different systems, pPRE only and pPRE in the presence of ferrous ions. The results shown in table 3.4.2.2(II) show a slight decrease in plaque forming units (pfu), but when an equal amount of Ferrous sulphate solution was added a higher reduction in pfu was observed table 3.4.2.2(I) as the concentration increased, see table 3.4.2.2(III). This suggested that the ferrous ion plays a part in potentiating the anti viral activity of pPRE. However statistical analysis showed that there was no significant difference between pPRE in the presence or absence of ferrous ions. But because of the antiviral activity present in pPRE on its own it also showed that the active molecule was not isolated, or that it has antiviral activity without active ferrous ions present. This showed that the active ingredient of the pomegranate was successfully purified and that the addition of Fe^{2+} potentiated the activity. Controls using only ferrous sulphate solution were done with each experiment but no reduction in pfu was observed. Ninety five percent kill of the phage particles was observed after three minutes contact time at a concentration of 10 μg/ml of pPRE with 0.5mM of Fe^{2+} added.

3.5 Assay of phagicidal activity by surface spread method

3.5.1 Materials

The same materials were used as listed in 3.2. unless stated below otherwise.

3.5.1.1Bacterial suspension

The inoculum was adjusted to give an optical density of 0.100 at 600nm using TSB and previously incubated bacterial suspension.

3.5.2 Method for surface spread assay

a. On a TSA plate three streaks of the inoculum were applied using a glass rod, each between 4 and 6 cm long and were allowed to set for 15 minutes

b. Autoclaved Eppendorf tubes were used to mix 100 µl of 10^3 of phage particles suspension with 100 µl of antiviral solution plus 100 µl of dilute ferrous sulphate solution if required in the experiment or 100 µl of sterilised water to keep the volume consistent and the three minutes contact time were allowed.

c. The Eppendorf tube was incubated in a water bath at 37 C^o. Three minutes contact-time was allowed. Volumes of 20 µl were then pipetted and applied as a drop on the streaked bacterial film.

d. Four to five dots of incubated test solution were applied to each streak

e. All experiments were done in 5 replicates and controls were done for ferrous sulphate without any anti viral solutions and blanks on the day to assure reproducibility. The concentration of phage and bacteria was constant throughout the experiment and only the concentrations of the plant extracts and ferrous sulphate were adjusted.

f. The plates were left to settle for at least 60 minutes before incubation at 37^oC for 16-18 hours.

g. Following incubation the plates could be analysed for two possible outcomes, clearing of the streak where the drop was applied or continuous growth the bacteria.

3.5.3 Results and discussion

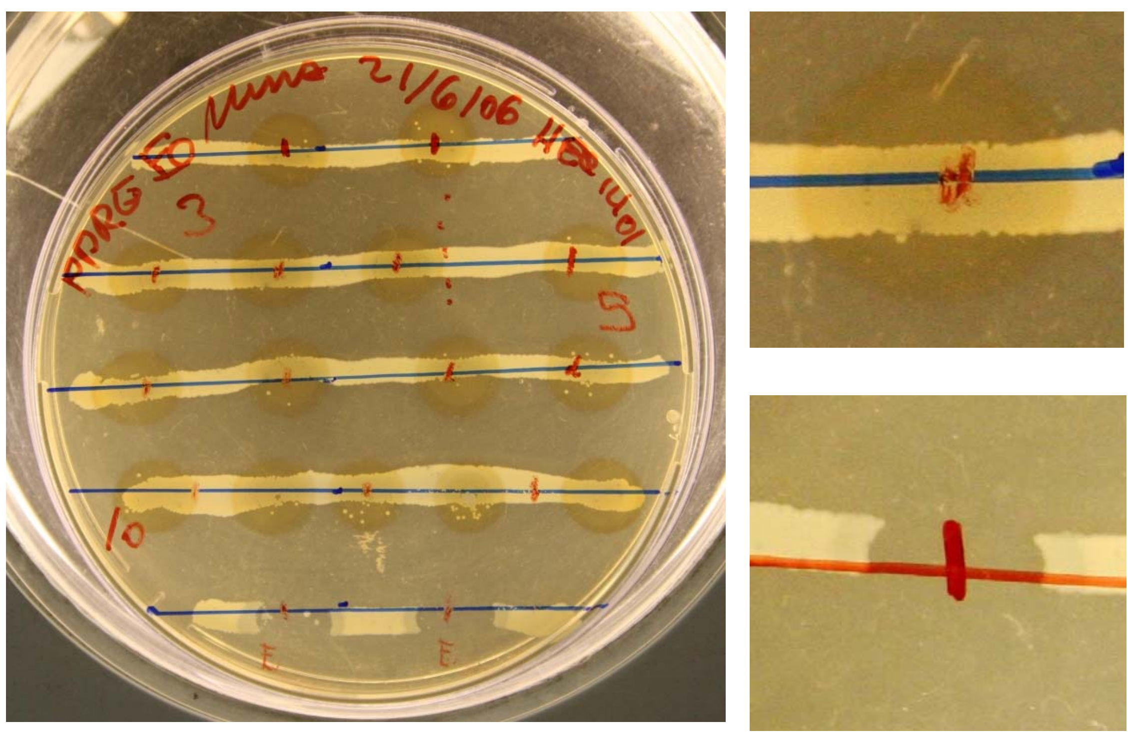
Table 3.5.1 Determination of phage propagation with increasing concentrations of pPRE in the presence of ferrous ions with 3 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
0.01mg/ml	✓	C	C	C	C	C
0.1mg/ml	✓	N	N	N	N	N
0.2mg/ml	✓	N	N	N	N	N
2mg/ml	✓	N	N	N	N	N

Table 3.5.2 Determination of phage propagation with increasing concentrations of pPRE with 3 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
0.01mg/ml		C	C	C	C	C
0.1mg/ml		C	C	C	C	C
0.2mg/ml		N	N	N	N	N
2mg/ml		N	N	N	N	N

Fig 3.5.3 Petri dish with streaking method applied, two possible outcomes: phage killed and bacterial lawn intact and phage survived bacterial lawn interrupted are magnified



The surface spread method made use of the optimisations carried out for the plaque counting assay (3.4.2). Due to the different way in which the bacteria was grown in this method, stock suspension was applied straight onto the plate rather then mixed with sloppy agar, changes in concentration had to be made. Test plates were done using various optical densities/concentrations of the inoculum. This was important as the bacterial lawn had to be the right thickness to allow for the 20 μl of test solution to give readable results. It was found that the optimum concentration of bacterial suspension was achieved when the optical density was adjusted to 0.100 using TSB measured at 600 nm. The phage suspension, pPRE, lambda buffer, sterile water and ferrous sulphate solution were all kept in a water bath at 37° C to minimise the risk of clumping of the phage particles. The results in table 3.5.1 and 3.5.2 show the results achieved using the surface spread method. They show the effect on phage propagation with increasing

concentrations of purified Pomegranate rind extract (pPRE) on its own and in the presence of ferrous ions at various concentrations with 3 minutes contact time allowed. The concentrations of pPRE were determined by doing a concentration titer.

The streaking method used can give 2 possible outcomes, see fig 3.5.3

- C = cleared; bacterial lawn disrupted, phage survived
- N = not cleared, bacterial lawn intact, phage killed.

It can be seen clearly that the reproducibility was good and that there was phagicidal activity both in the presence and in the absence of ferrous ions at concentrations higher than 0.2mg/ml. At a concentration of 0.1mg/ml of pPRE only the sample with added ferrous ions present showed phagicidal activity confirming the results from the plaque counting assay (3.4.3), that the presence of ferrous ions potentiates the activity of pPRE. The concentrations of the pPRE were slightly higher than those reported in the plaque counting (3.4.3) assay but that was due to complete kill of phage particles present required as partial kill could not be detected by this assay. The reproducibility of this assay was better than that of the plaque counting method. The lower sensitivity did not cause different overall outcomes confirming the modifications made for this method were successful.

3.6 Comparison of phagicidal activity of PRE and pPRE

3.6.1 Introduction to standardisation of PRE and pPRE by hplc

In order to compare the two pomegranate rind extracts, PRE and pPRE, the amount of the active anti viral component had to be deduced. The method of choice was hplc combined with a variable wavelength detector. The reason for that is that there were two different components of mobile phase, water and methanol, whose concentration gradient could be altered to increase separation. A problem when injecting crude plant extracts such as PRE into hplc systems is that due to the many constituents present the column can become overloaded. To reduce the impact of overloading the column diluted samples were used. A

different gradient compared to the one used in the analysis of pPRE was used in order to get broader and more comparable chromatograms.

3.6.1.1 Methods

Samples of PRE and pPRE were prepared according to the method described in sections 3.3.4 and 3.3.5 respectively. The pPRE was syringe filtered prior to analysis. PRE was previously filtered according to method 3.3.4. Samples were diluted and injected into a 200 µl injection loop and the flow rate was set at 1ml/min and the detector was set to286 nm. The column used was a C-18 reverse phase Phenomenex 250 x 4.60 mm, 5 microns particle size. The solvent gradient is shown in table 3.6.1. The height of the main peak was measured and dilutions of 1/10, 1/20 and 1/200 were done. The1/20 dilution of PRE was used as the resulting peak fitted perfectly onto the scale. To get an equivalent peak of pPRE a solution of 0.1 mg /ml was prepared. HPLC grade water was used for the dilutions.

Table 3.6.1 Mobile phase composition for the comparison of PRE and pPRE

Time/min	Duration/min	%water+ 0.1%TFA	%methanol+ 0.1%TFA
1	1	100	0
21	20	75	25
26	5	25	75
31	5	100	0

3.6.2 Comparison of PRE and pPRE activity

Table 3.6.2a(I) Determination of phage survival with increasing concentrations of pPRE in the presence of ferrous ions with 3 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
0.01mg/ml	✓	C	C	C	C	C
0.1mg/ml	✓	N	N	N	N	N
0.2mg/ml	✓	N	N	N	N	N
2mg/ml	✓	N	N	N	N	N

Table 3.6.2a(II) Determination of phage survival with increasing concentrations of pPRE with 3 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
0.01mg/ml		C	C	C	C	C
0.1mg/ml		C	C	C	C	C
0.2mg/ml		N	N	N	N	N
2mg/ml		N	N	N	N	N

Table 3.6.2b(I)) Determination of phage survival with increasing concentrations of PRE in the presence of ferrous ions with 3 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of PRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
PREx1/200	✓	C	C	C	C	C
PREx1/20	✓	N	N	N	N	N
PREx1/10	✓	N	N	N	N	N
PRE	✓	N	N	N	N	N

Table 3.6.2b(II)) Determination of phage survival with increasing concentrations of PRE in the absence of Fe^{2+}, with 3 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
PREx1/200		C	C	C	C	C
PREx1/20		C	C	C	C	C
PREx1/10		N	N	N	N	N
PRE		N	N	N	N	N

This method was used to compare the effect that different pomegranate rind extracts, PRE and pPRE, had on phage propagation. It is well established that PRE has antiviral activity as it has been used in the past by Stewart *et al* (1998) in the phage amplification assay as a virucide. The method herein was designed to compare the water extract PRE to the purified extract pPRE to show that the antiviral fraction was successfully purified. To determine the concentration ranges both extracts were syringe filter sterilised and then each extract was serially diluted. They were then injected into a HPLC to determine at what concentration similar peaks were seen. It was found that a 2 mg/ml solution of pPRE showed the largest peak (retention time = 19min 12 secs) with a peak height of 19.3cm. The equivalent peak of PRE solution measured 18.7cm. When PRE was prepared it was made up of 25% freeze dried rind. The pPRE was first extracted using a soxhlet extractor at a rate of 27% of freeze dried rind followed by purification using an Amberlite XAD-8 column at a rate of 15%. Overall 4% of the weight of freeze dried rind was present in pPRE extract. In terms of the weight of actual rind used in these two extracts that was 50.4 mg for pPRE and 250 mg for PRE in 1ml of solution which was equivalent to a ratio of 1 to 5. This allowed a comparison of their activities and showed that the compound of interest was present in both extracts, therefore confirming that the purification process was successful. When analysing the crude PRE many peaks were seen due to its complex composition but the main peaks were well resolved with the same retenetion times as in the pPRE. These peaks were observed at 16mins 28 secs and 19 mins 12 secs respectively. The smaller peaks measured 4.1 cm for pPRE and 3.8 cm for PRE. They were believed to be punicalagin which exists in two isomeric forms, ά and β which are readily interchangeable in aqueous solution due to the presence of the anomeric carbon on the glucose moiety. The two forms exist by opening of the glucose ring in aqueous solution and on ring closure the ά or β form is formed. The two anomeric isomers exist in an equilibrium of 25:75=ά: β (Mendez de Corao, 2002). If one anomer is isolated in aqueous form mutarotation occurs and within several hours the equilibrium is established again. Using HPLC analysis it was deduced on the height of the

corresponding peak that the concentration of one component, thought to be punicalagin, was the same in 1/20 of the original PRE and a 0.1 mg/ml of pPRE in de-ionised water. In this assay this relationship was used to compare the phagicidal activity at various concentrations to determine whether or not that component was responsible for this action. The results of pPRE and PRE in 3.6.1a and 3.6.1b respectively show antiviral activity and also enhanced activity if ferrous ions were provided. At a concentration of 0.1mg/ml of pPRE without ferrous ions present phage particles survived but when the metal ions were present the phage were killed, see 3.6.1a. This confirmed that ferrous ions potentiated the phagicidal activity which was already shown in section 3.5. When the PRE was examined it was found that at a certain concentration, in this case a dilution of 1:15 of the original water extract, there was no phage killing without ferrous ions present but 100% kill rate in the presence of the metal ions, see table 3.6.1b. This was exactly the same outcome that was observed with pPRE, showing that the HPLC analysis that was used to determine the amount of the antiviral component present was correct as well as the parameters such as wavelength, type of column and composition of the solvent gradient. The peak used to determine the concentration of the extract under examination was corresponding to the antiviral activity.

3.7 Validation of experimental conditions on phagicidal activity

Varying contact times were used in order to determine whether longer exposure increases kill rate.

3.7.1 Method

The Method was the same as in 3.5.2 except that the contact time between the plant extract and the phage preparation prior to the application onto the bacterial lawn was varied.

3.7.2 Determination of phagicidal activity of pomegranate rind extracts as a function of contact time

Table 3.7.1a(I) Determination of phage survival with increasing concentrations of pPRE in the presence of ferrous ions with 5 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
0.01mg/ml	✓	C	C	C	C	C
0.1mg/ml	✓	N	N	N	N	N
0.2mg/ml	✓	N	N	N	N	N
2mg/ml	✓	N	N	N	N	N

Table 3.7.1a(II) Determination of phage survival with increasing concentrations of pPRE with 5 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
0.01mg/ml		C	C	C	C	C
0.1mg/ml		C	C	C	C	C
0.2mg/ml		N	N	N	N	N
2mg/ml		N	N	N	N	N

Table 3.7.1b(I) Determination of phage survivial with increasing concentrations of PRE in the presence of ferrous ions with 5 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of PRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
PREx1/200	✓	C	C	C	C	C
PREx1/20	✓	N	N	N	N	N
PREx1/10	✓	N	N	N	N	N
PRE	✓	N	N	N	N	N

Table 3.7.1b(II) Determination of phage survival with increasing concentrations of PRE with 5 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
PREx1/200		C	C	C	C	C
PREx1/20		C	C	C	C	C
PRE*1/10		N	N	N	N	N
PRE		N	N	N	N	N

Table 3.7.2a(I) Determination of phage survival with increasing concentrations of pPRE in the presence of ferrous ions with 10 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
0.01mg/ml	✓	C	C	C	C	C
0.1mg/ml	✓	N	N	N	N	N
0.2mg/ml	✓	N	N	N	N	N
2mg/ml	✓	N	N	N	N	N

Table 3.7.2a(II) Determination of phage survival with increasing concentrations of pPRE with 10 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
0.01mg/ml		C	C	C	C	C
0.1mg/ml		C	C	C	C	C
0.2mg/ml		N	N	N	N	N
2mg/ml		N	N	N	N	N

Table 3.7.2b(I)) Determination of phage survival with increasing concentrations of PRE in the presence of ferrous ions with 10 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
PREx1/200	✓	C	C	C	C	C
PREx1/20	✓	C	C	C	C	C
PREx1/10	✓	N	N	N	N	N
PRE	✓	N	N	N	N	N

Table 3.7.2b(II) Determination of phage survival with increasing concentrations of PRE in the absence of ferrous ions with 10 mins contact time, C= cleared, phage survived, N= not cleared, phage killed

Concentration of pPRE	Fe^{2+} 0.5mM/l	I	II	III	IV	V
PREx1/200		C	C	C	C	C
PREx1/20		N	N	N	N	N
PRE*1/10		N	N	N	N	N
PRE		N	N	N	N	N

In the original patent by Stewart, 1998, 3 minutes contact time was used to kill all free phage left over. Mendez de Corao used the same contact time in her method and to determine whether or not that time interval was sufficient, two extended sets of time intervals 5 and 10 minutes were used, see tables 3.7.1 and 3.7.2 respectively. The method used was the surface spread method as described in 3.5.2 except extending the contact time of the phage and the antiviral solution. Comparing that to the results shown in table 3.5.2 and 3.6.2 it can clearly be seen that after three minutes the reaction was complete and that no increase in phagicidal activity was observed. The modifications in the assay increased sensitivity and precision and reduced the negative effects brought about by temperature changes that occurred during the longer assay procedure. One of the reasons why this method was much less prone to external changes was because of the number of samples that could have applied to one agar plate and therefore reducing the effect that different agar plates could have on the growth of bacteria, eg different amounts of growth medium and uneven setting when poured. Also because of the shorter time required to do a full set of experiments the impact that changes in temperature had, were reduced significantly.

3.8 General discussion

3.8.1 Comparison between methods used for assessing phagicidal activity of pomegranate extracts

The starting point for this assay was to establish a method for measuring the phagicidal activity of the pomegranate rind extracts PRE and pPRE and compare their activity. Both extracts gave positive results indicating that the purification process was successful in isolating the active constituents. The phage amplification assay by Stewart *et al* (1998) showed the phagicidal activity of PRE and that this was potentiated by the addition of ferrous ions. Phage particles were used to detect very small numbers of the host bacterium and were killed after the initial contact time by addition of a mixture of PRE and ferrous sulphate. A plaque counting method was then used to establish the presence of viable

bacteriophage and hence to deduce the presence of host bacteria in the original test sample. Mendez de Corao 2002 used this idea to design a quick screening method for phagocidal/virucidal activity. The phage amplification assay used a highly labour intense overlay method and Mendez de Corao sought to modify the method by making use of a simplified system in which the phage / bacteria cultures were incubated in 96-well plates. The objectives were to reduce the assay time and amount of reagents and materials consumed. Initially, it was expected that an automated plate reader would be used but this was not possible due to the nature of the culture conditions and a subjective reading of each well was carried out. There is also a problem that when using optical density to determine bacterial growth as there are no means of checking whether or not the particles that caused cloudiness were of bacterial origin.

A second disadvantage of de Corao's method involves the regrowth of bacteria within each well. The indicator for activity / lack of activity was opacity within the well and the presence of contaminating bacteria not sensitive to the phage or by resistant bacteria would give false negative results. If Petri dishes were used such interference could be detected due to the larger surface area compared to a well in a 96 well plate and because plaque-forming units were used to monitor activity rather then optical density. There was also more then one pfu so if there was 5% regrowth, for example, then it could still be determined that the phage survived whereas in a well, if regrowth occurred, the phage could wrongly be assumed inactive. All these assumptions seemed to make this method not very reliable and therefore a new method was designed trying to minimise assumptions and increase reliability.

Taking these considerations into account a new assay procedure was designed. A plaque counting method using an overlay technique was chosen as a starting point to see whether or not plaques could be seen confirming Mendez de Corao and Stewart's results (2001 and 1998). This method was accurate but also very labour and material intensive. The problem of regrowth was minimised as pfu were counted and compared and therefore the small percentage of overgrowth of plaques did not have the same influence on the outcome as in the 96 well plate

method. Due to the increased accuracy, the amounts of extract required and the increase in potency in the presence of ferrous ions to kill phages could be determined. Although the method was more accurate, there remained a high degree of variability in the results. The cause of this was identified as being due to the temperature used in preparation of the phage suspensions. Optimising the procedure resulted in more precise results. The full potential of this method could not be reached resulting in the need for a more reliable but less accurate procedure. Because of that this method was not suitable to compare the crude PRE and the pPRE that was obtained by the purification procedures described in chapter 3.

The surface spread method (3.5) was designed to improve on the plaque counting assay and to make it suitable for the reliable testing of the antiviral extracts PRE and pPRE.

Advantages of surface spread method:

- Reduced assay time
- Reduced labour intensity and material consumption
- Better reproducibility and reliability due to simplified monitoring system
- Increased accuracy due to only two possible outcomes
- Petri dishes allow for monitoring of regrowth

Disadvantages of surface spread method:

- Reduction in variety of possible experiments due to two outcomes only

A disadvantage of this method is that only two outcomes are available: either the phage is killed as evidenced by the bacterial streak remaining continuous or the phage is not killed and zones of non-growth on the bacterial streak can be seen where the phage suspension had been applied. Phagicidal activity of the test substance was inferred from the presence of an established bacterial lawn and hence the conclusion that the phage particles were either killed or rendered inactive in the process. The method is fast and can yield results in much shorter

time compared to the overlay method. The indicator is reliable as the outcome is measured by colonies formed where the bacterial lawn can be either seen or not. For the plaque-counting method (3.4) one agar plate provides a single result, whereas this method provides 15 results on one plate. In addition to the time savings this also results in reduction of consumables making it more economical then the plaque counting assay. It combines a higher throughput with a more reliable indicator than Mendez de Corao's quick screening method that made use of cloudiness. It was quicker than the overlay and but slower than the 96 well plate method but the superior monitoring technique gives it the edge over the 96 well plate method. Because there are only two possible outcomes the experiments that can be done are more limited compared to the plaque counting method. However on the upside it is less influenced by temperature as there are only two scenarios, phage killed or survived which increases the accuracy and also improves the reproducibility. The aim of this improved method was to measure the difference between the antiviral activity of PRE and pPRE and the effect of added ferrous ions to the extracts at various concentrations which was done successfully. Another application for this assay was to find out how extended contact times influenced the results as Stewart et al 1998 and Mendez de Corao 2002 both used 3 minutes. The results showed that there was no increase in phagicidal activity with extended contact times.

3.9 Conclusions

The work presented in this chapter has established an improved bio-assay for phagicidal activity for the pomegranate rind extracts. A number of bio-assays were used and improvements made to the methods available. A comparison between the various methods is presented and the advantages and disadvantages for each one discussed. It was conclusively shown using the plaque counting and surface spread method that PRE and pPRE have phagicidal activity which was potentiated by the addition of ferrous ions. The results also showed clearly that the pPRE possessed antiviral activity by reducing the

number of phage particles that infected the host bacteria. The method designed for this assay seemed suitable to monitor antiviral activity in a dose dependent manner, see Fig 3.4. It can also be seen that the addition of ferrous ions to the pPRE boosted the antiviral activity suggesting that it is involved in the mechanism of action. The involvement of ferrous ions suggests that free radicals and specifically hydroxyl radicals might be involved. The drawback of this method was that the increased accuracy achieved by getting only one reading per individual plate is that it is very labour intensive and results in low throughput. The increased accuracy also results in bigger influence of external conditions on the outcome of the experiment making it more difficult to get good reproducibility.

A comparison between the activity of PRE and pPRE was also carried out and using HPLC analysis, the relative concentrations of the phagicide were determined. These concentrations were then used successfully with the surface spread method and confirmed the presence of the same amount of phagicide present. As the main peak was used when comparing PRE and pPRE it is likely that one active component is responsible for the antiviral activity observed.

Chapter four

Studies on the mechanism of action of antiviral activity of PRE and pPRE

4. Studies on the mechanism of action of antiviral activity of PRE and pPRE

4.1 Introduction

4.1.1 Possible involvement of free radicals

Previous work done by Stewart et al (1998) and Mendez de Corao (2001) showed that when ferrous ions were added to PRE the anti viral activity increased. In the bio-assay these findings were confirmed and when pPRE was used the difference in activity between the extract on its own and in the presence of ferrous ions was increased. Ferrous ions are closely associated with Fenton chemistry and the Fenton reaction which results in the generation of hydroxyl radicals.

$H_2O_2+Fe^{2+}\rightarrow Fe^{3+}+OH^{-}+OH^{\bullet}$

In this reaction ferrous ions are oxidised to ferric ions. There is also a superoxide assisted Fenton reaction which uses superoxide radicals to recycle the inactive ferric ions back into the active ferrous state.

$Fe^{3+}+O_2^{\bullet-}\rightarrow Fe^{2+}+O_2$

Previous work by Stewart et al (1998) and Mendez de Corao (2001) clearly showed that the PRE extract did not have any effect on the host bacteria which was confirmed by the bio-assay. These findings support the theory that free radicals are involved in the virucidal activity of pomegranate rind extracts as bacterial cells have free radical defences.

4.1.2 Free radicals

A free radical is any species capable of independent existence that contains one or more unpaired electrons. An unpaired electron is one that occupies an atomic or molecular orbital by itself. A superscript dot after the formula is usually used to donate free radical species. In order to generate a free radical from a neutral molecule energy is required. This energy can be provided by a variety radiation such as heat, UV, x-ray, electrons, alpha-particles, neutrons or gamma rays (J.

M. Hay, 1974). The presence of one or more unpaired electrons in free radicals makes them highly reactive, although chemical reactivity varies over a wide spectrum, (Halliwell, 2003). However most entities commonly referred to as free radicals are relatively small molecules. In general the smaller a free radical the higher its reactivity as the electron deficiency can not be compensated for and therefore they are less stable on their own and highly reactive. They will react with a wide variety of molecules which in turn can become free radicals themselves. In the human body these reactions can result in damage to cells and have therefore been linked with the development and progression of various diseases such as heart disease congestive heart failure, hypertension, cerebrovascular accidents and hypertension. Damage caused by free radical attack is often referred to as oxidative stress. (Chen, 2002)

The most common biologically important free radicals are hydroxyl and superoxide radicals. They are implicated in many biological pathways, eg.: Krebs cycle and are present in various different parts of the human body. Enzymes like super oxide dismutase (SOD) and catalase are responsible for lowering the levels of free radicals and therefore reducing oxidative stress on cells. Catalase can help against H_2O_2 generated by dismutation of $O_2^{\bullet-}$ generated by haemoglobin auto oxidation (Winterbourn, 1995). Free radical scavengers are also present in our diet for example flavanols, flavanoids and β-carotene and these are useful for reducing oxidative stress, (De Bruyne, 1999) In the body a dynamic equilibrium exists between free radicals and free radical scavengers. Free radicals can also be produced by cells as a defence mechanism in order to defend themselves against pathogens. In the liver free radicals are used for detoxification. The presence of H_2O_2 increased the DNA binding ability of the transcription factor activator protein 1 (AP-1) 3 fold and if ascorbic acid was also present 5 fold. This suggested strongly that Fenton chemistry and therefore hydroxyl radicals were involved in the sharp increase of the protein`s activity. (Lunec, 2002)

$$H\text{-}O\text{-}O\text{-}H \xrightarrow{\text{UV light}} 2\ OH^{\bullet}$$

The hydroxyl radical is the most reactive oxygen radical that will react with almost every type of molecule found in living cells: sugars, amino acids, phospholipids, DNA bases and organic acids. This radical reacts with the cell membrane and lipoproteins in a process called lipid peroxidation. In low density lipoprotein this reaction plays an important role in the development of atherosclerosis. (Kerr, 1996) In recent years free radicals have been closely associated with oxidative stress which in turn has been linked with a variety of diseases including rheumatoid arthritis, cardiovascular disease, neurological disease and cancers. It is thought these are caused by the overproduction of reactive oxygen and nitrogen species, many of which are of free radical origin, resulting in an imbalance in favour of pro-oxidants. The reason for the imbalance is widespread as the overproduction of reactive oxygen species is influenced by many factors such as environment, diet, lifestyle and genetic constitution (Fleshner, 1999, Valko, 2005). The reactive oxygen species which include hydroxyl radicals, superoxides and peroxides have been strongly linked with various cancers including prostate cancer (Iguchi, 2008).

4.1.2.1 Free radical reactions and generation

Free radicals can react with other molecules in a number of different ways. If two free radicals meet they can combine their unpaired electrons to form a covalently bonded non-radical product. When a free radical reacts with a non-radical molecule there are two scenarios, a) it gives up its unpaired electron or b) it abstracts an electron. Either way the resulting product will be of a radical nature but larger radical molecules are usually more stable as they can spread the electron density deficit. This results in lower reactivity. There are many systems that are known to generate free radicals but the most commonly known one is the Fenton system. (Savaria, 2008) In 1876 Henry G. H. Fenton discovered that the mixing of tartaric acid with hydrogen peroxide and a ferrous salt produced a colour change which was due to the hydroxylation of the acid. The mechanism of

this reaction is still disputed as there are two theories. One involves the formation of hydroxyl radicals and was proposed by Haber and Weiss in 1934. The other approach proposed by Bray and Gorin 1932 involves the formation of a ferryl species as its intermediate that can donate oxygen to the substrate. (Chabot, 1998; Halliwell, 1990) However the Fenton reaction has been widely used for generation of hydroxyl radicals and it is therefore assumed hydroxyl radicals are involved in the hydroxylation of compounds. (Kunchandy, 1990)

Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$

However in biological systems the Fenton reaction is not thought to be a major contributor of hydroxyl radicals. The highly reactive ferrous ions occur mainly in bound form are therefore not available to take part in the reaction.

The Haber Weiss reaction also makes use of Fenton chemistry, it also involves the generation of hydroxyl radicals but by an interaction between the less reactive reactants superoxide and hydrogen peroxide. These two reactants can be generated enzymatically and are thought to be the major contributor of hydroxyl radicals in biological systems. (Kehrer, 2000)

Another important reaction that is closely associated with hydroxyl radical generation is the Udenfriend system. It involves the recycling of the inactive ferric ions back into the active ferrous state via a redox reaction involving ascorbic acid resulting in a consistent and constant hydroxyl radicals generation system. The Udenfriend system establishes a highly pro-oxidant mixture generating hydroxyl radicals that can oxidise almost any target molecule (Suh, 2003). This is due to the increased concentration of the reactive Fe^{2+} shifting the equilibrium of the fenton reaction to the right.

Udenfriend reaction: Ascorbate + $Fe^{3+} \rightarrow$ Ascorbate$^{\cdot}$ + Fe^{2+}

4.1.3 Detection of free radicals

4.1.3.1 Direct methods for the detection free radicals

Free radicals are highly reactive, short lived species. These characteristics make it very difficult to detect them. There is only one method available for the direct detection, this being electron spin resonance (ESR) also called electron paramagnetic resonance (EPR). This technique is specific to free radicals as it detects the presence of unpaired electrons. They can have a spin of either +0.5 or -0.5. When exposed to an external magnetic field they align either parallel or antiparallel. This results in two possible energy levels that are dependent on the strength of the external magnetic field applied. By supplying the right amount of electromagnetic radiation, usually in the microwave region of the electromagnetic spectrum, it will be absorbed by the electron promoting it from the lower to the higher energy level resulting in an absorption spectrum. ESR spectrometers are set up to display first-derivative spectra which do not show the absorbance but the rate of change of absorbance. The problem with this technique is that that it is too insensitive to detect highly reactive and therefore short-lived free radicals such as hydroxyl radicals in living systems. A method that can be used to detect short lived molecules, like hydroxyl radicals, in combination with ESR is to freeze a solution of organic radicals, as reactions are slowed down at low temperatures. The free radicals lifetime is prolonged and therefore the chance of their being detected is enhanced. (Kroger-Ohlsen, 1999).

4.1.3.2 Indirect methods for the detection of free radicals

4.1.3.2(I) Spin trapping of free radicals

One indirect approach to detect free radicals is called trapping. This method makes use of a trapping molecule that forms one or more stable products which can be detected and measured. A more novel method is called spin trapping, in which the free radicals react with the spin trap to form more stable radicals that accumulate to such an extent that they can be detected using ESR. (Halliwell, 2003) This method is an indirect method of detection as an intermediate is

formed and measured rather then the original free radical. Spin trapping makes use of a trap for example nitroso and nitrono compounds, which react with the free radical and form a more stable radical, for example, nitroxide that can be detected using ESR or HPLC. (Tosaki, 1992) The HPLC method was used by some groups in the past but due to improvements made in the field of ESR this technique is predominant in recent publications. The increased stability of this spin trap is due to the delocalisation of the electrons between the nitrogen and the oxygen atoms. With nitroso compounds the detected radical adds directly to the nitrogen whereas with nitrono compounds the detected radical adds to the carbon adjacent to the nitrogen atom. This causes adducts of the latter to be more stable and give a simpler, more reproducible spectrum, especially when oxygen radicals are trapped. (Evans, 1996)

Spin trapping methods can also be used in biological systems and are often used to detect the presence of various oxygen radicals and also the formation of organic radicals during lipid peroxidation. The most commonly used ones are the water soluble 5,5-dimethylpyrolline-*N*-oxide (DMPO) and the lipid soluble ά-phenyl-tert-butylnitrone (PBN), see fig 4.1.3.2. However none of the spin traps that are used at present are ideal. The perfect spin trap molecule should react instantly and specifically with the radical under investigation and form a product that is chemically stable, not metabolised by living systems and has a unique ESR spectrum.

An exception to these traps is azulenyl nitrone which is an azulene ring attached to a nitrone resulting in a molecule that is more stable when giving up an electron, see fig 4.1.3.2. Another advantage of this scavenger is that it is of green colour and once it reacts with the radical it gives a violet colour which can be detected colorimetrically. As with most nitroxide spin adducts this product also decomposes but the violet colour remains giving it a significant advantage over common nitrone spin traps. (Becker, 1996)

Fig 4.1.3.2 structure of spin traps DMPO, PBN and azulene nitrone

4.1.3.2(II) Hydroxylation of aromatic compounds

There are also many other trapping methods available that make use of more stable and detectable products, one of them being aromatic hydroxylation. The oxidation of aromatic compounds by metal ion-H_2O_2 systems has been known for 100 years by Fenton and has generated a vast chemical literature ever since the pioneering work by Merz and Waters in 1949. Hydroxylation and decarboxylation have been used for the detection of hydroxyl radicals. Depending on the pH and the conditions of the reaction a different hydroxylated products can be formed and in some case a different reaction will occur. Many hydroxylated aromatic products can be measured colorimetrically due to their capacity to form conjugated systems that often result in giving the products colours in the visible spectrum. These reactions are only semi quantitative as they can form isomeric hydroxylated forms due to the high reactivity of these compounds. For the quantitative detection of hydroxyl radicals by hydroxylated aromatic compounds separation techniques such as gas chromatography or high pressure liquid chromatography have to be incorporated into the system and coupled to electro chemical detection, fluorescence spectra and or mass spectrometry. (Marusawa, 2002) Frequently used aromatic detector molecules are salicylate and phenylalanine that react with hydroxyl radicals to form three tyrosine products that can be separated and detected (Halliwell and Kaur, 1994), see fig 4.1.3.2(II).

In the case of salicylate part of the reactant will give a decarboxylated product mainly at low pH and without metal ions present.

Fig 4.1.3.2(II) shows hydroxylation of phenylalanine and salicylate (Halliwell, 1998)

o-tyrosine
m-tyrosine
p-tyrosine
Phenylalanine
Catechol
2,3-Dihydroxybenzoate
2,5-Dihydroxybenzoate
Salicylate

In order to get more reliable results the reporter molecule chosen should only give a monohydroxylated product that can be detected easily. This is the case with para-nitro phenol (pNP) as the product has a distinguishable colour and can be detected spectrophotometrically (Chrastil, 1975). This reporter molecule has also been used successfully in a method yielding a fibre optic sensor capable of continuous monitoring of hydroxyl radicals. This method also has the advantage of being applicable over a range from pH=4 to pH=8 giving it the possible ability to monitor within biological systems (Naughton, 1993). A different approach detection wise is to use a reporter molecule that forms a fluorescent product that can be detected spectrofluorometrically (Barreto, 1994). A downside when using trapping molecules is that the radical products formed are more stable than the initial radical but they are still prone to degradation and therefore quantification of hydroxyl radicals using trapping methods is generally not recommended.

4.1.3.2i Fingerprinting

As an alternative to trapping a method called fingerprinting can be used. This method makes use of specific products of damage caused by free radicals,

therefore not measuring the species itself but the damage that they cause. Free radicals react in a specific way with DNA, proteins and other low molecular mass anti oxidants such as ascorbate or urate. The resulting products can therefore be regarded as fingerprints (or footprints).To ensure that this method is accurate it has to be assured that the products measured are specific to the species under investigation. Ascorbate combined with electron spin resonance has been used successfully as a bio indicator of oxidative stress (Halliwell and Kaur, 1998). Fingerprinting has been used to detect hydroxyl radical damage to polysaccharides in the ripening process of pears. In this case radioactively labelled polysaccharides were used to find the fingerprint products which were then separated by electrophoresis and analysed. In this case this method was successfully used to determine degradation of polysaccharides in the cell walls of ripening pear fruit. (Tabbi, 2001) Oxidative stress is the disturbance of pro-oxidant and anti-oxidant balance in favour of the former causing potential damage to the biological system due to a lack of the antioxidant defence. Pro-oxidants involve reactive oxygen and nitrogen species some of which are free radicals and they have been linked to a variety of disease like cancer, diabetes and cardiovascular disease. Free oxygen radicals have been used successfully in the free oxygen radical test (FORT) assay as markers for oxidative stress. In a novel colorimetric assay free oxygen radicals, alkoxyl- and peroxyl-radicals, are used as monitors for oxidative stress in blood samples. This method works by using blood or plasma samples and makes use of a buffered transition metal trap containing chromium, $Cr\text{-}NH_2$, that forms a very stable, coloured radical product. The intensity of the colour is directly proportional to the numbers of radicals present (Garelnabi, 2008)

4.2 Investigation into the possible involvement of superoxide radicals in the antiviral activity of pPRE

4.2.1 Introduction

The reason for using this assay was to find out whether or not superoxide radicals are responsible for the anti viral activity of pPRE or involved in the

mechanism of action. Nitro blue tetrazolium (NBT) has been used widely for the detection of superoxide radicals since the 1960's (Rajagopalan, 1964). The NBT assay gives an indirect measure of the rate of superoxide radical production that results from the interaction of the enzyme, xanthine oxidase, with its substrate, xanthine. (Goldstein, 1996) The assay measures the formation of formazan from 4-nitrotetrazolium chloride blue, NBT, which is an effective scavenger of superoxide. The method involves two reactions. The first one is the conversion of xanthine into urate by the enzyme xanthine oxidase yielding superoxide radicals. The second one is the reaction of superoxide radicals and NBT to form formazan at a steady rate. It is assumed that the rate of reaction between superoxide radical and NBT is significantly faster than the superoxide forming reaction and that the absorbance due to formazan is essentially a measure of the rate of formation of superoxide. Both end products can be detected spectrophotometrically; urate is detected at 295 nm and formazan at 550 nm (see figure 4.2.1). As a control the test substance has to be checked at 295 nm to ensure that no interference with the enzyme system occurs as this could lead to wrong interpretation of data. The slope of the plot of absorbance against time gives a measure of the rate of reaction between xanthine oxidase and xanthine, i.e. the formation of superoxide radicals. The gradient of the formazan formation against time is measured, the difference in gradient between the test solution and the control is used to determine superoxide scavenging or generating activity.

Fig 4.2.1 Reactions occurring in NBT assay, (Fisher, 2003)

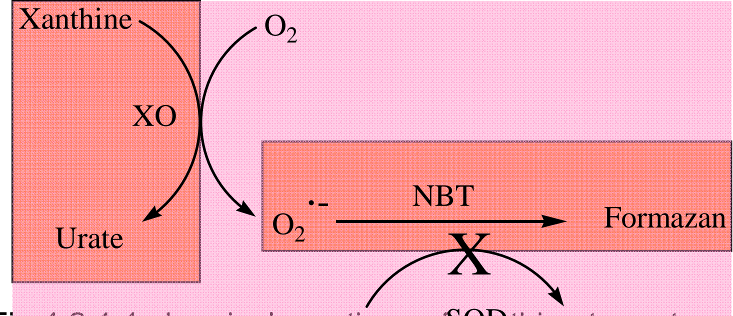


Fig 4.2.1.1 chemical reactions of Xanthine to urate

Xanthine oxidase

Fig 4.2.1.2 chemical reaction NBT to formazan

Nitro blue tetrazolium

Formazan

4.2.2 Materials

Xanthine oxidase from bovine milk: grade IV, ammonium sulphate suspension, 0.1-0.4 units/mg protein (Sigma-Aldrich, Gillingham)

Xanthine, 99% (Sigma-Aldrich, Gilingham)

Di-potassium orthophosphate, (AnalR, Poole)

Potassium dihydrogen orthophosphate (AnalR, Poole)

UV-Spectrophotometer: Shimadzu UV-1601 equipped with temperature regulated Quartz UV cell.

HPLC grade water obtained from an ELGA OPTION 3 reverse osmosis water purifier was used for all solutions.

4.2.3 Methods

4.2.3.1 Preparation of reagents

The potassium phosphate buffer, 50 mM at pH 7.8, was prepared by using 0.5934g di-potassium orthophosphate add 0.3130g potassium dihydrogen orthophosphate and dissolved in 500 ml of HPLC grade water

Stock solutions of NBT: 8.68mg of NBT, 10 ml of 0.5mM xanthine in water were placed in 100ml volumetric flask and made up using PBS buffer.

pPRE was dissolved in HPLC grade water to yield a stock solution with a concentration of 0.1 mg/ml.

Ferrous sulphate was dissolved in HPLC grade water that had been previously been degassed by bubbling nitrogen through it for 10 minutes to give a solution with a concentration of 0.1mM

4.2.3.2 Original method for the preparation of the enzyme solution

Xanthine oxidase, previously diluted using double de-ionised water, approximately 1:1.5, to give a change in absorbance at 550 nm of 0.025/min (10 µL), this corresponded to a flux of 1 µM $O_2^{\cdot -}$/min. The adjusted enzyme solution was kept on ice and was used with no delay. (Fisher, 2005)

4.2.3.3 Revised method for the preparation of enzyme solution

The same as original method but adjusted enzyme solution is left to equilibrate for 30 minutes on ice prior to use in the assay.

4.2.3.4 Control assay for the xanthine oxidase activity by monitoring consistent urate production

A volume of 3 ml of NBT stock solution that had been kept in the 25° C water bath for 5 minutes was placed in a quartz cuvette. The test solution was added along with water to keep the volume constant, followed by the dilute enzyme solution which started the reaction, see table 4.2.3 for composition. The cuvette was shaken and placed into the UV spectrophotometer. The added xanthine

oxidase started superoxide flux. Urate production was measured in a thermostatically controlled UV-visible spectrophotometer (25 °C) at 295 nm for 3 mins. The time intervals were every 0.1 of a second. The first 60 seconds were discarded due to fluctuation and only the last 120 seconds were used as valid data.

Table 4.2.3 Composition of all reactants involved

Test compound (μL)	Water (μL)	NBT reaction mixture (mL)	Xanthine Oxidase (μL)
0	30	3	20
10	20	3	20
20	10	3	20
30	0	3	20

4.2.3.5 Assay for superoxide generation

A volume of 3 ml of NBT stock solution that had been kept in the 25° C water bath for 5 minutes was placed in a quartz cuvette. The test solution was added along with water to keep the volume constant, followed by the dilute enzyme solution which started the reaction. The cuvette was shaken and placed in the UV spectrophotometer. The added xanthine oxidase started superoxide flux by converting xanthine into urate. The resulting NBT reduction to yield formazan was measured in a thermostatically controlled UV-visible spectrophotometer (25 °C) at 550 nm for 3 mins. The first 60 seconds were discarded due to fluctuation and only the last 120 seconds were used as valid data. The superoxide production and or scavenging by the test substance can be determined by a difference in the rate of formazan detected.

4.2.4 Results and Discussion

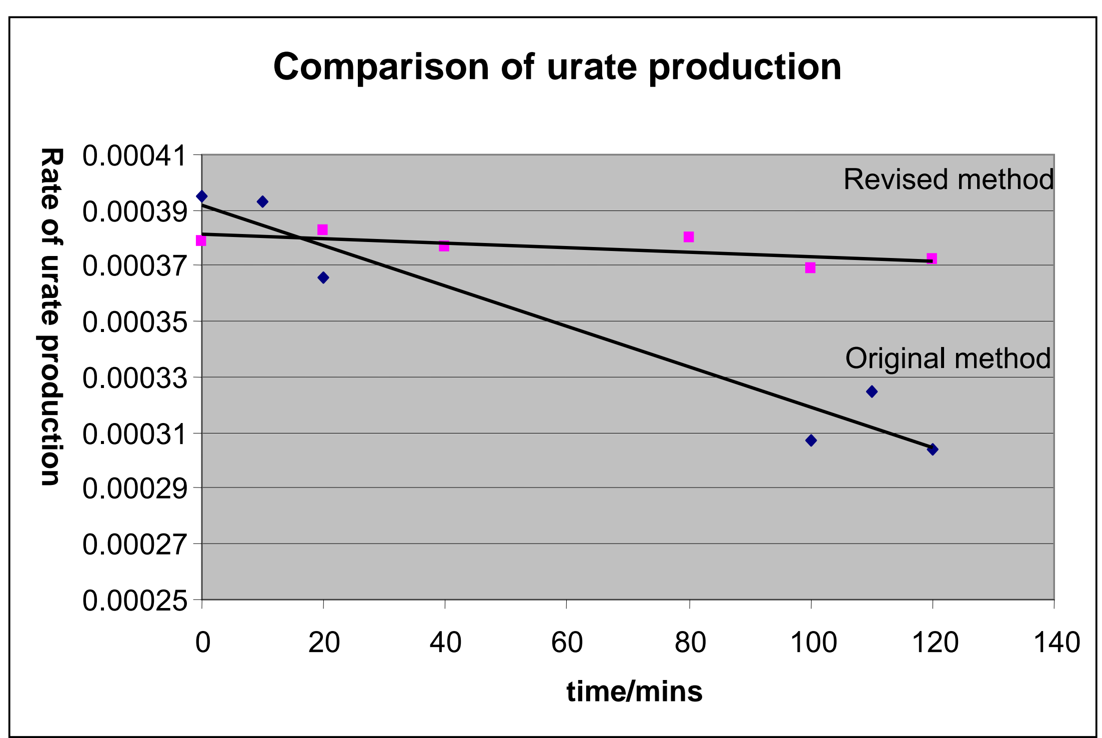
4.2.4.1 Standard method development

This assay makes use of the reaction of xanthine and the enzyme xanthine oxidase to produce a steady supply of superoxide radicals. Superoxide then reacted with the reporter molecule NBT to yield formazan, which is a dark purple colour. In order for this method to deliver accurate results the first reaction had to occur at a constant rate as the production of SO had to be constant. If this was not the case results obtained using this method could not be deemed accurate. If working correctly a steady increase in formazan concentration should be observed, the resulting data is plotted against time and the gradient is calculated. There were three possible scenarios that could occur when the test substance was added: less, more or the same amount of formazan was produced yielding a smaller, higher or the same gradient respectively. As a control the urate production had to be checked (295 nm) to see if the test substance interfered with the free radical producing system, the enzyme. This was absolutely necessary to ensure that no wrong conclusions were being drawn.

Table 4.2.4.1 Relating rates of urate production as measured by urate production at 295 nm as a function of time between the original and the revised method, the statistical analysis using a two tailed t-test showed no significant difference, r^2=0.8417

Time elapsed between assay runs /mins	Rate of urate production original method AUs^{-1}	Rate of urate production revised method AUs^{-1}
0	$3.94x10^{-4}$ s^{-1}	3.79 $x10^{-4}$ s^{-1}
10	3.93 $x10^{-4}$ s^{-1}	
20	3.66 $x10^{-4}$ s^{-1}	3.83 $x10^{-4}$ s^{-1}
30		
40		3.77 $x10^{-4}$ s^{-1}
80		3.80 $x10^{-4}$ s^{-1}
90		
100	3.07 $x10^{-4}$ s^{-1}	3.69 $x10^{-4}$ s^{-1}
110	3.25 $x10^{-4}$ s^{-1}	
120	3.04 $x10^{-4}$ s^{-1}	3.72 $x10^{-4}$ s^{-1}

Fig 4.2.4.1 Differences in the rate of urate production as a function of time of the original and the revised method



When the original method was used the reproducibility of results was poor, as a straight line should be seen on the graph if activity was constant. The results showed that mixing the enzyme with water and leaving it for 30 minutes (revised method) gave a much better reproducibility, see table and figure 4.2.4.1. This suggests that when mixed the enzyme needed some time to establish an equilibrium that gave constant enzyme activity. This could be due to the xanthine oxidase being suspended in 2.3M $(NH_4)_2SO_4$ containing 1mM Na salicylate and the water that was used to dilute the suspension lowered the concentration by 50%. This caused a slight decrease in activity over time but it seemed like in the first 30 mins the activity varied greatly, see fig 4.2.4.1. The reduction in activity from the first and the last measurement of the original method was 23% compared to 1.8% for the revised method showing a significant improvement. The decrease in activity in the original method could cause significant

interpretation errors. For example if the controls were done at the beginning an inhibition of the enzyme or superoxide scavenging activity could be deduced even though the substance tested had no activity. When the enzyme inhibition effect of the pPRE was tested, the inhibition of the first experiment did not exceed 18% which was less then the degradation of the enzyme in the original method after 100 minutes. Therefore it was a significant improvement of the method. A statistical analysis in the form of a Mann and Whitney test was carried out, it showed a significant difference between the two clusters of three from 80-120 minutes at 90% confidence limit with a p value of 0.0589.

4.2.4.2 Measure of XO inhibition by pPRE

Table 4.2.4.2 (i) Impact of increasing concentrations of pPRE with or without 0.1mM of Fe^{2+} present on the reaction between xanthine/ xanthine oxidase. Activity was measured by the difference of the rate of urate production compared to a blank run, measured at 295 nm

Sample	NBT	pPRE conc. in mg/ml	Fe^{2+}	Rate of urate/SO Production AUs^{-1}
1	✓	0		$3.68x10^{-4}$
2	✓	0	✓	$3.70x10^{-4}$
3	✓	$9.27x10^{-5}$		$3.13x10^{-4}$
4	✓	$9.27x10^{-5}$	✓	$3.20x10^{-4}$
5	✓	$1.85x10^{-4}$		$3.27x10^{-4}$
6	✓	$1.85x10^{-4}$	✓	$3.23x10^{-4}$
7	✓	$2.78x10^{-4}$		$3.07x10^{-4}$
8	✓	$2.78x10^{-4}$	✓	$3.02x10^{-4}$

Table 4.2.4.2(ii) Impact of increasing concentrations of pPRE with or without 0.1mM of Fe^{2+} present on the reaction between xanthine/ xanthine oxidase. The reactants were premixed with the enzyme for 5 minutes prior start of the reaction. Activity was measured by the difference of the rate of urate production compared to a blank run, measured at 295 nm

Sample	NBT	pPRE conc. in mg/ml	Fe^{2+}	Rate of urate/SO production AUs^{-1}
1	✓	0		3.67×10^{-4}
2	✓	0	✓	3.71×10^{-4}
3	✓	9.27×10^{-5}		3.25×10^{-4}
4	✓	9.27×10^{-5}	✓	2.74×10^{-4}
5	✓	1.85×10^{-4}		2.45×10^{-4}
6	✓	1.85×10^{-4}	✓	2.50×10^{-4}
7	✓	2.78×10^{-4}		2.51×10^{-4}
8	✓	2.78×10^{-4}	✓	2.62×10^{-4}

Table 4.2.4.2(iii) Percentage reduction of urate/SO production compared to blank with increasing concentrations of pPRE and with or without 0.1mM of Fe^{2+} present compared to a blank run measured at 295 nm.

Sample	pPRE conc. in mg/ml	Fe^{2+}	Percentage inhibition	Percentage inhibition (premixed for 5 mins)
1	$9.27x10^{-5}$		15.18	11.92
2	$9.27x10^{-5}$	✓	13.29	25.79
3	$1.85x10^{-4}$		11.38	33.60
4	$1.85x10^{-4}$	✓	12.49	32.27
5	$2.78x10^{-4}$		16.08	31.98
6	$2.78x10^{-4}$	✓	18.18	29.02

The NBT assay can be used to determine superoxide scavenging or generating activity of the substance tested. It has been suggested that free radicals are responsible for the anti-viral activity of pPRE. It was found that pPRE showed a small dose independent inhibition of the enzyme xanthine oxidase in converting xanthine into urate and therefore reducing the number of superoxide radicals produced, see table 4.2.4.2(i) and 4.2.4.2(ii). The inhibition did not increase with concentration or exceed 34% and was therefore thought to be non-specific. Four different experiments were conducted, pPRE only, pPRE and Fe^{2+}, pPRE and XO premixed for 5 mins and pPRE and Fe^{2+} premixed with XO for 5 mins and the reduction in activity was compared to a blank run done individually for each experiment, see table 4.2.4.2(iii).

Enzymes have many different active sites. If a molecule docks on at one of these sites the shape of other sites can be altered (Rang, 2003). This can cause non-specific enzyme inhibition by partial blockage of the active site of the enzyme and

therefore reducing its capability for the conversion of xanthine to urate yielding superoxide radicals.

The addition of ferrous ions and pPRE did not increase the overall inhibition of the enzyme. When the mixture was left for 5 minutes the inhibition increased but it did not exceed 34%. The functional groups present on puncalagin are phenolic groups, a hemiacetal group on the sugar moiety and ester groups. Glucose, which is the central sugar moiety in punicalagin, exists as two anomers in solution, axial and equatorial in a ratio of 1:3. (Clayden, 2001) Aldehyde groups have been reported to cause non-specific inhibition due to their high reactivity. However due to the fact that the aldehyde group is only present in trace amounts when the glucose ring opens before reforming it is probably not responsible for the inhibition. When fast screening methods like rapid elimination of swill are done they are filtered out because of their known reactivity with biological nucleophiles (Walters, 2002). The esters could be hydrolysed to yield alcohol and carboxylic acid groups. Punicalagin is part of the hydrolysable tannin family and the ester bond connecting the sugar moiety can be hydrolysed yielding ellagic acid which has been reported to induce apoptosis in various human cancer cell lines (Larrosa, 2005). The most likely reason for the non-specific inhibition of the enzyme is due to a tanning like reaction that occurs when the polyphenol groups bind to proteins present on the enzyme resulting in reduced activity.

4.2.4.3 Determination of superoxide generation interference by pPRE

It was found that the rate of production of superoxide as was deduced by the increase in absorbance due to formazan formation from NBT, shows a similar trend as was observed with the XO inhibition assay, see table 4.2.4.3(i)and figure 4.2.4.3. The reproducibility of this assay was poor which could be due to the yellow orange colour of the pPRE and the formation of a black precipitate when the phenol groups reacted with Fe^{2+} to form a complex. These results, see fig 4.2.4.3(ii), did not give any indication that pPRE/Fe^{2+} interferes directly with the production of superoxide radicals. Exclusion of xanthine oxidase from the experiment, i.e. reaction of pPRE/Fe^{2+} in the presence of NBT gave no

absorbance at 550 nm which indicated that the pPRE/Fe^{2+} did not involve the production of SO radicals.

Table 4.2.4.3(i) Effect on the superoxide production with increasing concentrations of pPRE with or without 0.1mM of Fe^{2+} present by measuring the rate of production of formazan as a function of time at 550 nm

Sample	NBT	pPRE conc. in mg/ml	Fe^{2+}	Rate of formazan/SO production AUs^{-1}
1	✓	0		2.41×10^{-4}
2	✓	0	✓	2.77×10^{-4}
3	✓	9.27×10^{-5}		2.04×10^{-4}
4	✓	9.27×10^{-5}	✓	2.46×10^{-4}
5	✓	1.85×10^{-4}		1.69×10^{-4}
6	✓	1.85×10^{-4}	✓	1.61×10^{-4}
7	✓	2.78×10^{-4}		1.97×10^{-4}
8	✓	2.78×10^{-4}	✓	1.99×10^{-4}

Figure 4.2.4.3 Production of formazan measured at 550 nm with increasing concentrations of pPRE and 0.1mM of Fe^{2+} present as a function of time, conc 1=9.27×10^{-5} mg/ml, conc 2=1.85×10^{-4} mg/ml, conc 3=2.78×10^{-4} mg/ml

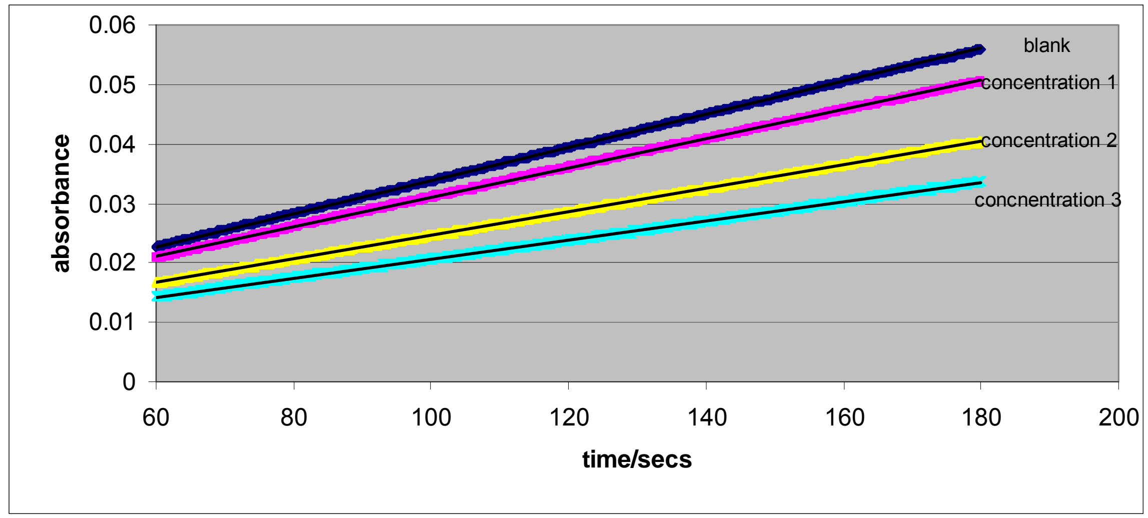


Table 4.2.4.3(ii) Increasing concentrations of pPRE with or without 0.1mM of Fe^{2+} present as a function of percentage reduction SO obtained from measuring rate of formazan production at 550 nm

Sample	pPRE conc. in mg/ml	Fe^{2+}	Percentage inhibition
1	9.27×10^{-5}		15.4
2	9.27×10^{-5}	✓	11.2
3	1.85×10^{-4}		29.9
4	1.85×10^{-4}	✓	42.1
5	2.78×10^{-4}		18.3
6	2.78×10^{-4}	✓	28.1

4.2.5 Conclusion

The reason for testing for superoxide was that the involvement of free radicals, especially hydroxyl radicals, was suspected. Increased phagicidal activity was observed when ferrous ions were added. This suggests Fenton and or Udenfriend chemistry could be involved. The Fenton reaction can involve superoxide in the so called superoxide assisted Fenton reaction, where superoxide is responsible for the reduction of the oxidised ferric ions back into the active ferrous state. Superoxide radicals can also react with water molecules to yield hydrogen peroxide which is the main reactant in the Fenton reaction alongside ferrous ions. This assay is an indirect method for the detection of superoxide using a reporter molecule, NBT, which reacts with superoxide to give purple coloured formazan. The original method showed a high degree of variability of urate production which could lead to misinterpretation of results. The results showed that once the method was revised the production of urate was steadier and constant for longer periods of time. The urate production was directly linked to the formazan production and better reproducibility was achieved. The modified method was used to show that non-specific inhibition of the enzyme occurred making this method unsuitable for the purpose of measuring superoxide scavenging activity of pPRE. However when the enzyme xanthine oxidase was left out NBT on its own could still be used to detect superoxide generation of the test substance but results showed that no formazan was produced. This indicated that pPRE and ferrous ions do not generate superoxide. A degree of interference of the pPRE-Fe^{2+} precipitate into the measurement of formazan was evident.

4.3 Investigation of the involvement of hydroxyl radicals in the antiviral activity of pPRE

4.3.1 Introduction

It is believed that the anti viral activity of punicalagin and other plant polyphenolics is due to the generation of free radicals. (Chattopadhyay, 2009, Khan, 2005) The problem with detecting the free radicals produced is that they are highly reactive and therefore short lived. Due to that fact the majority of techniques used are indirect methods of detection that require the formation of a more stable, detectable product formed by the free radicals and a substrate. The method chosen for this experiment was an aromatic hydroxylation trapping method with a coloured product that could be measured colorimetricaly. It made use of the hydroxylation of para nitro phenol (pNP), as this reaction is believed to be specific to hydroxyl radicals. (Chrastil, 1975) Due to the electrophilic nature of the free radicals a substitution reaction on the benzene ring occurs. The electron withdrawing properties of the nitro group activates the benzene ring at ortho, (2) and (4), position forming the product para-nitro-catechol (pNC), see fig 4.3.1.

Fig 4.3.1 possible mechanism for the hydroxylation of p-nitrophenol

p-Nitrophenol

4-Nitrocatechol

Because of the ortho activated ring mono-hydroxylated products are favoured and that gives it the edge over the more commonly used reporter molecules salicylate and phenylalanine that result in a variety of hydroxylated products, see

Fig 4.1.3.2(II). This method has also been successfully used by other members in the department for the detection of HR and was therefore the method of choice. (Naughton, 1993) The reactant pNP is yellow and the hydroxylated product pNC is blood red in alkaline conditions making it an easily detected reporter molecule. Due to this fact ultraviolet spectrophotometry can therefore be used to monitor the reaction as pNP absorbs at 400 nm and pNC at 510 nm respectively. The Fenton reaction is one of the best known reactions yielding hydroxyl radicals.

$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{-} + OH^{\cdot}$

This reaction was used to show that the reaction of hydroxyl radicals and pNP yields pNC which is detectable using a UV/VIS spectrophotometer.

4.3.2 Materials

Chemicals: para-nitro-phenol Aldrich Chemicals, Gillingham

Para-nitro-catechol Aldrich Chemicals Gillingham

Formic acid Fisher Scientific Loughborough

Sodium hydroxide Fisher Scientific, Loughborough

Hydrogen Peroxide Fisher Scientific, Loughborough

UV-grade cuvettes Fisher Scientific, Loughborough

All solvents used were purchased from Fisher scientific and of laboratory grade and the water used was of HPLC grade if not stated otherwise.

Equipment: UV-VIS Spectrophotometer VARIAN Cary 50 Scan

Rotary evaporator Büchi RE 111

Water bath, GRANT SU5

4.3.3 Preparation of reagents

A formate buffer (50mM) with a pH of 4.0 was prepared using 2.301 g of formic acid dissolved in 900 ml of deionised water and 10% NaOH was added drop wise with the appropriate pH was obtained using a pH meter.

Para-nitro-phenol (pNP) (5mM) was prepared by dissolving 0.0072 g of pNP in a 10 ml volumetric flask using HPLC grade water.

Para-nitro-catechol (pNC) (5mM) was prepared by dissolving 0.0078g of pNC in a 10 ml volumetric flask using HPLC grade water.

Hydrogen peroxide, H_2O_2, (25mM) and (250mM) was prepared by dissolving 0.02830 and 0.2830g of H_2O_2 (30%) respectively in a 10 ml volumetric flask using formate buffer

Ferrous sulphate hepta-hydrate, $FeSO_4$ $7H_2O$, (10mM) was prepared by dissolving 0.0590g of $FeSO_4$ $7H_2O$ in a volumetric flask using degassed HPLC grade water.

Purified pomegranate rind extract test solution (2mg/ml) was prepared by dissolving 0.0200g of pPRE, see chapter 3, in a 10 ml volumetric flask using HPLC grade water.

For the first experiment all solutions were degassed for 10 minutes using nitrogen.

4.3.4 Methods

4.3.4(I) Assay for $OH^{\cdot}$ production using paranitro-phenol (pNP)

The following method is adapted from Singh and Hider, 1988.

The reaction was carried out in triplicate using boiling tubes. A volume of 1 ml of each of the solutions of pNP, $FeSO_4$ $7H_2O$ and H_2O_2 plus 2 ml of formate buffer was added to give an overall volume of 5 ml. These were kept in a water bath for 1 hour at 37°C to ensure completion of the reaction. This represents concentrations of 1mM of pNP, 0.2mM of $FeSO_4$ $7H_2O$ and 5mM of H_2O_2. The reagents were placed in the water bath prior to use to ensure that they were at the appropriate temperature.1 ml of each reagent required was pipetted into a boiling tube with the $FeSO_4$ $7H_2O$ introduced last. In test and control experiments in which one or more reagents was excluded the final 5ml volume was maintained by the addition of 1 ml of buffer for each reagent

After the incubation period of 1 hour each sample was acidified using 20 drops of 10% sulphuric acid (H_2SO_4), lowering the pH to 1-1.5. The pNC will partition into an organic solution (chloroform). The pNC formed was then extracted using 5 portions of 10 ml of Chloroform in a separating funnel. The fractions were combined and the solvent was removed under reduced pressure. The residues were redisolved in 5 ml of 10% sodium hydroxide (NaOH) to give the same concentration that was used in the reaction and scanned using a UV-spectrophotometer from 300-600nm in standard plastic cuvettes using NaOH as a reference

4.3.4(II) Revised method

This was the same as the original method but as a result of only very small amounts of pNC being produced the concentration of H_2O_2 was increased 10 fold to give a concentration of 50mM.

4.3.5 Standardisation and optimisation of pNP and pNC concentrations

In order to find the appropriate peaks in the UV spectra of the substrate para-nitro-phenol (pNP) and the hydroxylated product para-nitro-catechol (pNC), separate solutions were prepared at a concentration of 1mM in 10% NaOH. The two solutions were then diluted 1 in 10 to a concentration where the maximum absorbance was less then 1 absorbance unit, 0.1mM.

The range of the UV spectrophotometer was 300-600 nm. pNP was found to have a maximum absorbance of 0.962 at 400 nm with no peak present at 510 nm. pNC showed maximum absorbance of 0.7093 at 514.1 nm and a smaller peak at 390 nm. This showed that pNP does not absorb at 514 nm and does therefore have no effect on the amount of pNC produced. 4.3.6 Results and discussion

4.3.6 Results and discussion

4.3.6.1 Measurement of the hydroxylation of paranitro-phenol using the Fenton reaction and/or pomegranate extracts

Table 4.3.6.1 Production of pNC using solutions that were previously degassed using nitrogen

Sample	H_2O_2	pNP	$FeSO_4$	pPRE	Absorbance	Mean
A	✓	✓	✓		0.885	
					0.612	0.743
					0.731	
B	✓	✓	✓	✓	0.901	
					0.641	0.783
					0.806	
C		✓	✓	✓	0.011	
					0.017	0.014
					0.014	
D			✓	✓	0.018	
					0.016	0.016
					0.013	

Table 4.3.6.1 b Statistical significance of the samples from table 4.3.6.1, shown as p values aquired using a t-test

	A	B	C	D
A	X			
B	0.155	X		
C	0.0121	0.0101	X	
D	0.0114	0.0095	0.0596	X

Table 4.3.6.2 Production of pNC using solutions that have not previously been degassed except Fe SO_4 $7H_2O$

Sample	H_2O_2	pNP	$FeSO_4$	pPRE	Absorbance	Mean
					0.727	
A	✓	✓	✓		0.544	0.627
					0.597	
					0.619	
B	✓	✓	✓	✓	0.520	0.652
					0.817	
					0.474	
C		✓	✓	✓	0.461	0.518
					0.620	
					0.014	
D			✓	✓	0.013	0.015
					0.017	

Table 4.3.6.2 b Statistical significance of the samples from table 4.3.6.2, shown as p values using a students t-test

	A	B	C	D
A	X			
B	0.7936	X		
C	0.3238	0.0799	X	
D	0.0079	0.0177	0.0096	X

4.3.6.2 Investigation into the hydroxyl radical generating activity of partially purified pomegranate rind extract (pPRE)

This method was used to show that hydroxyl radicals were produced by pPRE and Fe^{2+}. Scatter of the data indicates that this method was not especially precise but was a realistic model to show the hydroxylation of pNP. The same modified method used to determine the hydroxylation of pNP was used, the only difference being that pPRE was added, see table 4.3.6.1. The last three experiments in table 4.3.6.1 and 4.3.6.2 show that when pPRE and Fe^{2+} were added no absorbance at 514nm was observed. The data presented in table 4.3.6.1 were gathered under fully degassed conditions. It was evident that classic Fenton chemistry occured as pNP was converted into pNC. A control showed that pPRE in the presence of pNP did not generate any HR. This set of data established that the experimental conditions chosen were appropriate for measuring the product of the reaction, pNC.

The data presented table 4.3.6.2 was gathered under non-degassed conditions, however $FeSO_4$ was degassed in order to minimise oxidation of the reagent to Fe^{3+}. As this reagent was only 20% of the volume of the reaction this was of little practical relevance for the experiment. There was a major difference in the outcome of this experiment in correspondence to that in 4.3.6.1. pPRE and

ferrous ions on their own in non-degassed conditions produced hydroxyl radicals that then react with pNP to form a significant amount of pNC. However in degassed conditions these two reactants showed no activity giving the same absorbance as the controls. The statistical analysis shown in tables 4.3.6.1b and 4.3.6.2b respectively show that when A and C were compared that under degassed conditions there was no significant difference between the the two sets of data whereas under non-degased conditions there was a significant difference. This was intriguing as it seemed that atmospheric oxygen was required for the generation of hydroxyl radicals. From the results in table 4.3.6.2 it can be deduced that the ferrous ions seem to be the rate determining reactant as the same absorbances were observed whether pPRE was present along with H_2O_2 and ferrous ions or not giving mean absorbance of 0.652 compared to 0.627 respectively. However if no H_2O_2 was added the mean absorbance is 0.518 which was slightly lower then the values obtained in the previous experiments that contained H_2O_2. It would be expected that when H_2O_2, pPRE and ferrous ions are added that a higher absorbance than H_2O_2 on its own would be observed.

4.3.6.3 Investigation of add on effect of pPRE and H_2O_2 in the presence of ferrous ions

Concentrations of reactants:

H_2O_2: 125Mm (half the concentration than previously)

pNP : 5mM

pPRE: 1mg/ml (half the concentration than previously)

FeSO4: 20mM

The concentrations of H_2O_2 pPRE were halved to ensure that enough ferrous ions were present for both reactions to take place. The incubation period in a water bath at 37°C was 60 minutes and then the reaction was stopped by the addition of HCl.

Table 4.3.6.3 Production of pNC using solutions that had previously been degassed. An incubation period of 60 mins was used, a two tailed t-test showed significant difference between A and B, r^2=0.0058

Sample	H_2O_2	pNP	$FeSO_4$	pPRE	Absorbance	Mean
A	✓	✓	✓		0.872	
					0.912	0.902
					0.923	
B	✓	✓	✓	✓	1.213	
					1.199	1.200
					1.187	

Table 4.3.6.4 Production of pNC using solutions that had not previously been degassed except Fe SO_4 $7H_2O$. An incubation period of 90 minutes was used, a two tailed T-test showed significant difference between A and B, r^2=0.00061

Sample	H_2O_2	pNP	$FeSO_4$	pPRE	Absorbance	Mean
A	✓	✓	✓		0.914	
					0.925	0.932
					0.957	
B	✓	✓	✓	✓	1.392	
					1.406	1.400
					1.402	

Control experiments were done for all the reactants individually and no interference was observed. Absorption was in the 510 nm region

In the experiment 4.3.6.3 it was observed that H_2O_2 in the presence of ferrous ions produces hydroxyl radicals as predicted due to the Fenton reaction. Hydroxyl radical production was also observed when pPRE and ferrous ions were present. However when pPRE, H_2O_2 and ferrous ions were present no increase in hydroxyl radical production was observed compared to the previous two experiments. This indicated that not enough ferrous ions were present for both reactants. To find out if an add-on effect could be observed, the concentration of either H_2O_2 and pPRE had to be reduced or the amount of ferrous ions had to be increased. The ferrous concentration was at the solubility limit therefore the concentration of H_2O_2 and pPRE was halved. The results from this experiment can be seen in table 4.3.6.3. The results showed that a slight add-on effect occurred and the addition of pPRE increased the hydroxyl radical concentration by a third. When the reaction was incubated for 90 minutes instead of 60 the increase was more than half the amount observed when no pPRE was added, see table 4.6.4. This suggested that ferrous ions were required for the generation of hydroxyl radicals in both reactions. The reaction of H_2O_2 and ferrous ions was faster as no increase in pNC concentration was observed when the reaction mixture was incubated for 90 minutes instead of 60, see tables 4.3.6.3 and 4.3.6.4. The reaction of pPRE and ferrous ions was slower as an increase in overall concentration of pNC was observed when incubated for 90 minutes, see tables 4.6.3 and 4.6.4. The ferrous ions did seem to get recycled by pPRE as no increase in pNC was observed, table 4.3.6.1 or 4.3.6.2, when H_2O_2 and pPRE were tested in the presence of ferrous ions. It was shown by T. Lapidot, (2002) that hydroxyl radicals were produced by polyphenolics in the presence of ferrous ions, however the concentration of ferrous ions did not seem directly related to the number of hydroxyl radicals produced. This data indicated that there was no direct interaction between polyphenolics and ferrous ions for the production of H_2O_2, but indicated that some of the polyphenolics were possibly acting as iron chelators. Hodnick (1988) reported that the presence of an ion chelator such as EDTA was necessary to form redox-cycle interaction between flavanoids and ferrous ions in aqueous solution at physiological pH to

give H_2O_2. A redox-cycling interaction seemed likely as there was an increase in pNC when the mixture was incubated for 90 minutes compared to 60 minutes. The complexes formed between a metal and the chelate can result in a much more potent species than the metal ion on its own and only catalytic amounts are required. The ferrous ions that were added to the reaction mixture would have been oxidised after 60 minutes. This indicated that some ferrous ions must be recycled or form a complex with the polyphenolics, punicalagin, present in pPRE. The results in 4.3.6.1 and 4.3.6.2 showed that with a limited number of ferrous ions present no increase in pNC was observed when pPRE and H_2O_2 were present. This indicated that the reaction between H_2O_2 and ferrous ions was faster than the reaction of pPRE and ferrous ions. The reaction is so quick that most of the ferrous ions were used up by the H_2O_2 leaving only a few for the slower reaction with the punicalagin. This experiment showed that when the concentration of H_2O_2 was reduced more ferrous ions were left over to react with punicalagin to form HRs which then reacted with pNP to give pNC. When incubated for 90 rather then 60 minutes an increase in the pNC concentration was observed supporting the argument of a redox-cycle involving the polyphenolics and ferrous ions and maybe H_2O_2.

4.3.6.4 Potentiation of pomegranate rind phagicidal activity by ferrous ions

Stewart et al identified the increase in phagicidal activity that resulted from the addition of ferrous ions to the pomegranate rind extracts. The results from the phagicidal activity studies presented in this chapter indicate conclusively that this potentiation is both real and reproducible. There are a number of possible hypotheses for this potentiation, however Fenton or Fenton-like reactions which involve the production of hydroxyl radicals seem the most likely.

Iron is a transition metal which by definition has a partially filled d-subshell. This gives it the ability to have two stable oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}). The benefit that transition metals have is that there is only a small

difference between the energy levels of the two species and they can therefore be oxidised and reduced with relative ease. This gives it the ability to undergo one electron transfers easily making it into a powerful catalyst of autoxidation reactions such as the oxidation of adrenalin (Halliwell, 1990). Because of their relative stability transition metal ions can form complexes with so called ligands. This ability it has found numerous applications in nature for example in haemoglobin where it plays an essential role in binding oxygen. When free Fe^{2+} is oxidised into Fe^{3+}, the latter can be reduced back into the ferrous state with relative ease by the addition of a reducing agent such as ascorbic acid. Thereby recycling the product and providing a new reactant. This is a very important principle as it can provide a constant supply of the reactive ferrous ions, Fe^{2+}. Ascorbic acid has been found in many fruits including the pomegranate and could therefore be involved in recycling ferric ions providing ferrous ions and forming a redox reaction.

The involvement of Fe^{2+} suggests that free radicals, specifically hydroxyl radicals might be involved. Research has shown that if hydroxyl radicals are involved in biological systems they are usually generated from H_2O_2 via the Fenton reaction (Halliwell, 1992). There are two reactions that are frequently involved in the generation of free radicals. The first is the Fenton reaction and the second being the Udenfriend reaction. Fenton chemistry involves the oxidation of Fe^{2+} to Fe^{3+} which is coupled with the reduction of H_2O_2 giving OH- and $OH^{.}$. The Udenfriend system involves the same reaction of H_2O_2 and Fe^{2+} but combined with the addition of ascorbate which is there to reduce the ferric ion, Fe^{3+} back into the ferrous state recycling iron back to its active state. The presence of all three reactants can cause hydroxyl radical damage. It has been found that if metal ions and ascorbate are present at high levels in vitamin supplement pills they can generate high levels of HR when they dissolve (Kadiiska, 1995).

Figure 4.3.6.4 shows Fenton reaction and Udenfriend ferric ion recduction

$H_2O_2+Fe^{2+}\rightarrow Fe^{3+}+OH^{-}+OH^{.}$

Ascorbate + $Fe^{3+}\rightarrow Fe^{2+}$+Ascorbate.

Synergy occurs when the combined action of constituents is greater then would be expected from a consideration of individual components. Medical herbalists have insisted that often better results are obtained from whole plant extracts rather than isolated compounds. For example, side effects of pure ephedrine that were not found when an extract of the herb Ephedra was used (Williams, 2002). In this case it was found that the addition of ferrous ions increases the anti viral activity suggests a synergistic relationship between the ion and the active molecule. The crude extract showed activity on its own that was increased when ferrous ions were added suggesting that some iron might be present in the husk of the plant. Another possibility was that iron is present in a complexed form that allows it to partake in the reaction as it is unusual to find free ferrous ions due to their high reactivity.

4.3.7 Conclusion

The assay chosen for the detection of hydroxyl radicals uses indirect detection via a reporter molecule, para nitro phenol (pNP). PNP is slightly yellow in colour and when it reacts with HR it turns into para nitro catechol (pNC) which is blood red. By monitoring the amount of pNC produced it could be determined whether hydroxyl radicals were generated by the test system under investigation. This method also uses the Fenton reaction to check if hydroxyl radicals were generated. It was observed that pPRE on its own had no free radical generating activity in either previously degassed or standard conditions. However when ferrous ions were added pPRE in non-degassed conditions generated hydroxyl radicals whereas in degassed conditions no activity was observed. Hydrogen peroxide was added to the reaction and an add-on effect was seen. The presence of pPRE and ferrous ions increased the amount of hydroxyl radicals produced but the effect was smaller in de-gassed conditions. This indicates that atmospheric oxygen was involved in the generation of the hydroxyl radicals.

A method for the detection of hydroxyl radicals that was successfully used by Charlesuzor, 2008 involved a reporter molecule called terephthalic acid which produces a luminescent product that can be measured fluormetrically. It was

used to show that the leaves of *Camellia sinesis*, black tea, had hydroxyl radical generating ability. This method seems superior to the one used as only the product would be de detected fluormetrically making it more accurate and less prone to interference by by-products.

4.4 Discussion on free radical detection assays

The work described in this chapter is concerned with the detection of free radicals. Two separate assays were used for the detection of superoxide and hydroxyl radicals. Both used indirect detection of the FR in the form of reporter molecules. The reporter molecule reacted quickly with the FR to give a coloured compound which was used to deduce the FR generated.

The NBT assay was chosen for the detection ofsuperoxide, nitroblue-tetrazolium is converted into formazan which was then detected using a UV-spectrophotometer at 550 nm. This method used the substrate xanthine which was converted by the enzyme xanthine oxidase into urate and superoxide radicals. This should resulted in the steady generation of superoxide radicals and gave the assay the affinity to monitor superoxide radical generation and scavenging activity. The method was optimised by letting the diluted enzyme solution equilibrate for 30 minutes prior to use to give better reproducibility and a steadier generation of superoxide by the xanthine/xanthine oxidase system. The results showed that pPRE and ferrous ions did not generate superoxide radicals but the application of this method was limited by the non-specific inhibition of the enzyme xanthine oxidase.

The detection of hydroxyl radicals was done using para nitro phenol (pNP) as a reporter molecule. A reporter molecule was used because it forms a mono-hydroxylated product that has a distinct blood red colour when de-protonated using a base, NaOH. The reactant pNP was of a light yellow colour and reacts with one hydroxyl radicals to give para nitro catechol (pNC) which has a blood red colour and can be detected with a UV spectrophotometer at 514 nm. This method was optimised by increasing the H_2O_2 for the experiment as the original amount of pNC detected was too small. The Fenton reaction of H_2O_2 and ferrous

ions was used to check whether or not the detection was working and was also used in combination with pPRE in some parts. The assay showed that pPRE in the presence of ferrous ions generated hydroxyl radicals but if the reagents were de-gassed previously no hydroxyl radicals were detected indicating that atmospheric oxygen was required for the reaction to work. When pPRE, H_2O_2 and ferrous ions were combined an add-on effect was observed that resulted in a higher concentration of pNC than seen when only pPRE or H_2O_2 and ferrous ions were tested. This reaction was also done in de-gassed conditions which also showed an increase in the amount of pNC observed but to only half the amount observed with the non-degassed one in addition to the pNC produced by the Fenton reaction. The presence of atmospheric oxygen also contributed to the ability of pPRE, in the presence of ferrous ions, to generate hydroxyl radicals. Overall it was shown conclusively that pPRE and ferrous ions have the ability to generate hydroxyl radicals which may or may not be responsible for the phagicidal activity too. Stewart *et al.* (1998) reported that pomegranate rind extract showed phagicidal activity in the presence of ferrous ions and the addition of H_2O_2 increased that activity. They proposed that a Fenton like reaction was responsible for the generation of hydroxyl radicals which consequently was responsible for the phagicidal activity. The results from this chapter and the phage amplification assays in the last chapter support this theory.

Chapter five

Overall discussion

5. Overall discussion

The pomegranate contains many medically important constituents and extracts made from it have found a wide range of applications from vermifuge to aphrodisiac for more then 4000 years. Pomegranate rind extract has been attributed with antiviral properties and due to the lack of available antivirals this was the topic of interest. This extract has been successfully used as phagicide by Stewart et al in the phage amplification assay. Phage particles have been used as model systems for the study of viruses due to their high degree of similarities as they are bacterial viruses. They harbour a major advantage over viruses as they do not pose the danger of infection to the analyst and a quicker replication cycle. An assay for monitoring phagicidal activity has been established and applied successfully using *Acenitobacter* HER 1401 and its corresponding phage as a model system. It is called the streaking method and it has been developed for the comparison of the plant extracts and has got potential as a quick screening method for the determination of anti viral activity. Using this method it has been demonstrated that pomegranate rind extract (PRE) and a purified pomegranate rind extract (pPRE) have antiviral activity. In both cases this activity was potentiated by the presence of ferrous ions. The purified pomegranate rind extract (pPRE) was obtained by a multi step purification process with the main points being extraction of the freeze dried rind with a soxhlet extractor followed by column chromatography using an ion exchange resin called Amberlite XAD-8. Mass spectrometric analysis was used to determine the active component present in pPRE. It was suspected that the ellagotannin punicalagin was responsible and MICRO-TOF analysis confirmed that punicalagin was present in the purified extract. The next step was to determine the mechanism of action, the potentiation of the antiviral activity by the presence of additional ferrous ions suggested that free radicals and possibly hydroxyl radicals were involved. Most commonly associated with the generation of hydroxyl radicals is the Fenton reaction. This reaction involves the interaction of hydrogen peroxide and ferrous ions to yield hydroxyl radicals. It was investigated if superoxide radicals were involved as they have been known to contribute in the superoxide assisted

Fenton reaction. They are responsible for the reduction of ferric ions back into the more reactive ferrous ions. It was found that pomegranate rind extracts in the presence of ferrous ions did not generate or scavenge any superoxide radicals. The next step was to investigate the generation of hydroxyl radicals by the purified pomegranate rind extract (pPRE). This was done using aromatic hydroxylation of paranitro-phenol and it was found that hydroxyl radicals were generated. However it was shown that in the absence of atmospheric oxygen no HR were generated. In order to check the hydroxyl radical detection system hydrogen peroxide was used and it was found that when pPRE, hydrogen peroxide and ferrous ions were added more hydroxyl radicals were produced after an extended incubation period of 90 minutes rather then the standard 60 minutes. This indicates that some kind of recycling of the ferric ions occurred as any ferrous ions in solution would be oxidised into the non-active ferric state after 60 minutes. In order to find out if the pomegranate rind extracts produce hydrogen peroxide that in turn reacts with the added ferrous ions to yield hydroxyl radicals via the Fenton reaction and to confirm that hydrogen peroxide is definitely involved in the mechanism of action the use of the hydrogen peroxide scavenger peroxidase was investigated. Peroxidase was preferred over catalase as the latter produces oxygen. Problems were encountered as the enzyme did not function in the conditions of the pNP assay, catalase the second choice was also unsuccessful. The theory that hydroxyl radicals are responsible for the virucidal activity of PRE and pPRE was confirmed by bacteriophage assay as the host bacteria were not affected but the phage particles were killed. Most cells and bacteria have antioxidant defences whereas viruses and phage particles are very basic and lack a defence mechanism against free radicals and can therefore be killed by these highly reactive species. It seems that the generation of free radicals was partly responsible for the antiviral action of PRE and pPRE, the hydroxyl radicals involved are small in size and therefore of a highly reactive nature. Hydroxyl radicals are one of the most reactive free radical there are and are attributed to a high degree to oxidative stress. Mendez de Corea used electron microscopy to check on the the phages after treatment with PRE and

ferrous ions and found deformed phage particles. The phages showed similar damage to those treated with bleach supporting FR involvement. However in the bioassays it was clearly shown that pPRE with no added ferrous ions had phagicidal activity indicating that there might be more then one mechanism of action. As for the potential use of pPRE as an antiviral it has to be pointed out that it is not a good candidate for oral dosing as it fails lepinskis rule of 5. The aggressive nature of hydroxyl radicals that are likely to be involved in the antiviral activity reduce its use in vivo, as well as the size of the molecule, Mr=1085. However it could be used topicaly to treat herpes or warts where it can be applied straight on to the infected area. To compliment the research carried out it would be interesting to use a hydroxyl radical scavenger in the bio assay to establish whether activity was maintained. To further ensure the mechanism of action it should be investigated if the hydroxyl radical generation was still occurring in the presence of a hydrogen peroxide scavenger. Hydroxyl radical detection by terephthalic acid which gives a fluorescent product would be favourable over pNP as fluorescent spectroscopy has significant advantages over UV/vis spectroscopy. The main advantage being that interference which can lead to misinterpretation of the results is eradicated. In order to achieve pure punicalagin it has been reported that high speed counter current chromatography can yield 92% pure punicalagin so to use this method and use the resulting product in combination with the fluorescent method mentioned earlier would seem like the methods of choice.

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