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Patrick Ruddock
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Biology)
GRADE / DEGREE

Department of Biology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Phytochemical Mixtures and NHPs as Antimicrobials Against Antibiotic Resistant *Neisseria gonorrhoeae* and Other Pathogens

TITRE DE LA THÈSE / TITLE OF THESIS

J. T. Arnason
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

J. Dillon
CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

B. Foster

R. Kassen

J. Vierula

Gary W. Slater
LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES /
DEAN OF THE FACULTY OF GRADUATE AND POSTDOCORAL STUDIES

PHYTOCHEMICAL MIXTURES AND NHPs AS ANTIMICROBIALS AGAINST ANTIBIOTIC RESISTANT *Neisseria gonorrhoeae* AND OTHER PATHOGENS

Patrick Ruddock, B.Sc.

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Department of Biology
Faculty of Science
University of Ottawa

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ABSTRACT

Phytochemicals and garlic extracts were evaluated for their potential as complementary treatments against *Neisseria gonorrhoeae*. The minimum inhibitory concentrations (MICs) of the phytochemicals silibinin and dillapiol, and a panel of garlic natural health products were determined against a panel of resistant and susceptible *N. gonorrhoeae* strains using agar and broth dilution methods and bacterial killing curves. Combinations of subinhibitory concentrations of silibinin or dillapiol with penicillin elicited a significant reduction in MICs of ~50% and ~25% of the tested strains respectively. Ternary combinations of silibinin, dillapiol and penicillin yielded a significant MIC reduction in 88% of the tested strains, including some strains whose penicillin MICs were reduced below the penicillin resistance breakpoint. The garlic extracts exhibited discrepancies in product composition and antimicrobial activity, with few products having comparable activity to fresh garlic. The activity of these phytochemicals against mulitple *N. gonorrhoeae* resistance phenotypes makes them worthy of further investigation.

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It is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change. – Charles Darwin

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LIST OF ABBREVIATIONS

5'-MHC	5'-methoxyhydnocarpin-D
AGE	aged garlic extract
ATCC	American Type Culture Collection
AZI	azithromycin
CAA	casamino acids
CAM	complementary and alternative medicine
CFU	colony forming units
CIPRO	ciprofloxacin
CMRNG	chromosomally resistant *Neisseria gonorrhoeae*
C*n*	carbon chains (where *n* equals the number of carbons in the chain)
CO_2	carbon dioxide
Cu	copper
CYP3A4	cytochrome P450 3A4 enzymes
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
D-ala	D-alanine peptidoglycan subunit
D-lac	D-lactate peptidoglycan subunit
EDTA	ethylene diamine tetra-acetic acid
ERY	erythromyin
ESBL	extended spectrum β-lactamase
EtOH	ethanol
FIC	fractional inhibitory concentration
FICI	fractional inhibitory concentration index
g	gram
GC	guanine + cytosine [content]
GCMBK	gonococcal medium base agar + 1% Kellogg's supplement
GMP	good manufacturing practice(s)
GO	garlic oil
GP	garlic powder
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
kb	kilobase (pairs)
KCN	potassium cyanide
LAGE	gelatinous AGE
LGP	gelatinous GP
LPS	lipopolysaccharide
MBC	minimum bactericidal concentration
MDR	multi drug resistance
Mg	magnesium
$MgCl_2$	magnesium chloride
mg/g	milligrams per gram
mg/l	milligrams per litre
mg/ml	milligrams per millilitre

MH	Mueller Hinton (broth)
MIC	minimum inhibitory concentration
ml	millilitre
mM	millimolar
MRSA	methicillin resistant *Staphylococcus aureus*
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NCCLS	National Committee for Clinical Laboratory Standards
NHP	natural health product
nm	nanometre
NPN	1-N-phenylnapthylamine
OM	outer membrane
O/N	overnight [subculture]
PBP	penicillin binding protein
PPNG	penicillinase producing *Neisseria gonorrhoeae*
QRNG	quinolone resistant *Neisseria gonorrhoeae*
RFU	relative fluorescence units
RNA	ribonucleic acid
rpm	revolutions per minute
SAC	S-allylcysteine
SARS	severe acute respiratory syndrome
SPEC	spectinomycin
STD	sexually transmitted disease
STP	sewage treatment plant
TRNG	tetracycline resistant *Neisseria gonorrhoeae*
ul	microlitre
uM	micromolar
VISA	vancomycin intermediate *Staphylococcus aureus*
VRSA	vancomycin resistant *Staphylococcus aureus*
VRE	vancomycin resistant *Enterococcus faecalis*
WHO	World Health Organization
Zn	zinc
ZOI	zone of inhibition

CHAPTER 1 – INTRODUCTION

1.1 Introduction to the thesis

The problem of bacterial resistance to antibiotics has existed as long as antibiotics have been in use and continues to be a significant barrier to preventing the morbidity and mortality associated with bacterial infections. Currently, a number of bacterial pathogens including *Neisseria gonorrhoeae*, *Staphylococcus aureus* and *Enterococcus faecalis* represent a severe threat to human health by virtue of resistance to multiple antibiotics. Although the risk of antimicrobial resistance can be delayed or minimized by prudent antibiotic usage or using antibiotic combinations, the lack of new effective antibiotics necessitates the search for new sources of antimicrobial material.

The plant kingdom provides a wealth of biologically active material in the form of secondary metabolites, whose functions include protecting plants from the damage caused by bacterial infections. Such phytochemicals may exert their antibacterial effect alone or derive their effectiveness from the complex mixtures of phytochemicals naturally present in plant extracts. The concept of phytochemical mixtures working synergistically to elicit an antimicrobial effect is especially relevant in antibacterial therapy, since combinations of phytochemicals or phytochemicals and antibiotics may represent an effective way to treat antibiotic resistant organisms. The availability and lower cost of plant based remedies versus pharmaceuticals in developing countries also makes such an approach attractive. Thus, phytochemical mixtures represent a promising field in identifying potential new antimicrobial strategies for treating resistant bacterial pathogens.

1.2 Antimicrobial resistance in the 21st century

The last 100 years has seen a great advance in human understanding of infectious disease and pathogenesis. The discovery of penicillin by Alexander Fleming in 1929 ushered in the new era of antibiotics and other antimicrobials that significantly reduced, and in some cases almost eliminated, the morbidity and mortality associated with such long time scourges as tuberculosis or infected wounds acquired on the battlefield. Indeed, by the First World War, mortality rates due to infectious disease had improved little since scourges like the Black Death outbreak of the 13th century plagued Europe (caused by the bacterium *Yersinia pestis*, and still a problem in other parts of the world today) (Josko, 2004; Morens *et al.*, 2004). However, the changes witnessed in the latter half of the 20th century saw a great advance in the understanding of disease, pathogenicity, prevention and treatment. The discoveries of DNA and the nature of bacterial genetics provided a wealth of information, not only about how these seemingly simple organisms functioned, but also a better mechanistic understanding of how antibiotics kill bacteria and how bacteria use mechanisms of antibiotic resistance. The potential to discover specific new targets that could be exploited in designing and discovering new preventative and therapeutic drugs was another benefit.

Unfortunately however, with this new understanding came a period of complacency. By 1977, smallpox had been eradicated, major killers of antiquity such as tuberculosis and malaria were in decline, and the continued discovery and efficacy of new antimicrobial agents appeared to herald the "end of infectious disease" (Morens *et al.*, 2004). However, the reality was quite different, as evidenced by the continuing presence (and emergence) of diseases both new and old. Although the rates of morbidity and mortality are much lower

than 100 years ago, >25% of global deaths are still attributable to infectious disease, including a variety of age-old bacterial infections including diarrhoeal diseases, tuberculosis and acute respiratory infections among others (Feldmann *et al.*, 2002; Morens *et al.*, 2004). It is important to distinguish between the various factors responsible for the decline of infectious disease. While the discovery of new drugs and treatments is crucial, non drug-related factors such as the affordable access to these drugs, general improvement of food supplies and water quality, better basic access to medical care, improved surveillance and social/economic stability all have their roles to play (Morens *et al.*, 2004). These factors are usually lacking in the developing world, which partly explains why infectious disease continues to be so disproportionately pervasive in these settings despite the discovery of new drugs.

A survey of contemporary scientific and popular literature underscores how little the present day situation resembles what was predicted only 25 years ago. Terms such as "superbugs" (methicillin resistant *S. aureus* [MRSA] for example) have entered the vernacular, emerging diseases (e.g. *Escherichia coli* O157:H7, Ebola virus, SARS and HIV) and re-emerging (e.g. tuberculosis, gonorrhea) diseases dominate the headlines, and reports of resistance to previously effective drugs are commonplace (Feldmann *et al.*, 2002). While antimicrobial resistance is a phenomenon shared by bacteria, viruses and protozoans, the focus will hereafter be on bacteria for the purposes of this work.

A number of related factors have contributed to the rise of antibiotic resistance in recent times, some controllable and some not. The root causes of antimicrobial resistance are

the genetic flexibility, rapid generation times and environmental adaptability of bacteria under selection by single entity, highly active antibiotics. The development of resistance depends on selective pressure through exposure to antibiotics and/or through the acquisition of resistance genes from outside sources (extrinsic DNA). Resistance arises though single or multiple mutations of the bacterial genome and can then rapidly spread vertically (clonal spread of the parent strain) and horizontally (mobile genetic elements such as plasmids) through bacterial populations (Witte, 2000; Sefton, 2002). The transfer of mobile DNA elements such as plasmids through conjugation represents a significant source of acquired resistance, especially among Gram negative organisms (Sefton, 2002; Tenover, 2001). The rapid generation times of bacteria means that a particular resistance phenotype can spread from the parent bacterium to an entire bacterial population extremely quickly. Many bacteria (both animal and environmental microflora) also have a pronounced capacity to exchange genetic information between each other (both intra- and inter-species transfer), such that a resistance gene could spread from a pathogenic species to a related species that may be pathogenic or benign. For example, the *van*A gene cluster (which confers resistance to glycopeptide antibiotics) has been found in *Enterococcus faecalis* strains isolated from meat products, livestock, sewage, and both healthy and nosocomially infected people (Witte, 2000). Evidence suggests that this resistance determinant has spread through the exchange of plasmids between pools of resistant *E. faecalis* strains of both animal and human origin.

Exposure to antimicrobials is the main mechanism by which selection for resistance occurs in bacteria. It is important to note that antimicrobial use does not cause mutations *per se*, but exposure to an antimicrobial selects organisms with pre-existing mutations, which

through virtue of natural selection endure and multiply (Sefton, 2002). This fact elegantly demonstrates the capacity of bacteria to rapidly adapt to changing conditions as opposed to organisms with much longer generation times (notably humans). Thus, to a certain extent the onset of resistance cannot be prevented, since a small proportion of any treated bacterial population will inevitably survive. Consequently, it is in general not the antibiotics themselves, but their misuse that exacerbates this innate challenge. Acquired antimicrobial resistance can be classified into 4 general mechanisms: production of drug inactivating enzymes, target alteration or protection, reduced cell permeability and active efflux of a drug from the cell (Sefton, 2002). These mechanisms and how they relate to resistance among *Neisseria gonorrhoeae* isolates will be discussed in the next section.

The related factors that encourage the acquisition of resistance include prolonged prophylaxis and failure to complete antibiotic regimens (Jephcott, 1986), which can expose bacteria to sub-lethal antibiotic levels and increase the chance for selection of resistant strains. The widespread practice of doctors giving out antibiotics without knowing whether a bacterial infection was present (often at the behest of worried patients wanting a tangible treatment) also had obvious repercussions. For example, using antibiotics to medicate a patient with influenza (i.e. a virus) will not only fail to clear the infection, but may kill commensal microflora (allowing an opportunistic pathogen such as *E. faecalis* to take hold) and/or unnecessarily induce resistance in both benign and pathogenic bacteria that may be residing in the patient. In many developing countries, all these problems are further exacerbated by inadequate health care systems and unregulated access to antibiotics that are often obsolete, but still available (frequently without a prescription), due to their lower cost

versus more effective drugs (Tapsall, 2002). Conversely, the effectiveness of newer and more expensive antibiotics is also compromised in these settings because sub-clinical doses, incomplete therapies or pirated (i.e. unstandardized) versions of the drugs are often used to save money (Tapsall, 2002).

Another important and preventable factor, whose actual contribution to antibiotic resistance has still not been fully quantified, is the influence of antibiotic residues in the environment. The high amounts of antibiotics in use and persisting in the environment should not be underestimated. For example, in Denmark (a developed but relatively small country of about 5 million people) 38 tons of antibiotics were consumed for human treatment and 110 tons of antibiotics were used as growth promoters in livestock in 1995 (Halling Sorenson *et al.*, 1997). Because of this widespread antibiotic use in humans and their use as prophylactics and growth promoters in livestock, large amounts of antibiotic residues or metabolites end up in the water supply (through excretion by humans and animals and direct contamination through manufacturing runoff and discarded/unused antibiotics).

It is often assumed that antibiotics undergo spontaneous degradation or are metabolized both by the body and in sewage treatment plants (STPs) to more innocuous and inactive metabolites, but there is conflicting evidence regarding the processes that act on antibiotics during wastewater treatment. Elimination rates of antibiotics in STPs can vary from 60-90% efficiency, meaning that significant levels of biologically active antibiotics can enter the aquatic environment (Jones *et al.*, 2001). Although some antibiotics are

metabolized by the body (or indeed by some microorganisms) to more benign compounds, many metabolites are more active and toxic than the parent drug, or may be reactivated back to the parent compound (Hirsch *et al.*, 1999). For example, chloramphenicol glucuronide (an inactive metabolite) can be reactivated back to chloramphenicol (the active parent drug) in liquid manure (Halling Sorenson *et al.*, 1997; Witte, 2000). The threat such a situation creates is underscored by the high proportions (70-90% of the surviving bacterial population after STP treatment) of resistant bacteria found in STP effluents (Hirsch *et al.*, 1999). The fact that many antibiotics appear to be ubiquitous in the environment at very low levels is further cause for concern, as these sub-inhibitory levels could induce resistance that could then be transferred to a human pathogen (Rooklidge, 2003). Unfortunately, while some studies have measured the levels of antibiotics in the environment, only recently has the explicit question of what threat this contamination may pose been asked, and more work is needed to accurately determine the nature of this risk.

Research into new classes of antibiotics that utilize novel targets (preferable since the chance of cross resistance is minimized) or circumvent resistance mechanisms is ongoing (Payne, 2004). The uses of higer-dose or extended-release formulations of existing antibiotics represent other avenues to investigate. For example, animal studies and clinical trials have been performed with a new group of tetracycline derivatives (the glycylcyclines) that show potent activity against tetracycline resistant organisms, including MRSA and VRE (Zhanel *et al.*, 2004). Novel antibiotic targets currently under investigation include bacterial fatty acid synthesis enzymes and translocase I inhibitors (nucleoside antibiotics), which are both cell wall active agents (Payne, 2004; Kimura and Bugg, 2003). Another promising

approach consists of targeting resistance genes that reduce the anionic charge of the bacterial membrane, an effective resistance mechanism against the body's own cationic innate defenses (such as defensins or lysozyme) (Weidenmaier *et al.*, 2003). Nonetheless, the continued dearth of new antibiotics that are known to be effective and are currently available to treat most bacterial infections (including gonorrhoea) is worrisome.

One other approach to help prolong the usefulness of drugs to which bacteria are demonstrating resistance involves applying the idea of synergy with other agents, which has played a major role in scientific research and applied medicine over the past 100 years. Both synergy and antagonism (its opposite) describe the effects of interactions that can be broadly described as "working together" and "working against each other" respectively (Berenbaum, 1989). The operative term when considering synergy is that of "interactions", since by definition at least two factors are at work to elicit a synergistic effect that is greater than the simple cumulative effect of either factor alone. Thus, the most important concept is that of "no interaction" (i.e. the predicted effects of a drug combination where no interaction occurs), as synergy and antagonism are defined as departures from this model (Meletiadis *et al.*, 2003). The idea that a combination of two drugs may elicit a greater or lesser response than either agent individually has much relevance in treating drug resistant organisms for a number of reasons. First, the chance of therapeutic success is maximized in cases where one drug alone is insufficient to clear an infection due to resistance. Because lower drug concentrations are usually required, the chance of drug induced toxicity is also reduced (Krogstad and Moellering, 1986). Using drug combinations may also hinder the onset of resistance because multiple targets and mechanisms are used, making a focused bacterial

response more difficult (Bouza and Munoz, 2000; WHO, 2001). Antibiotic combinations are especially appropriate for treating drug resistant bacteria where treatment failure occurs when only one antibiotic is used (Acar, 2000). The main hinderance in such an approach is distinguishing between synergy *per se* and a simple additive (cumulative) effect, which does not share the favorable characteristics mentioned above.

Extensive research examining the *in vitro* and clinical nature of antibiotic combinations has proven the efficacy of such an approach for treating bacteria that are resistant to a single antibiotic (Acar, 2000; Bouza and Munoz, 2000). Similarly, combining a drug to which a bacterium has become resistant with another drug (which may or may not be effective on its own) may result in treatment success. A good example is the use of clavulanic acid (no antimicrobial effect on its own) which inhibits β-lactamases (which normally hydrolyze β-lactam antibiotics rendering them ineffective) and hence allows antibiotics such as penicillin to exert their effect on bacteria that would othewise demonstrate treatment failure (Krogstad and Moellering, 1986). The fact that plants contain complex mixtures of phytochemicals that often exhibit higher biological activity than any single constituent is another natural example of synergy.

A number of facts are clear at the dawn of the 21st century. Most importantly, the battle against bacteria has not been won, and likely never will be. Because most causes of antibiotic resistance are preventable, steps can be taken to minimize or at least delay the onset of antimicrobial resistance. Although new promising antibiotics that use novel targets or are effective against resistant organisms are being developed, it is doubtful that these will

be anything more than temporarily effective, while their high price makes their use in developing countries (where they are needed the most) unfeasible. Also, these steps do not reduce the rates of morbidity and mortality among the millions currently afflicted with resistant bacterial pathogens worldwide. This is why the search for new and affordable antimicrobials continues to be important. The challenge then arises as to where to search for new biologically active compounds with therapeutic potential, keeping in mind that most new synthetic antibiotics developed by pharmaceutical companies are variations on older antibiotic types. As will be discussed below, the plant kingdom represents a particularly promising source of such biologically active material.

1.3 Study organisms

1.3.1 *Neisseria gonorrhoeae*

N. gonorrhoeae, the Gram negative diplococcus that causes the sexually transmitted disease (STD) gonorrhoea, remains one of the most clinically significant and prevalent bacterial infectious diseases, with an estimated worldwide incidence of >60 million cases per year (Ison *et al.*, 1998; Tapsall, 2002). In all likelihood this number is an underestimate due to under reporting and syndromic diagnosing which are common in the developing world (Ison *et al.*, 1998; Tapsall, 2002). In comparison to severe nosocomial infections such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* (VRE), antibiotic resistant *N. gonorrhoeae* continues to represent a serious community aquired infection (Sefton, 2002). Acute infections are characterized by urethritis (men) or endocervicitis (women), while infections of other epithelial membranes (rectal, pharyngeal and ophthalmic) can also occur. Although death is rare, untreated infections can lead to serious complications and morbidity including sterility, pelvic inflammatory disease, disseminated gonococcal infection and ectopic pregnancy. As with many other STDs that adhere to and damage epithelial membranes, gonorrhoeal infections can amplify transmission of HIV in both men and women (Ison *et al.*, 1998; Tapsall, 2002), further underlining the importance of early diagnosis and rapid treatment. Controlling the spread of *N. gonorrhoeae* is problematic because many cases (especially among women and those with pharyngeal or rectal infections) are asymptomatic, which encourages spread of the disease (Tapsall, 2002).

N. gonorrhoeae is an exemplary resistant organism, as it demonstrates the inherent flexibility of bacteria to evolve to changing conditions (i.e. the successive development of resistance to multiple antibiotics). Resistance in gonococci arises through either chromosomal mutations or the acquisition of plasmids encoding resistance genes (Jephcott, 1986; Dillon and Yeung, 1989; Ison *et al.*, 1998; Tapsall, 2002). The onset of resistance among *N. gonorrhoeae* isolates has a long history. Although *N. gonorrhoeae* was intrinsically sensitive to antibiotics in the pre-antibiotic era, after only 10 years of therapeutic penicillin use, reduced susceptibility became apparant (Jephcott, 1986; Ison *et al.*, 1998). Of interest is that actual resistance (i.e. isolates responsible for total treatment failure) to penicillin likely arose in the Far East due to higher selection pressures and then spread to the West (Jephcott, 1986). This fact underlines that effective surveillance and control in the developing world is advantageous since resistance usually arises in such resource poor settings before spreading abroad (Tapsall, 2002). For gonorrhoea, antibiotics are usually excluded from use when single dose treatment failure is >5% (Tapsall, 2002).

Although penicillin resistance was initially due to chromosomal mutations, it was the onset of plasmid-mediated penicillinase producing *N. gonorrhoeae* (PPNG) that now precludes penicillin's use for treatment (Jephcott, 1986; Dillon and Pagotto, 1999). PPNG organisms have acquired the TEM-1 β-lactamase gene, whose product hydrolyses the β-lactam ring of penicillin and related antibiotics, rendering them inactive (Dillon and Yeung, 1989). Thus, this form of resistance falls under the class of drug inactivating enzymes. The β-lactamase gene, which causes high level penicillin resistance (penicillin MICs of >8 mg/l), is found on a related group of gonococcal plasmids (named after their original

epidemiological sources) including the Asian plasmid (7.2 kilobase pairs [kb]; first isolated in the UK), African plasmid (5.1 kb; first isolated in the USA), and Toronto/Rio plasmid (4.9 kb) (Dillon and Yeung, 1989; Dillon and Pagotto, 1999). These plasmids were first detected in 1976 (Jephcott, 1986; Ison *et al.*, 1998). Interestingly, the Asian and African plasmids are identical save the 2.1 kb fragment missing from the African plasmid (the 2.3 kb fragment missing from the Toronto plasmid also differs from this 2.1 kb fragment), indicating a common origin for these plasmids (Dillon and Yeung, 1989). The origin of these plasmids is of considerable interest due to their likely non-gonococcal origin. The Asian and African plasmids are respectively homologous to the 7.0 and 5.7 kb β-lactamase plasmids of *Haemophilus ducreyi* (the causative agent of chancroid), while the plasmids also have the characteristic GC content of *Haemophilus* species (Dillon and Yeung, 1989) which again shows the capacity for exchange of genetic material between bacterial genera. Although rates of PPNG resistance are falling in some areas (the Caribbean for example), its continuing prevalance worldwide effectively excludes penicillin from use for treating gonorrhea infections (Ison *et al.*, 1998; Dillon and Pagotto, 1999; Tapsall, 2002). Unfortunately, penicillin is still in use in some areas by virtue of its availability and lower cost versus more effective drugs.

Tetracycline resistant *N. gonorrhoeae* (TRNG) describes strains with another plasmid mediated form of high level resistance (tetracycline MICs of >8 mg/l), associated with a 25.2 MDa *tetM*-containing plasmid (TetM plasmid), originating from the association of the tetM determinant with an endogenous gonococcal conjugative plasmid (in comparison to the β-lactamase plasmids) (Greco *et al.*, 2003). This form of resistance was first identified

in 1985 in the United States (Turner *et al.*, 1999). The TetM protein protects the 70S ribosomal subunit from the inhibitory action of tetracycline, which normally inhibits bacterial protein synthesis and is thus an example of target protection. With few exceptions, all cases of TRNG worldwide have been linked to one of two related plasmid types, named American or Dutch (based on the original country of isolation) (Greco *et al.*, 2003). It is thought that the American and Dutch type plasmids originated in Africa and the Far East respectively (Turner *et al.*, 1999). Similar to PPNG, the prevalence of TRNG isolates worldwide has made treatment using tetracycline effectively obsolete (Turner *et al.*, 1999; Tapsall, 2002). Considerable geographic differences in TRNG prevalence have been noted however. For example, from 1992-1994, incidence of TRNG comprised 0% of isolates in Japan, but 98-100% of isolates in Indonesia and the Phillipines (Turner *et al.*, 1999). Because tetracycline therapy requires multiple doses, it is likely that this, coupled with inadequate dosages (i.e. the complete therapeutic dose was not taken), encouraged the rise of plasmid mediated tetracycline resistance (Tapsall, 2002; Greco *et al.*, 2003). It is likely the fall in TRNG rates that areas such as Canada have recently seen is due to curtailed tetracycline use (Greco *et al.*, 2003), which removes the selective pressure to maintain resistance.

While PPNG and TRNG represent plasmid-mediated high level resistance to penicillin and tetracycline, chromosomal resistance to both these antibiotics (and others) is another significant source of antimicrobial resistance in *N. gonorrhoeae*. Chromosomally resistant *Neisseria gonorrhoeae* (CMRNG) often involves mutations at several loci in the gonococcal genome that confer low-level resistance to one or multiple antibiotics (penicillin,

tetracycline, erythromycin and others), especially if the mutations affect membrane permeability (Ison *et al.*, 1998; Tapsall, 2002). Compared to plasmid- mediated resistance, CMRNG isolates demonstrate a variety of resistance mechanisms, which often work in concert to yield higher level resistance than any individual mutation alone. Most mutations related to penicillin resistance (e.g. *penA*, *penB*) encode altered penicillin binding proteins (PBPs) with reduced affinity for penicillin, and are thus an example of target alteration (Dillon and Yeung, 1989; Tapsall, 2002). The multi-drug resistance (MDR) phenotype is another chromosomally mediated type of resistance (caused by expression of the *mtr* operon) characterized by non-enzymatic mechanisms of resistance and cross resistance to multiple antibiotics after exposure to a single drug (Dillon and Pagotto, 1999). This can also include simultaneous low level resistance to dyes, detergents and fatty acids (Dillon and Yeung, 1989). The reason for this broad spectrum resistance is that the *mtr* operon codes for an outer membrane efflux pump and for altered membrane proteins which together work to exclude a wide variety of substrates from the cell (Dillon and Yeung, 1989; Dillon and Pagotto, 1999). Because individual mutations usually lead to only low level resistance, multiple genetic changes are required to affect more complete protection, and various combinations of resistance genes can lead to an additive effect in conferring resistance. For example, mutations of *penA2, penB2* and *mtr* collectivly give a 120 fold increase in resistance to penicillin, although the mutations individually only cause a 4 to 8 fold increase in resistance (Dillon and Yeung, 1989; Jephcott, 1986).

In contrast, single step high level chromosomal resistance to spectinomycin has also been identified. This mutation (*spc*) alters the 30S ribosomal subunit to which

spectinomycin normally binds (Dillon and Yeung, 1989). This form of resistance first appeared in Korea in the 1980s before spreading further afield (Tapsall, 2002). However, in areas where spectinomycin use was discontinued, resistant strains disappeared, underlining the sometimes dynamic nature of gonococcal resistance in response to environmental pressures, as also described above regarding TRNG. Of further importance is that these forms of resistance can co-exist (e.g. PP/TRNG, PP/CMRNG, etc), further complicating effective treatment. Spectinomycin (a single dose antibiotic) is still used in certain cases to treat gonorrhea (Tapsall, 2002). In contrast, macrolide antibiotics including azithromycin and erythromycin (chromosomal *ery* mutation) as well as aminoglycoside antibiotics are no longer front line therapies due to unacceptably high levels of resistance documented during treatment (Dillon and Yeung, 1989; Tapsall, 2002).

Unfortunately, this familiar pattern is now being repeated with currently used front line antibiotics. The preferred antibiotics in use today are all single dose to ensure patient compliance and minimize the chance of resistance developing (Tapsall, 2002). Fluoroquinolones such as ciprofloxacin and ofloxacin have long been recommended for use in treating gonorrheal infections and continue to demonstrate efficacy in some settings. Fluoroquinolones are also thought to have a plasmid curing effect (Dillon and Pagotto, 1999). However, the last decade has seen an alarming rise in quinolone resistant *N. gonorrhoeae* (QRNG). Although most strains in North America and Western Europe still demonstrate susceptibility to ciprofloxacin, QRNG prevalance is >50% in most Far East countries, with Thailand, the Phillipines and Hong Kong showing the highest incidence (Newman *et al.*, 2004; Dillon and Pagotto, 1999). More troublesome is the increasing

appearance of QRNG in Europe and North America (Dillon and Pagotto, 1999), which suggests that soon fluoroquinolones will also become obsolete.

Fluoroquinolones are unique among antibiotics that directly inhibit DNA synthesis of the bacterial genome because they show good selective toxicity towards prokaryotic enzymes. The enzymes DNA gyrase and topoisomerase IV (both responsible for regulating negative supercoiling of DNA during replication) are the primary targets of fluoroquinolones (Hooper, 2001). In gonococci, the primary enzyme target is the GyrA subunit of DNA gyrase (the ParC subunit of topoisomerase IV is a secondary target), and not surprisingly, the main form of resistance seen are chromosomal mutations of these target enzymes (Hooper, 2001; Tapsall, 2002). As with other forms of chromosomal resistance, multiple mutational events are required to elicit effective resistance. Alterations in drug permeability are another fluoroquinolone resistance mechansim common among Gram negative organisms including *N. gonorrhoeae* (porin changes and possibly overexpression of endogenous MDR efflux pumps) (Hooper, 2001; Tapsall, 2002).

The other main group of antibiotics still in use to treat gonorrheal infections is the 3rd generation cephalosporins (mainly ceftriaxone [injectable] and cefixime [oral]), in which a β-lactam ring is fused to a 6 membered dihydrothiazine ring. The single dose treatment regimens and favorable pharmacological properties of these antibiotics contributes to their continuing widespread use for gonorrheal (and many other bacterial) infections (Karlowsky *et al.*, 2002). Unlike other antibiotics, cephalosporins can cure all forms of gonorrhea (Tapsall, 2002). These antibiotics are resistant to TEM-1 and a number of other β-

lactamases, and thus many cases of cephalosporin resistance involve the rise of novel extended spectrum β-lactamases (ESBLs) (Karlowsky *et al.*, 2002). Other mechanisms of resistance include altered PBPs and reduced permeability to limit penetration of the antibiotic into the cell wall. A recent report from Japan demonstrates that reduced susceptibility of *N. gonorrhoeae* to oral cephalosporins (along with penicillin, tetracycline and fluoroquinolones) is increasing (Ito *et al.*, 2004). Although *de facto* cephalosporin resistance has only been reported in isolated cases, a general trend of reduced *N. gonorrhoeae* susceptibility to these antibiotics (especially in the Pacific region) is cause for concern (Tapsall, 2002).

A pattern can be detected when considering how *N. gonorrhoeae* has responded to the onslaught of antibiotics over the past few decades. Early treatment success is followed by sporadic, but generally increasing rates of reduced susceptibility, culminating in a more-or-less global spread of resistance and treatment failure. It is noteworthy that this progression has taken only about 10-15 years for each of the antibiotics described, an especially worrying fact considering that the long-used cephalosporins are the only widespread class of effective antibiotics still in use, and even here the seemingly inevitable rise of resistance is also occurring. It is notable that the more prudent use of cephalosporins might help explain their continued efficacy. *N. gonorrhoeae* has repeatedly shown itself to be eminently adaptable to the succession of antibiotic treatments used. As mentioned previously, human factors (especially in the developing world) have contributed to this rise in resistance, as evidenced by the Pacific region being a constant source of new resistance

phenotypes. However, with a dwindling choice of antibiotics available to treat gonorrhoeal infections, the search for new drugs or treatment protocols is paramount.

1.3.2 *Staphylococcus aureus* and *Enterococcus faecalis*

Two other organisms examined in my research were *S. aureus* and *E. faecalis*. These two Gram positive cocci are commensal organisms found in many healthy adults and both pose a significant health risk due to the development of resistance to antimicrobials used for therapy (Koch *et al.*, 2004; Lowy, 2003). In contrast to *N. gonorrhoeae*, which is primarily a community acquired infection, these pathogens are especially pervasive in nosocomial settings, where patients are often immunocompromised and/or taking antibiotics, presenting an ideal situation for such opportunistic pathogens to spread. Methicillin resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecalis* (VRE) isolates are currently leading causes of nosocomial infections worldwide (Muto *et al.*, 2003), and resistance to vancomycin, the "drug of last resort", raises the spectre of untreatable bacterial infections.

S. aureus is a versatile opportunistic pathogen that causes a wide spectrum of disease ranging from minor skin infections to life threatening bacteremia, for which mortality rates are 20-40% (Lowy, 2003). Approximately 20% of community acquired and nosocomial bacteremias in the US are caused by *S. aureus* (Tenover *et al.*, 2001), while MRSA is responsible for >50% of nosocomial infections in intensive care units (Walsh and Howe, 2002). As with *N. gonorrhoeae*, resistance to penicillins in *S. aureus* has a long history and can be both plasmid (*blaZ*; β-lactamase production) and chromosomally

mediated (Lowy, 2003). Resistance to methicillin, the first semi-synthetic penicillinase resistant antibiotic, is due to expression of the chromosomal *mecA* gene whose product encodes an altered PBP (Lowy, 2003). More serious are reports of vancomycin intermediate *S. aureus* (VISA) and more recently vancomycin resistant *S. aureus* (VRSA), since before the advent of antibiotics mortality from untreated staphylococcal infections was 70-80% (Lowy, 2003). Heterogeneously resistant VISA populations (*i.e.* populations in which not all cells demonstrate resistance) have been isolated in the US, Japan, and Western Europe, and are thought to have developed from preexisting MRSA infections, while hetero-VRSA strains have been reported in Western Europe and Hong Kong among other locales (Lowy, 2003; Tenover *et al.*, 2001).

Vancomycin, a glycopeptide antibiotic, interferes with bacterial cell wall synthesis by binding to the D-alanine-D-alanine peptide cross linkages of peptidoglycan, thus destabilizing the integrity of the cell wall (as opposed to beta-lactams, which inhibit the transpeptidase enzymes (PBPs) responsible for linking peptidoglycan chains together) (Walsh and Howe, 2002). For VISA strains, resistance is related to changes in peptidoglycan biosynthesis, seen morphologically as a thickened cell wall. These irregular thickened cell walls have reduced peptide cross linking, which effectively reduces the number of targets available to vancomycin (Walsh and Howe, 2002; Lowy, 2003). Also, excess D-ala-D-ala residues in the outer perimeter of the cell wall trap vancomycin before it can bind to the more critical residues near the cytoplasmic membrane that are actively involved in cross linking. A number of chromosomal mutations (*vraA-G*) have been implicated in this type of resistance (Walsh and Howe, 2002). Resistance in VRSA isolates is due to target alteration

of the terminal D-ala-D-ala residue to D-ala-D-lac, which occurs through acquistion of a conjugative plasmid containing the *vanA* gene cluster (Lowy, 2003). Of great interest is that this form of resistance is thought to have arisen when *S. aureus* acquired the *vanA* operon from a vancomycin resistant *E. faecalis* strain (Lowy, 2003; Koch *et al.*, 2004), which would represent a good example of prokaryote's propensity to exchange genetic material.

Like *S. aureus*, *E. faecalis* is an opportunistic pathogen that is part of the commensal microflora of many humans (as well as other mammals and birds, presenting an interesting example as to how resistance genes may be transferred between host species). Clinical manifestions are varied and include endocarditis, bacteremia, meningitis and urinary tract infections (Koch *et al.*, 2004). Although intrinsically less virulent than *S. aureus*, *E. faecalis* demonstrates intrinsic MDR resistance to many antibiotics and is the second leading cause of nosocomial infections in the US (Koch *et al.*, 2004). Intrinsic resistance to penicillins is due to the low affinity of enterococcal PBPs for β-lactams (Bouza and Munoz, 2000). Resistance to vancomycin (mediated in the same way as with *S. aureus* described above) and the rapid increase in VRE isolates in recent years, combined with evidence of the ability to transfer vancomycin resistance to other pathogens (e.g. *S. aureus*), represent a serious threat to effective treatment (Koch *et al.*, 2004; Muto *et al.*, 2003).

1.4 Phytochemicals as novel antimicrobials

The plant kingdom remains an attractive area to search for new antimicrobial strategies for a number of related reasons. First and foremost is the long history of plant use in traditional medicine by indigenous peoples worldwide to treat a variety of ailments,

including bacterial infections. It has been estimated that about 80% of the global population continues to use herbal remedies for primary healthcare (Bennett and Brown, 2000). This is especially significant in resource poor settings where the local availability, extensive knowledge, effectiveness and lower cost of medicinal plants versus pharmaceutical drugs make their use more pragmatic. While extensive research has been conducted into the antimicrobial activity of traditional remedies, many of these plants remain uninvestigated. Secondly, the plant kingdom collectively produces over 100,000 biologically active secondary metabolites (phytochemicals) that play roles in defense against infection by bacteria, fungi, and viruses, and herbivory by insects and other pests (Dixon, 2001). The recent interest and commercialization of traditional herbal remedies (i.e. natural health products (NHPs) (Ni *et al.*, 2002)), many with demonstrated clinical efficacy, provides strategies for complementary (i.e. using a phytochemical combined with a pharmaceutical) and alternative (i.e. using a phytochemical instead of a pharmaceutical) treatments for bacterial infections, an approach known as complementary and alternative medicine (CAM). For example, there are alternative treatments for ear infections, minor skin infections and diarrhea, whose therapeutic efficacies are supported by clinical research (Shadkchan *et al.*, 2004; Blumenthal *et al.*, 2000). For more serious conditions like STDs, complementary NHP treatments to hasten recovery and reduce the pharmaceutical drug load required are more appropriate.

Plants and bacteria are involved in chemically mediated co-evolutionary relationships that may be symbiotic or pathogenic. One example of the former relationship is use of isoflavonoids by leguminous plants to recruit soil bacteria of the genus *Rhizobium* to

colonize their root hairs and perform the critical function of atmospheric nitrogen fixation, while production of antibacterial phytochemicals to deal with bacterial infections represents the latter relationship. Both caffeine and nicotine (alkaloid phytochemicals that are heavily used by humans) are originally thought to have served defensive functions against insects and microorganisms.

A variety of approaches can be taken when investigating the antimicrobial activity of plant derived chemicals. Traditional medicine is practised most widely by indigenous peoples who have extensive knowledge based on oral tradition of the uses and activities of local flora, despite the lack of permanent records and a standardized analytical approach. Thus, the collection of such locally used plants is a good starting point, as they can be tested under controlled conditions to see if anecdotal and historical claims have any weight. Most ethnobotanical studies involve using a range of criteria (locality of the plant, systemic versus topical use, whether the plant has been previously characterized, etc) and the help of local healers (including their definitions of various diseases) to identify and select plants of interest that are worthwhile testing (Heinrich, 2000). For example, Kambizi & Afoloyan (2001) targeted plants traditionally used to treat STDs in Zimbabwe (using the local term of *njovhera* (STDs) as their criteria) to narrow their survey of local plants to ones that might be promising STD treatments. The majority of plants they examined had activity against both Gram positive and Gram negative organisms; methanolic extracts of *Acacia nilotica*, *Cassia abbreviata* and *Zanha africana* being most active (Kambizi and Afoloyan, 2001). Because most ethnopharmacology studies deal with crude extracts of medicinal plants, although the activity of plant can be confirmed, the exact phytochemicals responsible (alone or in

combination) remain unknown, until bioassay guided isolation has been undertaken. Also, quantitative measures of activity (MICs for example) cannot be determined this way since the extracts contain multiple phytochemicals. To elucidate the active compound(s), a bioassay guided fractionation approach is used.

Very few ethnobotanical studies have examined plants explicitly for activity against *N. gonorrhoeae*. Caceres *et al.* (1995) studied extracts of 44 plants (in 50% ethanol) used in Guatemala to treat gonorrheal infections. In a disk diffusion assay, it was found that 28% of them showed evident activity (defined as a zone of inhibition of >9 mm) against five strains of *N. gonorrhoeae*. The most active plants included those of the families Piperaceae, Leguminosae, Asteraceae and Rutaceae (Caceres *et al.*, 1995). In another study investigating 23 traditional remedies for gonorrhoea in Rwanda, 74% were found to have activity against *N. gonorrhoeae* (Van Puyvelde *et al.*, 1983), although the details of this study's experimental procedures and the plants involved (which may explain the higher proportion of active plants versus Caceres *et al.* (1995)) could not be located. Thus, for these studies the exact phytochemcals responsible for the plant extract's antimicrobial activity were not determined.

One complication to bear in mind is reconciling the traditional context of an herbal remedy with its activity in *in vitro* assays (which may use a variety of solvents and extraction methods). For example, a traditional use of a particular plant might consist of a water based tea extraction, but when both aqueous and organic solvent extractions are tested, the organic extraction may have much higher activity. Even though both extracts might have

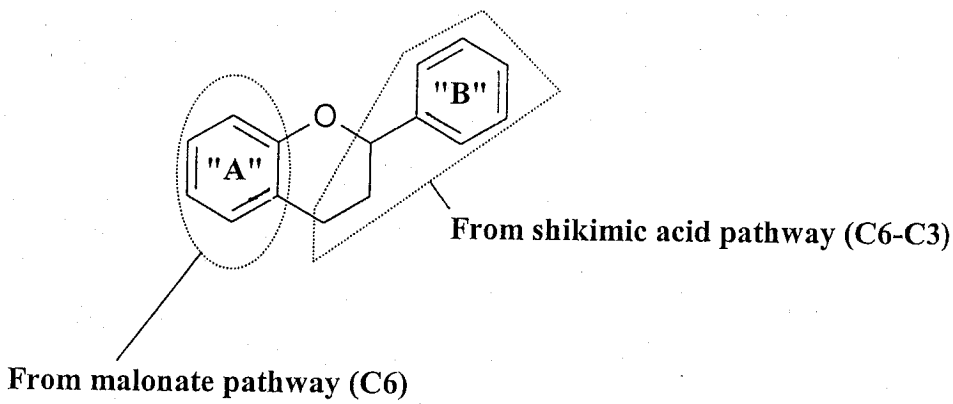
some activity, the organic extract may not be equivalent to the historical therapeutic use of the plant, since active compounds may not be present in the aqueous extract. Another factor to bear in mind is what part of the plant (flower, bark, root, whole plant, etc) is used to prepare the herbal remedy. Thus, accurate details of the preparation and use of herbal remedies are essential if a meaningful analysis is to be performed.

1.4.1 Phenolics: flavonoids

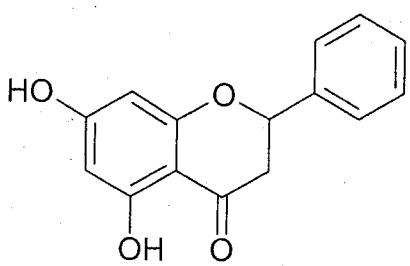
Phytochemicals encompass three major classes with a great diversity of activities: alkaloids, isoprenoids, and phenolics, as well as many products of minor biosynthetic pathways (Mbwambo *et al.*, 1996). A brief review of the precedents and some scientific studies involving phytochemicals follows, concentrating on the groups of phytochemicals examined in this study, all of which are phenolic compounds (except for the garlic constituents which are organo-sulfur compounds; see chapter 3). Phenolics are produced by the shikimic acid pathway and include over 10,000 known substances. Flavonoids are a group of phenolics that possess a characteristic C6-C3-C6 structure containing a benzene ring connected to a phenylpropanoid unit (Figure 1). Flavonoids are classified based on the degree of oxidation of the C3 bridge into chalcones, flavanones, flavones, flavonols, isoflavonoids, anthocyanidins, and catechins. These compounds may also be variously substituted with hydroxyl, methyl, prenyl, or other functional groups which further increases their structural diversity and activity. For example, many flavonoids are found naturally in a glycosylated form which renders them water soluble (Dewick, 2002). Flavonoids are the largest group of phenolic phytochemicals that are actively involved in a wide range of plant

Figure 1. General structure of flavonoids and pinocembin. (A) Ring A (one C6 ring; from the malonate biosynthetic pathway) is a benzene ring connected to a phenylpropanoid unit (C3 bridge and a second aromatic ring [ring B]) from the shikimic acid pathway. **(B)** Chemical structure of the flavanone pinocembrin, which has been found to possess moderate antibacterial activity (MICs of 64 mg/l) against susceptible and resistant phenotypes of *N. gonorrhoeae*.

(A)


"A"
O
"B"
From shikimic acid pathway (C6-C3)
From malonate pathway (C6)

(B)


HO
O
OH
O

metabolic processes (including energy transfer and control of respiration and photosynthesis), hormonal activity (such as growth regulators and sex determination) as well as pigmentation (Mbwambo *et al.*, 1996). By acting as analogues of cellular signal compounds or substrates (through mechanisms such as enzyme inhibition, catalyzing electron transport and antioxidation) flavonoids also play a vital role in defense against infection and herbivory (Mbwambo *et al.*, 1996; Reichling, 1999). Flavonoids can bind to many biological polymers such as enzymes and DNA, as well as divalent ions of many metals (Cu^{2+}, Zn^{2+}) (Mbwambo *et al.*, 1996). Given the importance of such polymers and metals for bacterial survival (Rohde and Dyer, 2003), such properties present interesting avenues to examine the antibacterial activity of flavonoids.

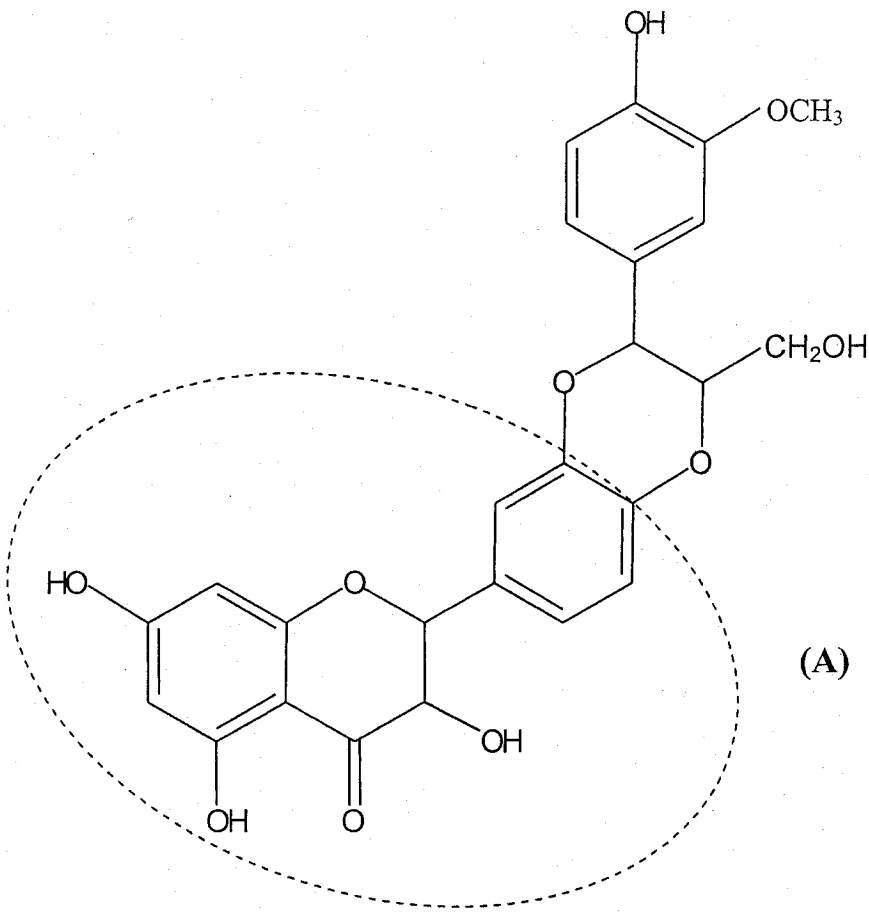
S. aureus, another bacterial pathogen demonstrating antibacterial resistance, is a more commonly tested organism than *N. gonorrhoeae* in ethnopharmalogical studies. A wide variety of flavonoids have been found to possess a spectrum of antibacterial activity against *S. aureus*. For example, the flavonols, quercetin and rutin (from seed extracts of *Zizyphus spina-christi*) possess marginal antibacterial activity against *S. aureus* (MICs of 500 mg/l) (Shahat *et al.*, 2001) while derivatives of the flavanone, kushenol (from methanolic root extracts of *Sophora flavescens*) have much higher anti-staphylococcal activity (MICs of 2.5-10 mg/l) (Kuroyanagi *et al.*, 1999). In another study, the activity of quercetin and related flavonols towards *Salmonella enteritidis* and *Bacillus cereus* was enhanced (2 fold to 8 fold higher activity) by combining them with rutin (Arima *et al.*, 2002), a good example of a possible synergistic interaction between two phytochemicals. Of great interest is a report that some glycosylated flavonols, including rutin, are selective

inhibitors of topoisomerase IV, which is also a target of fluoroquinolone antibiotics as described in the preceding section (Bernard *et al.*, 1997; Hooper, 2002). In this report purified topoisomerase IV from *E. coli* and *S. aureus* was examined, hence, although not performed in living cells, the fact that an antibiotic of proven efficacy shares a bacterial target with biologically active flavonoids makes them extremely promising candidates as potential novel drugs.

1.4.2 Phenolics: flavonolignans and neolignans

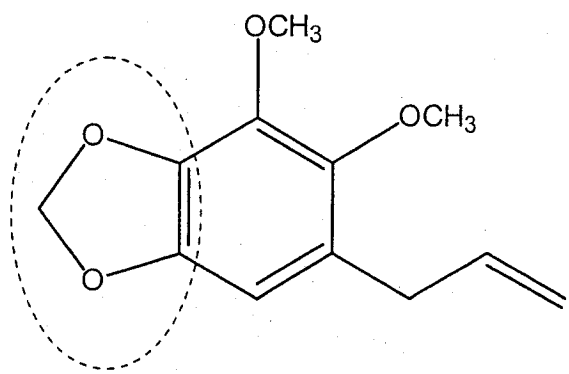
Silibinin (Figure 2A) belongs to a complex group of phytochemicals known as flavonolignans, in that their synthesis depends on a radical coupling of the B ring of a flavonoid with a molecule of coniferyl alcohol or a similar moiety (Guz and Stermitz, 2000). Due to their structural complexity, flavonolignans can be grouped as both flavonoids and phenylpropanoids. Silibinin is the principal isomer and main pharmacologically active compound of silymarin, the fruit extract of milk thistle (*Silybum marianum*). Flowers and leaves of this plant have long been used in preparing herbal remedies since antiquity (Sonnenbichler, 1999). Because most clinical studies involve crude extracts of silymarin (containing mainly silibinin and the related stereoisomers silichristin and silidianin), the biological activity is usually attributed to this mixture instead of solely silibinin (the main constituent) (Sonnenbichler *et al.*, 1999; Xi and Agarwal, 1999). Commerically prepared milk thistle NHPs are in widespread use outside of North America, at a recommended daily dose of ~900 mg of silymarin extract (Anonymous, 1999).

Figure 2. Chemical structures of the flavonolignan silibinin (A; flavonoid skeleton outlined) **and the neolignan dillapiol** (B; methylenedioxyphenyl active site outlined).



(A)

(B)



These flavonolignans have some unique hepatoprotective activities which are mediated via antioxidation (a common property of flavonoids), inhibition of lipid peroxidation, enhanced liver detoxification and protection of glutathione depletion (anon, 1999; Kurkin, 2003). Silibinin alone has also been shown to possess unique regenerative effects on both liver and kidney cells that are thought to be due to its indirect stimulation of total protein synthesis of eukaryotic cells. Specifically, silibinin strongly stimulates (*in vitro* and *in vivo*) synthesis of the enzyme DNA-dependent RNA polymerase I (which catalyzes the transcription of multiple rRNA subunits and promotes ribosome formation) and this leads to increased overall protein synthesis in treated cells (Sonnenbichler *et al.*, 1999). The main indications for treatment with milk thistle are hepatitis, cirrhosis, hypercholesterolemia and death cap mushroom poisoning, which all damage liver cells. Silibinin has also been found to inhibit cell growth of LNCaP (human prostate carcinoma) cell lines (it causes G_1 arrest of the cell cycle) (Xi and Agarwal, 1999), but these stimulatory or inhibitory effects have not yet been found in prokaryotic organisms (Sonnenbichler *et al.*, 1999).

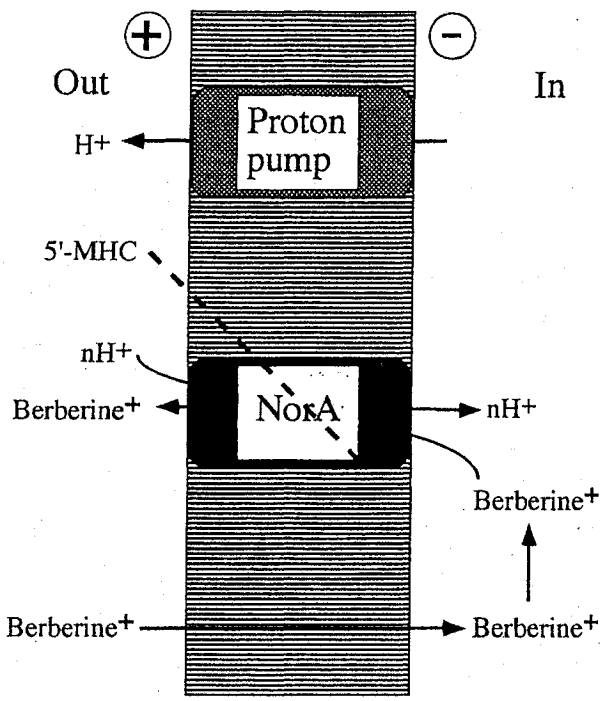
However, both silibinin and the related flavonolignan 5'-methoxyhydnocarpin-D (5'-MHC) have been shown to be a potent inhibitor of the NorA multidrug resistant (MDR) efflux pump of *Staphylococcus aureus* (Stermitz *et al.*, 2000). MDR efflux pumps are particularly effective resistance mechanisms because they can simultaneously confer resistance to a variety of antimicrobial compounds, complicating treatment. 5'-MHC can be considered a synergist, because although it possesses no antimicrobial activity alone, disabling this critical resistance mechanism allows antibiotics (in this study the natural alkaloid antibiotic berberine) to exert their effect (Stermitz *et al.*, 2000). Figure 3

demonstrates how 5'-MHC is thought to elicit this synergistic effect, which could provide a model for silibinin's mode of action.

Another interesting phenolic with therapeutic potential is the neolignan dillapiol (Figure 2B), an essential oil found in a number of tropical and temperate plant families (mainly Apiaceae and Piperaceae). As stated previously, crude extracts from plants of the family Piperaceae have been characterized as having anti-gonococcal activity (Caceres *et al.*, 1995). Dillapiol is a potent natural inhibitor of cytochrome P450 3A4 (CYP3A4) enzymes that are highly conserved among eukaryotes and detoxify an extremely wide range of substrates (Budzinski *et al.*, 2000). The active site of dillapiol responsible for this inhibition has been identified as a methylenedioxyphenyl group, characteristic of many biologically active lignans (Belzile *et al.*, 2000). CYP3A4 enzymes can confer resistance to insecticides by metabolizing them to products that are no longer toxic to the insect and are subsequently excreted. In an *in vitro* study involving over 30 different commercial ethanolic extracts and related pure compounds of herbal plants, dillapiol had the strongest inhibitory activity (Budzinski *et al.*, 2000). Indeed, the efficacy of dillapiol's CYP3A4 inhibition was demonstrated in a study that successfully used dillapiol as a synergist in an insect model with a variety of insecticides including DDT, pyrethrins, and carbaryl (Belzile *et al.*, 2000).

Pinocembrin is a flavonoid which was found to possess moderate antibacterial activity against *N. gonorrhoeae* (P. Ruddock, Honors thesis, 2002) (Figure 1). It was originally isolated from the leaves of *Piper lanceaefolium*, a medicinal plant used to treat cutaneous infections by indigenous peoples of the Colombian rainforest (Lopez *et al.*, 2001).

Figure 3. Model of 5'-MHC's (a flavonolignan) synergistic activity with berberine against the *S. aureus* NorA MDR efflux pump (from Stermitz *et al.*, 2000). 5'-MHC, which possess marginal antibacterial activity alone, inhibits the MDR NorA efflux pump that normally extrudes berberine from the cell before it reaches bactericidal concentrations (dotted line). Thus, 5'-MHC potentiates the antibiotic action of berberine (another phytochemical) an example of two phytochemicals exhibiting synergistic interactions against resistant bacteria.


Out
In
Proton pump
H+
5'-MHC
nH+
Berberine+
NorA
nH+
Berberine+
Berberine+
Berberine+

Thus, again we find a plant from the family Piperaceae containing antimicrobial phytochemicals. Much as with dillapiol and silibinin, very little data exists concerning pinocembrin's antimicrobial activity. Initial studies involving crude methanolic leaf extracts of this plant found some antibacterial activity against Gram positive organisms (*Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus faecalis*) but no activity against a variety of Gram negative organisms including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (Lopez *et al.*, 2001). Subsequent work by the Dillon lab (2001) identified *P. lanceaefolium* as the only plant (out of a total of 40 crude plant extracts tested) that possessed significant antimicrobial activity against *N. gonorrhoeae* without UV light activation. This unique property prompted the fractionation of the crude *P. lanceaefolium* extract into ten constituent compounds that were then tested against the panel of resistant *N. gonorrhoeae* strains. Three of the ten compounds were identified as being responsible for *P. lanceaefolium*'s antimicrobial activity; these fractions were pinocembrin (5,7-dihydroxyflavanone), pinocembrin chalcone (2',4',6'-trihydroxychalcone), and cyclolanceaefolic acid methyl ester.

1.5 Hypothesis and Objectives

N. gonorrhoeae, *S. aureus* and *E. faecalis* were chosen as the main test organisms due to the many strains with multiple drug resistance, their continued prevalence worldwide and (especially for *N. gonorrhoeae*) the paucity of data concerning the activity of phytochemicals or NHPs against these organisms. The main hypothesis for these studies was that since complex phytochemical mixtures have demonstrated antibacterial activity (both in natural settings where plants synthesize various phytochemicals in response to a bacterial

infection, and in *in vitro* and *in vivo* studies (of crude plant extracts and/or natural health products), such mixtures represent potential new antimicrobial treatments that could be useful in treating resistant pathogenic organisms. Fresh garlic, garlic NHPs, silibinin and dillapiol had not been tested against *N. gonorrhoeae* prior to this work. Thus, the general objective for this work was to establish the antimicrobial activity of both phytochemical mixtures, either in NHP form as found in garlic, or in more controlled mixtures of antibiotics with phytochemicals known to block efflux transporters and/or drug metabolism.

Indeed, the World Health Organization (WHO) has recommended the use of mixtures to combat malaria resistance. For example, malarone is a new binary antimalarial drug combining an atoquavone and proguanil hydrochloride, which is effective against the protozoan *Plasmodium falciparum*, the causative agent of malaria. A recent WHO report regarding new treatments for malaria advised that currently used synthetic drugs (i.e. quinine derivatives) should be used in combination with the phytochemical artemisinin and some related synthetic derivatives of artemisinin under development (WHO, 2001). The reasons for this conclusion included the greater efficiency, shorter treatment regimens and better tolerance versus single drug therapy. Another significant factor considered was the possible delay or slowing of the onset of resistance to currently used and effective antimalarials when using such a combination. While this example involves a protozoan parasite (i.e. not a prokaryote), the rationale behind these conclusions is certainly applicable to bacteria, which also demonstrate rapid onset of resistance to antimicrobials. This reasoning can be applied to both antibiotic/phytochemical mixtures and complex phytochemical mixtures.

Garlic, which since antiquity has been used as a medicinal agent, represents a good example to apply this approach of studying phytochemical mixtures as candidates for CAM therapies. Garlic NHPs are currently in widespread use globally and numerous studies have confirmed the *in vitro* antibacterial activity of a variety of garlic constituents, including allicin and S-allylcysteine. As with most NHPs, these products contain a variety of phytochemicals (or the phytochemical's precursors) found in the live plant and thus represent a natural mixture (albeit in processed form). However, few comprehensive studies have examined the antibacterial activity and efficacy of commercial phytochemical mixtures such as garlic NHPs.

The objectives for the garlic work were to evaluate 24 garlic preparations (19 commercial garlic NHPs, minced and crushed garlic (in vegetable oil) and three fresh garlics serving as standards) for their antibacterial effects against *N. gonorrhoeae*, *S. aureus*, and *E. faecalis*. The levels of active constituents present in each product were established with the hypothesis that products containing higher levels of the known principal constituents for a particular NHP would exhibit greater antibacterial activity. For GP-containing products, the exposure time of the extracts to water (which activates the allinase in GP products) (Amagase *et al.*, 2001) could affect their activity, since different extraction times should produce varying amounts of allicin. Thus, in the present study I investigated whether increasing the extraction times would lead to an increase in antibacterial activity.

Similarly, both silibinin (because it is an efflux inhibitor) and dillapiol (because it is an inhibitor of drug metabolism) have been implicated in synergistic interactions in various situations, albeit only silibinin involving prokaryotes. The activity of these phytochemicals alone and in combination with the antibiotic penicillin was examined. Clearly, a combination of these phytochemicals that would make a PPNG or CMRNG isolate susceptible to penicillin again would be of great interest. A secondary objective was to determine putative mechansims for dillapiol's antibacterial action.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Selection, growth and maintenance of bacterial cultures.

The majority of experiments involving *N. gonorrhoeae* used the strains WHO III, V and VII (WHO, 1999), and the "PR" panel of 30 strains comprising a representative selection of resistant gonococcal phenotypes (Table 1). The three WHO strains are internationally recognized World Health Organization *N. gonorrhoeae* control strains that are susceptible to all antibiotics and whose susceptibilities are well characterized. The other strains consisted of clinical isolates from Hong Kong (all $CIPRO^R$ isolates) and Venezuela (all AZI^R isolates), while the rest of the strains came from the Dillon laboratory collection of international and Canadian study strains. These 30 strains were selected for the diversity of their antibiotic resistance phenotypes and strain types. All non-PPNG and non-TRNG strains were subcultured on GC medium base agar (GCMB; Difco, Detroit MI, USA) supplemented with 1% Kelloggs defined supplement [formula/100 ml: 40 g glucose, 1 g glutamine, 10 ml 0.5% $Fe(NO_3)_3$, 1 ml 20% cocarboxylase] (GCMBK agar) (Kellogg *et al.*, 1963). PPNG and TRNG strains were subcultured on GCMBK agar plus 5 mg/l ampicillin or tetracycline (Sigma) respectively to ensure plasmid maintenance. Strains were grown in a Fisher isotemp CO_2 incubator at 35°C/5% CO_2 in a humid environment for 18-22 hours. For most assays, double overnight (O/N) subcultures were used. For extended testing, strains were subcultured daily. All frozen stocks were prepared after at least a 2 O/N subculture, with ~1/2 a plate's growth placed in 1 ml of brain heart infusion (BHI) broth (Difco) plus 20% glycerol (BDH, Poole, UK). Vials were only used until 0.5 ml was left at which point the stocks were discarded.

Table 1. Summary of *N. gonorrhoeae* strains used for testing against various phytochemicals. Strains are grouped according to their phenotypic resistance profiles.

Susceptibility phenotype[a]	PR #	Original strain	Auxotype[b]	Serovar[c]	MICs to (mg/L)[d]					
					PEN	TET	SPEC	CIPRO	ERY	AZI
TRNG	PR-1	IS-20	NR	IB-3	0.25	32[e]	32	0.004	1	nd[f]
	PR-2	IS-23	P	IB-7	0.125	16	32	0.004	2[e]	nd
	PR-3	IS-48	NR	IB-4	0.25	16	32	0.004	1	nd
PPNG	PR-4	IS-24	P	IA-6	128[e]	1	32	0.004	0.125	nd
	PR-5	IS-35	NR	IA-4	16[e]	0.5	32	0.004	0.5	nd
	PR-6	IS-37	O	IA-2	8	0.5	32	0.004	0.5[e]	nd
	PR-7	IS-53A	nd	IA-3/6; IB-9	32	1	32	0.004	1[e]	nd
	PR-8	IS-57	P	IB-2	16	1	32	0.004	0.5	nd
PP/TRNG	PR-9	IS-5	NR	IB-2	64[e]	16	16	0.004	0.5[e]	nd
	PR-10	IS-12	NR/Phe+	IB-2	16	16	16	0.004	0.25	nd
	PR-11	IS-30	NR	IB-3	8	16	16	0.004	0.5	nd
CMRNG	PR-12	IS-14	NR	IB-1	2	2	32[e]	0.016	4	nd
	PR-13	IS-18	O	IB-12/4	2	4	32	0.032	8[e]	nd
SPEC^R	PR-14	IS-36	P	Ab; IB-23	0.5	4[e]	>32	0.016[e]	1	1
	PR-15	IS-46	M	IB-9	0.016	0.25	>32	0.004	0.25	0.25
PP/CMRNG, ERY^R	PR-16	IS-6	NR	IB-22	128[e]	2	32	0.008	4	nd
	PR-17	IS-21	NR	IB-22	64	2	32[e]	0.016[e]	4	nd
	PR-18	IS-55	P	IB-5	128[e]	4	32[e]	0.016	2	nd
PP/CMRNG, SPEC^R, ERY^R	PR-19	IS-3	NR	IB-5	64	2	>32	0.008	4[e]	nd
PPNG, SPEC^R, ERY^R	PR-20	IS-38	P	IB-1	32[e]	1	>32	0.008	2[e]	nd
PP/CMRNG	PR-21	GC1-182	nd	nd	128	4	nd	nd	nd	nd
Susceptible strains	PR-22	WHO III	nd	nd	0.063	0.125	16	0.004	nd	0.032
	PR-23	WHO V	nd	nd	0.5	1	16	0.008	nd	0.125
	PR-24	WHO VII	nd	nd	0.016	0.25	16	0.004	nd	0.25
	PR-25	IS-26	P	IB-3	0.5	1	32	0.004	2[e]	nd
	PR-26	IS-13	PCU	IB-2	0.5	0.5	32	0.004	1[e]	nd
	PR-27	IS-17	OUH	IA-2	<0.008	0.25	32	0.004	0.5	nd
PP/CMRNG, CIPRO^R	PR-28	986002	P	IA-2	16	2	16	2	nd	0.25
	PR-35	986004	na[f]	IB-4	>128	4	16	0.5	nd	0.5
CMRNG, CIPRO^R	PR-29	986006	NR	IB-1	2	4	16	>2	nd	0.25
	PR-33	986001	na	IB-3	2	2	16	0.25	nd	0.125
	PR-34	986003	na	IB-5	2	4	16	0.25	nd	0.5
CIPRO^R	PR-36	986005	na	IA-6	0.25	1	16	1	nd	0.032
PP/CMRNG, SPEC^R, ERY^R, AZI^R	PR-30	961067	NR	IB-1	>128	2	>32	0.032	8	1
	PR-31	961033	NR	IB-1	>128	2	>32	0.008	2	1
	PR-32	961040	H	IB-1	>128	4	>32	0.032	8	1

[a]TRNG - Tetracycline resistant *N. gonorrhoeae*; PPNG - Penicillinase producing *N. gonorrhoeae*; CMRNG - Chromosomally resistant *N. gonorrhoeae*; CIPRO^R - Ciprofloxacin; SPEC^R - Spectinomycin; AZI^R - Azithromycin; ERY^R - Erythromycin

[b]isolates lacking the ability to synthesize certain essential nutrients. NR - non-requiring; P - proline; O - ornithine; M - methionine; PCU - proline, citrulline & uracil; OUH - ornithine, uracil & hypoxanthine; H - hypoxanthine

[c]describes taxonomic subdivisions of *N. gonorrhoeae* based on serotyping of cell surface antigens

[d]PEN - penicillin; TET - tetracycline; SPEC - spectinomycin; CIPRO - ciprofloxacin; ERY - erythromycin; AZI - azithromycin
Most of the MIC data was determined previously by the Dillon lab (1998). Note that PR number is the designation used for all these studies

[e]these MICs had a two fold difference between replicates, in these cases the higher MIC is given

[f]nd - not done; na - not available

2.2 Extract preparation.

Extracts were prepared as stock solutions in the appropriate solvent (usually pure ethanol). Pinocembrin (P5239) and silibinin (S0417) were purchased from Sigma-Aldrich (St. Louis MO, USA), while dillapiol (>98% pure) was obtained from the Arnason lab at the University of Ottawa. Because dillapiol is a thick oil (the other phytochemicals were powders), its density was determined (1100 mg/ml) and this value was used to prepare stock solutions. Ethanol solutions of silibinin were also sonicated for ~1-2 mins (with cooling) to improve dissolution of this compound. As these compounds are both light and temperature sensitive, they were stored in the dark at 4°C. Because the final concentration of ethanol could not be higher than 2% (so as not to affect microbial growth), stock solutions were prepared at least 50 times their final concentration prior to serial dilution and mixing with agar.

2.3 Agar dilution susceptibility testing.

For the majority of susceptibility testing involving polar phytochemicals (e.g. dillapiol, silibinin, pinocembrin), the agar dilution method following National Committee for Clinical Laboratory Standards (NCCLS) guidelines was used (NCCLS, 2003). Briefly, stock solutions (50 times final concentration) were used to perform serial dilutions in ddH_2O before adding a 1 ml aliquot of each tested concentration to 49 ml of molten (~45°C) GCMBK agar. This diluted the final ethanol concentration to 2% (due to additional dilution of the stock solution prior to serial dilution the concentration was often <1%). This 50 ml mixture (enough for 2 plates) was gently swirled for ~30 s before pouring. For larger experiments, the scale was simply expanded as needed (i.e. 100 ml for 4 plates, etc).

2.4 Synergy/combination assays

In cases where combinations of phytochemicals and antibiotics were studied, the phytochemical was added at a subinhibitory concentration (MIC-1 or -2) to each concentration of the antibiotic (i.e. penicillin) over its normally tested concentration range for *N. gonorrhoeae*. For these assays control penicillin and phytochemical plates (at subinhibitory concentrations) were also included. Because of the inherent variability of MICs (usually a 2-fold variation), at least a 4-fold reduction in MICs versus the antibiotic control was needed to be considered significant (Krogstad and Moellering, 1986), although this does not necessarily mean a synergistic interaction is responsible for the MIC reduction. To better quantitate the likelihood that an actual synergistic effect was at work, the fractional inhibitory concentration index (FICI) was applied to the experimental data. This value is calculated as follows (Krogstad and Moellering, 1986):

$$(\mathbf{FICI}) = \mathbf{FIC}A + \mathbf{FIC}B = [(\text{conc of drug A in combination})/(\text{conc of drug A alone})] + [(\text{conc of drug B in combination})/(\text{conc of drug B alone})]$$

Unfortunately, researchers use a variety of differing criteria to decide the cutoff FICI values representing synergy. For example, one system might conclude an FICI of 0.75 indicated "weak synergy" while another system would conclude "additive". For these purposes, a stringent definition of synergy was adopted from Krogstad and Moellering (1986): if the FICI value is ≤ 0.5 (only possible if the combination MICs are lower than the drug's MICs alone), then synergy is concluded. A value of 0.5-2 indicates no interaction (i.e. an additive effect, which is important to distinguish from synergy), while a value of >2

indicates antagonism. An alternative system (one of many) proposed by Bharadwaj *et al.* (2003) adds another category of FICIs between 0.5-1, which are classified as "weak synergy" or additive, while an FICI of 1-2 would be considered additive. For the results of this work, both of the above criteria were used to give an idea of the diversity of synergistic interpretations.

2.5 Inoculum preparation

All inocula were prepared according to NCCLS guidelines (NCCLS, 2003). Bacterial suspensions were prepared in MH broth (Difco), standardized to the 0.5 McFarland turbidity standard (Remel, Lenexa KS, USA; ~10^8 colong forming units [CFU]/ml), and diluted a further 10 times before loading into the wells of a Steers replicator block whose inoculating pins deliver a final inoculum size of approximately 10^5 CFU/ml onto an agar plate. The Steers replicator is a semi-automated device which allows up to 32 strains to be simultaneously inoculated onto one agar plate. After inoculation, the plates were allowed to dry (15-20 mins) before inverting and incubation. After 18-20 hours incubation at standard conditions, plates were examined visually and antibacterial activity recorded as the minimum concentration inhibiting growth (MIC) over the specified growth conditions and incubation period. Compounds were tested in duplicate two times and penicillin and 2% ethanol controls were included with all assays.

2.6 Bacterial time kill curves.

This approach was used to gain a more dynamic view of the killing rate of dillapiol and pinocembrin (versus the single timepoint determination of the MIC agar dilution

assays). Experimental combinations were prepared in glass test tubes containing (total volume) 10 ml of broth. Briefly, 9 ml aliquots of the compounds were prepared at 1.11 times their final concentration in GC broth + 1% Kelloggs. Bacterial suspensions were prepared from a 0.5 McFarland turbidity standard, which was diluted 10 times in GC broth. 1 ml of this suspension was then added to the 9 ml of broth containing the compounds, giving an initial inoculum size of 10^5-10^6 CFU/ml. To this was added 1% 80 mg/ml $NaHCO_3$ (an additional CO_2 source). The tubes, including a negative growth control (no added compounds or antibiotics) and positive antibiotic control (penicillin at the MIC for the strain), were incubated at standard *N. gonorrhoeae* growth conditions (plus shaking; ~200 rpm). For colony determinations, 100 uL was removed from each tube at chosen timepoints (usually at t = 0 hours, ½ hour, and hourly timepoints thereafter). 10 fold serial dilutions were then performed in microtubes using 0.7% casamino acids (CAA; Difco) as the diluent (100 uL suspension into 900 uL 0.7% CAA). Dilutions were normally 10^0, 10^{-1}, 10^{-2}, 10^{-3} and sometimes 10^{-4} (*i.e.* 1/10,000 dilution from the initial 100 uL aliquot). From each of these dilutions, two 10 uL aliquots were placed on a GCMBK plate and incubated under standard conditions. The following day, the number of colonies (from the lowest countable dilution) was determined visually as the average of the two aliquots. By taking the dilution factors into account, the total CFU/ml at each timepoint for each combination could then be determined. For example, if an average of 15 colonies was counted at the 10^{-1} dilution, then the actual CFU/ml was: (15/0.1)/0.01 = $1.5x10^4$ CFU/ml, where 0.1 represents the 10^{-1} dilution and 0.01 represents the 10 uL spot (0.01 ml). Tabulation of the various experimental combinations could then be represented graphically (as reduction in log CFU/ml versus time) using Microsoft Excel software.

2.7 1-N-phenylnaphthylamine (NPN) assays

2.7.1 Preparation of bacterial suspensions.

Overnight subcultures of *S. typhimurium* ATCC-14028 were grown in LB broth (Difco) to an optical density of OD_{630} 0.5 ± 0.05 (mid-log phase growth) at 37°C with shaking (~250 rpm). Overnight subcultures of *N. gonorrhoeae* WHO V were grown in GC broth (plus 1% Kelloggs plus 1% 0.95 M $NaHCO_3$) (Piekarowicz and Stein, 2002) to an optical density of OD_{630} 0.5 ± 0.05 at 35°C/5% CO_2 in a humid atmosphere with shaking (~250 rpm). All growth was monitored using a Beckman DU640B spectophotometer. Cells were then centrifuged at ~1000g for 5 mins, the supernatant removed, and the cells resuspended in a half volume of 5 mM HEPES buffer for *S. typhimurium*, or 0.7% CAA for *N. gonorrhoeae*. This suspension was added to the microplates at 100 uL/well.

2.7.2 NPN uptake assays.

NPN stock solutions (10 mM) were prepared weekly in acetone. For a standard assay, the wells (4 wells/sample) of a white fluoresence microplate (Corning Costar, Acton MA, USA) were prepared as follows: (1) 100 uL of bacterial suspension in the appropriate buffer; (2) 50 uL of 40 uM NPN (in buffer); (3) 50 uL of the test substance(s) in buffer. Portions (2) and (3) were added at 4 times their final concentration to the wells prior to adding the bacterial suspension. Dillapiol (50 mg/ml in ethanol) and EDTA (Sigma; 40 mM in ddH_2O) were diluted in buffer; the concentrations of ethanol and acetone never exceeded 1% and 0.5% (respectively) of the total volume in the wells. Any supplements (e.g. $MgCl_2$ or KCN) were added to portion (3) prior to adding the inoculum. After the cells were added, the plate was placed in the Cytofluor 4000 microplate fluorescence reader (Framington MA,

USA) and fluorescence readings were taken within 3 mins. Standard settings were a mix time of 3 sec, and three cycles of scanning the plate (1 scan/cycle, except for assays involving different photomultipler tube gains). For each test substance (at all concentrations tested), it was ensured that alone or with 10 uM NPN, there was no significant fluroescence increase compared to NPN in buffer alone.

2.7.3 Analysis of results.

Raw data was saved as an Excel file (Microsoft) from the Cytofluor software and all subsequent manipulation of data was performed in Excel. Analyzing the results of this assay consists of subtracting the various control and experimental combinations of buffer, cells, and the test substance(s) with or without NPN, to determine the fluorescence minus the background fluorescence. These background corrected fluorescence values could then be used to determine the NPN fluorescence ratio as a ratio of background corrected fluorescent values of the bacterial suspensions and buffer respectively (effectively the fluorescence ratio). It should be noted that all measurements were taken in relative fluorescence units (RFU), thus all results are presented relative to NPN fluorescence in buffer alone. One complication of this assay is that there is no clear cutoff for what fluorescence ratios should be considered significant. Helander and Mattila-Sandholm (2000) reported that an uptake increase of 4.2 (from 6.6 RFU [cells] to 10.8 RFU [cells + 1 mM EDTA]) was indicative of permeabilization. This is a fold increase of 1.6 over the background cell NPN uptake value. Thus, for the purposes of this study, any change less than 1.5 was considered insignificant, although the subjective nature of this criterion should be noted.

CHAPTER 3 – ANTIMICROBIAL ACTIVITY OF GARLIC (*Allium sativum*) NATURAL HEALTH PRODUCTS

3.1 Introduction

Between 4 and 10% of people surveyed in North America, in 1999, reported the use of natural health products (NHPs) (Ni *et al.*, 2002). Garlic (*Allium sativum* L. (Liliaceae)) is among the top ten NHPs and has been consumed for centuries as a food, spice and traditional medicine, and is one of the most often used herbal remedies (Ni *et al.*, 2002; Amagase *et al.*, 2001; Matsuura, 1997). Over 3000 studies have validated the traditionally recognized health benefits of garlic, including its antithrombotic, antiarthritic, anticancer and antimicrobial activities (Amagase *et al.*, 2001; Matsuura, 1997).

Commercial garlic products comprise a number of different formulations including garlic powder (GP), gelatinous suspensions of GP (LGP), oils of steam-distilled garlic (GO), or aged garlic extract (AGE), which is also prepared as a gelatinous suspension (LAGE) (Amagase *et al.*, 2001; Ross *et al.*, 2001). Allicin (allyl-2-propene thiosulfinate) is the primary constituent associated with garlic's antimicrobial activity, along with other related allyl thiosulfinates (Matsuura, 1997; Lawson, 1990). The specific organosulfur compounds present in or released from each type of garlic product varies due to the spontaneous and enzymatic transformations that take place in allicin and γ-glutamyl-*S*-allylcysteine when garlic is commercially processed (Lawson, 1990). After intact garlic tissues are damaged, (i.e. by cutting or crushing), the enzyme allinase is released, converting the inactive precursor alliin into the volatile active component allicin. GP is a preparation of sliced, dried

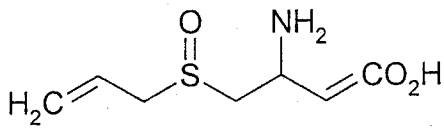
and pulverized garlic cloves which forms allicin upon the addition of water (allinase is present in these products) (Amagase *et al.*, 2001).The distillation of GO typically converts allicin to various volatile garlic sulfides (mainly diallyl sulfides and diallyl disulfides) (Amagase *et al.*, 2001; Ross *et al.*, 2001). AGE preparation involves aging a water/diluted alcohol garlic extract for up to 20 months; during this time most of the precursor substance γ-glutamyl-S-allylcysteine is naturally converted to non-volatile water soluble compounds, primarily S-allylcysteine (SAC) (Amagase *et al.*, 2001) (see Figure 4).

Fresh garlic preparations and isolated garlic constituents have been shown to have antimicrobial activity against a variety of Gram positive and Gram negative pathogenic bacteria (Amagase *et al.*, 2001; Sivam *et al.*, 1997; Ankri & Mirelman, 1999; Lee *et al.*, 2003; Dikasso *et al.*, 2002, Ross *et al.*, 2001), which provides a good basis for examining the antibacterial activity of garlic NHPs. Only one study to date has examined the activity of garlic NHPs against microorganisms (Ward *et al.*, 2002). However, the activity of garlic (fresh or commercial preparations) has not been examined against *Neisseria gonorrhoeae*. In addition to *N. gonorrhoeae*, the two clinically relevant Gram positive pathogens *Staphylococcus aureus* and *Enterococcus faecalis* were selected to examine the antibacterial activity of commercial garlic NHPs. Consequently, garlic represents an important herbal remedy and NHP, with much historical and scientific evidence attesting to its wide range of biological activity. However, the lack of concrete data concerning the antibacterial activity of commercial preparations (not to mention the paucity of *in vivo* evidence), combined with unproven claims on labels and lack of standardization, represent continued unknowns. On the positive side, these garlic formulations are currently in wide use in a somewhat

Figure 4. Chemical structures of the principal organosulfur compounds found in garlic. (A) Diagram showing simplified biosynthesis of allicin (found in garlic powder products) from alliin, and diallyl disulphide, a typical garlic sulfide (garlic oil products). **(B)** S-allylcysteine (SAC), the primary constituent of aged garlic products.

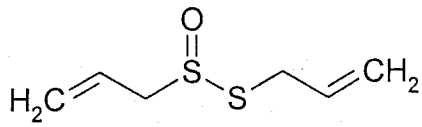
(A)

Alliin



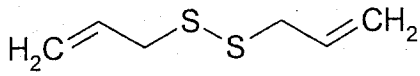
Allinase

Allicin



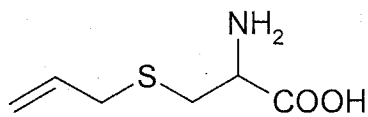
Heat

Diallyl disulphide



(B)

S-allylcysteine (SAC)



standardized form (Ni *et al.*, 2002), which both reduces the likelihood of widespread complications and facilitates their development into a potential new use (since they are already in use versus a clinically unproven drug). However, most of the work to date on plant based remedies has focussed on crude or isolated plant extracts, not commerical preparations. Thus, the *in vitro* activity of an isolated garlic component (e.g. allicin) tells us little about the *in vivo* activity of a crude garlic mixture taken as an NHP. Indeed, because manufacturing and quality control standards for the preparation of garlic NHPs are often either inadequate or non-existant, the levels of constituents can vary between products (Amagase *et al.*, 2001; Lawson *et al.*, 1990), underscoring the need to properly study and classify these products. Nonetheless, the large amount of *in vitro* data attesting to garlic's antimicrobial activity warrants further investigation into garlic's potential in CAM strategies.

3.2 Materials and Methods

3.2.1 Microorganisms.

The susceptible control strains *N. gonorrhoeae* WHO V, *S. aureus* ATCC-29213 and *E. faecalis* ATCC-29212 were used for this work. *N. gonorrhoeae* WHO V was subcultured as previously described (see Chapter 2; "Selection, growth and maintenance of bacterial cultures") on GCMBK agar. *S. aureus* and *E. faecalis* were subcultured on MH agar (Difco) at 37°C for 18-20 hours.

3.2.2 Garlic extract preparation.

Nineteen garlic NHPs were purchased from local pharmacies or natural health product outlets. This panel of products comprised commonly available garlic NHPs from 8 manufacturers in Canada and the original specimens have been retained. These garlic NHPs included GP (4 products; 2 were tablets and 2 were encapsulated), LGP (6 products), GO (3 products), AGE (5 products) and LAGE (1 product) capsules (Table 2). Minced and crushed garlic (in vegetable oil) and 3 fresh garlic bulbs (sold as American, Chinese and Thai varieties) were purchased from local groceries to use as standards. The garlic products were stored at 4°C and were used within their stated expiry dates. Garlic extracts were prepared by combining 4 units of each product to obtain a representative sample prior to preparing the final extract concentration. Garlic tablets and fresh garlic bulbs were wrapped in aluminum foil and crushed with a hammer. Gelcaps were opened with a sterile scalpel and their contents squeezed out. Using the product's label information, extract solutions were prepared to a final concentration of 200 mg/ml by calculating the amount of each garlic NHP that was equivalent to 200 mg of fresh garlic (Table 2). This was necessary because the products had varying unit weights and potencies and simply measuring out 200 mg of product would have produced significant variation in the stock solution concentrations. The garlic equivalents were standardized at 200 mg to minimize the variability inherent in testing such products.

Aqueous and EtOH extracts of the garlic NHPs were prepared to ensure the recovery of garlic's various constituents, most of which demonstrate good water solubility (except GO, as the water-soluble compounds and allicin are eliminated or converted to allyl sulfides

Table 2. Overview of manufacturer's advised dosing regimens & advertised equivalents for fresh garlic (as taken from the label), and calculated 200 mg equivalents used to standardize stock solution concentrations.

Product no	Form[a]	mg of fresh garlic/unit	Recommended daily dose (mg/day)	Average content weight[b] (mg)	200 mg equivalent (mg)
1	GP	1500	4500	484.5	64.6
2	GP	400	1200	650.5	325.4
3	GP	500	3000	829.5	110.6
4	GP	1500	3000	515	68.6
5	LGP	500	3000	227.25	91
6	LGP	500	3000	188	75.2
7	LGP	500	3000	179	71.6
8	LGP	500	2000	181	72.4
9	LGP	500	1500	173	69.2
10	LGP	1500	4500	256.75	34.2
11	GO	1500	1500-6000	226.75	30.2
12	GO	1500	4500	233.25	31.1
13	GO	1500	1500-3000	224	29.8
14	AGE	350	350	345	197
15	AGE	350	350	354.5	202.6
16	AGE	300	1200	491.25	327.5
17	AGE	350	1400	496.25	283.6
18	AGE	300	600	490	326.6
19	LAGE	500	1500	178.5	71.4
20	crushed	n/a[c]	n/a	100	200
21	minced	n/a	n/a	100	200
22	fresh	n/a	n/a	100	200
23	fresh	n/a	n/a	100	200
24	fresh	n/a	n/a	100	200

[a]GP - garlic powder; LGP - gelatinous suspension of GP; GO - garlic oil;
AGE - aged garlic extract; LAGE - gelatinous suspension of AGE

[b]These values were the "fresh garlic equivalents" taken directly from the NHP labels

[c]The removable contents of each product, calculated as the average of 4 units

[d]Calculated by equating the content weight to the "fresh garlic equivalents" stated on the labels

[e]n/a - not available

during distillation) (Ross *et al.*, 2001). The 200 mg fresh garlic equivalent was added to 5 ml of ddH_2O or 5 ml of 55% EtOH and vortexed for 1 min. Minced and fresh garlic samples were also polytroned for 1 min to homogenize them. In addition, the aqueous extracts were maintained at room temperature for 5, 10 and 15 mins before rapid cooling to 4°C in a salt-water ice bath (~5 g NaCl/100 ml water:ice) to decrease alliinase activity and minimize volatility. Extracts were then centrifuged for 18 minutes at 13,000 rpm at 4°C (Foster *et al.*, 2001). Supernatants were removed in 250 µl aliquots, frozen by immersion in liquid nitrogen and stored at -70°C until use. Aliquots were not refrozen or reused after being thawed for testing.

3.2.3 Garlic product analysis

Aqueous extracts of fresh garlic, the GP products and garlic minced in vegetable oil were analyzed for yield of total allyl thiosulfinates by Larry Lawson (Plant Bioactives Research Institute, Orem, UT, USA). See Table 3 The thiosulfinates were analyzed by HPLC as previously described, using allicin synthesized from diallyl disulfide (Lawson *et al.*, 1991a). Due to alliinase damage that usually occurs during processing, GP products and garlic minced in vegetable oil were also analyzed for alliin content. Alliin was analyzed by HPLC after extraction with 10 mM carboxymethoxylamine (alliinase inhibitor) and derivatization with *o*-phthaldialdehyde (Ziegler and Sticher, 1989). The oil products (steam-distilled or minced in vegetable oil) were analyzed by HPLC after extraction with acetonitrile for total allyl sulfides (diallyl sulfide, diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide, allyl methyl sulfide, ajoene, and vinyldithiins) using previously described

Table 3. Chemical analysis of the garlic NHPs examined. Analytical results are in mg/g product content and represent the average of duplicate analyses (2 extractions/sample).

Product no	Form	Allicin [Allicin:alliin ratio]	Total allyl thiosulfinates[a]	S-allylcysteine	Total sulfides
1	GP	1.61 [0.089]	2.21	n/a[b]	n/a
2	GP	4.83 [0.46]	6.09	n/a	n/a
3	GP	1.33 [0.14]	1.50	n/a	n/a
4	GP	4.61 [0.48]	5.34	n/a	n/a
5	LGP	<0.002	<0.002	n/a	n/a
6	LGP	<0.002 [<0.017]	<0.002	n/a	n/a
7	LGP	0.24 [0.38]	0.29	n/a	n/a
8	LGP	<0.002 [<0.015]	<0.002	n/a	n/a
9	LGP	<0.002 [<0.017]	<0.002	n/a	n/a
10	LGP	<0.002 [<0.007]	<0.002	n/a	n/a
11	GO	n/a	n/a	n/a	8.26
12	GO	n/a	n/a	n/a	7.40
13	GO	n/a	n/a	n/a	8.90
14	AGE	n/a	n/a	0.92	n/a
15	AGE	n/a	n/a	0.89	n/a
16	AGE	n/a	n/a	0.73	n/a
17	AGE	n/a	n/a	0.55	n/a
18	AGE	n/a	n/a	0.59	n/a
19	LAGE	n/a	n/a	0.0036	n/a
20	crushed	nd[c]	nd	nd	nd
21	minced	<0.002 [<0.013]	<0.002	n/a	0.26
22	fresh	4.49	7.19	n/a	n/a
23	fresh	4.38	7.49	n/a	n/a
24	fresh	4.65	7.31	n/a	n/a

[a]Total allyl thiosulfinates = allicin +allyl methyl THSs + allyl -1-propenyl THSs.

[b]n/a - not available.

[c]nd - not determined.

methods (Lawson *et al.*, 1990). AGE products were analyzed for *S*-allylcysteine by HPLC after extraction with water as previously described (Lawson *et al.*, 1991b).

3.2.4 Determination of MICs.

MICs were determined using a modification of the broth microdilution methodology recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2003). Briefly, the garlic extract aliquots were quickly thawed at room temperature, and serial 10-fold dilutions were prepared in U-bottomed 96-well microplate wells (Corning Costar, Acton, MA, USA) in ddH_2O for a final concentration range of 0.1-100 mg/ml (aqueous extracts) or 0.002-2 mg/ml (ethanol extracts). This lower concentration range with the EtOH extracts was used since an initial 50-fold dilution of the stock solution was required to lower the volume of organic solvent to <2% so as not to inhibit bacterial growth.The final volume per well was 180 µl (90 µl extract and 90 µl inoculum). The bacterial inocula were prepared in double strength broth [for *N. gonorrhoeae*, GCMBK broth with 1% 80 mg/ml $NaHCO_3$ (Piekarowicz and Stein, 2002); for *S. aureus* and *E. faecalis*, Mueller Hinton broth], standardized to a 0.5 McFarland turbidity standard (Remel) and added to the wells for a final cell concentration of ~$5x10^5$ CFU/ml. Double strength broth was used so that the garlic extracts could be diluted in the appropriate solvent (ddH_2O or 55% EtOH) instead of in broth. MIC determinations were performed in duplicate and the antimicrobial susceptibility of each strain to penicillin, as a control, was ascertained for every test. Microplates, covered with their low evaporation lids, were wrapped with parafilm and incubated as described above, except that *N. gonorrhoeae* cultures were shaken (250 rpm). Growth was measured by absorption at 595 nm using a microplate reader (Tecan Shell

Spectra, Maennedorf, Switzerland). MICs were considered to be the concentration completely inhibiting growth after both visual inspection and OD analysis. Minimum Bactericidal Concentrations (MBCs) were ascertained by sampling 25 µl aliquots from microplate wells and culturing them on either GCMBK or MH agar under the appropriate conditions.

3.3 Results

3.3.1 Active ingredients of garlic NHPs vary by product.

Chemical analysis of the garlic products showed that the concentration of active garlic constituents varied both within and between products (Table 3). The levels of allicin in the GP products (nos 1-4) ranged from 1.33-4.83 mg/g, with only product nos 2 and 4 having allicin levels comparable (4.49-4.65 mg/g) to the fresh garlic preparations (nos 22-24; 4.38-4.65 mg/g). The levels of total allyl thiosulfinates for the GP products ranged from 1.5-6.1 mg/g, in contrast to the fresh garlic preparations (7.19-7.49 mg/g). LGP products (nos 5-10) showed mostly undetectable levels of allicin or allyl thiosulfinates. In GO products (nos 11-13), allicin is generally converted to various garlic sulfides and the concentrations of these compounds ranged from 7.40-8.90 mg/g in these products. Interestingly, garlic sulfides were also detected in the minced garlic product (no 21), while allicin or other allyl thiosulfinates were not. SAC is the principal constituent of AGE products (nos 14-18), and levels of this compound ranged from 0.59-0.92 mg/g. In contrast, the LAGE product (no 19) contained only 3.6 ug/g of SAC.

3.3.2 Aqueous garlic NHP extracts are active against *N. gonorrhoeae.*

The 3 fresh (nos 22-24) garlic preparations, minced garlic (no 20), crushed garlic (no 21) and 47% of the garlic NHPs inhibited the growth of *N. gonorrhoeae* (Table 4). As a group, the GP products (nos 1-4) were the most active of the commerical preparations tested, with all 4 products showing activity (MICs of 0.1-100 mg/ml). In fact, GP products 2 and 4 (MICs of 0.1-1.0 mg/ml) were the only two products in this study which had higher activity than the fresh garlic preparations (nos 22-24; MICs of 1-10 mg/ml). By contrast,

Table 4. Susceptibility of *Neisseria gonorrhoeae* strain WHO V to garlic NHPs.

Product no	Form	MICs [MBCs] (mg/ml) of active garlic NHPs		
		5 min	10 min	15 min
1	GP	10-100 [>100]	100 [>100]	10-100 [100]
2	GP	1-10 [10]	1 [1]	1 [1]
3	GP	10-100 [>100]	10 [>100]	10 [10]
4	GP	0.1 [1]	10 [10]	10 [10]
8	LGP	10-100 [>100]	100 [>100]	10 [10]
11	GO	10-100 [100]	10 [100]	10 [100]
14	AGE	100 [100]	>100	>100
15	AGE	100 [100]	>100	>100
17	AGE	10 [10]	10-100 [>100]	10 [10]
20	crushed	10 [10]	10 [10]	10 [10]
21	minced	10 [10]	10 [10]	10 [>100]
22	fresh	10 [10]	1-10 [10]	1 [10]
23	fresh	10 [10]	1-10 [10]	10 [>100]
24	fresh	10 [10]	1-10 [10]	10 [>100]
5,6,7,9,10	LGP	>100	>100	>100
12,13	GO	>100	>100	>100
16,18	AGE	>100	>100	>100
19	LAGE	>100	>100	>100

only 1/6 of the LGP products (no 8), 1/3 of the GO products (no 11), and 3/6 of the AGE/LAGE products (nos 14, 15, 17) showed activity against *N. gonorrhoeae* at the tested concentrations (MICs of 10-100 mg/ml) (Table 4). In general, the MBCs were equal to, or within a 10-fold concentration of the MICs.

There was a modest trend to lower MICs for *N. gonorrhoeae* with longer product extraction times. Including the fresh garlics (nos 22-24), 8/14 products (57%) with activity against *N. gonorrhoeae* had MICs $\leq$ 10 mg/ml after 5 mins of aqueous extraction, while 11 products (79%) had these MICs after 15 minutes of extraction. However, products no 4 (GP) and 14 & 15 (AGE) exhibited only a modest increase in MICs in the 10 and 15 min extracts. Some products (i.e. 1-3 (GP), 8 (LGP)) showed slightly increased activity, as indicated by a 1-2 order of magnitude reduction in MBCs, with the longer extraction times.

3.3.3 Aqueous NHP garlic extracts are less active against *S. aureus* and *E. faecalis*.

Aqueous garlic extracts were less active against *S. aureus* ATCC-29213 (Table 5). Only GP products nos 1, 2, 4 and the fresh garlics (nos 22-24) inhibited the growth of *S. aureus* (MICs of 10-100 mg/ml). The length of extraction had no effect on activity. The MBCs of the garlic extracts against *S. aureus* generally exceeded 100 mg/ml, except for the fresh garlic extracts (nos 22-24) in which the MBCs were 100 mg/ml after 5 or 10 minutes of extraction. Similarly, few aqueous garlic extracts showed activity against *E. faecalis* ATCC-29212 (Table 6). Only the GP products nos 1-4 and 22-24 (the fresh garlics) demonstrated activity comparable to that against *S. aureus* (10-100 mg/ml). No significant

Table 5. Susceptibility of *Staphylococcus aureus* strain ATCC-29213 to garlic NHPs.

		MICs [MBCs] (mg/ml) of garlic NHPs		
Product no	Form	5 min	10 min	15 min
1	GP	100 [>100]	100 [>100]	100 [>100]
2	GP	10 [>100]	10 [100]	10 [>100]
4	GP	100 [100]	100 [>100]	100 [>100]
22	fresh	10-100 [100]	10-100 [>100]	10 [>100]
23	fresh	10-100 [100]	10-100 [100]	10 [>100]
24	fresh	10-100 [100]	10-100 [100]	10 [>100]
3	GP	>100	>100	>100
5,6,7,8,9,10	LGP	>100	>100	>100
11,12,13	GO	>100	>100	>100
14,15,16,17,18	AGE	>100	>100	>100
19	LAGE	>100	>100	>100
20	crushed	>100	>100	>100
21	minced	>100	>100	>100

Table 6. Susceptibility of *Enterococcus faecalis* strain ATCC-29212 to garlic NHPs.

		MICs [MBCs] (mg/ml) of garlic NHPs		
Product no	Form	5 min	10 min	15 min
1	GP	>100	100 [>100]	100 [>100]
2	GP	100 [>100]	10 [>100]	10-100 [>100]
3	GP	>100	100 [>100]	>100
4	GP	100 [>100]	100 [>100]	100 [>100]
22	fresh	10-100 [100]	10-100 [>100]	100 [>100]
23	fresh	10 [10]	10-100 [>100]	100 [>100]
24	fresh	100 [>100]	10-100 [>100]	100 [>100]
5,6,7,8,9,10	LGP	>100	>100	>100
11,12,13	GO	>100	>100	>100
14,15,16,17,18	AGE	>100	>100	>100
19	LAGE	>100	>100	>100
20	crushed	>100	>100	>100
21	minced	>100	>100	>100

differences were observed with different extraction length timepoints. The MBCs for most extracts exceeded 100 mg/ml.

3.3.4 Ethanol garlic NHP extracts exhibit marginal activity against *N. gonorrhoeae*, *S. aureus*, and *E. faecalis*.

Table 7 shows that none of the ethanol NHP extracts showed activity against *S. aureus* or *E. faecalis*, while only product nos 16 (AGE), 21 (minced) and 22-24 (fresh) were active against *N. gonorrhoeae* (all MICs of 2 mg/ml).

3.3.5 Active products have higher levels of garlic constituents.

A general trend towards increased antibacterial activity was noted *in vitro* among those products containing higher levels of garlic constituents. For example, product nos 2 and 4 (GP), which showed the highest activity of any NHPs in our study, contained >4.5 mg allicin/g product content, the only two NHPs to have similar levels of allicin as the fresh garlic products (Table 3). Similarly, NHPs containing low or undetectable levels of the garlic constituents possessed low or no activity (for example most of the LGP products). With the AGE products, although product nos 14 and 15 contained the highest levels of SAC (0.92 and 0.89 mg/g respectively), product no 17 (containing the lowest SAC levels; 0.55 mg/g) demonstrated the highest activity (10 mg/ml) for these products (Table 3). It is also notable that one product containing undetectable levels of the garlic constituents assayed for did possess antimicrobial activity (product no 8).

Table 7. Susceptibility of *N. gonorrhoeae* WHO V (NG) , *S. aureus* ATCC-29213 (SA) and *E. faecalis* ATCC-29212 (EF) to 55% EtOH extracts of garlic NHPs. Concentrations tested were 0.002, 0.02, 0.2 and 2 mg/ml.

		MICs [MBCs] (mg/ml) of garlic NHPs		
Product no	Form	NG	SA	EF
16	AGE	2 [>2]	>2	>2
21	minced	2 [2]	>2	>2
22	fresh	2 [>2]	>2	>2
23	fresh	2 [>2]	>2	>2
24	fresh	2 [>2]	>2	>2
1,2,3,4	GP	>2	>2	>2
5,6,7,8,9,10	LGP	>2	>2	>2
11,12,13	GO	>2	>2	>2
14,15,17,18	AGE	>2	>2	>2
19	LAGE	>2	>2	>2
20	crushed	>2	>2	>2

3.4 Discussion

The results of this work represent the first report of the antibacterial activity of fresh garlic and garlic NHPs against *N. gonorrhoeae*, while extending observations on garlic's activity (especially garlic NHPs) against *S. aureus* and *E. faecalis*. *N. gonorrhoeae* exhibited a markedly higher susceptibility to the garlic products than either *S. aureus* or *E. faecalis*. In addition, excluding the minced, crushed, and fresh garlics (nos 20-24), a larger proportion of garlic-based NHPs (47%) were active against *N. gonorrhoeae,* while only products 1, 2 and 4 (16%) possessed activity against all three species. Especially noteworthy is the observation that the 5 min aqueous extract of GP product no 4 exhibited a MIC of 0.1 mg/ml against *N. gonorrhoeae*, the highest activity of any product that was tested.

Most studies investigating the antimicrobial properties of garlic have used isolated garlic constituents such as allicin in their susceptibility assays, while here crude NHP extracts from products used by consumers were examined; thus, direct comparisons between studies is difficult. *S. aureus* has been commonly used in such assays, while the testing of *E. faecalis* has been less frequent. In one study, concentrations of up to 0.16 mg/ml (fresh garlic extract of standardized thiosulfinate concentration) were insufficient to inhibit staphylococcal growth (Sivam *et al.*, 1997), similar to the findings reported here that much higher concentrations were indeed required (10 mg/ml). In another study, in which pure GO and pure GP were tested against *E. faecalis*, MICs of 0.34 mg/ml GO were required to the inhibit growth of *E. faecalis* while GP was ineffective (although the maximum concentration of GP tested was unclear) (Ross *et al.*, 2001). The higher activity observed as compared to this study is likely due to the testing of pure GO activity against *E. faecalis*. Another report

found that crude fresh garlic extracts tested against the Gram positive bacteria *Bacillus subtilis* produced MICs of 100 mg/ml, similar to the present findings of MICs of 10-100 mg/ml against the Gram positives *S. aureus* and *E. faecalis* (De *et al.*, 1999).

The antibacterial activity of garlic has been established for Gram negative bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Helicobacter pylori* (Ward *et al.*, 2002; Sivam *et al.*, 1997; Tsao and Yin, 2001; Dikasso *et al.*, 2002). One study found concentrations of 24.38 mg/ml of crude garlic extract to be inhibitory against *K. pneumoniae* (Dikasso *et al.*, 2002), which is similar (~2.5 fold difference) to our MICs of 10 mg/ml for our crude fresh garlic extracts against *N. gonorrhoeae*. However, it should be noted that in general, the variety of pure compounds tested in the various studies and differences in methodology makes meaningful comparisons difficult.

Only one other report has investigated the antibacterial activity of garlic NHPs (Ward *et al.*, 2002). In contrast to these results, no activity was found among six garlic NHPs tested against *S. aureus* ATCC-29213; this may have been because the concentrations in the study were reported as percentages of product dosages and hence were a function of the extract's concentrations (and inconsistencies), whereas extracts in the present study were standardized to fresh garlic equivalents. More significant is the finding that, when combined with ampicillin or norfloxacin, the garlic NHPs decreased the susceptibility of *S. aureus* to ampicillin 8-32 fold with a 4 fold decrease in susceptibility to norfloxacin (Ward *et al.*, 2002). This finding underlines the point that regardless of antibacterial (or any other

biological) activity, garlic NHPs should not be considered as benign agents with no effects on other commonly used antibiotics.

Interestingly, both the most and least active NHPs in the present study were the GP-containing products, underlining the variability of commercial garlic preparations. Discrepancies between advertised and actual levels of allicin (usually reported as allicin potential on the label) were noted in several products. For example, products no 4 (GP) and no 5 (LGP) are ostensibly the same products: they both contain garlic powder and recommend a total daily intake of an equivalent of 3000 mg of fresh garlic of their products. However, while product no 4 possessed high levels of allicin and significant antibacterial activity, product no 5 (along with all LGP products except no 8) exhibited no activity and our chemical analysis showed undetectable levels of allicin (Table 3), similar to previous findings for LGP products (Lawson *et al.*, 1990). It is notable, however, that LGP product no 8 showed activity against *N. gonorrhoeae* in all three aqueous extracts despite its lack of allicin content. Garlic contains a large variety of secondary metabolites, such as steroids, terpenoids and flavonoids, in addition to organosulfur compounds which could also be antibacterial (Amagase *et al.*, 2002).

The garlic NHPs evaluated in this study were not only inconsistent in composition and activity, but also in the allicin:alliin ratio. This ratio is a good measure of alliinase activity and provides a benchmark for the activity of the GP products. For products that have sufficient allinase activity to convert alliin to allicin, this should be in the range of 0.35-0.46 (Lawson, 1996). As Table 3 indicates, all of the manufactured products, except for product

nos 2 and 4 (the two most active NHPs), were below this value, indicating highly damaged alliinase activity among the garlic products (Lawson, 2001).

To my knowledge, this is the first study in which aqueous garlic extracts with differing extraction times were tested for antibacterial activity. Among the aqueous extract time points, a trend towards heightened activity with increasing time was seen against *N. gonorrhoeae* with products nos 1-3 (GP), 8 (LGP) and 22 (fresh). Excluding product 22, these were all GP-containing products and generally contained higher levels of allicin. Despite a conflicting result with GP product no 4, this suggests that raising the alliin:alliinase exposure time may indeed increase the levels of allicin, and hence the antibacterial activity of the products, at least *in vitro*. Conversely, different extraction time points did not affect the antibacterial activity of the NHPs against *S. aureus* and *E. faecalis*.

Excluding the fresh garlic ethanol extracts (nos 22-24), only extracts no 16 (AGE) and 21 (minced) exhibited activity at the highest concentration tested (2 mg/ml), and only against *N. gonorrhoeae*. Interestingly, although extract no 16 shared similarly high levels of SAC with the other two active AGE products (nos 14 and 15), this does not likely explain this extract's activity, since only aqueous extracts of the other AGE products demonstrated any activity, and indeed, AGE products contain mainly water soluble components (e.g. SAC) (Amagase, 2001). This suggests there may be other constituents in this (and possibly the other) AGE products responsible for these NHP's antibacterial activity. The activity of extract no 21 is also somewhat surprising since this product had far lower levels of total sulfides than the GO products (0.26 mg/g versus 7.40-8.90 mg/g). The GO products would

have been expected to show better activity in the ethanol extracts since they contain more organic-soluble constituents (Ross *et al.*, 2001). While the ethanol extracts thus showed somewhat lower activity than expected, these results could represent a simple concentration dependant effect, since the highest tested ethanol extract concentration was only twenty times higher than the lowest tested aqueous extract concentration (0.1 mg/ml).

The bioavailibility of garlic's active ingredients is important when considering their potential in CAM approaches. While the antibacterial activity of allicin is well characterized *in vitro*, its *in vivo* effects require more study. In fact, the bioavailibility of allicin, especially absorption from the intestines to the circulatory system, may be quite poor (Amagase *et al.*, 2002). This is not surprising considering allicin's instability (both acid- and heat-labile) and its transient nature as an intermediate for other (more stable) garlic compounds. However, Klein (1999) has suggested that allicin-containing garlic extract, which is bactericidal towards the major bacterial pathogens (*Haemophilus influenzae*, *Pneumococcus* spp. and *Moraxella* spp.) that cause acute otitis media (ear infections), has potential as an alternative therapy if used topically. In contrast, SAC, the major constituent of AGE products, is both biologically active and bioavailable (Matsuura, 1997). In addition, SAC has 30-fold less toxicity than allicin in humans (Amagase *et al.*, 2002), and its biological properties have been better characterized (Lee *et al.*, 2003).

It is somewhat surprising that despite the therapeutic potential and claims attributed to garlic NHPs, very few *in vivo* and/or controlled clinical trials have been performed to evaluate the clinical efficacy of these products. A few studies do suggest the *in vivo*

potential of garlic NHPs or constituents in treating infections. Shadkchan *et al.* (2004) investigated the efficacy of pure allicin in a murine model of disseminated aspergillosis (induced in this case by the fungus *Aspergillus fumigatus*). They found that 5 mg/kg/day of pure allicin (administered intravenously) caused a significant increase in the mean survival time (from 7.7 to 21.3 days) and a significant (10-fold) reduction in the fungal burden of infected mice. However, none of the allicin treatments examined in this study were as effective as the antifungal agent amphotericin B, which was used as a comparative standard. Tsao *et al.* (2003) showed that garlic extract, diallyl sulfide and diallyl disulfide were effective in inhibiting MRSA infection in a murine model. The garlic constituents appeared to be active in the blood, liver, kidney and spleen, suggesting good penetration of garlic to internal organs. Thus, these results do show that allicin elicits some antimicrobial effect *in vivo* against both fungal and bacterial pathogens and represent a promising starting point for further study of allicin's (and garlic's) *in vivo* antibacterial activity.

A review of controlled clinical trials identified two qualifying studies examining the efficacy of garlic preparations in eradicating *H. pylori* infections (Martin and Ernst, 2003). One trial involved dyspeptic patients who took capsules containing an oil macerate of garlic (much like product nos 20 and 21 in the present study) or the same capsules combined with the antibiotic omeprazole (i.e. a complementary approach). The other compared the effect of 10 fresh sliced cloves of garlic (taken with meals) to bismuth subsalicylate (e.g. a Pepto-Bismol type remedy). Both studies reported no change in outcomes (when compared to controls) and no reduction of symptoms. However, the fact that a persistent organism found

in a highly acidic environment (the stomach) was the target pathogen, as well as the small sample size and non-randomized nature of the trials, may explain the lack of positive results.

CHAPTER 4 – ANTIMICROBIAL ACTIVITY OF DILLAPIOL, SILIBININ AND ANTIBIOTIC COMBINATIONS

4.1 Introduction

The flavonolignan silibinin and the neolignan dillapiol were originally chosen for investigation for two reasons. Both are novel phytochemicals that have significant biological activity in eukaryotes (silibinin in stimulating protein synthesis and dillapiol in inhibiting CYP450 enzymes), and prior to this work, neither had been tested for activity against prokaryotic organisms. The synergistic effect of the related flavonolignan 5'-MHC in inhibiting the NorA MDR efflux pump of *S. aureus* provided another motive for investigation of silibinin for similar effects in *N. gonorrhoeae*.

Previously, both silibinin and dillapiol were tested alone against the PR panel of 30 *N. gonorrhoeae* strains. This work was performed as part of my honors research project (2001-2002). Both phytochemicals were prepared in 100% EtOH and tested over a concentration range of 1-256 mg/l. Table 8 shows the cumulative inhibition of strains from the PR panel towards silibinin. At 64 mg/l, 27% of the strains (n = 30) were inhibited by silibinin, while at 128 mg/l and 256 mg/l, the proportion of strains inhibited were 80% and 93% respectively. Table 9 shows the cumulative inhibition of inhibited strains from the PR panel towards dillapiol. Dillapiol was clearly more active, as evidenced by the higher proportion of inhibited strains at 64 and 128 mg/l (both 96%; n = 26) versus silibinin.

Table 8. Cumulative percent of tested *N. gonorrhoeae* strains inhibited at minimum inhibitory concentrations (MICs) of silibinin. Percentages are rounded to the nearest whole number and represent the proportion of strains inhibited at each MIC from two independent experiments (2 replicates/experiment).

		Cumulative % inhibition at each tested concentration (mg/l)									
Susceptibility phenotype[a]	n	<1	1	2	4	8	16	32	64	128	256
Susceptible	6	0	0	0	0	0	0	17	33	83	100
TRNG	3	0	0	0	0	0	33	33	33	100	100
PPNG (PR4, 5, 6, 8)[b]	5	0	0	0	0	0	20	60	60	100	100
PP/TRNG (PR9, 11)	2	0	0	50	50	50	50	50	50	100	100
CMRNG	2	0	0	0	0	0	0	0	0	50	50
PP/ CMRNG	1	0	0	0	0	0	0	0	0	100	100
CMRNG, CIPRO^R (PR29)	1	0	0	0	0	0	0	0	0	0	100
PP/CMRNG, CIPRO^R (PR28)	1	0	0	0	0	0	0	0	0	100	100
SPEC^R	2	0	0	0	0	0	0	0	0	100	100
PP/CMRNG, SPEC^R, ERY^R, AZI^R (PR31)	1	0	0	0	0	0	0	0	0	100	100
PP/CMRNG, ERY^R	3	0	0	0	0	0	0	33	33	33	100
PPNG, SPEC^R, ERY^R	1	0	0	0	0	0	0	0	0	0	0
PP/CMRNG, SPEC^R, ERY^R	1	0	0	0	0	0	0	0	0	100	100
Totals	30	0	0	3	3	3	10	23	27	80	93

[a]refer to Table 1 for abbreviations

[b]where complete phenotype groups were not tested (see Table 1), the specific strains that were included are listed

Table 9. Cumulative percent of tested *N. gonorrhoeae* strains inhibited at minimum inhibitory concentrations (MICs) of dillapiol. Percentages are rounded to the nearest whole number and represent the proportion of strains inhibited at each MIC from two independent experiments (2 replicates/experiment).

		Cumulative % inhibition at each tested concentration (mg/l)									
Susceptibility phenotype[a]	n	<1	1	2	4	8	16	32	64	128	256
Susceptible	6	0	0	0	0	0	0	33	83	83	100
TRNG	3	0	0	0	0	0	0	0	100	100	100
PPNG (PR4, 5, 6, 8)[b]	4	0	0	0	0	0	0	25	100	100	100
PP/TRNG	3	0	0	0	0	0	0	100	100	100	100
CMRNG	2	0	0	0	0	0	0	0	100	100	100
PP/CMRNG, CIPRO^R (PR28)	1	0	0	0	0	0	0	0	100	100	100
SPEC^R	2	0	0	0	0	0	0	50	100	100	100
PP/CMRNG, SPEC^R, ERY^R, AZI^R (PR29)	1	0	0	0	0	0	0	0	100	100	100
PP/CMRNG, ERY^R (PR16, 17)	2	0	0	0	0	0	0	0	100	100	100
PPNG, SPEC^R, ERY^R	1	0	0	0	0	0	0	100	100	100	100
PP/CMRNG, SPEC^R, ERY^R	1	0	0	0	0	0	0	0	100	100	100
Totals	26	0	0	0	0	0	0	31	96	96	100

[a]refer to Table 1 for abbreviations

[b]where complete phenotype groups were not tested (see Table 1), the specific strains that were included are listed

4.2 Results

4.2.1 Binary combinations of dillapiol or silibinin with penicillin & tetracycline

Subsequently, combination studies were performed in which subinhibitory concentrations of silibinin or dillapiol (determined from the previous work, see Tables 8 and 9) were added to a range of penicillin (0.008-32 mg/l) and tetracycline (0.063-32 mg/l) concentrations. The MICs of this phytochemical/antibiotic combination towards the PR panel could then be compared to the antibiotic alone; a reduction would suggest an interaction. The specific hypothesis for this work was that PPNG strain might be made susceptible to penicillin again if a penicillin/phytochemical combination reduced the strain's MIC to below the breakpoint of penicillin resistance (2 mg/l for *N. gonorrhoeae*) (NCCLS, 2003).

For the penicillin and tetracycline combinations, addition of 64 mg/l silibinin resulted in at least a four fold reduction in MICs for 68% (with penicillin) and 41% (with tetracycline) of the strains (n = 22; Table 10). Two strains (PR16 (PP/CMRNG, ERY^R) and PR21 (PP/CMRNG)) showed a 16-fold reduction in penicillin MICs (both from 128 mg/l to 8 mg/l), while the PPNG strain PR5 showed a 32-fold reduction in penicillin MICs from 128 mg/l to 4 mg/l (Table 10). Although these reductions are significant, they were not large enough to make these strains sensitive to penicillin again (MICs of <2 mg/l), although these three strains all exhibit high level resistance (penicillin MICs of 128 mg/l). Table 11 shows that adding 16 mg/l dillapiol to penicillin and tetracycline resulted in at least a four fold reduction in MICs for 27% (with penicillin) and 3% (with tetracycline) of the tested strains (n = 30). Although the proportion of strains with significant MIC reductions was thus lower

Table 10. Combinations of penicillin or tetracycline with 64 mg/l silibinin (subinhibitory) eliciting an x-fold reduction in Minimum Inhibitory Concentrations (MICs) against tested *N. gonorrhoeae* strains.

		fold reduction in MICs vs PEN or TET controls[b]									
		PEN						TET			
Susceptibility phenotype[a]	n	none	2	**4**	**8**	**16**	**32**	none	2	**4**	**8**
Controls (susceptible)	4	1	1	**1**	**1**				2	**2**	
TRNG	3			**2**	**1**			2	1		
PPNG	2				**1**[c]		**1**		2		
CMRNG	2		1		**1**			1		**1**	
PP/CMRNG	1					**1**[c]			1		
CMRNG, CIPRO^R	1			**1**						**1**	
PP/CMRNG, CIPRO^R	1			**1**						**1**	
SPEC^R	1				**1**				1		
PP/CMRNG, SPEC^R, ERY^R, AZI^R	3	3							1	**2**	
PP/CMRNG, ERY^R	3			**2**[c]		**1**[c]			2	**1**	
PP/CMRNG, SPEC^R, ERY^R	1		1[c]							**1**	
Total	22	4	3	**7**	**5**	**2**	**1**	3	10	**9**	**0**
				(68%)[d]						**(41%)[d]**	

[a]refer to Table 1 for abbreviations

[b]PEN - penicillin (range: 0.008-32 mg/l); TET - tetracycline (range: 0.063-32 mg/l)

[c]for these strains, the reduction was *at least* the value indicated

[d]percentage of strains whose MICs were reduced ≥ 4-fold by the silibinin & PEN or TET combination

Table 11. Combinations of penicillin or tetracycline with 16 mg/l dillapiol (subinhibitory) eliciting an x-fold reduction in Minimum Inhibitory Concentrations (MICs) against tested *N. gonorrhoeae* strains.

		fold reduction in MICs vs PEN or TET controls[b]								
		PEN					TET			
Susceptibility phenotype[a]	n	none	2	**4**	**8**	**16**	none	2	**4**	**8**
Controls (susceptible)	6	2	2	**1**	**1**		6			
TRNG	3		2	**1**			1	2		
PPNG	5	3	1		**1**		5			
CMRNG	2		1			**1**		1	**1**	
PP/CMRNG	1	1						1		
PP/TRNG	2	1		**1**			2			
CMRNG, CIPRO^R	1			**1**				1		
PP/CMRNG, CIPRO^R	1		1				1			
SPEC^R	2		1		**1**		1	1		
PP/CMRNG, SPEC^R, ERY^R, AZI^R	2	2					2			
PP/CMRNG, ERY^R	3	2	1				2	1		
PPNG, SPEC^R, ERY^R	1	1					1			
PP/CMRNG, SPEC^R, ERY^R	1	1						1		
Total	30	13	9	**4**	**3**	**1**	21	8	**1**	**0**
				(27%)[c]				**(3%)[c]**		

[a]refer to Table 1 for abbreviations

[b]PEN - penicillin (range: 0.008-32 mg/l); TET - tetracycline (range: 0.063-32 mg/l)

[c]percentage of strains whose MICs were reduced ≥ 4-fold by the dillapiol & PEN or TET combination

with dillapiol, one strain (PR12; CMRNG) was made susceptible to penicillin (a 16 fold reduction from 16 mg/l to 1 mg/l), an especially promising result (Table 11). Overall, silibinin alone was much more effective than dillapiol at significantly reducing either penicillin or tetracycline MICs.

4.2.2 Ternary combination of silibinin, dillapiol and penicillin

The promising results of the two way combinations between penicillin and silibinin or dillapiol prompted an investigation of combining all three chemicals together. For this experiment, the same concentrations of dillapiol (16 mg/l) and silibinin (64 mg/l) were used as before, and both of these were added to a standard range of plates containing penicillin. Dillapiol/penicillin and silibinin/penicillin controls were also included and the PR panel of strains was again used. Table 12 shows the 3 way combination was much more effective at eliciting MIC reductions than either 2 way combination alone. While the penicillin/silibinin and penicillin/dillapiol combinations resulted in at least a 4-fold MIC reduction in 45% and 20% of the strains respectively (n = 20), the 3 way combination caused 88% of the strains to show a 4 fold reduction (n = 17). Of greater interest is that the MICs of 36% of the CMRNG or PPNG strains included in this assay (n = 11) were reduced below the susceptibility breakpoint for penicillin resistance (data not shown). In other words, with the triple combination these resistant strains were rendered susceptible to penicillin again at therapeutically relevant levels (at least *in vitro*). Clearly, there is some inherent variability in such an assay, as the spread in proportion of strains with at least a 4-fold reduction in MICs by penicillin/silibinin and penicillin/dillapiol combinations varied from 45-68% and 20-27% between experiments.

Table 12. Combinations of penicillin, silibinin and dillapiol eliciting an x-fold reduction in Minimum Inhibitory Concentrations (MICs) against tested N. gonorrhoeae strains. An MIC reduction of at least 4-fold versus the antibiotic control is considered to be significant (values in bold).

		fold reduction in MICs vs PEN control[a]														
		Silibinin (64 mg/l)					Dillapiol (16 mg/l)						Silibinin:Dillapiol[b]			
Resistance	n	none	2	**4**	**8**	**16**	none	2	**4**	**8**	**16**	(n)	none	2	**4**	**8**	**16**
Controls (susceptible)	4	2	1	**1**			3	1				3	1		**1**	**1**	
TRNG	3		3					3				3			**3**		
PPNG	2			**1**	**1**			1		**1**		2					**2**
CMRNG	2		1	**1**				1	**1**			2				**1**	**1**
PP/ CMRNG	1		1				1					1			**1**		
CMRNG, CIPRO^R	1			**1**				1	**1**			2			**1**	**1**	
PP/CMRNG, CIPRO^R	1			**1**													
SPEC^R	1			**1**					**1**			1				**1**	
PP/CMRNG, ERY^R	3	1	1		**1**		1	2				2		1	**1**		
PPNG, SPEC^R, ERY^R	1			**1**				1				0					
PP/CMRNG, SPEC^R, ERY^R	1		1				1					1				**1**	
Totals	20	3	8	**7**	**2**	**0**	6	10	**3**	**1**	**0**	(17)	1	1	**7**	**5**	**3**
				(45%)[c]					**(20%)[d]**						**(88.2%)**		

[a]PEN - penicillin (0.063-128 mg/l)

[b]Silibinin concentration used: 64 mg/l (sub-inhibitory to 70% of tested strains)
Dillapiol concentration used: 16 mg/l (sub-inhibitory)

[c]Previous independent expt found inhibition of ~68% (n=22; see Table 10)

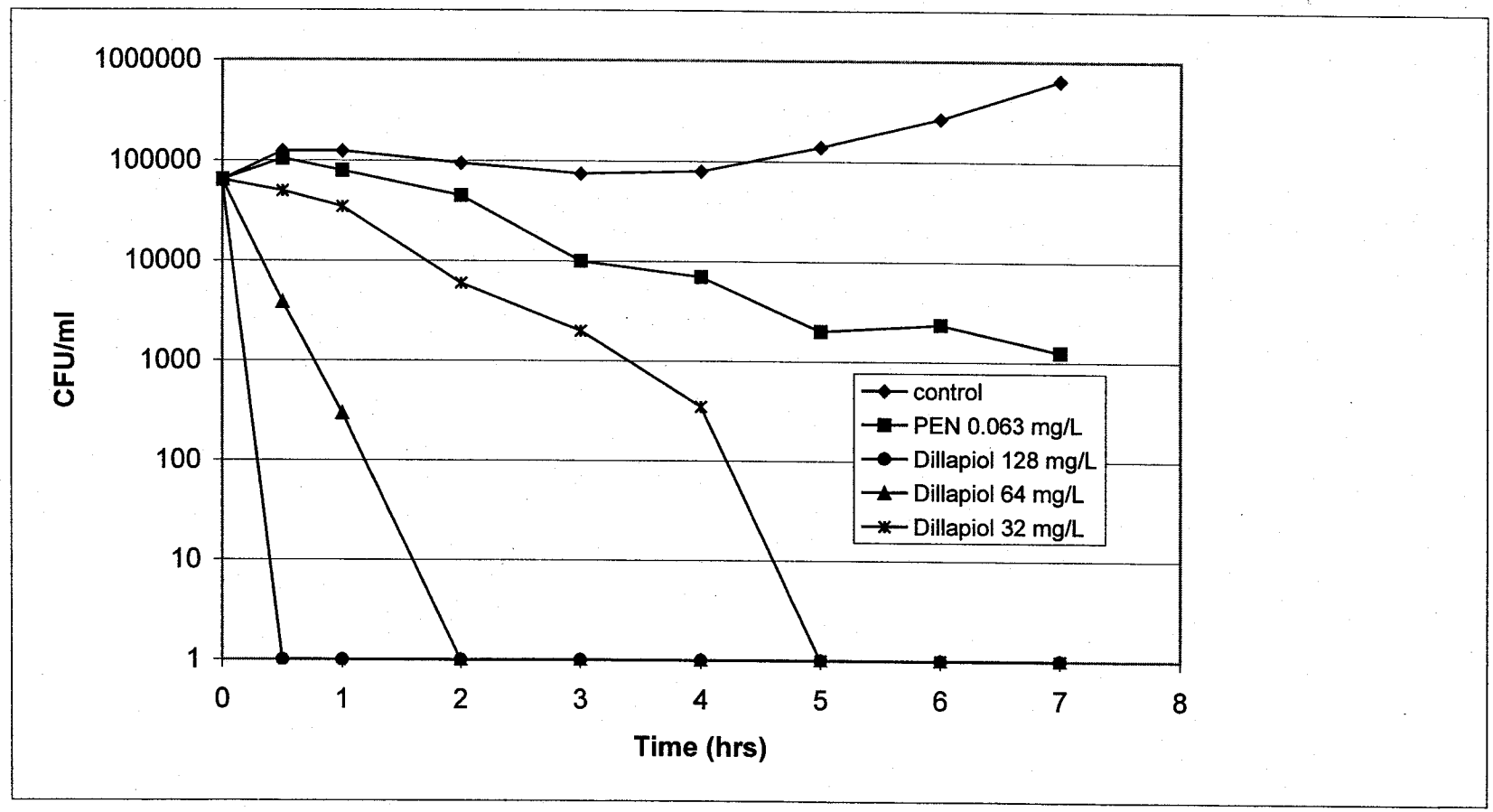
[d]Previous independent expt found inhibition of ~27% (n=30; see Table 11)

4.2.3 Dillapiol time kill curves

Bacterial time kill curves show the killing rate of a substance over a specified time period and are thus more comprehensive than the single 18-24 hour timepoint of MIC assays. The more dynamic picture that this approach gives is also more therapeutically relevant (Krogstad and Moellering, 1986). For these experiments, *N. gonorrhoeae* strain WHO III was used. Figure 5 shows a 7 hour time kill curve performed using dillapiol at three concentrations: 32 mg/l (MIC), 64 mg/l (MIC +1) and 128 mg/l (MIC +2). The rate of killing at the MIC +1 and MIC +2 concentrations was very rapid, with the viable count of cells falling to 0 CFU/ml within 2 hours and 30 mins respectively. At 32 mg/l, the viable count of cells dropped to 0 at the 5 hour timepoint, indicating that this concentration is bactericidal. When compared to 0.063 mg/l of penicillin (the equivalent MIC of this antibiotic), dillapiol's rate of killing at the MIC is much more pronounced, as there were still >1000 CFU/ml at the 5 hour timepoint with penicillin. Another time kill curve (data not shown) was performed over a two hour time period using WHO III and dillapiol at MIC +1 and MIC +2 concentrations, which showed the viable count of cells falling to 0 CFU/ml within 1 hour and 15 minutes respectively.

A single time kill curve experiment was also performed using combinations of dillapiol and penicillin to try and better discern what, if any, synergistic interactions might be occurring. According to Krogstad and Moellering (1986), synergy can be defined here as $\geq$ 100 fold increased killing by the combination versus either drug alone (as measured by CFU/ml). It is especially important to use subinhibitory concentrations of both drugs to

Figure 5. 7 hour time kill curve with 32 mg/l (MIC), 64 mg/l (MIC +1) and 128 mg/l (MIC +2) dillapiol against *N. gonorrhoeae* WHO III. Note that an equivalent concentration of penicillin (0.063 mg/l) kills more slowly than 32 mg/l dillapiol (compare at the 5 hour timepoint). At 128 mg/l and 64 mg/l, the viable count of cells has fallen to 0 CFU/ml within 30 mins and 2 hours respectively.


1000000
100000
10000
1000
100
10
1
CFU/ml
0
1
2
3
4
5
6
7
8
Time (hrs)
control
PEN 0.063 mg/L
Dillapiol 128 mg/L
Dillapiol 64 mg/L
Dillapiol 32 mg/L

ensure a synergistic interaction is not confused with an additive one. For this experiment, the PPNG strain PR6 (MIC to penicillin: 8 mg/l; MIC to dillapiol: 64 mg/l) was used. Figure 6 shows the kill curves for penicillin and dillapiol alone at their MICs towards PR6. This figure represents the benchmark from which penicillin/dillapiol combinations are compared. It can be seen that dillapiol is more effective at killing strain PR6 than penicillin at the equivalent MIC concentration. For the combinations, a subinhibitory concentration of dillapiol (32 mg/l; MIC -1) was combined with 4 (MIC-1), 2 (MIC-2) and 1 (MIC-3) mg/l of penicillin, with corresponding concentrations of penicillin alone used as controls. Figure 7 shows the results of the 4 mg/l penicillin and 32 mg/l dillapiol combination. For each substance alone, the killing rate appears to be quite similar up to 3 hours, after which penicillin causes a higher drop in the viable count. The viable count appears to begin rising again around the 3 hour mark with both dillapiol situations.When these two concentrations are combined, the rate of killing is increased significantly, and at the 3 hour timepoint, the viable count is approximately 2 orders of magnitude lower than either dillapiol or penicillin alone. Thus, by Krogstad and Moellering's definition, this would be considered synergy. However, after 3 hours, the viable count begins to climb again, suggesting some other interaction may be at work. An extended time period (10+ hours) is required to better characterize this. Figure 8 shows the results of the 2 mg/l penicillin and 32 mg/l dillapiol combination. The killing profile of either substance alone is roughly equivalent, while the combination elicits about a 10 fold drop in the viable count. Similar to the combination with 4 mg/l penicillin, after 3 hours the viable count begins to increase, and the rate of increase is higher as well. A similar pattern was observed for the 1 mg/l penicillin and 32 mg/l dillapiol combination (Figure 9).

Figure 6. 5 hour dillapiol time kill curve performed against *N. gonorrhoeae* strain PR6 (PPNG; MIC to penicillin: 8 mg/l; MIC to dillapiol: 64 mg/l) using both dillapiol and penicillin at their MIC concentrations. All subinhibitory combinations (Figures 7-9) are compared to these kill curves.

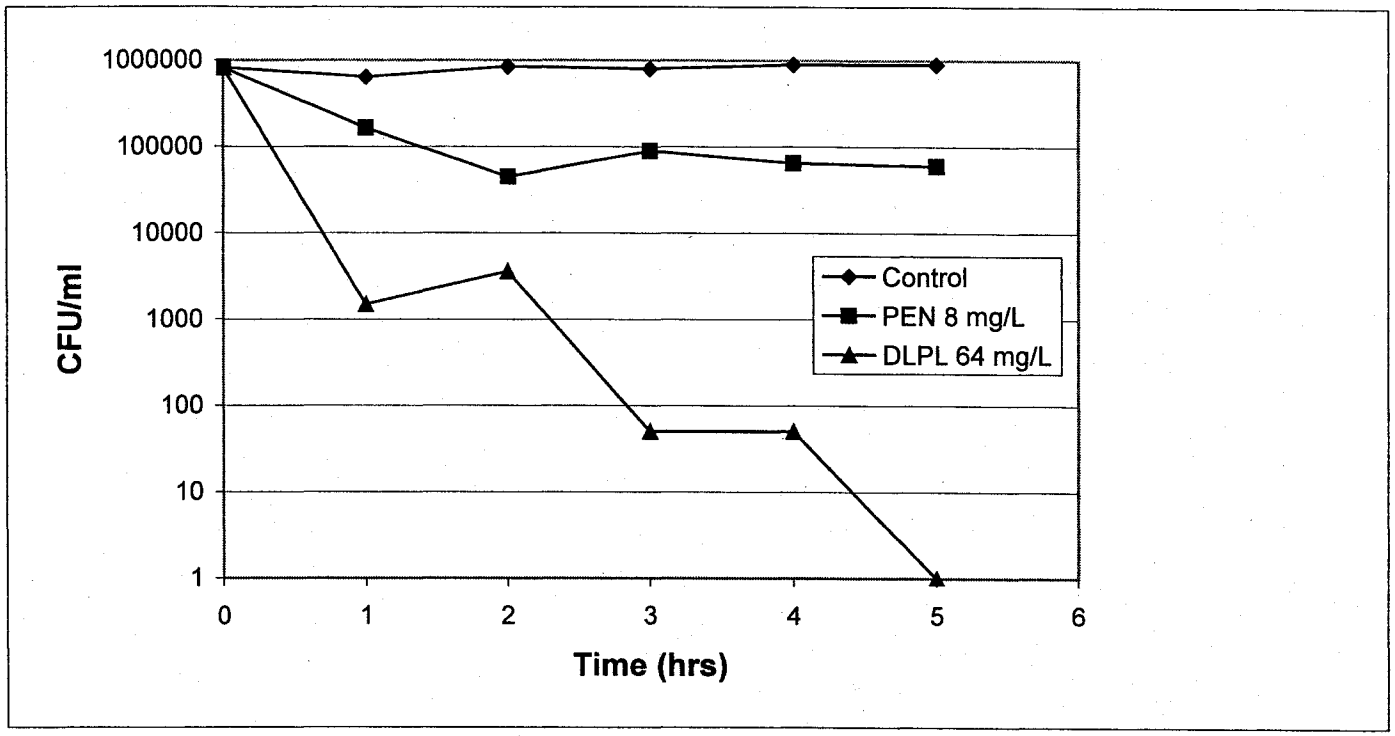

1000000
100000
10000
1000
100
10
1
CFU/ml
0
1
2
3
4
5
6
Time (hrs)
Control
PEN 8 mg/L
DLPL 64 mg/L

Figure 7. 5 hour dillapiol time kill curve performed against *N. gonorrhoeae* strain PR6 at concentrations of 32 mg/l dillapiol (MIC -1) and/or 4 mg/l penicillin (MIC -1). Note that the killing profile of either dillapiol or penicillin at the MIC -1 concentration is very similar for the first 3 hours, while the combination of these two elicits a significant drop in the viable count of cells.

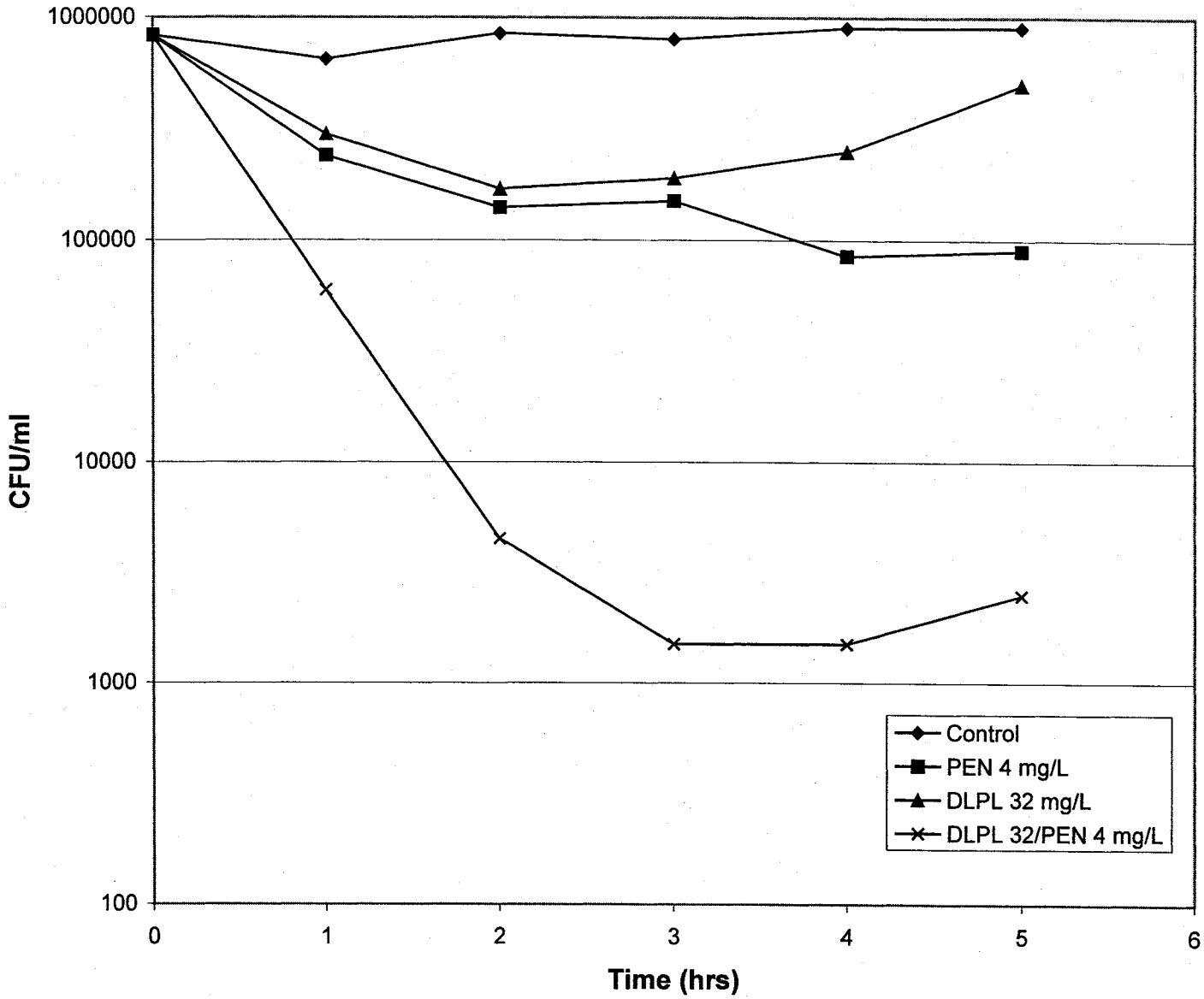

1000000
100000
10000
1000
100
CFU/ml
0
1
2
3
4
5
6
Time (hrs)
Control
PEN 4 mg/L
DLPL 32 mg/L
DLPL 32/PEN 4 mg/L

Figure 8. 5 hour dillapiol time kill curve performed against *N. gonorrhoeae* strain PR6 at concentrations of 32 mg/l dillapiol (MIC -1) and/or 2 mg/l penicillin (MIC -2). The killing rate of either dillapiol or penicillin individually at these concentrations are quite similar, while the combination is somewhat more effective.

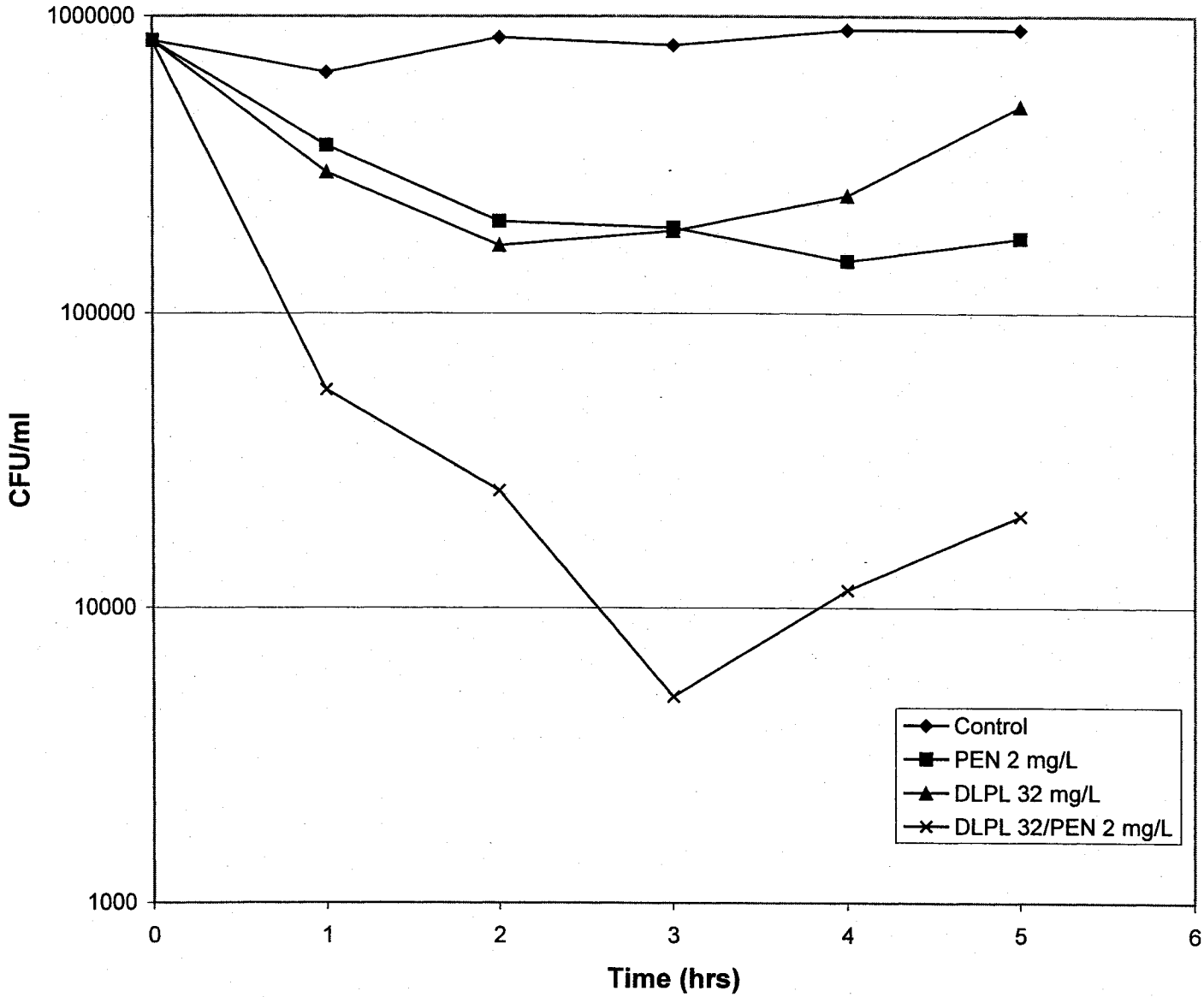
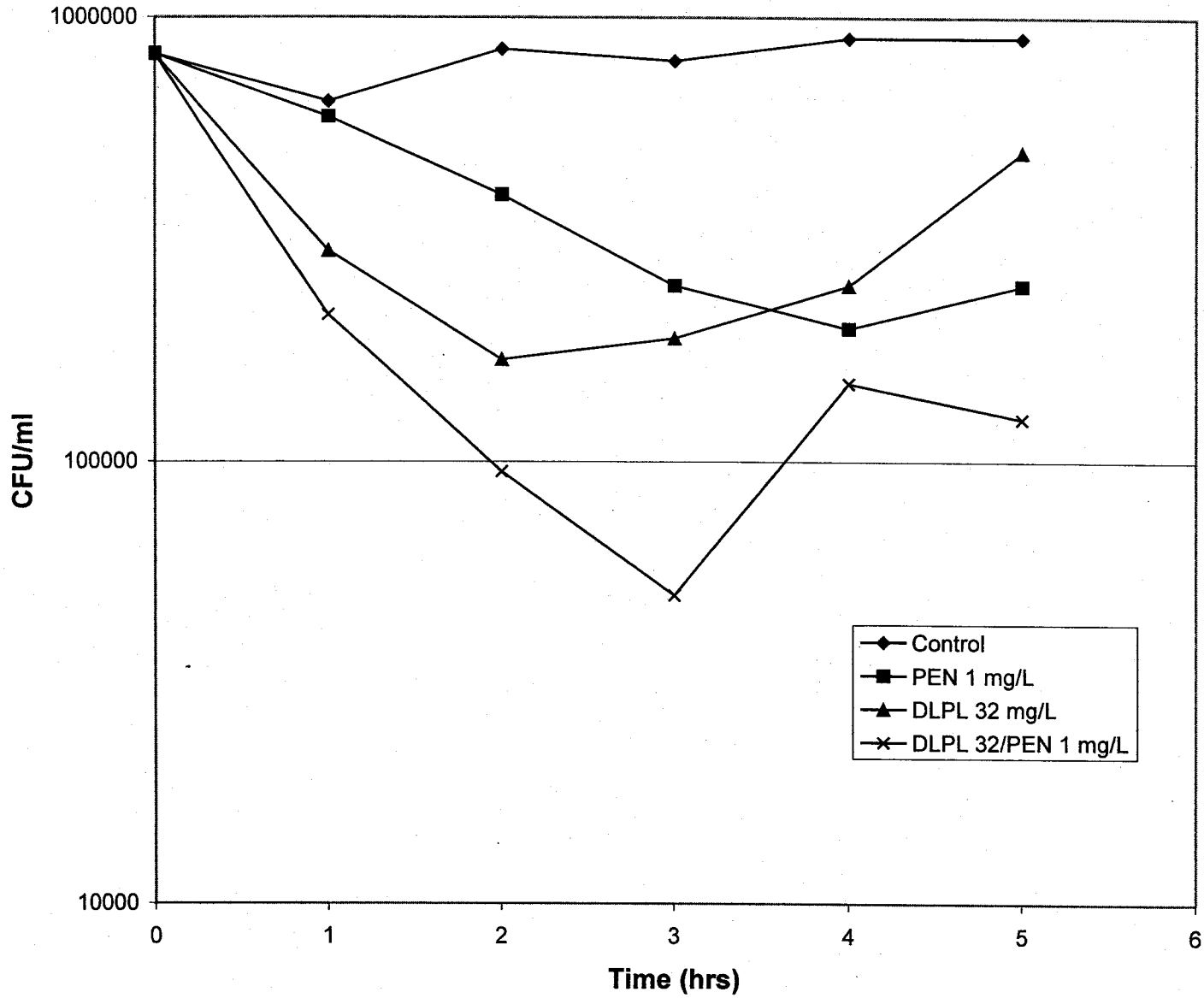

1000000
100000
10000
1000
CFU/ml
0
1
2
3
4
5
6
Time (hrs)
Control
PEN 2 mg/L
DLPL 32 mg/L
DLPL 32/PEN 2 mg/L

Figure 9. 5 hour dillapiol time kill curve performed against *N. gonorrhoeae* strain PR6 at concentrations of 32 mg/l dillapiol (MIC -1) and/or 1 mg/l penicillin (MIC -4).


1000000
100000
10000
CFU/ml
0
1
2
3
4
5
6
Time (hrs)
Control
PEN 1 mg/L
DLPL 32 mg/L
DLPL 32/PEN 1 mg/L

4.3 Discussion

The combination studies show that significant reductions in the MICs of both susceptible and resistant *N. gonorrhoeae* strains can be achieved using antibiotic/phytochemical combinations, with the silibinin/dillapiol/penicillin combination being the most effective. Although silibinin alone had lower antimicrobial activity than dillapiol, the silibinin/penicillin combination was also more effective than the dillapiol/penicillin combination. Two important issues must be addressed when considering such experimental combinations. First is whether the combination has actually reduced the MIC of a resistant strain (to a particular antibiotic) to the point that the strain is effectively no longer resistant (e.g. <2 mg/l for penicillin). As the above results show, this was seen with one strain in the dillapiol/penicillin combination and 4 strains in the ternary combination (all CMRNG and/or PPNG strains).

The second issue is whether an actual synergistic interaction is taking place (versus a simple additive effect) since this has therapeutic relevance. It is important to note that the 4-fold reduction criterion means only that the reduction is *significant*, not necessarily that a synergistic interaction between the phytochemical and the antibiotic is taking place. However, the fractional inhibitory concentration index provides a useful method of quantitating such reductions by comparing the change(s) in MICs between two drugs alone and in combination (see chapter 2.4). Table 13 shows the results of applying the FICI calculation to the penicillin/silibinin combination and Table 14 shows the same for the penicillin/dillapiol combination. It should be noted that if a strain was inhibited by 64 mg/l silibinin alone, it could not be included in the FICI calculation because the combination MIC

Table 13. FICI values calculated for silibinin (SLBN)/penicillin (PEN) combination controls of 3 way combination experiment. Only interpretable strains included (n = 15; 75% of tested strains).

	PEN MICs (mg/l)			SLBN MICs (mg/l)					
Strain (PR)	**combo**[a]	alone	FIC_A	**combo**[a]	alone	FIC_B	FICI	Interpretation 1[b]	Interpretation 2[c]
2	0.125	0.25	0.5	64	128	0.5	1	A	A
3	0.125	0.25	0.5	64	128	0.5	1	A	A
8	4	16	0.25	64	128	0.5	0.75	A	WS
12	1	4	0.25	64	128	0.5	0.75	A	WS
13	2	4	0.5	64	256	0.25	0.75	A[d]	WS
14	0.25	1	0.25	64	128	0.5	0.75	A	WS
16	64	128	0.5	64	256	0.25	0.75	A	WS
17	64	64	1	64	256	0.25	1.25	A	A
19	32	64	0.5	64	128	0.5	1	A	A
20	4	16	0.25	64	256	0.25	0.5	S[d]	S
21	16	32	0.5	64	128	0.5	1	A	A
23	0.25	0.5	0.5	64	128	0.5	1	A	A
25	0.25	1	0.25	64	256	0.25	0.5	S	S
28	4	16	0.25	64	128	0.5	0.75	A	WS
29	0.5	2	0.25	64	256	0.25	0.5	S	S

[a]refers to the same experimental combination of silibinin and penicillin

[b]Interpretive criteria of Krogstad and Moellering (1986) (A - additive; S - synergy)

[c]Interpretive criteria of Bharadwaj *et al.* (2003) (A - additive; S - synergy; WS - weak synergy)

[d]because these two strain's silibinin MICs were >256 mg/L, the FICI is at most the value indicated and may be lower

Table 14. FICI values calculated for dillapiol (DLPL)/penicillin (PEN) combination controls of 3 way combination experiment. Only interpretable strains included (n = 18; 90% of tested strains).

	PEN MICs (mg/l)			DLPL MICs (mg/l)					
Strain (PR)	**combo**[a]	alone	FIC_A	**combo**[a]	alone	FIC_B	FICI	Interpretation[b]	Interpretation[c]
1	0.125	0.25	0.5	16	64	0.25	0.75	A	WS
2	0.125	0.25	0.5	16	64	0.25	0.75	A	WS
3	0.125	0.25	0.5	16	64	0.25	0.75	A	WS
5	8	16	0.5	16	64	0.25	0.75	A	WS
8	8	16	0.5	16	64	0.25	0.75	A	WS
12	1	4	0.25	16	64	0.25	0.5	S	S
13	2	4	0.5	16	64	0.25	0.75	A	WS
14	0.25	1	0.25	16	32	0.5	0.75	A	WS
16	64	128	0.5	16	64	0.25	0.75	A	WS
17	64	64	1	16	64	0.25	1.25	A	A
19	64	64	1	16	64	0.25	1.25	A	A
20	8	16	0.5	16	32	0.5	1	A	A
22	0.063	0.063	1	16	32	0.5	1.5	A	A
23	0.25	0.5	0.5	16	64	0.25	0.75	A	WS
24	0.063	0.063	1	16	256	0.0625	1.0625	A	A
25	1	1	1	16	64	0.25	1.25	A	A
27	0.063	0.063	1	16	64	0.25	1.25	A	A
28	8	16	0.5	16	64	0.25	0.75	A	WS

[a]refers to same experimental combination of penicillin and dillapiol

[b]Interpretive criteria of Krogstad and Moellering (1986) (A - additive; S - synergy)

[c]Interpretive criteria of Bharadwaj *et al.* (2003) (A - additive; S - synergy; WS - weak synergy)

would be higher than the MIC alone. This is why the FICI could only be used on 15 of the strains from the silibinin combination study. This approach found that of the 15 interpretable strains that showed a reduction in MICs, 20% were synergistic while the remaining 80% were additive (Table 13). For the dillapiol/penicillin combination (18 interpretable strains), 5% were synergistic while the remaining 95% were additive (Table 14).

If the criterion of Bharadwaj *et al.* (2003) were used, then for the silibinin/penicillin combination, the same 20% of strains would be considered synergistic, while the remaining 80% would become weak synergy instead of no interaction. For the dillapiol/silibinin combination, of the 95% considered additive by the Lorian criteria, 61% would be classified as weak synergy. The FICI was not applied to the 3 way combination of penicillin/silibinin/dillapiol because the mathematical derivation for such a 3 part combination could not be readily determined. However, it is likely that if this was done, the proportion of synergistic interactions would be significantly higher.

It should also be noted that because a relatively high silibinin concentration was used, the $FIC_{SILIBININ}$ was rarely less than 0.5. For this reason, even if the FICI was applied to the strain showing a 32-fold MIC reduction in the other independent silibinin/penicillin experiment (see Table 10), the FICI would still only be ~0.53, and would not thus be considered synergy according to some criteria, although clearly if synergy is happening anywhere it is with just such a strain and phytochemical/antibiotic combination. Thus, one way to better resolve such experiments in the future might be to try a lower concentration of

silibinin in the combinations (MIC-2 instead of MIC-1) as this would require a smaller antibiotic MIC reduction to classify as synergy.

The dillapiol time-kill curves showed that dillapiol, at its MIC, killed *N. gonorrhoeae* at a greater rate than an equivalent concentration of penicillin. This suggests that whatever dillapiol's mechanism is, it is clearly more rapid than the mechanism employed by penicillin. It is difficult to conclude that a synergistic effect was seen with the combination time-kill curves. Only with the MIC -1/MIC -1 combination of dillapiol and penicillin was a large enough drop in viable counts seen to qualify as synergy (Figure 7). Using longer timepoints and perhaps a lower dillapiol concentration (e.g. 16 mg/l) could help. The results could then be compared to the agar dilution data (Tables 11 and 12) to see if any CFU reductions are reflected by MIC reductions at the time (~20 hours) that MIC results are normally recorded. The rise in the viable count towards the end of the time period is an interesting phenomenon that requires more investigation. This could represent selection of *N. gonorrhoeae* cells for adaptation to dillapiol exposure. Since the agar dilution methods show that lower dillapiol concentrations (16 mg/l) can yield a concrete reduction in penicillin MICs, perhaps this increase is only temporary.

CHAPTER 5 – ANTIMICROBIAL MECHANISM OF DILLAPIOL

5.1 Introduction

Permeabilization assays using the nonpolar hydrophobic fluorescent probe 1-N-phenylnapthylamine (NPN) were undertaken to better characterize dillapiol's possible mechanism of action. Permeabilization was considered to be a good candidate as dillapiol's putative antibacterial mechanism, due to its wide spectrum of activity against various *N. gonorrhoeae* resistant phenotypes (i.e. likely some general mechanism is at work) and anecdotal evidence that it permeabilizes eukaryotic cells. The lipopolysaccharide (LPS)-containing outer membrane (OM) of Gram negative bacteria provides a hydrophilic surface that allows small non-polar molecules to enter but serves as an effective permeability barrier to larger or more polar ones (Nikaido, 1999). It has thus been proposed that the OM (which also serves to anchor many antibiotic efflux pumps) confers higher resistance to the insult of many phytochemicals, which makes the activity of dillapiol more intriguing if it does indeed mediate its effects this way (Tegos *et al.*, 2002; Nikaido, 1999).

NPN fluoresces weakly in an aqueous environment and strongly in a hydrophobic environment (such as a bacterial membrane) and is hence an ideal probe for investigating membrane permeabilization as higher fluoresence indicates membrane permeabilization. Upon exposure to a permeabilized cell membrane, NPN partitions into the hydrophobic membrane and increases its fluorescence (Helander & Mattila-Sandholm, 2000; Loh *et al.*, 1984). Loh *et al.* (1984) describes an early application of this probe for examining the effect of aminoglycosides against the OM of *Pseudomonas aeruginosa.* Various studies since have used modifications of this approach for similar uses. Helander *et al.* (1998) used this

approach to analyze the permeabilizing ability of various essential oils on the OM of *E. coli* and *S. typhimurium*, making this a logical assay to try with dillapiol against *N. gonorrhoeae*. Helander and Mattila-Sandholm (2000) report using a fluoresence microplate reader with NPN to perform this assay, which is advantageous as it allows the simultaneous measurement of the various experimental combinations necessary to analyze this assay. This simplifies comparison of controls as all experimental combinations are analyzed at essentially the same time (within 1-2 minutes).

After reviewing the literature available on the subject, and evaluating the most cost effective method considering the equipment and time available, it was decided to proceed using a modification of Helander and Mattila-Sandholom's (2000) approach. Because *N. gonorrhoeae* had never been tested in such a fluoresence assay, a number of changes to this protocol, as well as the necessary method validation, was performed prior to the actual experiments. The first step was to repeat Helander and Mattila-Sandholm's experiment with our equipment to compare any differences in the results. The original study had used *Salmonella typhimurium* ATCC-13311 as the test organism, a Fluoroskan Ascent FL microplate fluorometer and the following filters: 355 ± 38 nm (excitation) and 405 ± 50 nm (emission). For the comparison assay, *Salmonella typhimurium* ATCC-14028 was used as the test organism, with a Cytofluor 4000 microplate fluorometer and the following filters: 360 ± 40 nm (excitation) and 409 ± 20 nm (emission). Also, the gain (intensity) of the photomultipler tube that feeds the filters was set at three different intensities to see if this would have any effect on the relative fluorescent values observed (see Appendix B.1 for

further details of this work). Besides these changes, the assay was performed essentially as in Helander and Mattila-Sandholm (2000).

A number of supplements can be added to this assay to increase this assay's resolution. For example, divalent metal cations (e.g. Ca^{2+}, Mg^{2+}) are critical for stabilizing the outer bacterial membrane by modulating the overall charge of the membrane. These ions are attracted to the bacterial OM and stabilize it by inserting between negatively charged LPS molecules, allowing closer packing of membrane components (i.e. reducing electrostatic repulsion) (Maisch et al., 2004). Thus, if dillapiol does permeabilize the OM, then addition of such cations should abrogate dillapiol's effects, which would be seen as a reduction in fluorescence.

5.2 Results and Discussion

5.2.1 Initial *N. gonorrhoeae* trial

Table 15 shows the results of combining 64 and 128 mg/l dillapiol against WHO V in the NPN assay system. This was actually a repeat of a previous experiment, except that 0.7% CAA was used as the buffer instead of 5 mM HEPES. This change was done due to the abnormally high fluorescence ratios seen with HEPES buffer, which is known to lyse *N. gonorrhoeae* cells and hence was probably creating the artificially high ratios (see Appendix B for more details). As Table 15 indicates, the fluorescence ratio for the cells + NPN was 7, compared to 12.2 when HEPES was used instead of 0.7% CAA (see Table 15). Also, the NPN fluorescence ratios for 64 and 128 mg/l dillapiol with no cells in 0.7% CAA (5.3 and 17.6 respectively) corresponded well to the equivalent values in 5 mM HEPES (5.6 and 14.9

Table 15. Fluorescence (F) values obtained in an NPN uptake assay using Cytofluor 4000 system (filters 360/40 (EX) and 409/20 (EM); gain 50) and *N. gonorrhoeae* WHO V as the test organism. Methods follow those of Helander & Mattila-Sandholm (2000).

Sample	NPN	F value (mean ± SD)[a]	F value after background subtraction	F ratio[b]	Corrected F ratios[c]
0.7% CAA (buffer)[d]	-	90.8 ± 0.8			
	+	122.8 ± 0.3	32	1	
+ cells	-	103.5 ± 0.8			
	+	329 ± 1	225.5	7	
+ 128 mg/L dillapiol	-	94 ± 0.7			
	+	657.1 ± 5.2	563.1	17.6	
+ cells + 128 mg/L dillapiol	-	115.3 ± 0.6			
	+	1299.6 ± 4.6	1184.3	37	19.4
+ 64 mg/L dillapiol	-	89.7 ± 1.0			
	+	258.9 ± 0.1	169.2	5.3	
+ cells + 64 mg/L dillapiol	-	105.8 ± 0.7			
	+	921 ± 0.6	814.3	25.5	20.2
+ cells + 1mM EDTA	-	111.7 ± 0.7			
	+	362.7 ± 2.1	251	8.1	
+cells + 0.1 mM EDTA	-	107.9 ± 1.1			
	+	373.8 ± 0.5	265.9	8.6	

[a]All values represent average F values of 3 cycles on the Cytofluor 4000 (4 parallel wells/sample/cycle) [n = 12]

[b]represents ratio of background-subtracted F values to that of the buffer value

[c]represents the total fluorescence seen minus the contribution from dillapiol itself

[d]CAA - casamino acids

respectively). The absolute fluorescence of the dillapiol + cell mixtures in 0.7% CAA were approximately twice as high as in 5 mM HEPES, however, once the background fluorescence contributed by dillapiol was taken into account, the NPN uptake values for 64 and 128 mg/l dillapiol were 20.2 and 19.4 respectively, compared to 27.2 and 20.5 respectively when 5 mM HEPES was used. Thus, it seems the discrepancy in NPN fluorescence ratios seen with HEPES (with the lower dillapiol concentration leading to apparantly higher uptake) is avoided when 0.7% CAA is used. Interestingly, 0.1 mM and 1 mM EDTA (used as positive controls) caused only a marginal increase in NPN uptake (from 7 to 8.6 and 8.1 respectively).

5.2.2 *N. gonorrhoeae* strains PR6 and PR8.

The Cytofluor assay system was then tested on two PPNG strains, PR6 and PR8 (MICs to penicillin of 8 mg/l and 16 mg/l respectively). The setup was as previously described and 0.7% CAA was used as the buffer. As Table 16 shows, the NPN fluorescence ratios for 128 mg/l of dillapiol (no cells) were slightly higher than the previous trial using 0.7% CAA (21.2 versus 17.6) while for 64 mg/l dillapiol (no cells), the uptake was almost three times higher (15.8 versus 5.3). For PR6 plus 64 mg/l and 128 mg/l dillapiol, the corrected NPN fluorescence ratios were 29.4 and 25.4 respectively, while for PR8 the corrected NPN fluorescence ratios were 35.2 and 30.4 respectively. Thus, when compared to the cell plus buffer combinations for PR6 and PR8 (19.1 and 18 respectively) these values are highly suggestive of permeabilization. It is notable that the NPN uptake for the strains alone are about 2.5 times greater than found with WHO V in the previous experiment. Since the cell densities (as measured by absorbance) was similar between these two experiments

Table 16. Fluorescence (F) values obtained in an NPN uptake assay using the Cytofluor 4000 system (filters 360/40 (EX) and 409/20 (EM); gain 50) and *N. gonorrhoeae* PR6 and PR8 as the test organisms.

Sample	NPN	F value (mean ± SD)[a]	F value after background subtraction	F ratio[b]	Corrected F ratios[c]
CAA	-	86.8 ± 0.8			
	+	110.3 ± 0.6	23.5	1	
+ 128 mg/l dillapiol	-	94.7 ± 0.9			
	+	592.8 ± 18.8	498.1	21.2	
+ 64 mg/l dillapiol	-	88.2 ± 0.9			
	+	458.5 ± 9.6	370.3	15.8	
+ cells (PR6)	-	103 ± 1.3			
	+	551.2 ± 9.3	448.2	19.1	
+ cells (PR6) + 128 mg/L dillapiol	-	108.4 ± 0.7			
	+	1202.8 ± 1.0	1094.4	46.6	25.4
+ cells (PR6) + 64 mg/L dillapiol	-	114 ± 0.8			
	+	1176 ± 4.1	1062	45.2	29.4
+ cells (PR8)	-	102.3 ± 1.0			
	+	525.3 ± 10.7	423	18	
+ cells (PR8) + 128 mg/L dillapiol	-	105.5 ± 0.9			
	+	1318.5 ± 1.6	1213	51.6	30.4
+ cells (PR8) + 64 mg/L dillapiol	-	107.7 ± 0.6			
	+	1306.2 ± 3.6	1198.5	51	35.2

[a]All values represent average F values of 3 cycles on the Cytofluor 4000 (4 parallel wells/sample/cycle) [n = 12]

[b]represents ratio of background-subtracted F values to that of the buffer value

[c]represents the total fluorescence seen minus the contribution from dillapiol itself

(0.51-0.55), this is likely not the cause of the increased fluorescence. It may simply be a strain specific difference (*i.e.* genetic variation).

5.2.3 Addition of $MgCl_2$ to the test system.

If dillapiol is permeabilizing the membrane, then addition of excess $MgCl_2$ should provide the divalent magnesium ions necessary to stabilize the permeabilized membrane. First, 10 mM $MgCl_2$ was added to the test system (WHO V and 0.7% CAA) with 128 mg/l dillapiol. Table 17 presents the results of these combinations, with (corrected) NPN fluorescence ratios of 15.6 (cells), 25.9 (cells + 128 mg/l dillapiol) and 21.8 (cells + 128 mg/l dillapiol + 10 mM $MgCl_2$). While the addition of 10 mM $MgCl_2$ did reduce the NPN uptake in the cell + 128 mg/l dillapiol combination (from 25.9 to 21.8), it was still significantly higher than the cell's NPN uptake alone (21.8 versus 15.6).

This experiment was repeated using 20 mM $MgCl_2$ in the same system. Table 18 shows the results of these combinations (two independent experiments), with NPN fluorescence ratios of 16.7 (cells), 32.6 (cells + 128 mg/l dillapiol) and 18.2 (cells + 128 mg/l dillapiol + 20 mM $MgCl_2$) for the first experiment. The addition of 20 mM $MgCl_2$ appears to almost completely abrogate the effects of dillapiol, as the NPN uptake (18.2) is very close to the unpermeablized cell value of 16.7. In a repeat of this experiment (see also Table 18) the values obtained were 17.8 (cells), 27 (cells + 128 mg/l dillapiol) and 21.3 (cells + 128 mg/l dillapiol + 20 mM $MgCl_2$). Although the reduction in NPN fluorescence ratios was not as large as the first trial, the $MgCl_2$ supplemented value (21.3) is still quite close to the cell control (17.8). These results strongly suggest that dillapiol's damage is

Table 17. Fluorescence (F) values obtained in an NPN uptake assay using the Cytofluor 4000 system (filters 360/40 (EX) and 409/20 (EM); gain 50) with *N. gonorrhoeae* WHO V and 10 mM $MgCl_2$ added to the system.

Sample	NPN	F value (mean ± SD)[a]	F value after background subtraction	F ratio[b]	Corrected F ratios[c]
CAA	-	92.8 ± 0.6			
	+	108.5 ± 0.5	15.7	1	
+ cells	-	106.9 ± 0.6			
	+	352 ± 0.9	245.1	15.6	
+ 128 mg/L dillapiol	-	94.3 ± 0.9			
	+	459.5 ± 15.5	365.2	23.3	
+ cells + 128 mg/L dillapiol	-	116.9 ± 0.8			
	+	888.7 ± 6.1	771.8	49.2	25.9
+ 64 mg/L dillapiol	-	91.8 ± 1.1			
	+	361 ± 8.8	269.2	17.2	
+ cells + 64 mg/L dillapiol	-	109.1 ± 1.0			
	+	819.2 ± 1.6	710.1	45.2	28
+ 128 mg/L dillapiol + 10 mM $MgCl_2$	-	90.8 ± 0.7			
	+	429.3 ± 18.6	338.5	21.6	
+ 128 mg/L dillapiol + 10 mM $MgCl_2$ + cells	-	106.1 ± 1.0			
	+	787 ± 8.7	680.9	43.4	21.8

[a]All values represent average F values of 3 cycles on the Cytofluor 4000 (4 parallel wells/sample/cycle) [n = 12]

[b]represents ratio of background-subtracted F values to that of the buffer value

[c]represents the total fluorescence seen minus the contribution from dillapiol or any other additives themselves

Table 18. Fluorescence (F) values obtained in an NPN uptake assay using the Cytofluor 4000 system with N. gonorrheoeae WHO V and 20 mM $MgCl_2$ added to the system.

Sample	NPN	F value (mean ± SD)[a]	F value after background subtraction	F ratio[b]	Corrected F ratios[c]
CAA	-	83 ± 0.8			
	+	98.1 ± 0.4	15.1	1[d]	
	-	92.4 ± 1.0			
	+	107.8 ± 0.4	15.4	1[d]	
+ cells	-	104.2 ± 0.8			
	+	356.8 ± 2.6	252.6	16.7	
	-	106.4 ± 0.6			
	+	379.8 ± 1.0	273.4	17.8	
+ 128 mg/L dillapiol	-	84.3 ± 0.6			
	+	516.8 ± 11.6	432.5	28.6	
	-	95.3 ± 0.9			
	+	454.3 ± 21.8	359	23.3	
+ cells + 128 mg/L dillapiol	-	113.2 ± 0.8			
	+	1037.3 ± 2.1	924.1	61.2	32.6
	-	118.8 ± 1.0			
	+	892.9 ± 12.6	774.1	50.3	27
+ 128 mg/L dillapiol & 20 mM $MgCl_2$	-	83.3 ± 0.7			
	+	415.3 ± 9.3	332	22	
	-	92.5 ± 1.0			
	+	390.8 ± 16.9	298.3	19.4	
+ 128 mg/L dillapiol + 20 mM $MgCl_2$ + cells	-	105.7 ± 0.6			
	+	713 ± 6.0	607.3	40.2	18.2
	-	110 ± 0.9			
	+	736.9 ± 12.5	626.9	40.7	21.3
+ 20 mM $MgCl_2$	-	81.4 ± 0.8			
	+	95.5 ± 0.4	14.1	<1[e]	
	-	90.3 ± 0.5			
	+	109.5 ± 0.5	19.2	1.3[e]	
+cells + 20 mM $MgCl_2$	-	102.8 ± 0.8			
	+	342.7 ± 3.0	239.9	15.9	15.9
	-	105.9 ± 0.8			
	+	382.7 ± 4.6	276.8	18	16.7

[a]All values represent average F values of 3 cycles on the Cytofluor 4000 (4 parallel wells/sample/cycle)

[b]represents ratio of background-subtracted F values to that of the buffer value

[c]represents the total fluorescence seen minus the contribution from dillapiol or any other additives themselves

[d]Each data pair represents NPN uptake values from two independent experiments

[e]these values are low enough to be negligible to the overall total fluorescence

occurring by displacement of cations in the bacterial OM, since the addition of Mg^{2+} significantly reduces NPN uptake caused by dillapiol.

5.2.4 Addition of 1 mM KCN to the test system.

Cyanide was added to the test system to more explicitly determine what the increase in NPN uptake represents. No change in NPN uptake when KCN is added would indicate that NPN uptake is measuring actual OM permeabilization instead of secondary membrane weakening (through inhibition of cellular metabolism) (Helander and Mattila-Sandholm, 2000). *N. gonorrhoeae* strain WHO III was used for this assay. Table 19 shows a higher NPN fluorescence ratio (19.6) for the cells in buffer alone, which was very close to the NPN fluorescence ratio for 128 mg/l dillapiol in buffer (19.9). Indeed, when background fluorescence is taken into account, the cell + 64 or 128 mg/l dillapiol combinations with or without KCN were very close (26.8 versus 25.8 for 64 mg/l dillapiol and 20.9 versus 21.4 for 128 mg/l dillapiol respectively). Interestingly, the NPN uptake for cells + 1 mM KCN (no dillapiol) was lower (17.1) than the cells alone (see above), suggesting that secondary weakening of the membrane does not affect the NPN uptake values elicited by dillpiol.

It is difficult to compare these results to the literature due to the variety of methods used to perform and interpret these assays, as well as the diversity of substrates tested. However, Helander *et al.* (1998) examined the effect of essential oils (similar to dillapiol) on the OMs of *E. coli* and *S. typhimurium*. They found that 0.5 mM and 1 mM concentrations of the essential oil thymol (equivalent to about 75 mg/l and 150 mg/l respectively) caused a (corrected) increase in fluorescence values of ~22 and ~44 respectively for *E. coli*. The

Table 19. Fluorescence (F) values obtained in an NPN uptake assay using Cytofluor 4000 system with *N. gonorrhoeae* WHO V with 1 mM KCN added to the system.

Sample	NPN	F value (mean ± SD)[a]	F value after background subtraction	F ratio[b]	Corrected F ratios[c]
CAA	-	84.8 ± 0.7			
	+	104.3 ± 0.1	19.5	1	
+ cells	-	118.5 ± 0.7			
	+	499.9 ± 0.1	381.4	19.6	
+ 128 mg/L dillapiol	-	87.8 ± 1.5			
	+	476.5 ± 14.4	388.7	19.9	
+ 128 mg/L dillapiol + cells	-	122.8 ± 0.8			
	+	889.6 ± 4.3	766.8	39.3	19.4
+ 64 mg/L dillapiol	-	88.2 ± 0.9			
	+	264.7 ± 3.1	176.5	9.1	
+ 64 mg/L dillapiol + cells	-	130.8 ± 0.6			
	+	811.3 ± 2.4	680.5	34.9	25.8
+ 1 mM KCN	-	83.7 ± 0.5			
	+	102.5 ± 0	18.8	<1	
+ 1 mM KCN + cells	-	120 ± 0.8			
	+	453.8 ± 5.7	333.8	17.1	17.1
+ 128 mg/L dillapiol/ 1 mM KCN	-	86.9 ± 0.3			
	+	445.5 ± 13.5	358.6	18.4	
+ 128 mg/L dillapiol/ 1 mM KCN + cells	-	121.1 ± 1.0			
	+	887.2 ± 1.3	766.1	39.3	20.9
+ 64 mg/L dillapiol/ 1 mM KCN	-	86.6 ± 0.4			
	+	354.3 ± 5.1	267.7	13.7	
+ 64 mg/L dillapiol/ 1 mM KCN + cells	-	121.8 ± 0.9			
	+	910.8 ± 3.1	789.7	40.5	26.8

[a]All values represent average F values of 3 cycles on the Cytofluor 4000 (4 parallel wells/sample/cycle)

[b]represents ratio of background-subtracted F values to that of the buffer value

[c]represents the total fluorescence seen minus the contribution from dillapiol or any other additives themselves

essential oil carvacrol at the same two concentrations elicited an increase of ~15 and ~38 respectively against *E. coli*. Similar fluorescence ratios were also obtained for *S. typhimurium*. These two concentrations are close to the 64 mg/l and 128 mg/l concentrations of dillapiol that were tested against *N. gonorrhoeae*, obtaining fluorescence ratios of ~15-40 (depending on the concentrations and strains used). Hence, these values correspond well to those obtained by Helander *et al.* (1998).

CHAPTER 6 – GENERAL DISCUSSION

This thesis presents the first evidence of the antimicrobial activity of silibinin and dillapiol combinations, and garlic/garlic NHPs against *N. gonorrhoeae*. While susceptibility testing of phytochemicals against bacteria is common, *N. gonorrhoeae* is a rarely tested organism. In fact, besides a few ethnobotanical studies examining crude plant extracts (Van Puyvelde *et al.*, 1983; Caceres *et al.*, 1995), there are few examples of studies employing *N. gonorrhoeae* as a test organism, and no studies involving isolated phytochemicals.

The moderate activity of both pinocembrin and dillapiol (MICs of 64 mg/l) against both resistant and susceptible strains of *N. gonorrhoeae* makes them promising candidates as potential new antimicrobials. For example, their activity in terms of MIC concentrations is comparable to spectinomycin (still in use against gonorrhea at similar concentrations); the resistance breakpoint of *N. gonorrhoeae* to this antibiotic is two fold higher (128 mg/l) than dillapiol or pinocembrin's activity (NCCLS, 2003). Of course, whether such concentrations can be attained at the site of infection in humans remains an unanswered question. Since most cases of uncomplicated gonorrhea are localized to epithelial sites of the urogenitary system, pharmacokinetic and pharmacodynamic studies would be necessary to determine the fate of these phytochemicals taken orally versus a topical application. The current use of milk thistle extract suggests that such studies could be feasible (Anonymous, 1999). Unpublished pharmacokinetic studies of dillapiol from Dr Arnason's lab suggests movement to all major organs in a rat model. The combination results are especially promising because lower therapeutic doses of dillapiol or silibinin (combined with a known antibiotic) would be required and likely more attainable. Also, the fact that these phytochemicals have similar

levels of activity against strains possessing a variety (and often combinations) of resistance mechanisms suggests that they may act through a novel, and likely general (as opposed to specific inhibition of an enzyme for example), mechanism to elicit an antibacterial effect.

Indeed, the fluorescence assays performed with dillapiol against *N. gonorrhoeae* strain WHO V strongly suggest permeabilization of the OM as a possible mechanism of action. This was demonstrated by significantly higher fluorescence values in cell suspensions containing dillapiol and the fluorescent probe NPN than in suspensions containing only the probe, which indicates higher penetration of the probe into the bacterial cell wall. Dillapiol is a relatively small non-polar compound and this is a common property of many chemicals that can penetrate bacterial membranes. It is also likely that the high fluorescence of dillapiol (when added to NPN in the absence of cells) is due to its lipophilic nature as an essential oil The presumed mechanism is thus that dillapiol inserts into the OM and somehow destabilizes its structure leading to collapse of the membrane. Supplementing the test system with $MgCl_2$ reduced the NPN induced fluoresence, which also indicates that membrane destabilization is a likely mechanism, since Mg^{2+} and other such divalent metal ions are well known to be crucial to membrane stability (Helander and Mattila-Sandholm, 2000; Maisch *et al.*, 2004). As the dillapiol time kill experiments indicate, at MIC levels dillapiol kills *N. gonorrhoeae* more rapidly than pencillin (another compound that acts on bacterial cell walls) and it would be interesting to further examine the exact nature of dillapiol's interaction with the cell wall.

The antibacterial activity of silibinin was lower than that of either dillapiol or pinocembrin. Although the related flavonolignan 5'-MHC inhibited an MDR efflux pump in *S. aureus* (potentiating the toxicity of berberine) it is not clear how silibinin exerts its directs antibacterial effect against *N. gonorrhoeae* with no coadministered component. Rifampicin is an antibiotic that, like the fluoroquinolones, inhibits bacterial DNA synthesis (in this case by inhibiting bacterial DNA-dependant RNA polymerase) (Sefton, 2002). Interestingly, silibinin stimulates eukaryotic DNA-dependant RNA polymerase I (responsible for rRNA transcription) (Sonnenbichler *et al.*, 1999). Whether such dissimilar action between two somewhat similar enzymes is significant remains an unanswered question. However, both rifampicin and silibinin are similarly sized large planar molecules (although silibinin contains more ring structures), so perhaps silibinin interacts with bacterial RNA polymerase in some way.

The silibinin/penicillin combination was more effective at reducing MIC towards tested *N. gonorrhoeae* strains than was the dillapiol/penicillin combination. The ternary combination of all three compounds was more effective than either combination alone, although there are currently no satisfactory explanations as to how this reduction is mediated. The fact that only 5% of strains showed definite synergy in the silibinin/penicillin combination does not rule out a simple additive effect, although more work is warranted to better characterize this. Perhaps silibinin is able to inhibit the *mtr* efflux pump of *N. gonorrhoeae* much as 5'-MHC inhibits the NorA efflux pump of *S. aureus* (Stermitz *et al.*, 2000). Measuring the change in transcription and translation levels of the components of the *mtr* efflux pump (using CMRNG strains) would be one way to examine this. Of course, it is

doubtful whether this would improve the activity of penicillin since, being a cell wall inhibitor, it does not need to penetrate the interior of the cell. Concerning the dillapiol/penicillin combination, the permeabilization of the outer membrane by dillapiol could facilitate the entry of penicillin into the membrane, although how effective this would be against a PPNG strain (where the resistance is enzymatically mediated) is uncertain. The possibility that silibinin and/or dillapiol interact with as yet unidentified enzymes (as they certainly do among eukaryotes) cannot yet be ruled out. The inhibition of drug metabolizing enzymes in insects by dillapiol opens an interesting question of whether similar interactions exist in bacteria. For example, if dillapiol somehow inhibited beta-lactamases, this would represent another plausible mechanism.

Few studies have been performed examining the antibacterial activity of flavanones such as pinocembrin (5,7-dihydroxyflavanone). Based on an analysis of quantitative structure activity relationships (QSAR) among a variety of chalcones and flavanones (including pinocembrin), Alcaraz *et al.* (2000) proposed that OH groups at the 2' position of chalcones and the 5 position of flavanones were required for high anti-MRSA activity, while OCH_3 groups in these positions reduce such activity. However, Popova *et al.* (2001) identified two chalcones (2',3'$(OH)_2$-dimethoxychalcone and 2',3',4$(OH)_3$-4'methoxychalcone) containing methoxy groups as having "significant" activity against methicillin susceptible *S. aureus*. Unfortunately, it used the more ambiguous disc diffusion assay with only two interpretive criteria for zone of inhibition sizes (active or not active), and did not include antibiotic controls (Popova *et al.*, 2001). Despite using the same bacterium, this makes a meaningful comparison between Alcaraz *et al.*'s conclusion

regarding the relative activity of flavonoids and Popova *et al.*'s statements difficult to compare, and underlines the difficulty in reconciling such general conclusions when different methodologies are used. While clearly such conclusions are not yet warranted (nor equivalent) for *N. gonorrhoeae*, such a QSAR approach would be a logical next step for investigating the activity of all the phytochemicals tested here. Better understanding of how a particular functional group leads to improved antibacterial activity is useful for both mechanistic purposes and for designing more effective compounds in the future. For example, increasing hydroxylation at certain points around either aromatic ring of a flavonoid has been linked with greater antimicrobial activity (Alcaraz *et al.*, 2000), while the effect of adding methoxy or other short chain alkyl groups remains inconclusive (see above). The presence of prenyl groups or glycosylation is associated with higher antimicrobial activity (Kuroyanagi *et al.*, 1999; Bernard *et al.*, 1997). As with dillapiol, bacterial time kill curves indicate that pinocembrin kills *N. gonorrhoeae* much more quickly than penicillin at its equivalent MIC. Further investigation of pinocembrin's mechanism of action is warranted.

Overall, all the tested phytochemicals show promising evidence of antibacterial activity, and inconclusive evidence of synergy with penicillin. Nonetheless, the fact that in the 3 way combination the majority of PPNG resistant strains were made susceptible to penicillin is a promising result, as this opens the possibility of using penicillin as part of a viable complementary therapy to treat gonorrhoea. More work is needed specifically to understand the antibacterial mechanisms employed, as well as the fate of these phytochemicals in the human body. Silibinin has been studied in this respect since it is

currently in use in Europe as an NHP. Hopefully, the paucity of data regarding the activity of phytochemicals against *N. gonorrhoeae* and the promising results described here will prompt further work with this pathogen.

While the discovery of antimicrobials and insight into the mechanisms of pathogenesis was undoubtedly one of the more significant achievements of human medicine in the 20th century, the hubris that followed is all the more ironic considering the situation today. The anthropocentric view of many people that humankind is superior to nature's insults and that through we are destined to prevail betrays some fundamental misunderstandings. It is not as much that we don't have the power to discover new drugs or understand in fine detail how a disease may spread or an organism may acquire resistance, but that our innate biases towards bacteria as primitive vessels of disease may be detrimental to synthesizing new strategies to combat antibiotic resistance and discovering new treatments which are not necessarily drug related (certainly not exclusive fields). Thus, while some might claim we now live in the "age of man", the reality is of course that we live (and have always lived, as has all of life) in the "age of bacteria". It is a pity that such a simple realization has been clouded by the belief that humans have a great advantage over our microscopic foes, when in fact bacteria have had three billion years to evolve and place themselves in positions that may be alternatively essential to our survival (for example commensal *E. coli* in our digestive system, or the mitochondrial and chloroplast organelles of prokaryotic origin that are essential to the survival of any eukaryotic cell) or extremely dangerous (*Y. pestis* for example). It is hoped that by shifting perspectives to stress the pervasiveness of bacteria among all forms of life and the complexity of both

human/bacterial interactions and their different reactions to antibiotic exposure, more coherent and integrated strategies (such as those outlined in the work presented here) can be developed to combat antibiotic resistance.

In conclusion, unifying the fields of modern science and traditional medicine has the possibility to provide new antimicrobial strategies. The widespread use of silibinin and garlic as NHPs, and the activity and favourable pharmacokinetic properties of dillapiol attests to such a potential. As the WHO malaria report (WHO, 2001) and my garlic work (Chapter 3) emphasizes, the concept of integrating NHPs into CAM therapies is a viable strategy. More work is necessary to characterize the nature of both dillapiol's and silibinin's interactions (with *N. gonorrhoeae* and antibiotics) with an eventual goal of possibly integrating these phytochemcials into a complementary drug regimen to treat both susceptible and resistant isolates of *N. gonorrhoeae*. As alluded to earlier, garlic and garlic NHPs (particularly those containing SAC or high amounts of allicin) have a greater likelihood to become alternative therapies which would probably be most successful with self limiting infections such as ear or skin infections, which have the added advantage of being easily targeted (i.e. through topical medications). The most important factors to bear in mind when considering using phytochemicals in CAM therapies are the pharmacokinetic/pharmacodynamic properties of the phytochemicals individually and in combination with antibiotics, the potential for drug interactions, and whether bactericidal concentrations are attainable at the site of infection (one reason why an alternative use of garlic for a gonorrheoal infection would not be appropriate). However, the garlic NHPs could be considered prodrugs, as it is possible that the body metabolizes garlic's

organosulfur constituents to compounds that have higher activity *in vivo*. Thus, much as bacteria have been previously discounted, contributions from the plant kingdom should not be overlooked when looking for novel drugs, especially considering the long history of using plants to treat infections.

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Mr. Patrick Ruddock, B.Sc., M.Sc.
97 Ontario St, Ottawa, Ontario, K1K 1K8
home: (613) 867-2908 pat1918@hotmail.com

DEMONSTRATED SKILLS

- Experience independently planning, conducting & supervising experimental research
- Extensive experience with resistant bacterial pathogens (*Neisseria gonorrhoeae*, *Staphylococcus aureus* (including MRSA), *Enterococcus faecalis* (including VRE), etc.), natural health products (NHPs) and quality control monitoring
- Effective collaboration with government on two NHP oriented studies (Health Canada) and a strain quality control study (Centers for Disease Control); projects completed on time and budget
- Experience as lead author (peer-reviewed journal articles & government reports), effective communication of research progress to superiors & presentation of results at various conferences
- Excellent teamwork and interpersonal skills gained through supervising and training employees/colleagues/students in both a laboratory & business setting
- Managed a profitable summer painting business as a Student Works Painting franchisee

EDUCATION

M.Sc. Biology (University of Ottawa, Ottawa, ON, Canada). To be completed Spring 2005.

- Studied novel approaches to combating and reversing antibiotic resistance in pathogenic bacteria by examining the potential of phytochemicals (*in vitro*) in complementary & alternative medicine (CAM) therapy
  - Antimicrobial susceptibility assays (MIC testing, time-kill curves, disk diffusion assays) used to identify several phytochemicals with activity against a panel of susceptible & resistant strains of *N. gonorrhoeae* (28 strains)
  - Synergy/interactions between phytochemicals & antibiotics also examined
  - Mechanistic studies of these phytochemical's mode of action

- Garlic NHP study & Canadian botanicals study (in collaboration with Health Canada)
  - Evaluated the antibacterial activity and efficacy of commercial garlic products & of crude extracts of commonly used Canadian botanical remedies
  - Designed & wrote novel standard operating procedure (SOP) for garlic study
  - Garlic work to be published in *Phytotherapy Research* (in press)
  - Both projects completed on time and on budget

- *N. gonorrhoeae* strain study examined antibiotic resistance profiles of 60 unknown isolates for potential future use as reference strains
  - Part of an effort coordinated by the Centers for Disease Control (Atlanta, GA, USA) and involving other international collaborators

- Supervised undergraduate honors and summer students (Fall 2003/Summer 2004)
  - Trained students in basic and advanced microbiological techniques, guided their experiments & oversaw research progress

B.Sc. (Honors) Biology (University of Ottawa, Ottawa, ON, Canada). 2002.

- Honors Thesis: Discovered antimicrobial activity in several novel plant-derived compounds towards antibiotic-resistant strains of *N. gonorrhoeae*
  - Performed laboratory quality control testing and reporting of strains

- Microbiological techniques including preparation of media & laboratory culture stocks, growth & identification of human pathogens and antimicrobial susceptibility testing

WORK EXPERIENCE

2002-04 **Teaching Assistant for undergraduate science laboratory courses**
University of Ottawa, Ottawa, ON, Canada

- Introductory Chemistry (1st year) & Animal Form and Function (2nd year)
- Responsible for explaining outline of labs and directing approximately 20 students in experiments & dissections
- Also in charge of leading group discussions, evaluating student's progress and marking class assignments/lab exams

2000-02 **Senior Telephone Research Interviewer**
Opinion Search Incorporated, Ottawa, ON, Canada

- Collected respondent's feedback & opinions on a variety of commercial, government, and customer satisfaction surveys conducted over the telephone
- Performed data entry for selected projects and recruiting for focus groups
- Trained new interviewers during their first shift in the call center

Summer 1999 **Franchise Manager**
Student Works Painting, Ottawa, ON, Canada

- Set up and managed an incorporated summer business ("Patrick Ruddock Painting")
- Recruited, trained and supervised three employees
- Estimated painting projects and presented proposals to clients
- Responsible for planning, organizing and managing production; customer relations & cost control of business, including keeping projects on time and budget; purchasing equipment and quality control
- Gross sales of $25,000; net profit of $3,500
- One of only 3 (out of 20) Student Works Painting franchise businesses to report a profit in the Ottawa area

PUBLICATIONS & CONFERENCES

- **PS Ruddock**, MM Liao, BC Foster, LD Lawson, JT Arnason, JR Dillon.* 2005. "Antimicrobial activities of garlic and garlic natural health products against *N. gonorrhoeae*, *S. aureus*, and *E. faecalis*." Accepted for publication in *Phytotherapy Research* (in press)
- **PS Ruddock**, S Ramirez, M Charland, A Lopez, GHN Towers, JT Arnason, JR Dillon. "Antimicrobial activity of 43 Colombian medicinal plants, and particularly *Piper lanceaefolium*, against *N. gonorrhoeae*". To be submitted to *Journal of Ethnopharmacology*.
- **PS Ruddock**, I Scott, JT Arnason, JR Dillon. "Antimicrobial activity and putative mechanism of action of the neo-lignan dillapiol against *Neisseria gonorrhoeae*" In preparation.
- **PS Ruddock** *et al.**. "Antimicrobial activity of 24 garlic natural health products against *Neisseria gonorrhoeae*" poster presented at **25th International Society for Sexually Transmitted Disease Research (ISSTDR) conference** (Ottawa, ON, Canada; July 27-30, 2003)
- **PS Ruddock** *et al.**. "Activity of commercial garlic NHPs against *Neisseria gonorrhoeae* and other pathogenic bacteria" poster presented at **Biodiversity and Health Symposium: Focusing Research to Policy (Tropical Conservancy)** (Ottawa, ON, Canada; October 25-28, 2003)

REFERENCES AVAILABLE UPON REQUEST

APPENDIX A – PINOCEMBRIN

A.1 Previous work with pinocembrin

Table 20 shows the cumulative percent inhibition of pinocembrin at various concentrations against the PR panel of *N. gonorrhoeae* strains. The activity of pinocembrin was similar to dillapiol, with 100% of the strains inhibited at a concentration of 64 mg/l. The activity of pinocembrin chalcone and cyclolanceaefolic acid methyl ester was lower, with 100% and 48% of the strains inhibited at 128 mg/l respectively (data not shown). Another study examining the activity of natural and synthetic chalcones (n=11), flavanones (n=4) and flavones (n=3) against MRSA identified a number of chalcones as having significant and higher antibacterial activity (MICs of 16 mg/l [2'-hydroxychalcone] and ~35 mg/l [2',4'-dihydroxychalcone and 2',4-dihydroxychalcone]) than pinocembrin (MIC of 125 mg/l) in contrast to our results, although a different organism (Gram positive *S. aureus*) was examined (Alcaraz *et al.*, 2000). This study concluded that anti MRSA activity varied approximately as follows: chalcones > flavones ≈ flavanones. Using quantitative structure activity relationships (QSAR) A separate experiment confirmed Alcaraz *et al.*'s (2000) report of pinocembrin's moderate MRSA activity against the methicillin susceptible strain *S. aureus* ATCC-29213 and a clinical MRSA strain (MICs of 128 mg/l; data not shown). Due to pinocembrin's higher activity and the lack of testable material of the other two compounds, further studies were conducted with pinocembrin only. Although these initial experiments were performed with pinocembrin isolated from *P. lanceaefolium*, the subsequent time kill assays used commercially available pinocembrin (Sigma, P-5329).

Table 20. Cumulative percent of tested *N. gonorrhoeae* strains inhibited at minimum inhibitory concentrations (MICs) of pinocembrin. Percentages are rounded to the nearest whole number and represent inhibition at each MIC from two independent experiments (2 replicates/experiment).

		Cumulative % inhibition at each tested concentration (mg/l)							
Resistance[a]	n	≤1	2	4	8	16	32	64	128
Susceptible	6	0	0	0	0	25	50	100	100
TRNG	3	0	0	0	0	0	50	100	100
PPNG	4	0	25	25	25	25	100	100	100
PP/TRNG	3	84[b]	84	84	100	100	100	100	100
CMRNG	2	0	0	0	0	0	25	100	100
PP/ CMRNG	1	0	0	0	0	0	50	100	100
CMRNG, CIPRO^R	1	0	0	0	0	0	0	100	100
PP/CMRNG, CIPRO^R	1	0	0	0	0	0	50	100	100
SPEC^R	2	0	0	0	0	0	100	100	100
PP/CMRNG, SPEC^R, ERY^R, AZI^R	1	0	0	0	0	0	50	100	100
PP/CMRNG, ERY^R	2	0	0	0	0	0	25	100	100
PPNG, SPEC^R, ERY^R	1	0	0	0	0	0	100	100	100
PP/CMRNG, SPEC^R, ERY^R	1	0	0	0	0	0	50	100	100
Totals	28	9	13	13	14	20	63	100	100

[a]TRNG - Tetracycline resistant *N. gonorrhoeae*; PPNG - Penicillinase producing *N. gonorrhoeae*; CMRNG - Chromosomally resistant *N. gonorrhoeae*; CIPRO^R - Ciprofloxacin; SPEC^R - Spectinomycin; AZI^R - Azithromycin; ERY^R - Erythromycin

[b]84% of PP/TRNG strains were also inhibited at the lowest concentration tested (0.063 mg/L)

A.2 Pinocembrin time kill curves

See "Bacterial time kill curves" in chapter II for a description of the methodology used for the following figures. Figure 10 shows the aggregate data (means ± standard deviation) for two 6 hour time curves performed using 32 mg/l (MIC-1), 64 mg/l (MIC) and 128 mg/l (MIC+1) of pinocembrin against *N. gonorrhoeae* WHO V. At the MIC of pinocembrin (64 mg/l), the rate of killing is much more rapid than the equivalent concentration of penicillin (0.5 mg/l). Indeed, pinocembrin appears to be more effective than penicillin, as a subinhibitory concentration of pinocembrin (32 mg/l) is only marginally less effective than penicillin at its MIC. Figure 11 shows the same assay performed using a PPNG strain (PR8). Again, at the subinhibitory concentration of 32 mg/l, pinocembrin is as effective as penicillin at its MIC, while at 64 mg/l of pinocembrin, the viable count of cells has fallen to 0 CFU/ml within 2 hours (within 1 hour at 128 mg/l pinocembrin).

Figure 10. Pinocembrin kill curve - WHO V (Means +/- std deviation)

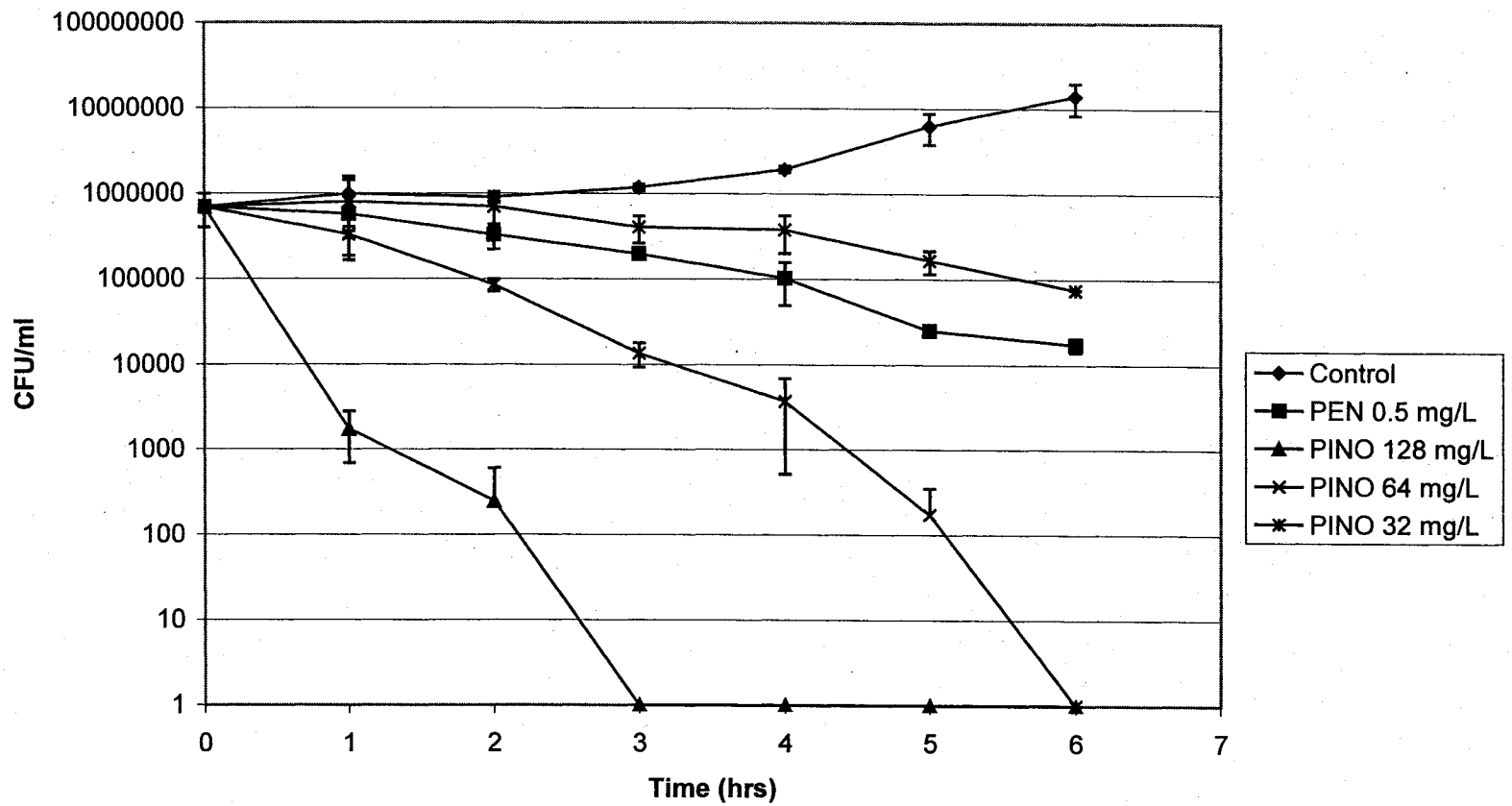
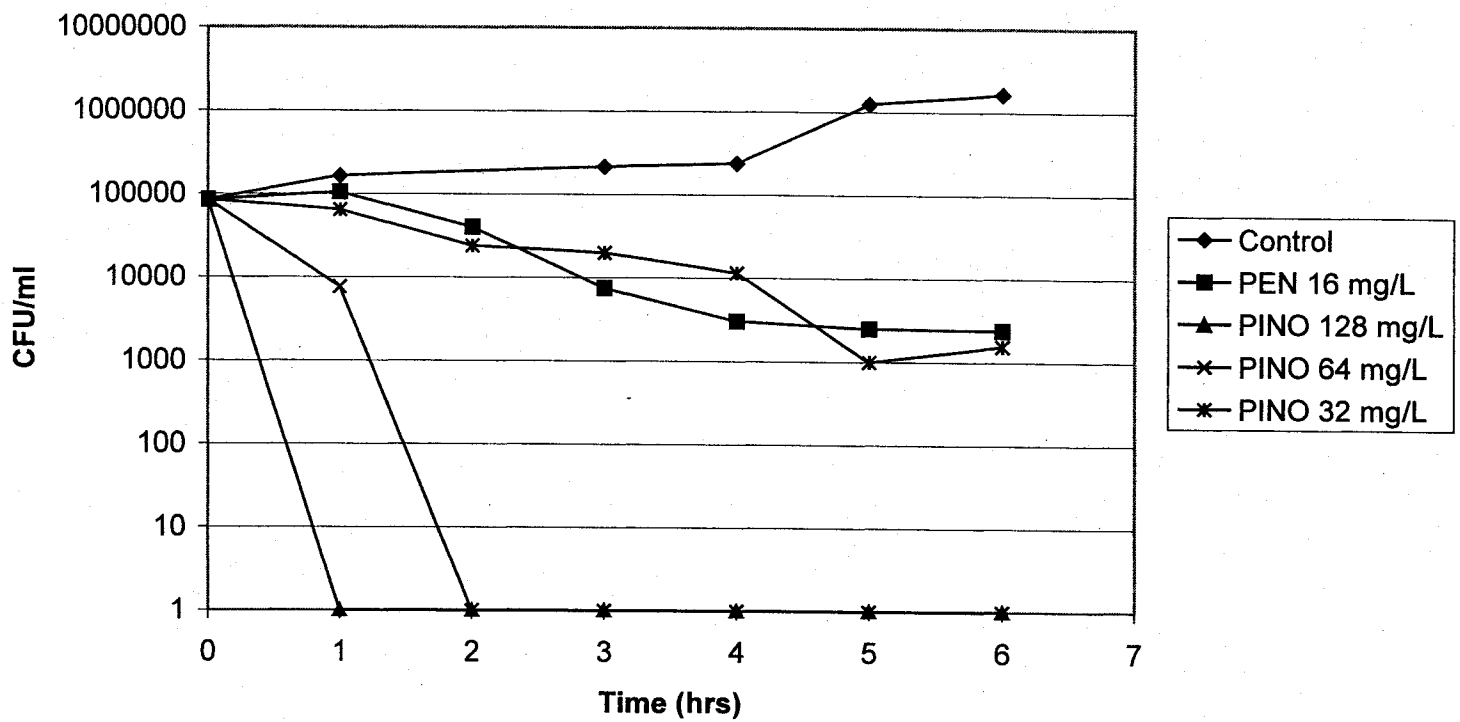


Figure 11. Pinocembrin kill curve - PR8 (PPNG)



APPENDIX B – NPN ASSAY METHOD DEVELOPMENT

B.1 Method validation.

Table 21 shows the summary of the initial trial conducted as a repeat of Helander and Mattila-Sandholm's (2000) experiment with *S. typhimurium.* There was a clear relationship between the photomultipler gain and the relative fluorescence values seen; the higher the setting the higher the values, although relative to each other they remained quite consistent. The combination of buffer (5 mM HEPES) and cells elicited an NPN fluorescence ratio of 5.6-6.0 (depending on the gain) compared to Helander & Mattila-Sandholm's uptake value of 7.3. When 1 mM EDTA was added, the NPN fluorescence ratio varied from 6.7-7.0 compared to Helander & Mattila-Sandholm's 10.5. Similarly, addition of 0.1 mM EDTA elicited an fluorescence ratio of 8.4-8.6 compared to Helander & Mattila-Sandholm's value of 10.7. Thus, the absolute values of fluorescence were lower than Helander & Mattila-Sandholm, as were the increases in NPN fluorescence ratios (increase of 1.1 versus 3.2 with 1mM EDTA and an increase of 2.7 versus 3.4 with 0.1 mM EDTA). Interestingly however, it was found that the lower concentration of EDTA elicited marginally higher fluorescence in concordance with Helander & Mattila-Sandholm. Based on comparison of the various gain settings, it was decided that a gain of 50 would be used for subsequent experiments, as this setting gave the most comparable results to Helander & Mattila-Sandholm (2000). A possible explanation for these discrepancies could include the different *S. typhimurium* strain tested (these bacteria tend to yield higher NPN uptake values than other bacteria, so perhaps inter strain differences have a significant effect), the different fluorometer, and

Table 21. Fluorescence (F) values obtained in an NPN uptake assay using Cytofluor system (using filters 360/40 (EX) and 409/20 (EM)) using *S. typhimurium* ATCC-14028 as the test organism.

Sample	Gain	NPN	F value (mean ± SD)[a]	F value after background subtraction	F ratio[b]	Helander *et al.*, 2000
Buffer	50	-	41.7 ± 0.4			
		+	54.1 ± 0.9	12.4	1	1
	40	-	8.3 ± 0.4			
		+	10.8 ± 0.1	2.5	1	
	60	-	174 ± 1.6			
		+	228.3 ± 3.8	54.3	1	
+cells	50	-	78.4 ± 0.8			
		+	152.1 ± 4	73.7	5.9	7.3
	40	-	15.7 ± 0.3			
		+	30.8 ± 1	15.1	6	
	60	-	328.6 ± 1.8			
		+	634.2 ± 13.2	305.6	5.6	
+cells +1mM EDTA	50	-	84.2 ± 0.8			
		+	170.4 ± 2.1	86.2	7	10.5
	40	-	16.9 ± 0.1			
		+	34.4 ± 0.6	17.5	7	
	60	-	353.5 ± 2.2			
		+	715.4 ± 9	361.9	6.7	
+cells +0.1mM EDTA	50	-	75.8 ± 0.5			
		+	182.5 ± 1.4	106.7	8.6	10.7
	40	-	15.3 ± 0.3			
		+	36.4 ± 0.5	21.1	8.4	
	60	-	317.9 ± 2.7			
		+	773.4 ± 10.5	455.5	8.4	

[a]All values represent average F values of 3 cycles on the Cytofluor 4000 (4 parallel wells/sample/cycle) [n = 12]
Methods follow those of Helander & Mattila-Sandholm (2000). Readings were taken at 3 different photomultiplier tube gain settings.
[b]represents ratio of background-subtracted F values to that of the buffer value

the different filter set. However, since this is a comparative assay, it was decided that the tested setup was adequate for these testing purposes.

B.2 Initial *N. gonorrhoeae* and buffer trials

Following this initial trial, *N. gonorrhoeae* WHO V was assayed against 64 mg/l and 128 mg/l dillapiol in HEPES buffer. Table 22 shows a summary of the results for this experiment. The NPN fluorescence ratio for *N. gonorrhoeae* cells in HEPES was approximately twice as high as that for *S. typhimurium* (12.2 versus 6 respectively); this is likely a result of the lower membrane integrity of *N. gonorrhoeae*, which would explain the higher incorporation of NPN into the membrane. The NPN uptake of 128 mg/l dillapiol in HEPES (no cells) was marginally higher than the NPN uptake of *N. gonorrhoeae* (14.9 versus 12.2), meaning this fluorescence must be taken into account when examining the combined fluorescence of 128 mg/l dillapiol plus the cells. For example, the fluorescence of [128 mg/l dillapiol + cells] was 32.4; thus the corrected NPN uptake value is 32.4-14.9 = 17.5. Similarly, the corrected NPN uptake for 64 mg/l dillapiol was 27.2. However, there were a number of problems with these results. The higher NPN uptake (almost twice as high) for the lower dillapiol concentration is a puzzling result. A potentially more serious problem is the effect that 5 mM HEPES has on *N. gonorrhoeae* cells. According to Elmros *et al.* (1976), *N. gonorrhoeae* cells lyze when placed in HEPES buffer, although examination of *N. gonorrhoeae* WHO V exposed to HEPES for 30 mins (~6 times longer than required for this assay) under phase contrast microscopy did not show obviously lyzed cells (data not shown).

Table 22. Fluorescence values obtained in an NPN uptake assay using the Cytofluor 4000 (Ex: 360/40; Em: 409/20) and *N. gonorrhoeae* WHO V as the test organism.

Sample	NPN	F value (mean ± SD)[a]	F value after background subtraction	F ratio[b]	Corrected F ratios[c]
HEPES	-	41.1 ± 0.4			
	+	54.8 ± 0.3	13.7	1	
+ cells	-	47.5 ± 0.3			
	+	215.1 ± 3.2	167.6	12.2	
+ 128 mg/l dillapiol	-	42.6 ± 0.1			
	+	247.2 ± 7.5	204.6	14.9	
+ cells + 128 mg/l dillapiol	-	58.3 ± 0.4			
	+	502.2 ± 0.9	443.9	32.4	17.5
+ 64 mg/l dillapiol	-	40.6 ± 0.4			
	+	117.5 ± 0.7	76.9	5.6	
+ cells + 64 mg/l dillapiol	-	50.8 ± 0.4			
	+	500.1 ± 0.5	449.3	32.8	27.2

[a]All values represent average F values of 3 cycles on the Cytofluor 4000 (4 parallel wells/sample/cycle)

[b]represents ratio of background-subtracted F values to that of the buffer value

[c]represents the total fluorescence seen minus the contribution from dillapiol or any other additives themselves

However, it is quite likely that suspending the cells in HEPES buffer would be enough to permeabilize, or at least sensitize, the cells to subsequent permeabilization by dillapiol.

To test this theory, a number of different buffer/cell combinations were analyzed in the cytofluor with or without NPN and *N. gonorrhoeae* cells. 5 mM HEPES was compared to Mueller Hinton (MH) broth and 0.7% casamino acids (CAA), which are two of the few broths for preparing bacterial suspensions that are known to not affect *N. gonorrhoeae* permeability or viability. Table 23 shows that HEPES and MH broth exhibited similar NPN fluorescence ratios (19-21), while 0.7% CAA showed the lowest uptake value (12.4). The high NPN uptake in MH broth is unexpected as this buffer should not affect the permeability of *N. gonorrhoeae*. A possible explanation is that the absolute value of fluorescence for MH broth (with or without NPN or cells) was much higher than the other two buffers. For the combination of buffer + NPN + cells, the value for HEPES was >1000 RFU compared to 306 and 288 RFU for HEPES and CAA respectively. Even for the buffer alone (no NPN and no cells added), the value was >850 RFU for MH broth compared to 42 and 95 RFU for HEPES and CAA respectively. Perhaps this high background fluorescence (and apparent autofluoresence) explains the high fluorescence ratio. Thus, because CAA showed the lowest NPN fluorescence ratios for *N. gonorrhoeae*, as well as comparable RFUs to HEPES, it was chosen as the buffer for all subsequent experiments.

Table 23. Fluorescence (F) value comparison of HEPES buffer, Mueller Hinton broth (MH), and 0.7% Casamino Acids (CAA) with and without *Neisseria gonorrhoeae* WHO V.

Sample	NPN	F value (mean ± SD)[a]	F value after background subtraction	F ratio[b]
HEPES	-	42.2 ± 0.4		
	+	55.3 ± 0.4	13.1	1
HEPES + cells	-	44.7 ± 0.3		
	+	306.6 ± 3.0	261.9	19.9
MH	-	871.3 ± 8.1		
	+	878 ± 4.9	6.7	1
MH + cells	-	955.8 ± 3.9		
	+	1097.8 ± 4.3	142	21.2
CAA	-	94.9 ± 1		
	+	109.3 ± 0.3	14.4	1
CAA + cells	-	109.1 ± 0.9		
	+	288 ± 0.3	178.9	12.4

[a]All values represent average F values of 3 cycles on the Cytofluor 4000 (4 parallel wells/sample/cycle)

[b]represents ratio of background-subtracted F values to that of the buffer value

APPENDIX C – STATISTICAL CONSIDERATIONS

C.1 Control MIC variability

As mentioned earlier in this thesis, most bacterial strains show some variability in MICs. This inherent variability does not depend on extraneous factors such as growth conditions or growth media. Thus, all things being equal, strains can be expected to show a 2-fold difference in MICs when tested against commonly used control antibiotics (this can be considered the standard accepted variability). Only differences in MICs that are greater than 2-fold are considered to be significant. This is why at least a 4 fold reduction in MICs was considered to be significant in the antibiotic:phytochemical combinations reported here. To examine the spread of MIC data (penicillin controls) from the work presented here, the standard error was calculated for the MICs of six representative strains (WHO III, WHO V, PR8, PR12, PR14, PR20) that were tested on multiple occasions.

Strain	Standard Error	n
WHO III	0.013	6
WHO V	0	6
PR8 (PPNG)	5.34	4
PR12 (CMRNG)	3.2	5
PR14 ($SPEC^R$)	0.801	4
PR20 (PPNG, $SPEC^R$, ERY^R)	5.34	5

These standard errors give a good idea of the variability encountered when testing any bacterial strain. It is important to note that the errors will vary depending on the MIC

values of the strains being examined. For example, consider two strains that are being examined which have standard MIC ranges to penicillin of 16-32 mg/l and 0.25-0.5 mg/l. If the following four values are obtained in an experiment for each strain [32,32,32,16] and [0.5,0.5,0.5,0.25] the standard errors are 4 and 0.063 respectively, even though the spread of the MICs (i.e. three higher values and one lower value) is the same for both strains.

C.2 Synergy and statistical interpretations

The review of Greco *et al.* (1995) gives a good idea of the variety of approaches that can be used in statistically analyzing combination data. In general, the concept is to construct dose response curves (i.e. effect of drug versus concentration of drug) for each agent in the combination individually, and to use these curves to create a hypothetical "no interaction" surface to which experimental data can be compared. However, such analysis was not possible for this work because only one effect level (MIC_{100} or the concentration that kills 100% of the cell population being tested) was examined. Performing experiments to determine the MIC_{25}, MIC_{50}, MIC_{75} and MIC_{90} for penicillin and the phytochemicals would be sufficient to make such curves. The two dose response curves are then placed perpendicularly together to form a three dimensional graph (i.e. sharing the effect (y) axis). Joining the two curves together creates a 3 dimensional surface which represents the "no interaction" effect of combining the two agents in question together. Experimental data can then be placed on the same graph and compared to the hypothetical surface to determine if any observed differences are statistically significant.