Antimicrobial compounds as side products from the agricultural processing industry

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Antimicrobial compounds as side products from the agricultural processing industry

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Antimicrobial compounds as side products from the agricultural processing industry

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Chapter 1

General introduction

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1.1 Antimicrobials used in human medicine

Antimicrobial chemotherapy has been an important medical treatment since the first investigations of antibacterial dyes by Ehrlich in the beginning of the twentieth century. However, by the late 1940s bacteria resistant to antimicrobials were soon recognized as a serious problem in clinical environments, such as hospitals and care facilities [Martin, 1998]. Bacterial resistance forces the research community to develop methods of altering structures of antimicrobial compounds to avoid their inactivation, yet structural modifications alone are not enough to avert bacterial resistance. The increasing use of household antibacterial products and agricultural antimicrobials fosters resistance to drugs specific for human therapy, and may have huge consequences for particularly children and elderly [Levy, 2001; Shea, 2003]. Antimicrobials contained in manure and biosolids may enhance selection of resistant bacteria by entering the aquatic environment through pathways of diffuse pollution [USEPA, 2002]. Surface water and shallow groundwater are commonly used for drinking water, and antimicrobials are now found to pollute many aquatic sources [Rooklidge, 2004]. Antimicrobials are used worldwide in human medicine, food, agriculture, livestock and household products. In many cases the use of antibiotics is unnecessary or questionable. Consumption of antibiotics is linked to bacterial resistance. In hospitals, most common resistant bacteria include methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci and gram-negative rods, including the *Enterobacteriaceae* and *Pseudomonas aeruginosa* [Beović, 2006].

Many medicinal plants are considered to be potential antimicrobial crude drugs as well as a source for novel compounds with anti-microbial activity, with possibly new modes of action. This expectation that some naturally occurring plant compounds can kill antibiotic-resistant strains of bacteria such as *Bacillus cereus*, *Escherichia coli*, *Micrococcus luteus* and *S. aureus* has been confirmed, for example, by Friedman et al. [2006]. In the past few decades, the search for new anti-infection agents has occupied many research groups in the field of ethnopharmacology. A Pubmed search for the antimicrobial activity of medicinal plants producesd a 115 articles from the period between 1966 and 1994. However, in the following decade between 1995 and 2004, this number more than doubled, to 307. In these studies one finds a wide range of criteria related to the discovery of antimicrobial compounds in plants. Many focus on determining the antimicrobial activity of plant extracts found in folk medicine, essential oils or isolated compounds such as alkaloids, flavonoids, sesquiterpene lactones, diterpenes, triterpenes or naphthoquinones. After detection of antimicrobial activity in the plant extract, some of these compounds were isolated or obtained by bioassay-guided isolation. A second block of studies focuses on the random screening of natural flora of a specific region or country and the third relevant group of papers is made up of in-depth studies of the activity of a plant or plant compound against a specific pathological microorganism [Ríos and Recio, 2005].

The goals of using plants as sources of therapeutic agents are *a)* to isolate bioactive compounds for direct use as drug, e.g., atropine, scopolamine, digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine; *b)* to produce bioactive compounds from novel or known structures, using them as lead compounds for (semi)synthesis of novel patentable entities with better activity and/or lower toxicity (examples are shown in Table 1.1); *c)* to use natural products as pharmacological tools, e.g., lysergic acid diethylamide, mescaline, strychnine, yohimbine; and *d)* to use the whole plant or part of it as a herbal remedy, e.g., cranberry, Echinacea, feverfew, garlic, *Ginkgo biloba*, St. John's wort and saw palmetto.

The number of higher plant species (angiosperms and gymnosperms) is estimated between 215,000 and 500,000 species. Of these, only about 6% have been screened for biological activity, and a reported 15% have been evaluated phytochemically [Fabricant and Farnsworth, 2001, Verpoorte, 2000].

Table 1.1 Some (semi)synthetic bioactive compounds derived from natural compounds but which demonstrate better activity and/or lower toxicity.

(semi)synthetic compounds	natural compounds
cocaine	morphine
metformin	galegine
nabilone	Δ^9–tetrahydrocannabinol
oxycodon (and other narcotic analgesics)	morphine
taxotere	taxol
teniposide	podophyllotoxin
verapamil	khellin
amiodarone	khellin

1.2 Antimicrobials used in food and food packaging

Research and development of antimicrobial materials for food applications such as packaging and other food contact surfaces is expected to grow in the next decade with the advent of new polymer materials and antimicrobials. Antimicrobial packaging can take several forms such as addition of sachets containing volatile antimicrobial agents into packages; incorporation of volatile and non-volatile antimicrobial agents directly into polymers; coating or adsorbtion of antimicrobials onto polymer surfaces; immobilization of antimicrobials to polymers by ion or covalent linkages; and use of polymers that are inherently antimicrobial. Recent food-borne microbial diseases are driving a search for innovative ways to inhibit microbial growth in food while maintaining quality, freshness and safety [Appendini and Hotchkiss, 2002].

Campylobacter and *Salmonella* are the most commonly reported bacterial causes of human food-borne infections and increasing proportions of these pathogens are becoming resistant to medically important antimicrobial agents, imposing a burden on public health. Acquisition of resistance to antibiotics affects the adaptation and evolution of *Salmonella* and *Campylobacter* in various environments [Threlfall, 2002; Zhang et al., 2006]. Angulo et al. [2004] found that antimicrobial resistance is increasing in the food-borne pathogens, *Salmonella* and *Campylobacter*. Many resistance-conferring mutations entail a biological fitness cost, while others (e.g. fluoroquinolone resistance in *Campylobacter*) have no cost or even enhance fitness. In *Salmonella*, the fitness disadvantage due to antimicrobial resistance can be restored by acquired compensatory mutations, which occur both *in vitro* and *in vivo*. The compensated or even enhanced fitness associated with antibiotic resistance may facilitate the spread and persistence of antimicrobial-resistant *Salmonella* and *Campylobacter* in the absence of selection pressure, creating a significant barrier for controlling antibiotic-resistant food-borne pathogens [Zhang et al., 2006]. Strains of *Salmonella enterica* resistanced to antimicrobial drugs are now widespread in both developed and developing countries. Since the early 1990s, a multi-drug resistant strain of *S. enterica*, serovar Typhimurium definitive phage type 104, displaying resistance to six commonly used antimicrobials, has gained particular importance. The incidence of human *Campylobacter* infection is increasing worldwide, as well as the proportion of isolates resistant to fluoroquinolones and/or macrolides, the drugs of choice to treat campylobacteriosis. Antimicrobial-resistant *Campylobacter* strains appear to cause more prolonged or more severe illness than do antimicrobial-susceptible strains [Moore et al., 2005 and Threlfall, 2002]

Antimicrobial packaging is a form of active packaging that could extend the shelf-life of products and provides microbial safety for consumers [Rooney, 1995]. Several compounds have been proposed for antimicrobial activity in food packaging, including organic acids, enzymes such as lysozyme, and fungicides such as benomyl, imazalil and natural antimicrobial compounds such as spices [Tharanathan, 2003; Weng and Hotchkiss, 1992]. Spices are rich in phenolic compounds, such as phenolic acids and flavonoids [Dadalioglu and Evrendilek, 2004]. Generally, the essential oils possessing the strongest antibacterial properties against food-borne pathogens contain higher concentrations of phenolic compounds such as carvacrol, eugenol (2-methoxy-4-(2-propenyl) phenol) and thymol [Burt, 2004]. Essential oil fractions of oregano and pimento are effective against various food-borne bacteria such as *Salmonella* and *E. coli* 0157:H7. The extracts from oregano, sage, rosemary, garlic, thyme and pimento are also reported to possess antioxidant properties [Dorman and Deans, 2000; Hammer et al., 1999]. Seydim and Sarikus [2006] found that the packaging films containing oregano essential oil was the most effective against *E. coli*, *S. aureus*, *Salmonella enteritidis*, *Listeria monocytogenes* and *Lactobacillus plantarum* compared to rosemary and garlic essential oils. A Japanese spice, wasabi (*Wasabi japonica*) is traditionally used on raw fish such as sashimi in Japan. This spice is known to have antimicrobial effects against several bacteria including *Vibrio parahaemolyticus* and is believed to contribute to the safety of eating raw seafood [Hasegawa et al., 1999]. The antimicrobial effects of 18 different herbs and spices were examined on the food-borne pathogen, *V. parahaemolyticus*, using different combinations of temperatures and nutrient levels. The results suggest that the spices and herbs, such as basil, clove, garlic, horseradish, marjoram, oregano, rosemary and thyme can protect seafood from contamination by *V. parahaemolyticus* [Yano et al., 2006].

1.3 Antimicrobials used in agriculture

Benomyl, captan and chlorothalonil are considered to be non-selective and are commonly used to control a broad range of plant diseases [Chen et al., 2001]. Imazalil (an imidazole fungicide) and triadimefon (a triazole derivative) are both used in agriculture to control a wide range of fungi on fruit and vegetables. These compounds interfere with the cellular permeability of pathogenic fungi [Ortelli et al., 2005; Vanden Bossche et al., 1989]. Chemical fungicides and insecticides used in agriculture can be detected at relatively high concentration in local water, sediments and biota. Their uncontrolled use may have a long-term negative impact on natural aquatic environments [Pennati et al., 2006].

The development of antimicrobial compounds from natural sources is considered to be a promising approach. Manohar et al. [2001] analyzed origanum commercial oil against *Candida albicans*. Zygadlo and Grosso [1995] tested *Salvia gilliessi*, *Satureja parvifolia* and *Lippia junelliana* against *Alternaria solani*, *Sclerotium cepivorum* and *Colletotrichum coccodes*. Dubey et al. [2000] tested *Ocimum gratissimum*, *Zingiber cassumunar*, *Cymbopogon citratus* and *Caesulia axilliaris* against *Aspergillus flavus*. They reported that these oils can be used in the management of fungal contamination, although large scale trials are required for registration as formulations for botanical antifungal agents. Singh et al. [1998] determined the fungitoxicity of extracts from 11 higher plants against a range of sugarcane pathogenic fungi such as *Rhizoctonia solani*. Okemo et al. [2003] found that the extract of *Maesa lanceolata* var. goulungensis was very active against the fungal plant pathogens: *Phytophthora cryptogea*, *Trichoderma virens*, *Aspergillus niger*, *Phoma* sp., *Fusarium oxysporium*, *Cochliobolus heterostrophus*, *Sclerotium rolfsii* and *Pyrenophora teres*.

1.4 Antibiotics used in livestock

At least 17 classes of antimicrobial agents, including tetracyclins, penicillins, macrolides, lincomycin (an analog of clindamycin), and virginiamycin (an analog of quinupristin/dalfopristin) are approved for growth promotion (also called improved feed efficiency) of livestock. Dietary enhancing feed additives (growth promoters) are also incorporated into the feed of animals reared for meat in order to improve their growth rates [Boxall et al., 2003]. Such agricultural use of antimicrobial agents can have an impact on the treatment of human disease. To understand the human health consequences of the agricultural use of antimicrobial agents, it is important to evaluate the quantity of antimicrobial agents used in food animals [Angulo et al., 2004]. The use of such antimicrobial agents in food animals increases the likelihood that humans pathogenic bacteria that have food animal reservoirs, such as *Salmonella* or *Campylobacter*, will develop cross-resistance to drugs approved for use in human medicine. Resistance determinants may also be transmitted from food animals to humans through the food supply with bacteria that are usually commensal such as *E. coli* and enterococci. Antimicrobial resistant bacteria are frequently isolated from livestock and farms. Several European countries have demonstrated that restricting the use of antimicrobial agents in food animals can decrease antimicrobial resistance in humans without compromising animal health or significantly increasing the cost of production [Angulo et al., 2004].

The presence of antimicrobial-resistant non-pathogenic commensal bacteria on farms is considered a problem, as it provides a pool of transferable resistance genes [Defrancesco et al. 2004]. To replace the currently used antibiotics in fodder, folk veterinary medicine is interesting for finding novel antimicrobial substances. Among the plants used in folk veterinary medicine in Italy, the most common medicines concerned the digestive system (96 plants) and skin (82 plants). Fifty three plants were used for wounds and inflammations and 49 plants as digestives, 23 plants against diarrhea, 20 plants for respiratory ailments, 16 plants in connection with labor and delivery, and 15 plants as laxatives and purgatives. In this traditional pharmacopoeia, there are well-known genera such as *Allium*, *Artemisia*, *Clematis*, *Echium*, *Euphorbia*, *Fraxinus*, *Hedera*, *Helleborus*, *Malva*, *Mercurialis*, *Salix*, *Urtica*, *Verbascum* and also unusual species as well as species and whole genera, relatively unknown from a medicinal viewpoint, such as *Berula*, *Coriaria*, *Cynoglossum*, *Kicxia*, *Micromeria*, *Muscari*, *Pulicaria* and *Scorpiurus*. The various animals treated with plants were cattle, sheep, horses, poultry, pigs, dogs and rabbits. Some are also known for human use [Viegi et al., 2003]. A study was done on the ethnoveterinary medicines for cattle (*Bos indicus*) in Bulamogi, Uganda. The 38 plants species in this study such as *Vernonia amygdalina*, *Balanites aegyptiaca*, *Cannabis sativa*, *Chenopodium opulifolium*, *Senna occidentalis*, *Tephrosia vogelii* and *Harrisonia abyssinica* were distributed over 37 genera and 28 families. They were used to treat common cattle diseases for example cough, east cost fewer, measles, diarrhea and skin disease. Most of these plants are indigenous shrubs. The plant parts most frequently used for treating cattle are roots and leaves. Medications are usually prepared as infusions and seldom as decoctions [Tabuti et al., 2003]. Approximately 75% of rural livestock owners in the Eastern Cape province of South Africa use plants or plant based remedies to treat their livestock. Prominent among these plants are *Combretum caffrum*, *Salix capensis* and *Schotia latifolia*. The methanol and acetone extracts of these plants showed activity against gram-positive bacteria and fungi [Masika and Afolayan, 2002].

1.5 Antimicrobials used in household products

Antimicrobial coating of household products has obtained a wide acceptance in the past years. To control the growth of microorganisms, antimicrobials are used in cotton fibers and a wide range of plastic applications, such as telephones, PVC (Poly-Vinyl Chloride) leather for furniture, wall covering, flooring, escalator rails, roof and pool liners, film and sheathing They are also used in plastic products where infection is a concern, such as hospital furniture [The Biocide Information Services (BIS), 2001]. Pyridine derivatives used as antifungal or antibacterial agents in many common products, are known to cause contact dermatitis [Huh et al., 2001]. Recently, non-leaching, permanent, sterile-surface materials have been developed in which one end of a long-chained hydrophobic polycation containing antimicrobial monomers is attached covalently to the surface of a material such as cotton or plastic [Lewis and Klibanov, 2005]. Barnes et al. [2006] synthesized an *N*-halamine siloxane monomer precursor to coat the surfaces of cotton fibers. Antimicrobial chemical compounds are also applied in buildings and houses in paint, wallpaper, ceiling boards and glass panels, which frequently become infested by fungi. Fungal growth results in biodeterioration and discolouration of these substrates. Buildings affected by fungi have yielded 28 identified species. Among them are species of *Aspergillus*, *Cephalosporium*, *Cladosporium*, *Curvularia*, *Fusarium*, *Penicillium*, *Pithomyces*, *Trichoderma*, *Verticillium* and a number of sterile non-sporing isolates. The most abundant and most often encountered was *Aspergillus fumigatus* followed by *Cladosporium cladosporioides* and *Curvularia lunata*. *Fusarium decemcellulare* was abundant on ceiling boards and *Fusarium solani* on wallpaper. Some antifungal chemical compound used in commercial paints were tested for inhibition of fungi. The best fungicide was 8-hydroxyquinoline [Lim et al., 1989].

Until 2004, chromate copper arsenate (CCA) was used to preserve wood for house construction and furniture. Since then, the European and US Environmental Protection Agency no longer allow the use of this compound for wood treatment. These new regulations and the concern about environmental contamination have brought about an urgent need to develop new chemical formulations which will not harm either the environment or humans. In the past decade, several new chemical formulations, such as ammoniacal copper quaternary (ACQ), Tanalith-E, Wolmanit CX-8 and copper dimethyldithiocarbamate (CDDC) have been developed and currently used in building constructions, children's play structures, decks, picnic tables and other items [Yildiz et al., 2004]. However, novel antimicrobial coatings of household products from natural sources should be an interesting target as it would present a sustainable resource, and particularly in the atmosphere of rising oil prices such renewable resources are of great interest. Natural products are more beneficial for the environment and human health.

1.6 Aim of this thesis

The plant kingdom is a very rich resource for discovering new antimicrobial compounds for human medicine as well as many other applications such as food preservation, disease management in agriculture, veterinary disease control and the coatings of household products.

The goal of this work was to screen plants for (novel) antimicrobial compounds and particularly to find antifungal compounds for the inhibition of wood rot fungi.

Chapter 1 is a general introduction about antimicrobials used in human medicine, food, agriculture and household products as well as antibiotics used in livestock. Medicinal plants are a promising source for novel antimicrobial compounds that are active against antibiotic resistant microorganisms. **Chapter 2** is a review of the most common mechanisms of action of antibiotics followed by an overview of possible assays which can be used to discover active compounds from natural sources. **Chapter 3** describes the use of such assays for screening antimicrobial activity from plant extracts. **Chapter 4** deals with the fractionation of active plant extracts to isolate and elucidate the structure of pure active compounds. **Chapter 5** is a study on the effect of plant extracts and pure compounds on the induction of fungal cell wall stress using *Aspergillus niger* as a model. **Chapter 6** is focused on the inhibition of a target enzyme, anthranilate synthase, which generally occurs in microorganisms and plants but not in mammals. **Chapter 7** reports the results of screening for anti-wood rot plant extracts and compounds. Cellulase was used as a possible key enzyme to learn more about the mode of action of wood rot fungi.

Chapter 2

Developing antimicrobial compounds from natural sources

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Natural products from plants are of interest for the discovery of antimicrobial compounds (see general introduction). Assays used in the identification of antimicrobial compounds are reviewed in this chapter. The measurement of growth inhibition of microorganisms by diffusion or dilution assays is used for screening antimicrobial compounds and plant extracts. For drug discovery, microbial growth inhibition is not sufficient. Additional studies are required on the mode of action in pathogenic microorganisms such as effects on bacterial cell membranes, fungal cell wall synthesis, DNA replication and repair, ribosome binding, protein synthesis and metabolic enzymes. It is therefore important to study the mode of action of plant antimicrobial compounds after positive screening for microbial growth inhibition. This chapter discusses first the most common mode of action of antibiotics followed by an overview of possible assays which can be used as tools to find antimicrobial compounds and discover novel leads for drug development.

2.1 Mode of action

From the discovery of penicillin in 1928 and during the four decades after World Wall II, many advances were made in antimicrobial therapy. Today, the pace of antimicrobial discovery has slowed. During the 20-year period from 1983 to 2002, the FDA's (Food and Drug Administration) approval of new antibacterial agents decreased by 56%. Between 2004 and 2006, only three new antibacterial agents have been approved [Mukhopadhyay and Peterson, 2006]. Most antimicrobial agents used for the treatment of bacterial infections may be categorized according to their principle mode of action. The most common modes of action are interference with the cell membrane and cell wall, interference with nucleic acids, and enzyme interactions [Lambert and O'Grady, 1992; Hugo and Russell, 1992; Neu, 1992; Tenover, 2006].

2.1.1 Cell membrane and cell wall interactions

Disruption of the bacterial membrane structure by antimicrobial compounds has not yet been well characterized in term of the mode of action. It is postulated that polymyxins exert their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial cell contents. Lipopeptides consist of a linear or cyclic peptide sequence, with either a net positive or negative charge, to which a fatty acid moiety is covalently attached to its N-terminus. They are a class of antibiotics which are highly active against multidrug resistant bacteria. Some lipopeptides also display anti-fungal activity. In the anionic lipopeptide class, the first naturally occurring compound discovered was amphomycin over fifty years ago. Additional members of this class of compounds include crystallomycin, aspartocin, glumamycin, laspartomycin, tsushimycin, and, by far the most studied, daptomycin. They neither inhibit cell wall synthesis by interacting with ribosome subunits nor do they inhibit protein synthesis. Rather, they are thought to target and bind to the bacterial membrane directly, and cause rapid depolarization of the antibacterial membrane potential as well as eventually death of the bacterium [Storm et al., 1977; Carpenter and Chambers, 2004; Straus and Hancock, 2006].

The fungal cell wall is a unique structure that is essential for the survivor of fungi. It differs from the mammalian cells and consequently presents an attractive target for new antifungals. The fungal cell wall is a vital and complex structure containing mannoproteins, chitins and glucans. Chitin and glucan components of the cell wall should be good drug targets because they are unique and essential to fungi [Georgopapadakou and Tkacz, 1995]. Any disruption in cell wall integrity should affect growth. The echinocandins are cyclic hexapeptides, members of a new class of antifungal agents. They appear to inhibit the synthesis of 1,3-β-D-glucan, a major cell wall component which provides structural integrity and osmotic stability in most pathogenic fungi [González et al., 2001]. Caspofungin is a noncompetitive inhibitor of the enzyme *β*-(1,3)-glucan synthase, which catalyzes the polymerization of uridine diphosphate-glucose (UDP-glucose) into *β*-(1,3)-glucan, a structural component of the fungal cell wall responsible for maintaining integrity and rigidity. When *β*-(1,3)-glucan synthesis is inhibited, ballooning out of the weakened cell wall occurs as a result of the high osmotic pressure of the protoplast and causes cell lysis [Stone et al., 2002].

More recently, studies focused on the search for water-soluble inhibitors of fungal 1,3-*β*-D-glucan synthase, an enzyme critical for the synthesis of 1,3-*β*-D-glucan, a major component of the cell wall of a number of key pathogenic fungi. Aerothricin lipopeptidolactones and Sankyo lipopeptides have been identified as novel members of liposaccharide glucan synthesis inhibitors. Aerothricins, like natural product molecules, act as antifungal drugs that inhibit the formation of the *β*-1,3-D-glucan component of the cell wall, but they are less water soluble than the related semi-synthetic molecules. The semi-synthetic molecules contain various basic amino acids and a large series of aminoalkyl groups [Schwartz, 2001].

Bacterial cell walls have only a single layer of peptidoglycan. A single unit of peptidoglycan is a combination of alternatively *β*-(1→4) linked disaccharides of *N*-acetylglucosamine (NAG) and *N*-acetyl muramic acid (NAM) and four amino acids such as L-alanine, D-isoglutamic acid, L-lysine and D-alanine attached through peptide bonds at the NAM residue [Rai et al., 2003]. Antibacterial drugs that work by inhibiting bacterial cell wall synthesis are the *β*-lactams (e.g. penicillins, cephalosporins), carbapenems, monobactams, glycopeptides, vancomycin and teicoplanin. *β*-Lactams inhibit synthesis of bacterial cell walls by interfering with the enzymes required for the synthesis of the peptidoglycan layer. Vancomycin and teicoplanin bind to the terminal D-alanine residues of the nascent peptidoglycan chain, thereby preventing the cross-linking steps required for stable cell wall synthesis [McManus, 1997].

2.1.2 Nucleic acid interactions

Fluoroquinolones exert their antibacterial effects by disturbing DNA synthesis and causing lethal double-strand DNA breaks during DNA replication [Drlica and Zhoa, 1997; Yao and Moellering, 2003; Petri, 2006]. The 4-quinolones are antibacterial agents that have two essential bacterial enzymes, DNA gyrase and DNA topoisomerase IV, as targets. DNA gyrase controls DNA supercoiling and relieves topological stress arising from the translocation of transcription and replication complexes along DNA; topoisomerase IV is an enzyme that resolves interlinked daughter chromosomes following DNA replication. Both enzymes are required for cell growth and division. It is thus not surprising that the quinolones are bactericidal. However, these compounds do not simply eliminate topoisomerase function: trapping of gyrase and topoisomerase IV on DNA probably leads to the lethal release of double-strand DNA breaks. For three decades, the quinolones have been used for a variety of physiological studies, serving as convenient inhibitors of DNA synthesis and as probes for the study of topoisomerase-DNA interactions [Drlica and Zhao, 1997]. Chloramphenicol has an inhibitory effect on DNA synthesis [Chen et al., 1996]. The common antibacterial drug combination of TMP (a folic acid analogue) with sulfamethoxazole (SMX, a sulfonamide), inhibits two steps in the enzymatic pathway for bacterial folate synthesis [Petri, 2006; Tenover, 2006].

Bacterial ribosomes differ in structure from their counterparts in eukaryotic cells. These differences can be used to selectively inhibit bacterial growth. Aminoglycosides, a large family of water-soluble polycationic amino sugars, are used as broad spectrum antibacterial agents. Aminoglycosides target the microbial ribosome by direct interaction with ribosomal RNA, and they affect protein synthesis by inducing codon misreading and by inhibiting translocation of the tRNA-mRNA complex [Hobbie et al., 2006; Neu, 1992; McManus, 1997]. Antibacterial agents like aminoglycosides, macrolides and tetracyclines bind to the 30S subunit of the ribosome, whereas chloramphenicol binds to the 50S subunit [Tenover, 2006].

2.1.3 Enzyme interactions

There are many possible target enzymes in microorganisms. The gram-negative bacterium *Pseudomonas aeruginosa* is an important pathogen of plants and animals. Given the high prevalence of antibiotic resistant strains of *P. aeruginosa*, it is desirable to design new chemotherapeutic agents against this opportunistic pathogen, which is a growing human health problem because of the susceptibility to infection in the increasing number of immuno-suppressed people. Betaine aldehyde dehydrogenase (BADH) is a target enzyme for inhibition of *P. aeruginosa* growth. Glycine betaine, the product of the BADH catalyzed reaction, is an effective osmoprotectant and most likely acts as such in bacterial cells growing in the hyperosmotic environment of infected tissues. It has been found that *P. aeruginosa* is able to thrive under osmotic stress if glycine betaine, choline, or choline precursors are present. Indeed, the virulence of this bacterium has been correlated with its ability to adapt to osmotic stress and to express phospholipase C, the first enzyme in the pathway from phosphatidylcholine to glycine betaine. BADH from *P. aeruginosa* therefore might be a key enzyme for the survival of the pathogen and thus a potential target for chemotherapeutic agents. Velasco-García et al. [2006] suggested that the growth inhibition is due to the accumulation of the BADH substrate, betaine aldehyde, which is highly toxic. However, they found that disulfiram destabilized the quaternary structure of BADH and promoted irreversible aggregation of this enzyme. Inhibition of glutamate dehydrogenase and 2-ketoglutarate reductase, the first enzymes in the 2-ketoglutarate pathway of glutamate catabolism by *Fusobacterium nucleatum*, the oral anaerobes, were assayed. Benzimidazoles and lansoprazole were found to be antimicrobial against *F. nucleatum* by inhibition of those enzymes [Sheng et al., 2006].

Most clinically useful antifungal agents inhibit the biosynthesis of ergosterol or interact directly with ergosterol in membranes. Ergosterol is the principle sterol in yeast and fungi, except the Oomycete genera *Pythium* and *Phytophthora*, which do not synthesize any sterol. Beuchet et al. [1998] reported that the synthetic compound 6-β-aminocholestanol inhibits the biosynthesis of ergosterol. The azole antifungal agents, such as fluconazole, itraconazole and azolylmethyloxolane derivatives with modified sterol side-chain structures, inhibit cytochrome P450 14α-demethylase (14DM) and Δ^{24}-sterol methyltransferase (24-SMT) which are the key enzymes involved in fungal ergosterol biosynthesis [Chung, et al., 1998; Chung et al., 2000]. Amorolfine inhibits Δ^{14} reductase and $\Delta^{7,8}$ isomerase which are part of the ergosterol biosynthesis pathway [Polak-Wyss, 1995]. The α-bisabolol in chamomile interfered with zymosterol and prevents the formation of fecosterol from zymosterol which is the first fungal specific step in ergosterol biosynthesis [Pauli, 2006].

2.2 General screening

There are many different assays for screening antimicrobial activity. Many publications report the antimicrobial activity of plants using general screening assays for microbial growth inhibition which are *in vitro*. The standard general screening assays are diffusion assays, dilution assays and bioautographic assays.

2.2.1 Diffusion assays

2.2.1.1 Disc diffusion assay

Paper disc diffusion assays are generally used for screening of antibacterial and antifungal activities from natural extracts and compounds [Quiroga, et al., 2001; Ahmad et al., 2005; Pyun and Shin, 2005]. However, the diffusion method is not appropriate for testing non-polar samples or samples that do not easily diffuse into agar if the inhibition diameter has to be measured [Cos et al., 2006]. Plant extracts are dissolved in organic solvents such as ethanol, methanol or ethyl acetate [Moreno et al., 1999; Pyun and Shin, 2005; Eldeen et al., 2005]. The concentration of bacterial or fungal inoculum used for the tests is between 10^4-10^8 CFU (Colony

Forming Units) /mL. The inoculi are spread on the agar surface or mix into the agar media [Pyun and Shin, 2005; Eldeen et al., 2005]. Sterile filter paper discs, Whatman No.4 or No.1, 5 mm or 8 mm diameter,r are the most often used [Moreno, et al., 1999; Quiroga, et al., 2001; Ahmad et al., 2005; Pyun and Shin, 2005].

2.2.1.2 Well diffusion assay

The well diffusion assay is suitable for aqueous extracts because they are difficult to dry on paper discs [Vlietinck, et al., 1995; Fazeli et al., 2007; Magaldi, et al., 2004; Tadeg, et al., 2005]. However, the leaking of sample under the agar layer must be considered. Wells with 8 mm diameter are cut in the agar plate using a cork borer and 100 µL of sample is loaded into the well [Fazeli et al., 2007; Patton et al., 2006]. Microbial cell suspension is used in a similar way to the disc diffusion assay and the inhibition diameter is measured after incubation.

2.2.2 Dilution assays

Dilution assays are standard methods used to compare the inhibition efficiency of antimicrobial agents. The test extracts or compounds are mixed with suitable media that has been inoculated with the test microorganism. It can be carried out in liquid media (broth dilution assay) or in solid media (agar dilution assay). Growth inhibition is expressed as Minimal Inhibitory Concentration (MIC) which is defined as the lowest concentration able to inhibit any visible microbial growth. The Minimal Bactericidal or Fungicidal Concentration (MBC or MFC) is determined by plating-out samples of completely inhibited dilution cultures and assessing growth after incubation [Cos et al., 2006; Yin and Tsao et al., 1999; Salie et al., 1996]. The inoculate concentrations of bacterial or fungal cultures are between 10^4-10^8 CFU/mL [Camporese et al., 2003; Karaman et al., 2003]. In the agar plate dilution assay, the microbial cell suspension is spread over the surface of the agar plate [Verástegui et al., 1996], inoculated on the center of the agar surface [Sato et al., 2000; Quiroga, et al., 2001], by the streak method [Kumar et al., 2006] or mixed with the media as in the broth dilution assay [Navarro and Delgado, 1999; Cos et al., 2002; Pyun and Shin, 2005].

2.2.3 Bioautographic assays

There are three different approaches for bioautography to localize antimicrobial activity on a TLC chromatogram [Cos et al., 2006]. In direct bioautography, the microorganism grows directly on the thin-layer chromatographic (TLC) plate [García, et al., 1997; Yff et al., 2002]. In contact bioautography (biogram assay), the antimicrobial compounds are transferred from the

TLC plate to an inoculated agar plate through direct contact. In the agar overlay bioautography, agar media is applied directly onto the TLC plate [Silva et al., 1996; Chomnawang et al., 2005; Schmourlo et al., 2005]. Those assays supply a quick screen for new antimicrobial compounds through bioassay-guided isolation. The concentrations of bacterial or fungal inoculates are 10^6 CFU/mL [Moreno et al., 1999].

2.3 Advanced screening on modes of action

To discover antimicrobial compounds with multiple applications, the mode of action in a microorganism must be considered as the drug target. The advanced screening on mode of action can be divided into two groups: assays on microbial cells *in vivo* and assays on molecular targets *in vitro*.

2.3.1 Assays on microbial cells

2.3.1.1 Viability of cells

The fluorescent viability test uses fluorescein diacetate (FDA) and ethidium bromide (EB) which show a strong contrast between living and dead cells. The living cells show a green fluorescence as fluorescein diacetate can pass through the membrane into the cell where it is hydrolyzed into fluorescein and acetate by esterases. Due to their polarity, intact fluoresceins cannot traverse the cell membranes. Dead cells show a bright red fluorescence due to ethidium bromide penetration into the dead cells in which esterases were inactive. The fluorescence can be observed under a fluorescent microscope [Aquino, et al., 2005].

2.3.1.2 Microbial cell membrane and cell wall targets

Electron microscopy was used to investigate the mechanism of action of biocides in pathogenic microorganisms. Scanning and transmission electron microscopy (SEM and TEM) were used to observe membrane damage and leakage of intracellular materials in *Aspergillus fumigatus*, *Candida albicans*, *P. aeruginosa*, *Serratia marcescens* and *Staphylococcus aureus* after treatment with polyquaternium-1 (PQ-1) and myristamidopropyl dimethylamine (MAPD) [Codling et al., 2005].

Yang et al. [2006] studied the mode of action of antimicrobial compounds on the bacterial membrane using a membrane depolarization assay. *Staphylococcus aureus* and *Escherichia coli* were grown and incubated with the inhibitors. The collapse of the cytoplasmic membrane potential was monitored using a spectrofluorometer at 622 nm excitation wavelength and 670 nm emission wavelength.

The bacterial cell membrane integrity can be examined by determination of the release of material absorbing at 260 nm, which is monitored by UV spectrometry. Outer membrane permeabilization is determined by the NPN (1-*N*-phenyl-naphthylamine) assay, in which fluorescence of NPN is recorded using a fluorescence spectrophotometer. Enhanced fluorescence is due to NPN uptake by *E. coli*. The inner membrane permeabilization assay is measuring the release of cytoplasmic β-galactosidase from *E. coli* into the culture medium using *O*-nitrophenyl-β-D-galactoside (ONPG) as the substrate. The production of *O*-nitrophenol over time is determined by monitoring the change in absorbance (420 nm) using a spectrophotometer [Je and Kim, 2006].

The depolarization of the cytoplasmic membrane of yeast and *S. aureus* by antimicrobial peptides is determined using the membrane potential sensitive cyanine dye $DiSC_3$-5 (3,3'- dipropyl-2,2'-thiadicarbocyanine iodide). Fluorescence is monitored by a fluorescence spectrometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Membrane depolarization is determined by an increase in fluorescence units as a function of antimicrobial peptide concentration [Friedrich, et al., 2000; Zhu, et al., 2006].

A commercially available Live/Dead Bacterial Viability Kit (Molecular probes, Inc., Eugene, Oregon, USA) is rapid test for distinguishing membrane-active antibacterial agents. This method utilizes two fluorescent nucleic acid stains, SYTO9 (stains all cells green) and propidium iodide (stains cells with damaged membrane red) for the drug-treated bacterial cells. The cells are then either examined visually by fluorescence microscopy or their fluorescence emissions are recorded using a multi-label plate reader set to measure emissions at two different wavelengths [Singh, 2006].

Straus and Hancock [2006] determined the interaction of an inhibitor with bacterial membranes using differential scanning calorimetry in model membranes of calorimetry lipid films, DiPoPE (dipalmitoleoyl phosphatidylethanolamine). The interaction between the inhibitor and the bacteria was detected by NMR analysis of Ca^+ level which is involved in bacterial membrane damaged.

Aspergillus fumigatus was incubated with wheat germ agglutinin fluorescein isothiocyanate (WGA-FITC). An intense fluorescence all along the hyphal wall was observed for the negative control. The labeling was detected when the fungi was grown in the presence of caledonixanthone E, an antifungal compound. WGA-FITC recognizes chitin, a structural polysaccharide of the fungal cell wall, and the reduction of the chitin content in hyphae after exposure to caledonixanthone E was observed under a fluorescence microscope [Larcher, et al., 2004].

2.3.2 Assays on molecular targets

2.3.2.1 Nucleic acid targets

DNA replication is a well known target for screening of antibiotics. *Escherichia coli* DnaG primase is a single-stranded DNA-dependent RNA polymerase. The primase catalyzes synthesis of a short RNA primer to initiate DNA replication at the origin and to initiate Okazaki fragment synthesis for the lagging strand. *Escherichia coli* DnaG and DnaB, which overexpressed primase and helicase, respectively are used. The SPA primase assay is monitored using the topcount instrument which is assessed by comparison to a filter-binding method. DnaB helicase activity is monitored by a FRET method in which the fluorescence of a double-stranded, forked DNA substrate, labeled on the 5′ ends with the fluorochrome Texas Red, is internally quenched by a Dabcyl moiety located on the complementary strand [Zhang et al., 2002].

DNA microarray assays can be used to study gene expression profiles of *Saccharomyces cerevisiae* treated with ergosterol biosynthesis inhibitors. It leads to the identification of a subset of genes that are up- or down-regulated in response to these compounds, and to the determination of the mode of action of an unknown compound based on the similarity of its gene expression profile to those of an ergosterol biosynthesis inhibitor [Bammert and Fostel, 2000 *In* Kagen et al., 2005].

Mycobacterium smegmatis, a non-pathogenic microorganism, carries two rRNA operons, rrnA and rrnB, which allow for mutagenesis of its ribosomal nucleic acids. One of the two chromosomal rrn operons is usually inactivated by insertion-deletion mutagenesis, which results in cells carrying homogenous populations of mutant ribosomes. This model has provided an important tool in the investigation of drug-target activity of ribosomal inhibitors [Hobbie, et al., 2006].

2.3.2.2 Enzyme targets

Transgenic *S. cerevisiae* strains are used to determine the inhibition of ergosterol biosynthesis. The specific target enzymes in ergosterol biosynthesis, lanosterol C-14 demethylase, C-14 reductase, Δ^{8}- Δ^{7}-isomerase, C-3 ketoreductase or squalene epoxidase are encoded by ERG11, ERG24, ERG2, ERG27 and ERG1 gene of *S. cerevisiae*, respectively [Daum et at., 1998; Bammert and Fostel, 2000; Gachotte et al., 1999; Mercer, 1991 *In* Kagen et al., 2005]. The crude extract of *S. cervisiae* is used to determine the inhibition of Δ^{14}-reductase and Δ^{8}- Δ^{7}-isomerase, two enzymes in the pathway of ergosterol biosynthesis by IC_{50} values [Polak-Wyss, 1995].

Barrett [2002] reported an assay for the inhibition of the enzyme 1,3-β-D-glucan synthase from *C. albicans* 6406. Membrane and cell wall targets in *A. fumigatus* were studied using transgenic *A. fumigatus* which overexpress the β-(1,3)-glucanosyltransferase (GEL) gene [Beauvais and Latgé, 2001].

Betaine aldehyde dehydrogenase (PaBADH) is a target enzyme for inhibition of *P. aeruginosa*, a plants and animals pathogen. *Escherichia coli* strains were transformed with a mutant plasmid to express PaBADH. Enzyme activity was assessed by spectrophotometer [Velasco-García et al., 2006].

Glutamate dehydrogenase and 2-ketoglutarate reductase, the first 2 enzymes in the 2-ketoglutarate pathway of glutamate catabolism by *F. neucleatum* were assayed for screening of antimicrobial activity of bacteria cell extracts. Enzyme activities were assayed following the procedure described by Fujimura and Makamura [1987] with use of L-X-glutamyl-*p*-nitroanilide as a substrate [Sheng, et al., 2006].

2.4 Methods used in this thesis

In this thesis both general and advanced screening assays were used in order to find antimicrobial compounds for application in anti-wood rot preparations. The disc diffusion assay was the general method used to select the active crude extract and compounds. The agar plate dilution assay was used to evaluate the MIC and MFC values of anti-wood rot compounds. The broth dilution assay was used to determine MIC value of active compounds against *Aspergillus niger* for further study on the mode of action in fungi. The contact bioautographic assay (biogram assay) was used for fast screening after separation of crude extracts.

Two additional advanced screening assays were used to further investigate the mode of action of natural products in microorganisms. Anthranilate synthase, one of the enzymes in tryptophan biosynthesis pathway, is a new enzyme target for inhibition due to its present in microorganisms, plants and some parasites, but not in mammals. 1,3-α-D-glucan is the prominent component in the cell wall of many fungal species, and was used as a target in a study of the induction of fungal cell wall stress in transgenic *A. niger*.

Chapter 3

Screening for antimicrobial activity

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Abstract

Flowers of *Cannabis sativa* and *Humulus lupulus* as well as sawdust of the tropical hardwoods *Tectona grandis*, *Xylia xylocarpa, Shorea obtusa, Shorea albida* and *Hopea odorata*, were screened for antimicrobial activity. The *Cannabis sativa* extract and fractions inhibited growth of *Bacillus subtilis* and *Escherichia coli* in the paper disc diffusion assay, inhibition was found to be stronger against *B. subtilis*. The strongest inhibition was found in a fraction derived from the *C. sativa* flower $CHCl_3$-MeOH (1:1) extract. This fraction was compared with reference cannabinoids in the biogram assay and it was found that the cannabinoid acids, THCA, CBDA and CBGA, have activity. *Humulus lupulus* flower extract ($CHCl_3$-MeOH, 1:1) showed inhibition of *Aspergillus niger* in the broth dilution assay, with a MIC of 100 ppm. The tropical hardwoods, *T. grandis*, *X. xylocarpa, S. obtusa, S. albida* and *H. odorata* extracts ($CHCl_3$-MeOH, 1:1) were tested for inhibition of *A. niger* in a broth dilution assay. Only *T. grandis* extract caused clear inhibition (MIC=25 ppm).

3.1 Introduction

Extracts of the flowers of two Cannabaceae plants (*Cannabis sativa* L. and *Humulus lupulus* L.) and sawdust of five tropical hardwoods (*Tectona grandis* L.f., *Xylia xylocarpa* Roxb., *Shorea obtusa* Wall. ex Blume, *Shorea albida* Symington and *Hopea odorata* Roxb.) were screened for antimicrobial activity. *Cannabis sativa* and *H. lupulus* have been reported to have pharmacological and also antimicrobial effects [Polunin, 1969; Baker et al., 2003; Hartwell, 1971; Foster, 1996; Langezaal et al., 1992]. Their flower extracts contain acid compounds such as cannabinoid acids and hop bitter acids as major constituents, respectively [Padua, 1999; Simpson and Smith, 1992]. As these compounds are easy to produce on a large scale and are all available as pure compounds, it is of interest to further test their antimicrobial activity with both general and specific microorganisms, as well as to study their mode of action in microorganisms. Cannabinoids are used for medical purposes and hop bitter α-acids are mainly used in beer processing. Hop *β*-acids are already used as antimicrobial compounds in the sugar industry.

The waste material remaining after the isolation of economically useful products from the agricultural processing industry is a potential source for the development of novel products, and would add extra value to the production process. Additionally, tropical hardwood sawdust could be an interesting source for screening antimicrobial activity because hardwoods are known to be resistant against termites and fungi.

The family Cannabaceae consist of two genera, *Cannabis* and *Humulus*. *Cannabis sativa* is the only species in *Cannabis* with several varieties. It is an erect herb, with leaves palmately divided into long, lanceolate and serrate leaflets. Trichomes are of various types but two-armed hairs are absent. The flowers are unisexual. Male flowers occur in short, dense cymes, united into foliate, terminal panicles with very shortly pedicelled. Female flowers inflorescences are congested series of false spikes with solitary flowers instead of cymes. The separation of sex in flowers is perfect [Kubitzki et al., 1993; Padua, 1999]. *Cannabis sativa* is normally dioecious but monoecious cultivars have been bred. The two sexes are normally indistinguishable before flowering [Padua, 1999].

Cannabis has been domesticated for about 8,500 years to obtain fibers from the stems, oil of the seeds and an intoxicating resin from the epidermal glands. The earliest recorded medicinal use of *C. sativa* is found in a 4,700 year old Chinese pharmacopoeia. The most significant group of compounds are the cannabinoids, of which many individual compounds are known [Padua, 1999]. Cannabinoids are highly concentrated in small droplets of sticky resin produced by glands at the base of the fine hairs that coat the leaves and particularly the bracts of the female flower head. The medicinally useful pharmacological effects of Cannabis are well recognized. There has for example been a steady stream of medical claims throughout history that cannabis eases limb-muscle spasms, migraine and pain [Polunin, 1969]. Because of its psycho-activity, mildly euphoric and relaxing effects, it is widely used as a recreational drug although it might have intoxicating effects [Ameri, 1999; Baker et al., 2003]. In some limited cases, cannabis can induce unpleasant transient effects, such as anxiety, panic and paranoia. It might also lead to acute transient psychosis involving delusions and hallucinations. Cannabis also induces an increase in heart rate, lowers blood pressure due to vasodilatation, stimulates appetite, and causes dry mouth and dizziness [Baker et al., 2003].

Humulus lupulus (Hop) is a twining perennial herb. Leaves are palmately lobed or simple. Two-armed hairs are present on stems and petioles. The greenish flowers are dioecious. Male flowers are growing from the axils of leaves on racemiform branches and possess five stamens. Female flower inflorescence is pendent and conelike. The cones are spikes in which 2-6 flowered cymes are condensed. Cones originate in the axis of stipular bracts with reduced leaf blades, pale green, papery and overlapping oval bract. Cones are used to give a bitter flavour to beer and help to preserve it [Kraemer, 1910; Kubitzki et al., 1993; Polunin, 1969]. *Humulus lupulus* has been used for brewing beer since the 8th century in Europe, and since the 1300's it has been in cultivation [Kubitzki et al., 1993].

Hop cones are frequently used as phytomedicine, e.g. as a bitter tonic, sedative or hypnotic, and for promoting healthy digestion. Sometimes they are used to treat cancer and ulcerations [Hartwell, 1971]. Hop tea is used as a mild sedative and remedy for insomnia [Weiss, 1988]. A poultice of hops is used to topically treat sores and skin injuries and to relieve muscle spasms and nerve pain [Foster, 1996]. It has been reported in many articles that *H. lupulus* preparations have an antimicrobial effect. Hop extracts and essential oils showed activities against gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and fungi (*Trichophyton mentagrophytes*) but almost no activities against gram negative bacteria (*Escherichia coli*) and yeast (*Candida albicans*) [Langezaal et al., 1992]. The bitter acids from hop plants have an antimicrobial activity against *Lactobacillus brevis* and monovalent cations enhanced the antibacterial activity of trans-isohumulone [Simpson and Smith, 1992]. Growth of *Listeria monocytogenes* was found to be inhibited in culture media and in certain foods by four different hop extracts containing varying concentrations of α- and *β*-acids [Larson et al., 1996]. Iso-α-acids have antibacterial activity against gram positive bacteria [Sakamoto and Konings, 2003].

Tectona grandis (Teak, Verbenaceae) occurs naturally in peninsular India, Myanmar, Thailand and Laos. It was most probably introduced to Java several hundred years ago and now occurs more or less naturally. It is cultivated on a large scale both inside and around the Malaysian region. It is a medium-sized to large tree growing up to 50 m tall, bole straight and branchless for up to 20(-25) m, with a diameter up to 150(-250) cm, sometimes fluted or with low buttresses at the base. The bark surface with longitudinal cracks is grayish brown and the inner bark is red and has a sticky sap. Leaf shape is broadly ovate, with (11-)20-55 cm x (6-)15-37 cm (and much larger on suckers). Flowers are 3-6 mm long, the corolla is white with pink on the lobes. The fruit is enclosed by an inflated calyx. Several morphological forms have been distinguished, principally by leaf characters. *Tectona grandis* generally occurs in deciduous forest on fertile and well-drained soil up to an altitude of a 1000 m. Teak is a well-known and very good general-purpose timber. Its favorable properties make it suitable for a wide variety of purposes. It is used extensively for houses, rails, bulwarks, latches, weather doors, etc. Teak is an excellent timber for bridge building and other constructions in constant contact with water such as docks, quays, piers and floodgates in fresh water. In house building teak is particularly suitable for interior and exterior joinery (windows, solid panel doors, framing) and is used for floors exposed to light and to moderate pedestrian traffic. It is also used quite extensively in the manufacture of both indoor and garden furniture. The root bark and the young leaves produce a yellowish-brown or reddish dye which is used for paper, clothes and matting. Sawdust from teak wood is used as incense in Java. In traditional medicine a wood powder paste has been used against bilious headaches and swellings, and internally against dermatitis or as a vermifuge. The charred wood soaked in poppy juice and made into a paste is used to relieve swelling of eyelids. The bark has been used as an astringent and the wood oil as a hair tonic [Soerianegara and Lemmens, 1993].

Xylia xylocarpa (Leguminosae) occurs in India, Myanmar, Indo-China and Thailand. It is also planted within its natural area of distribution, occasionally in Singapore and Malaysia but rarely outside this region. *Xylia xylocarpa* is a deciduous, medium-sized tree up to 25(-40) m tall, bole straight and cylindrical, sometimes fluted, branchless for up to 12(-25) m and up to 75(-120) cm in diameter. The bark surface is flaky with small lenticels, grayish to reddish or yellow-brown, the inner bark is pinkish. Leaves are arranged spirally, bipinnate with 1 pair of pinnae, rachis and pinnae glandular. Leaflets are opposite, 3-6 pairs per pinna. Flowers are in stalked globose heads, male or bisexual, penta-merous. Fruits have a boomerang-shaped, flat, woody pod. The seeds are ellipsoid flat, the testa hard and brown, with pleurogram. *Xylia xylocarpa* occurs in dry evergreen forest, mixed deciduous forest and dry deciduous dipterocarp forest, on well-drained, sandy and rocky soils, up to an altitude of 850 m. The hard and durable wood of *X. xylocarpa* is used for heavy construction, e.g. for posts and flooring, bridges, marine pilings, railway and boat construction, freshwater locks, paving blocks, rubbing fenders, chutes and for furniture, carvings and household implements. The bark and fruits are used in the local medicine of Indo-China in a decoction against haemoptysis. The hardwood is very resistant to treatment with preservatives, but the sapwood is readily treatable. The wood is susceptible to longhorn and buprestid beetle attack while resistant to termites and marine borers [Sosef et al., 1998].

Shorea obtusa (Dipterocarpaceae) is distributed in Myanmar, Cambodia, Laos, Thailand and Southern Vietnam. It is a small to medium size tree growing up to 30 m, bole branchless for up to 15 m and up to 65 cm in diameter. The bark is scaly, thick and brown. Leaves are variable and generally oblong 7.0-11.5 cm x 3.5-9.5 cm, sparsely pubescent below, with 15-20 pairs of secondary veins. *Shorea obtusa* is common in dry deciduous dipterocarp forest at altitude between 200-1,000 m. It is an important source of balau timber used for high-grade outdoor constructions. The bark has tanniferous properties [Soerianegara and Lemmens, 1993].

Shorea albida (Dipterocarpaceae) occurs in North-western Borneo. It is a medium-sized to very large tree up to 70 m tall, with a long bole up to 190 cm in diameter, prominent buttresses, up to 5 m high, compressed twigs. Leaves are oblong-elliptical, 7.5-15 cm x 4.5-6.5 cm. The fruit calyx is lobed, up to 8 cm x 1.4 cm. *Shorea albida* occurs typically in peat swamp forest and locally on podzolic soils in heath forest up to 1200 m altitude. It is an important source of dark red meranti timber. Comparatively heavy timber is sometimes traded as 'alan batu' which is similar to red balau. Lighter material is called 'alan bunga'[Soerianegara and Lemmens, 1993].

Hopea odorata (Dipterocarpaceae) is distributed in Bangladesh, Burma, Laos, southern Vietnam, Cambodia, Thailand, the Andaman Islands and northern Peninsular Malaysia. It is a medium-sized to large tree growing up to 45 m tall, bole straight, cylindrical, branchless for up to 25 m, with a diameter of up to 120 cm and prominent buttresses. The bark surface is scaly and dark brown. The outer bark is rather thick, the inner bark is a dull yellow and the sapwood is resinous. *Hopea odorata* is a riparian species and usually occurs on deep rich soils up to an altitude of 600 m. The wood is suitable for rollers in the textile industry, piles and bridge construction, and as an alternative to maple for shoe and boot lasts. The bark has high tannin content, and is suitable for tanning leather. The Burmese use this bark to make a varnish, and use as paint by mixing with ink. It is also used to caulk boats. The bark is medicinally applied to sores and wounds. In Indo-China the bark has been used as a masticatory [Soerianegara and Lemmens, 1993].

In the experiments reported here, the extracts of dry flowers of *C. sativa* and *H. lupulus* and dry sawdust of *T. grandis*, *X. xylocarpa, S. obtusa, S. albida* and *H. odorata were used* for the screening of antimicrobial activity against the gram positive bacteria, *Bacillus subtilis*, the gram negative bacteria *Escherichia coli,* and the filamentous fungi, *Aspergillus niger,* using paper disc diffusion assays, biogram assays and broth dilution assays.

3.2 Materials and Methods

3.2.1 Plant extracts

All the solvents for the extraction and isolation of plant compounds were obtained from Biosolve B.V. (Valkenswaard, the Netherlands). *Cannabis sativa* (SIMM 4) flowers were collected July 12, 2002 by The Institute of Medical Marijuana, The Netherlands. Flowers of *Humulus lupulus* were collected on September 16, 2003 from the garden at the Institute of Biology, Leiden University, Sterrenwachtlaan, Leiden, the Netherlands. A supercritical carbon dioxide extract of *H. lupulus* flowers was received from Botanix (Paddock Wood, Kent, UK). Tropical hardwood sawdust was collected on February 13, 2004 from a wood processing company, Bangkok, Thailand.

Samples of *C. sativa*, *H. lupulus* and the tropical hardwoods, *Tectona grandis*, *Xylia xylocarpa, Shorea obtusa, Shorea albida* and *Hopea odorata* were each extracted twice with chloroform-methanol ($CHCl_3$-MeOH, 1:1). *Cannabis sativa*, *T. grandis* and *X. xylocapa* were also extracted with 80% MeOH. All extracts were sonicated (Sonicor, New York, USA) for 2 hours. The crude extracts were dried using an evaporator. The *Cannabis sativa* $CHCl_3$-MeOH (1:1) extract (F1) was fractionated with *n*-hexane-90% MeOH (to get fractions F2 and F3) and the 80% MeOH extract (F4) was fractionated with $CHCl_3$-water (to get fractions F5 and F6).

3.2.2 Paper disc diffusion assay

The dried cannabis flower extracts (F1 to F6) were dissolved in dimethylsulfoxide (DMSO) [Gülerman et al., 2001] to a final concentration of 100 mg/mL. DMSO was used as a negative control and 1 mg/mL of Chloramphenicol (Sigma, St. Louis, USA) was used as a positive control for this assay. Sterile filter paper discs (Schleicher & Schuell type 602 H, Dassel, Germany) 5 mm in diameter, were impregnated with 2 mg (15 µL) of plant extract.

Escherichia coli (LMD 72.2) and *Bacillus subtilis* (NCCB 89157) were used to determine the antibacterial activities. Cultures of *E.coli* and *B. subtilis* were stored in CASO-Bouillon broth (Merck, Darmstadt, Germany) with 70% glycerol (Acros organics, Geel, Belgium) at -80 °C before inoculating 50 mL of CASO-Bouillon broth, and incubating 37 °C overnight. 250 µL of bacterial cell suspension (at a concentration of 10^8 CFU/mL) was spread onto the surface of

CASO-Bouillon agar media in 9 cm diameter Petri dishes before additional of the impregnated papers discs.

The diameter of an inhibition zone around the discs was measured after incubating bacterial plates at 37 °C in the dark for 24 h. The values were recorded with the average (mm) of two diameter measurements per disc taken in two directions, roughly perpendicular. This assay was done in 5 replicates.

3.2.3 Biogram assay

The biogram assay was used for determining antimicrobial activity of all extracts. Cannabis flower extracts (F1 to F5) and cannabinoids were dissolved in ethanol and spotted on TLC plates (aluminum sheet silica gel 60 F_{254}, Merck, Darmstadt, Germany) in duplicate sets (one as the reference, and one for the assay). The TLC plates were developed in TLC chambers with a solution of $CHCl_3$ and $CHCl_3$-MeOH (20:1) then air-dried in a fume hood. The reference TLC plate was sprayed with Anisaldehyde-sulphuric acid reagent to evaluate the R_f-value of the bands present. For the assay plate, *Bacillus subtilis* was grown by spreading 500 µL of bacterial cell suspension (at the concentration of 10^8 CFU/mL) on a CASO-Bouillon agar Petri dish (12 cm diameter). The TLC plate was placed on the bacteria-inoculated agar Petri dishes and incubated at 4 °C overnight for diffusion of the bands into the agar media. After removing the TLC plate, the Petri dishes were incubated at 37 °C for 24 h for bacterial growth. The determination of inhibition zones are reported as the R_f-value on the reference TLC plates.

A solid phase column (Strata SI-1 Silica) was used to separate the compounds from fraction F3 of the cannabis flower extract, before testing the activities of $F3_1$ to $F3_9$ against *B. subtilis* by the biogram assays. The mobile phase solvents for this separation were *n*-hexane-diethyl ether in the ratios of 100:0, 50:1, 25:1, 10:1, 5:1, 2:1, 1:1 and 0:100, respectively. Methanol was used to wash the column for the last fraction. The fractions from this separation were spotted on TLC plates in triplicate. The reference TLC plates were sprayed with Anisaldehyde-H_2SO_4 reagent. And the test TLC plate was used for the biogram assay with *B. subtilis*. This assay was done in triplicate.

In order to confirm that malt extract (ME) agar media (Fluka, Spain) and complete media (CM) can used in this assay, five hundred µL *Aspergillus niger* N402 (wild type) spore suspension with a concentration of 10^7 CFU/mL was mixed with 50 mL CM [Bennett and Lasure, 1991] or ME and poured into a 15 cm Petri dish to make the final concentration of 10^6 CFU/mL. *Tectona grandis* $CHCl_3$-MeOH (1:1) dry extract was dissolved in methanol at a concentration of 5 mg/mL and 4 µL was used per spot (each spot had 20 µg of the extract). Both $CHCl_3$-MeOH

(5:1) and $CHCl_3$-MeOH (19:1) were used to develop each TLC plate. For 2D-TLC, two different developing systems were used: in system 1, the first dimension (y) was developed with $CHCl_3$-MeOH (19:1) and the second dimension (x) with *n*-hexane-ethyl acetate (*n*-hexane-EtOAc, 19:1); in system 2, the first dimension (y) was developed with $CHCl_3$-MeOH (19:1) and the second dimension (x) with *n*-hexane-EtOAc (5:1). All TLC plate treatments were made in duplicate with one replication used as a reference plate. The reference plate was observed under UV light (254 and 366 nm) and then sprayed with Anisaldehyde-sulphuric acid reagent.

3.2.4 Broth dilution assay

Aspergillus niger N402 (wild type) spores were grown on a CM agar plate for 3 days at 30 °C until sporulation. A spore suspension was created by adding physiological salt (0.9 % NaCl) to the plates and lightly scraping the surface of fungal growth. The spores were collected and pipetted to mira cloth for removing the fungal mycelium. The spore suspension was then diluted and the number of spores counted per mL using hemocytometer. Stock of *A. niger* spores was made at 10^7 CFU/mL and kept refrigerated at 4 °C. Spore stock can be used for two weeks after harvesting.

The microplate assay was done in a 96 well microplate. 200 µl of plant extract (200 µg/mL) was added to the first well and twofold dilutions were made with sterile water to concentrations of 100, 50, 25 and 12.5 µg/mL. A 100 µL hydrogen peroxide (H_2O_2) solution (80 mM) was used as the positive control to make a concentration of 40 mM, while 100 µL sterile water and DMSO (concentrations of 2.5, 1.25, 0.625 and 0.312 %) were used as the negative controls. The *A. niger* stock spores were diluted in CM to a concentration of 2 x 10^5 CFU/mL before adding into each well. The wells were inoculated with 100 µL of *A. niger* spore stock to have the final concentration of 10^5 CFU/mL. The total volume in each well is 200 µL. The microplate was incubated at 37 °C in the dark, and measured every hour for 40 hours by a microplate reader (HTS 7000 Bio Assay Reader, Perkin Elmer, USA). The absorbance in each well was read at the wavelength of 590 nm. This assay was done in 4 replications.

3.3 Results and discussion

3.3.1 Paper disc diffusion assay

Escherichia coli and *B. subtilis* were used to determine the antibacterial activities of *C. sativa* flower extract (F1 to F6) by disc-diffusion assay. Clear inhibition zones were found at 24 h after incubation at 37 °C (Figure 3.1). *Cannabis sativa* extract, F1 to F3, showed strong inhibition against *B. subtilis* and lower activity was found in F4 and F5. No inhibition was observed for F6 against either *B. subtilis* or *E. coli*. *Escherichia coli* showed weak inhibition by cannabis extract, F1 to F5. The negative control (DMSO) had no effect on bacterial growth while the positive control (Chloramphenicol) showed a strong inhibition of both *E. coli* and *B. subtilis*.

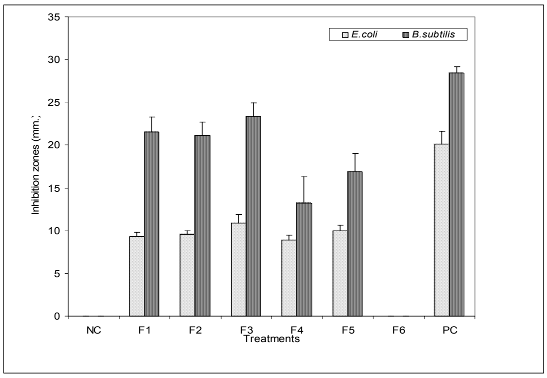

Figure 3.1 *In vitro* antibacterial activities of *Cannabis sativa* flower extracts (F1 to F6) against *Escherichia coli* and *Bacillus subtilis* in disc-diffusion assays. Data shown as mean (n=5) with standard deviation as error bars; significant difference ($p<0.05$). NC, negative control; PC, positive control.

3.3.2 Biogram assay

After TLC separation of fraction F3, the plates were studied by means of the biogram method. Several active spots were observed. Strongest activity was at R_f 0.37 (0.17-0.57) using $CHCl_3$-MeOH as the mobile phase. Comparing fraction F3 with reference compounds showed that the cannabinoid acids, cannabigerolic acid (CBGA), cannabidiolic acid (CBDA) and tetrahydrocannabinolic acid (THCA) are the active bands at R_f 0.25, 0.35 and 0.37, respectively (Figures 3.2 and 3.3). Since the cannabinoid acids partially overlapped in the TLC, fraction F3 was separated on a solid phase column (Strata SI-1 Silica) to separate the cannabinoid acids from each other and any other compounds. Nine fractions ($F3_1$ to $F3_9$) were collected from this separation using a gradient of *n*-hexane and diethyl ether as mobile phase. Each fraction was tested for inhibition on *B. subtilis* growth. Inhibition zones were observed for $F3_1$ and $F3_2$ but not in the other fractions (Table 3.1). After spraying with AS reagent the compounds in both fractions were identified as cannabinoid acids.

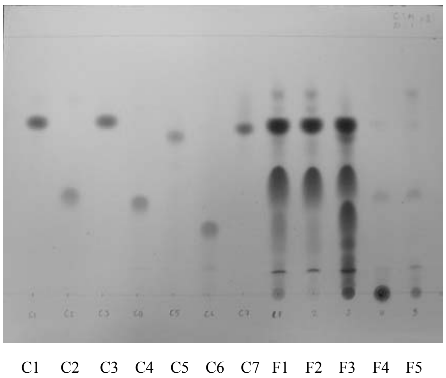

Figure 3.2 Seven cannabinoids (C1-C7) and *Cannabis sativa* extracts F1 to F5 on TLC plate after spraying with anisaldehyde-H_2SO_4 reagent. C1, Δ^9-THC; C2, THCA-A; C3, CBD; C4, CBDA; C5, CBG; C6, CBGA; C7, CBN.

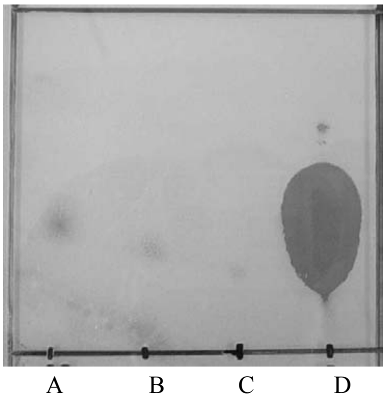

Figure 3.3 Inhibition zones of THCA (A), CBDA (B), CBGA (C) and *Cannabis sativa* $CHCl_3$-MeOH (1:1) extract Fraction F3 (D).

Table 3.1 *In vitro* antibacterial activity of *Cannabis sativa* $CHCl_3$-MeOH (1:1) extract against *Bacillus subtilis* on biogram assays after solid phase fractionation.

Fraction	Mobile phase solvents*	Inhibition zone
$F3_1$	*n*-hexane	+
$F3_2$	*n*-hexane-diethyl ether 50:1	+
$F3_3$	*n*-hexane-diethyl ether 25:1	-
$F3_4$	*n*-hexane-diethyl ether 10:1	-
$F3_5$	*n*-hexane-diethyl ether 5:1	-
$F3_6$	*n*-hexane-diethyl ether 2:1	-
$F3_7$	*n*-hexane-diethyl ether 1:1	-
$F3_8$	diethyl ether	-
$F3_9$	methanol	-

*Each fraction included 6 washes with solvent; + Shows inhibition zone; - No inhibition zone.

The dried crude extract of *T. grandis* used in this assay was prepared by dissolving in methanol. The biogram results showed that the active compounds are present in two areas which are non polar (R_f 0.76 in $CHCl_3$-MeOH (5:1) and R_f 0.7 in $CHCl_3$-MeOH (19:1). However, both areas consist of many compounds that are close together. A 2D-TLC plate was used to solve this problem. A better separation was obtained by 2D-TLC and the inhibition zones are shown in Table 3.2. The reference TLC plate was observed under UV 366 and showed orange coloured spots.

Table 3.2 Bands R_f values and their inhibition zone from *Tectona grandis* $CHCl_3$-MeOH (1:1) extract against *Aspergillus niger* using biogram assay with 2D-TLC.

System 1*			**System 2***		
R_f values		Inhibition zone	R_f values		Inhibition zone
1st dimension (y)	2nd dimension (x)		1st dimension (y)	2nd dimension (x)	
0.08	0	-	0.08	0	-
0.18	0	-	0.18	0	-
0.28	0	-	0.28	0	-
0.41	0	+	0.41	0	-
0.52	0	+	0.52	0.10	-
				0.16	-
0.73	0	-	0.73	0.02	-
	0.08	-		0.20	-
	0.16	+		0.36	+
0.77	0.64	-	0.77	0.65	-
0.81	0.45	-			

* System 1, the first dimension (y) was developed with $CHCl_3$-MeOH (19:1) and the second dimension (x) with *n*-hexane-EtOAc (19:1); system 2, the first dimension (y) was developed with $CHCl_3$-MeOH (19:1) and the second dimension (x) with *n*-hexane-EtOAc (5:1).

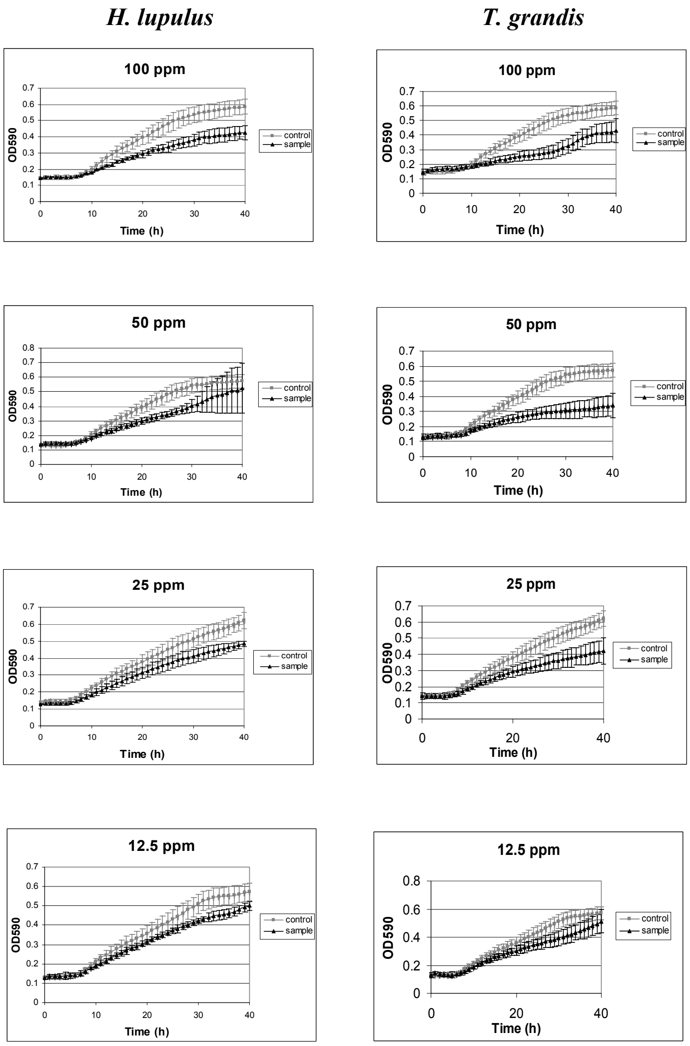

Figure 3.4 The effect of *Humulus lupulus* and *Tectona grandis* $CHCl_3$-MeOH extract, at the concentrations of 100, 50, 25 and 12.5 ppm, on *Aspergillus niger* growth. The error bars show the standard deviation (SD) from the mean (n=3) ($p<0.05$).

3.3.3 Broth dilution assay

The broth dilution assay showed that *C. sativa, X. xylocarpa* ($CHCl_3$-MeOH and 80% MeOH extract)*, S. obtusa, S. olbida* and *H. odorata* ($CHCl_3$-MeOH extract) did not inhibit growth of *A. niger* even at a concentration of 100 ppm. *Humulus lupulus* and *T. grandis* ($CHCl_3$-MeOH) extract inhibited growth of *A. niger* at MIC values of 100.0 and 25.0 ppm, respectively (Figure 3.4). *Tectona grandis* 80% MeOH extract showed the same result as $CHCl_3$-MeOH extract. The DMSO negative control (2.5, 1.25, 0.625 and 0.312 %) had no effect on growth of *A. niger* when compared with the sterilized water negative control. No *A. niger* growth was found in the positive control (H_2O_2).

3.4 Conclusion

Cannabis sativa, *H. lupulus* and *T. grandis* extracts have antimicrobial effects. *Cannabis sativa* extract fraction F3 ($CHCl_3$-MeOH, 1:1 extract, fractionated with 90% MeOH) showed this strongest inhibition of *B. subtilis* growth in the paper disc diffusion assay. When compared with the reference compounds in biogram assay, we found that the cannabinoid acids, THCA, CBDA and CBGA were active. *Humulus lupulus* and *T. grandis* extracts inhibited growth of *A. niger* in the broth dilution assay (MIC=100 and 25 ppm, respectively). Several compounds in the *T. grandis* extract showed inhibition zones in the biogram assay. Further studies on the isolation of these active compounds from this plant are described in the next chapter.

Chapter 4

Isolation and elucidation of quinones in Tectona grandis

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Abstract

The compounds deoxylapachol, tectoquinone, 2-hydroxymethylanthraquinone, 3'-OH-deoxyisolapachol (2-[(1*E*)-3-hydroxy-3-methylbut-1-enyl]naphthoquinone), hemitectol (2,2-dimethyl-2*H*-benzo[*h*]chromen-6-ol) and tectol were isolated from *Tectona grandis* sawdust $CHCl_3$-MeOH (1:1) extract. Centrifugal partition chromatography was used to separate these compounds using *n*-hexane-MeOH-H_2O (50:47.5:2.5) as a solvent system. All compounds except tectol showed antifungal activity in a biogram assay against *Aspergillus niger*.

4.1 Introduction

Teak (*Tectona grandis*) wood is commonly used for house construction and furniture in the Indochina region, because this strong hardwood has a beautiful surface and is resistant to mite and fungal damage. For possible new applications, we studied sawdust for the occurrence of interesting phytochemicals. Teak wood contains naphthoquinones (lapachol, deoxylapachol, 5-hydroxylapachol), naphthoquinone derivatives (α-dehydrolapachone, β-dehydrolapachone, tectol, dehydrotectol), anthraquinones (tectoquinone, 1-hydroxy-2-methylanthraquinone, 2-methyl quinizarin, pachybasin) and also obtusifolin, betulinic acid, trichione, β-sitosterol and squalene [Thomson, 1957; Hegnauer, 1973; Singh et al., 1989; Khan and Mlungwana, 1999]. Naphthoquinones have been reported to have antimicrobial activity [Guiraud, et al., 1994; Gafner, et al., 1996]. Lapachol has antitumour activity [Rao, et al., 1968; Rao and Kingston, 1982]. In Chapter 3, it was shown that *T. grandis* sawdust $CHCl_3$-MeOH (1:1) extract has antifungal activity. Here the structures of the active compounds found in this material are isolated and elucidated.

4.2 Materials and Methods

Centrifugal partition chromatography (CPC type LLN, Sanki engineering limited Kyoto, Japan) was used to separate the *T. grandis* chloroform-methanol ($CHCl_3$-MeOH, 1:1) extract using *n*-hexane-methanol-water (*n*-hexane-MeOH-H_2O, 50:47.5:2.5) as a solvent system with *n*-hexane as the mobile phase and MeOH-H_2O as the stationary phase. Ascending mode was used with 2.5 mL/min pump flow rate and 800 rpm rotation speed. After 80 fractions were collected (3 mL per fraction) the system was changed to the descending mode and collecting of fractions continued (fractions 81-90). All fractions were spotted on TLC plates (aluminum sheets silica gel 60 F_{254}, Merck, Darmstadt, Germany). The TLC plates were developed with $CHCl_3$-MeOH (19:1) or petroleum ether-acetone-acetic acid (75:25:1.5) and observed under UV 254 and 366 nm. The TLC plates were sprayed with Anisaldehyde-sulphuric acid reagent or 5% methanolic potassium hydroxide (KOH) [Sherma and Fried, 1991]. The biogram assay was used to test the activity of fractions using *Aspergillus niger* spore suspensions. The active compounds were measured by ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, HSQC and HMBC experiments with 300.13 MHz, (Bruker DPX-300), 399.68 MHz, (Bruker DPX-400) and 600.13 MHz (Bruker DPX-600) spectrometer. LC/MS (Agilent, USA) was used to determine the molecular weight of the active compounds. A C_{18} column (length 70 mm, 5 μM particle diameter, Macherey-Nagel, Germany) was used for the analysis with a gradient elution system of water containing 0.1% formic acid (v/v) (solvent A), and methanol or ethanol containing 0.1% formic acid (v/v) (solvent B). The gradient range was 50-100% of solvent B in 11 min using a flow rate of 0.5 mL/min with an injection volume of 5 µL. All mass spectrometric analyses were performed in a positive atmospheric pressure chemical ionization (APCI) mode.

4.3 Results and discussion

After CPC separation of *T. grandis* extract using *n*-hexane-MeOH-H_2O as the solvent system, it was found that fraction 19-21 (compound I), 25-29 (compound II), 82 (compound III), 84 (compound IV) and 87 (containing both compound V and VI) inhibited *A. niger* growth in the biogram assay. Inhibition was not found with compound VI, which was present in small amounts in fraction 87.

Fraction 19-21 showed an orange colour under UV 366 nm at R_f 0.72 in $CDCl_3$–MeOH (19:1) and R_f 0.44 in petroleum ether-acetone-acetic acid (75:25:1.5). It also showed a violet colour after spraying with 5% methanolic KOH. The UV spectrum showed absorption at λ_{max} (MeOH + 0.1% formic acid): 205, 250 and 335 nm. The ^1H NMR ($CDCl_3$, 300 MHz) showed signals at: δ=1.60 (3H, s, H-5'), 1.71 (3H, s, H-4'), 3.21 (2H, d, J=7.3 Hz, H-1'), 5.16 (1H, tm, 7.3 Hz, 1.4 Hz, H-2'), 6.70 (1H, t, J=1.7 Hz, H-3), 7.66 (2H, m, H-6, H-7), 7.99 and 8.03 (2H, m, H-5, H-8). These data and data from LC/MS: 227 $[M+H]^+$, correspond with deoxylapachol (Figure 4.1).

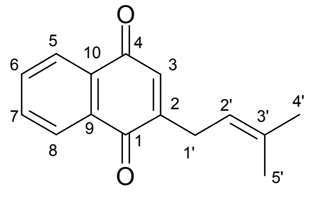

Figure 4.1 The structure of deoxylapachol (compound I)

Fraction 25-29 showed a red-orange colour under UV 366 nm at R_f 0.72 in $CDCl_3$ – MeOH (19:1) and R_f 0.37 in petroleum ether-acetone-acetic acid (75:25:1.5). The UV spectrum showed absorption at λ_{max} (MeOH + 0.1% formic acid): 205, 255 and 330 nm. The ^1H NMR ($CDCl_3$, 300 MHz) showed signals at: δ=2.47 (3H, s, H-1'), 7.53 (1H, d, J=7.9 Hz, H-3), 7.72

(2H, m, H-6, H-7), 8.04 (1H, s, H-1), 8.14 (1H, d, *J*=7.9 Hz, H-4), 8.23 (2H, m, H-8, H-5). These data and the result from LC/MS: 223 $[M+H]^+$, correspond with tectoquinone (Figure 4.2).

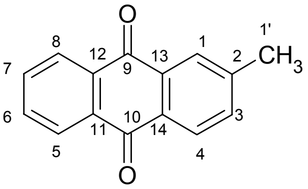

Figure 4.2 The structure of tectoquinone (compound II)

Fraction 82 contained active compound III at $R_f = 0.29$ which presented a bright yellow colour on TLC silica gel plate when developed with $CDCl_3$–MeOH (19:1) and showed a bright orange colour under UV 366 nm, a purple colour after spraying with AS, and a red colour after spraying with 5% methanolic KOH. The UV spectrum showed absorption at λ_{max} (EtOH + 0.1% formic acid): 205, 257 and 330 nm. ^1H NMR and ^{13}C NMR data and ^1H-^1H COSY, HSQC and HMBC experiments ($CDCl_3$, 600 MHz) were measured with 600 MHz Bruker DPX-600 spectrometer (Tables 4.1 and 4.2). These data and the data from LC/MS: 240 $[M+H]^+$, correspond with 2-hydroxymethylanthraquinone is proposed (Figure 4.3).

Table 4.1 COSY ($CDCl_3$, 600 MHz) correlations of 2-hydroxymethyl anthraquinone (compound III)

H		3	6 and 7	1'
	δ_H	7.82 (1H, m)	7.81 (2H, m)	4.90 (2H, s)
1	8.29 (1H, s)			*
3	7.82 (1H, m)			*
6 and 7	7.81 (2H, m)			
4, 5 and 8	8.32 (3H, m)	*	*	
1'	4.90 (2H, s)			

Table 4.2 HSQC (*) and HMBC (Δ) ($CDCl_3$, 600 MHz) correlations of 2-hydroxymethylanthraquinone (compound III)

C/H		1	3	6 and 7	4, 5 and 8	1'
	δ_C/δ_H	8.29 (1H)	7.82 (1H, m)	7.81 (2H, m)	8.32 (3H, m)	4.90 (2H, s)
1	124.91	*	Δ			Δ
2	147.58					Δ
3	131.97	Δ	*			Δ
4	127.73				*	
5	127.27			Δ	*	
6	134.19			*	Δ	
7	134.11			*	Δ	
8	127.25			Δ	*	
9	183.17	Δ			Δ	
10	182.93				Δ	
11 and 12	133.53 and 133.54			Δ	Δ	
13	133.64				Δ	
14	132.72	Δ	Δ			
1'	64.40					*

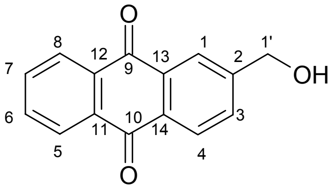

Figure 4.3 The structure of 2-hydroxymethylanthraquinone (compound III)

Fraction 84 contained active compound IV at R_f 0.31 which showed a bright yellow colour on the TLC silica gel plate when developed with $CDCl_3$ –MeOH (19:1), a dark orange colour under UV 366 nm and a dark red colour after spraying with 5% methanolic KOH. The UV spectrum showed absorption at λ_{max} (EtOH + 0.1% formic acid): 208, 252 and 300, 345 nm. ^1H NMR and ^{13}C NMR data and ^1H-^1H COSY, HSQC and HMBC experiments ($CDCl_3$, 600 MHz) were measured with 600 MHz Bruker DPX-600 spectrometer (Tables 4.3 and 4.4). LC/MS showed 225 $[M+H-18]^+$. Based on the spectral data, the novel structure IV (Figure 4.4) is proposed (2-[(1*E*)-3-hydroxy-3-methylbut-1-enyl] naphthoquinone), which we gave the trivial name 3'-OH-deoxyisolapachol.

Table 4.3 COSY ($CDCl_3$, 600 MHz) correlations of 3'-OH-deoxyisolapachol (compound IV)

H		6 and 7	1'
	δ_H	7.75 (2H, m)	6.81 (1H, d, J=16.3 Hz)
3	6.98 (1H, s)		
5	8.07 (1H, m)	*	
6 and 7	7.75 (2H, m)		
8	8.12 (1H,m)	*	
1'	6.80 (1H, d, J=16.3 Hz)		
2'	6.83 (1H, d, J=16.3 Hz)		*
4' and 5'	1.44 (6H, s)		

Table 4.4 HSQC (*) and HMBC (Δ) ($CDCl_3$, 600 MHz) correlations of 3'-OH-deoxyisolapachol (compound IV)

C/H		3	5	6 and 7	8	1'	2'	4' and 5'
	δ_C/δ_H	6.98 (1H, s)	8.07 (1H, m)	7.75 (2H, m)	8.12 (1H,m)	6.80 (1H, d, J=16.3 Hz)	6.83 (1H, d, J=16.3 Hz)	1.44 (6H, s)
1	185.40					Δ		
2	143.81	Δ					Δ	
3	131.28	*						
4	184.90	Δ						
5	125.93		*	Δ				
6	133.90			*	Δ			
7	133.77		Δ	*				
8	126.77				*			
9	132.30		Δ	Δ				
10	132.40	Δ		Δ	Δ			
1'	118.52	Δ				*		
2'	147.44						*	Δ
3'	71.61							Δ
4' and 5'	29.55							*,Δ

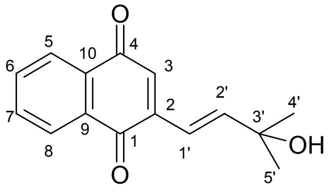

Figure 4.4 The structure of 3'-OH-deoxyisolapachol (compound IV)

Fraction 87 showed activity and contained two compounds (compound V and VI). Compound V at R_f 0.47 showed a violet colour under UV 366 nm, a blue-gray colour after spraying with AS and a red-purple colour after spraying with 5% methanolic KOH. The UV spectrum showed absorption at λ_{max} (EtOH + 0.1% formic acid): 222, 273 and 360 nm. ^{1}H NMR and ^{13}C NMR data and ^{1}H-^{1}H COSY, HSQC and HMBC experiments (MeOD, 400 MHz) were measured with 400 MHz Bruker DPX-400 spectrometer (Tables 4.5 and 4.6). LC/MS showed 227 $[M+H]^+$. Based on the spectral data, the structure of the novel compound (Figure 4.5, compound V) is proposed (2,2-dimethyl-2*H*-benzo[*h*]chromen-6-ol). This compound was given the trivial name hemitectol.

Table 4.5 COSY (MeOD, 400 MHz) correlations of hemitectol (compound V)

H		6 and 7	2'
	δ_H	7.39 (2H, m)	5.68 (1H, d, *J*=9.6 Hz)
3	6.53 (1H, s)		
5 and 8	8.04 (2H, m)	*	
6 and 7	7.39 (2H, m)		
1'	6.36 (1H, d, *J*=9.6 Hz)		*
2'	5.68 (1H, d, *J*=9.6 Hz)		
4' and 5'	1.45 (6H, s)		

Table 4.6 HSQC (*) and HMBC (Δ) (MeOD, 400 MHz) correlations of hemitectol (compound V)

C/H		3	5 and 8	6 and 7	1'	2'	4' and 5'
	δ_C/δ_H	6.53 (1H, s)	8.04 (2H, m)	7.39 (2H, m)	6.36 (1H, d, J=9.7 Hz)	5.68 (1H, d, J=9.7 Hz)	1.45 (6H, s)
1	142.13	Δ			Δ		
2	116.74				Δ	Δ	
3	106.99	*			Δ		
4	147.80	Δ					
5	123.13		*	Δ			
6	126.49		Δ	*			
7	125.81		Δ	*			
8	122.45		*	Δ			
9 and 10	126.95 and 127.14						
1'	123.97	Δ			*		
2'	131.27					*	Δ
3'	77.07				Δ	Δ	Δ
4' and 5'	27.69 and 27.84					Δ	*, Δ

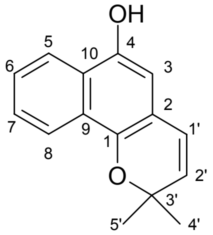

Figure 4.5 The structure of hemitectol (compound V)

Compound VI at $R_f = 0.77$ showed a violet colour under UV 366 nm, a gray colour after spraying with AS, and a violet colour after spraying with 5% methanolic KOH. The UV spectrum showed absorption at λ_{max} (EtOH + 0.1% formic acid): 230, 275 and 362 nm. ^{1}H NMR and ^{13}C NMR data and ^{1}H-^{1}H COSY, HSQC and HMBC experiments ($CDCl_3$, 600 MHz) were measured with 600 MHz Bruker DPX-600 spectrometer (Tables 4.7 and 4.8). LC/MS showed 451 $[M+H]^+$. The spectral data correspond with tectol (Figure 4.6).

Table 4.7 COSY ($CDCl_3$, 600 MHz) correlations of tectol (compound VI)

H		6 and 7	2'	5'
	δ_H	7.46 (2H, m)	5.56 (1H, d, *J*=9.8 Hz)	1.46 (3H, s)
5	8.15 (1H, d, *J*= 7.9 Hz)	*		
8	8.18 (1H, d, *J*=7.7 Hz)	*		
6 and 7	7.46 (2H, m)			
1'	5.86 (1H, d, *J*=9.8 Hz)		*	
2'	5.56 (1H, d, *J*=9.8 Hz)			
4'	1.50 (3H, s)			*
5'	1.46 (3H, s)			

Table 4.8 HSQC (*) and HMBC (Δ) ($CDCl_3$, 600 MHz) correlations of tectol (compound VI)

C/H		5	8	6 and 7	1'	2'	4'	5'
	δ_C/δ_H	8.15 (1H, d, *J*= 7.9 Hz)	8.18 (1H, d, *J*=7.7 Hz)	7.46 (2H, m)	5.86 (1H, d, *J*=9.8 Hz)	5.56 (1H, d, *J*=9.8 Hz)	1.50 (3H, s)	1.46 (3H, s)
1	143.20		Δ		Δ			
2	117.40					Δ		
3	114.00				Δ			
4	145.30	Δ						
5	123.57	*		Δ				
6	126.46		Δ	*				
7	126.78	Δ		*				
8	122.68		*	Δ				
9 and 10	127.17 and 127.27			Δ				
1'	122.37				*			
2'	130.92					*	Δ	Δ
3'	76.73				Δ	Δ	Δ	Δ
4'	27.84					Δ	*	Δ
5'	27.67					Δ	Δ	*

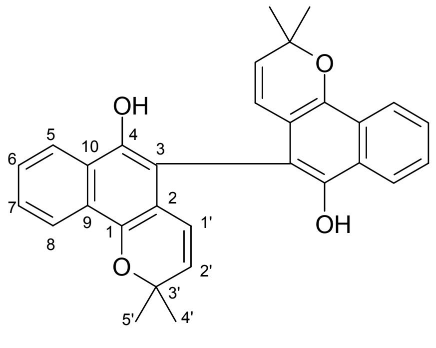

Figure 4.6 The structure of tectol (compound VI)

4.4 Conclusion

Centrifugal partition chromatography (CPC) was used successfully to separate the active compounds from *T. grandis* extract. The biogram assay was used for fast screening of the antifungal compounds from CPC fractions. Six compounds were found and were identified as deoxylapachol, tectoquinone, tectol, hemitectol, 2-hydroxymethylanthraquinone and 3'-OH-deoxyisolapachol, all of which except tectol showed zones of inhibition in the biogram assay. Some of these compounds were tested further for antimicrobial activities (Chapters 5 and 7).

Chapter 5

Induction of fungal cell wall stress

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Abstract

The effect of *Humulus lupulus* and *Tectona grandis* extracts on a transgenic *Aspergillus niger* was studied, in order to learn more about the possible mode of action. The transgenic strain that was used is a cell wall damage model. It shows induction of 1,3-*α*-D-glucan synthase gene by coupling it to a green fluorescent protein (GFP) marker encoding sequence. Induction of the gene encoding the glucan synthase is detected as fluorescence in the fungal cells. The results show that *T. grandis* extract, fraction 87 (hemitectol + tectol) and deoxylapachol, which were derived from this plant extract, induce fungal cell wall stress.

5.1 Introduction

The fungal cell wall is an attractive target for the development of new antifungal agents because it is essential for the viability of fungal cells, and the fungal cell wall has no counterpart in mammalian cells. The fungal cell wall is composed 80-90% of carbohydrates. The main structural polysaccharides include cellulose, chitin, mannan and glucan [Hugo and Russell, 1992]. 1,3-α-D-glucan synthase is a prominent component in the cell walls of many fungal species such as *Schizosaccharomyces pombe* [Bacon et al., 1968; Sietsma and Wessels, 1990], *Aspergillus nidulans* [Bull, 1970, Zonneveld, 1972] and *Aspergillus niger* [Horisberger et al., 1972; Johnston, 1965]. The first 1,3-α-D-glucan synthase gene, named *ags1,* was identified and analyzed in *S. pombe* [Hochstenbach, et al., 1998]. Damveld and coworkers [2005] identified a family of five 1,3-α-D-glucan synthase-encoding genes in *A. niger*.

To further study the antifungal activity of *Humulus lupulus* and *Tectona grandis* extracts shown in Chapter 3 the mode of action was tested. A transgenic *Aspergillus niger* is used as a model for the induction of fungal cell wall stress. By coupling a green fluorescence protein marker encoding sequence to the 1,3-α-D-glucan synthase gene, this cell line will show fluorescence when cell wall synthesis is induced after cell wall damage.

5.2 Materials and Methods

Humulus lupulus flowers and *Tectona grandis* sawdust were extracted with chloroform-methanol ($CHCl_3$-MeOH, 1:1) as described in Chapter 3. The isolation of active compounds from *T. grandis* extract is described in Chapter 4.

Three transgenic *A. niger* strains were developed by the Fungal Genetics Group, Clusius Laboratories, Institute of Biology, Leiden University, Leiden, The Netherlands. *Aspergillus niger* MA 26.1 (PgpdA-H_2B-GFP) was used as a GFP positive control (the GFP gene is continuously expressed) while *A. niger* N402 was used as a GFP negative control (not containing the GFP gene). *Aspergillus niger* RD 6.47 (PagsA-H_2B-GFP) and *A. niger* JD 1.1 (PagsA-GFP-Mc) were used for measuring the induction of fungal cell wall stress by coupling a green fluorescence protein marker encoding sequence to the glucan synthase gene (expression of the reporter gene resulting in the presence of GFP in the nucleus and cytoplasm, respectively) [Damveld, et al., 2005]. These fungi were grown and the fungal spores were harvested as previously described in Chapter 3. The detection of green fluorescence was done in a 96 well optical Etm Plt PolymerBase (Nalge Nunc International, New York, USA). A 200 µL solution of plant extract or compound was added and a two fold dilutions with sterile water were made to the concentrations of 100, 50, 25, 12.5 ppm. Dimethylsulfoxide (DMSO) was used as the negative control (the concentrations of 5.0, 2.5, 1.25 and 0.625 %) while 100 ppm calco fluor white (CFW) (Sigma, Steinheim, Germany) and 50 ppm sodium dodecyl sulfate (SDS) (MP Biomedicals, Eschwege, Germany) were used as the positive control. *Aspergillus niger* spore suspension was diluted to the concentration of 2 x 10^4 CFU/mL in the complete media (CM) [Bennett and Lasure, 1991]. One hundred µL of *A. niger* (RD 6.47 or JD 1.1) was added to each well after adding the plant extracts, compounds and controls. *Aspergillus niger* N402 was used to indicate the GFP non-expression while *A. niger* MA 26.1 was used to indicate the expression of GFP. Spores of this latter strain was only diluted in CM (concentration of 2 x 10^4 CFU/mL) and added to the wells containing sterile water only. The final volume in each well was 200 µL. The microplate was incubated at 30 °C in the dark for 16 hours. A fluorescence microscope (Zeiss, Germany) was used to determine the induction of fungal cell wall stress by measuring the fluorescence caused by expression of the GFP genes via the *agsA* promoter. The liquid in the microplate was removed by turning the microplate upside down onto thick absorption paper. Pictures were taken of the fungal mycelium. The growth shapes were observed and the intensity of green fluorescence in each treatment was compared with the control.

5.3 Results and discussion

Humulus lupulus and *Tectona grandis* ($CHCl_3$-MeOH, 1:1) extracts were used to test the induction of fungal cell wall stress using *A. niger* RD 6.47 and *A. niger* JD 1.1 with expression of the reporter gene resulting in the presence of GFP in the nucleus and cytoplasm, respectively. The results showed that *H. lupulus* extract did not induce fungal cell wall stress in either strain up to the concentration of 100 ppm. *Tectona grandis* extract at the concentrations of 100, 50, 25 and 12.5 ppm induced fungal cell wall stress in *A. niger* JD 1.1 (Figure 5.1) while concentrations of 100 and 50 ppm induced *A. niger* RD 6.47. Positive controls (SDS and CFW) induced fungal cell wall stress while a negative control (DMSO) did not induce the fungal cell wall stress. *Aspergillus niger* MA 26.1 (GFP positive control) expressed GFP while *A. niger* N402 (GFP negative control) did not express GFP. After isolating the compounds from *T. grandis* sawdust extract, the induction of fungal cell wall stress was tested with the isolated compounds deoxylapachol, tectoquinone and fraction 87 (hemitectol + tectol). It was found that deoxylapachol induced fungal cell wall stress in *A. niger* JD 1.1 and RD 6.47 at the concentrations of 100, 50, 25 and 12.5 ppm, while tectoquinone precipitated and quenched fluorescence, making it difficult to observe the fluorescence. Fraction 87 (hemitectol + tectol) induced fungal cell wall stress in *A. niger* RD 6.97 and JD 1.1 at concentrations of 100, 50, 25 and 12.5 ppm, but some precipitation and quenching of fluorescence was observed as well.

Apparently, from the work on the isolation and structure elucidation described in Chapter 4, hemitectol in fraction 87 is unstable. Deoxylapachol at high concentration (100 ppm) gave a similar result as SDS. The effects of deoxylapachol and SDS on fungal cell wall stress were compared at the same concentration (1mM). The results show that deoxylapachol induces fungal cell wall stress and inhibits fungal growth more than SDS. Moreover, an unusual *A. niger* mycelium growth with branching was observed in deoxylapachol treatment after spore germination (Figure 5.2).

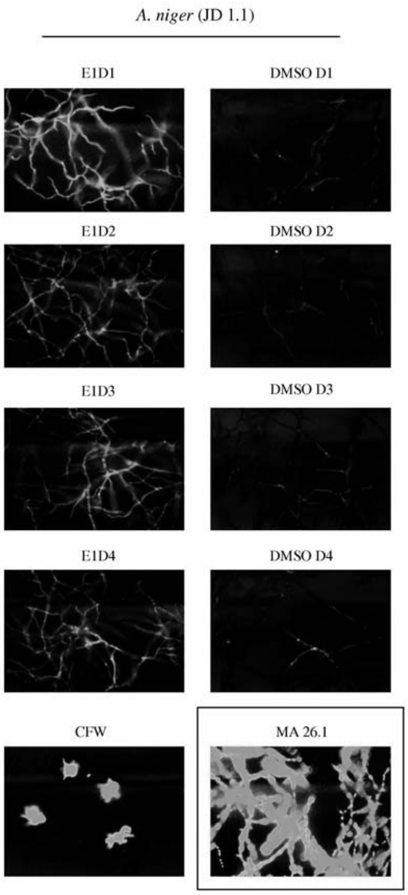

Figure 5.1 GFP expression in *Aspergillus niger* (JD 1.1) mycelium after treatment with *Tectona grandis* extract (E1) at the concentrations of 100 ppm (D1), 50 ppm (D2), 25 ppm (D3) and 12.5 ppm (D4), compared with the negative controls; DMSO 5% (D1), 2.5% (D2), 1.25% (D3) and 0.625% (D4) and the positive controls CFW and *Aspergillus niger* (MA 26.1).

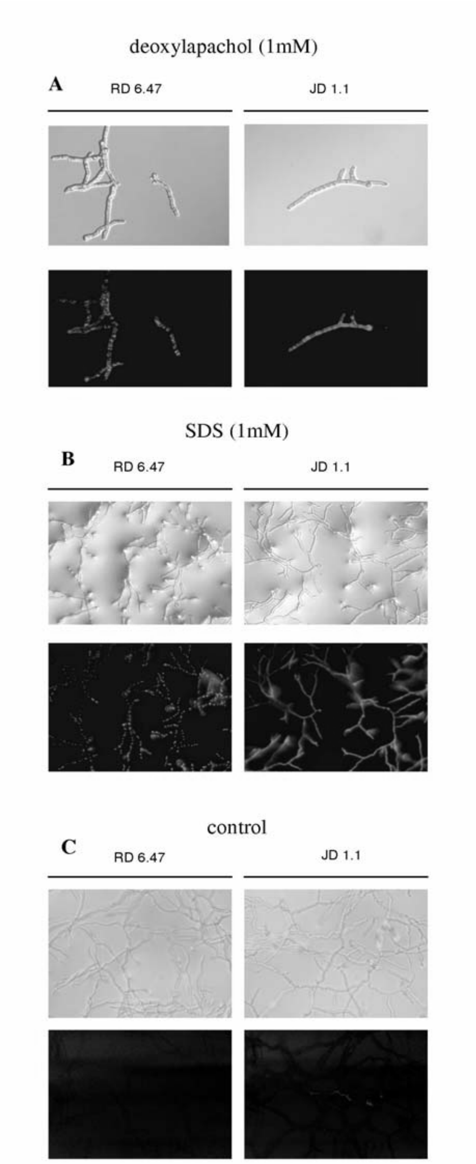

Figure 5.2 The growth of *Aspergillus niger* (RD 6.47 and JD 1.1) and GFP expression in their mycelium after treatment with deoxylapachol (1 mM) (A), SDS (1 mM) (B) and control (5% DMSO) (C).

5.4 Conclusion

From the results, we conclude that deoxylapachol might be an interesting natural compound which can be isolated from *Tectona grandis* sawdust for application as an antifungal compound. *Tectona grandis* wood is one of the most valuable materials for house construction and furniture in South-East Asia. The plant material used for this study was sawdust, which is a waste material from wood factories. Deoxylapachol could become a new product from this waste material, adding new value to the waste. The transgenic *Aspergillus niger* cell lines proved to be an excellent system to confirm antifungal activity of compounds and learn more about their the mode of action.

Chapter 6

Anthranilate synthase inhibition

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Abstract

Anthranilate synthase (AS) is an enzyme in the biosynthetic pathway of tryptophan, and is used as a target enzyme for the study of the inhibitory growth of microorganisms. An HPLC assay was used to measure inhibition of a plant AS that was produced by a transgenic *Escherichia coli* strain. *Cannabis sativa* flower extracts showed the highest inhibition of AS, compared with two kinds of *Humulus lupulus* flower extracts. Among cannabinoids, cannabigerolic acid (CBGA) showed the highest inhibition followed by tetrahydrocannabinolic acid (THCA). Also hop bitter acids inhibited AS. The strongest AS inhibition was shown by adhumulone followed by *β*-acids and humulone. Iso-*trans*-adhumulone showed the highest inhibition compared with other iso-*α*-acids and iso-*cis*-adhumulone.

6.1 Introduction

Anthranilate synthase (AS) is a key enzyme in the biosynthesis of the amino acid tryptophan. This pathway occurs in microorganisms, plants and some parasites, but not in mammals. It is thus an interesting target for developing anti-microbial compounds. Anthranilate synthase catalyses the first committed step in the sequence of reactions which lead to the biosynthesis of tryptophan from chorismate. Tryptophan is an essential amino acid which is utilized in microorganisms and plants as a substrate for protein biosynthesis. In almost all microbial species, AS consists of nonidentical subunits designated as the AS α–subunit (ASI or component I) and AS β-subunit (ASII or component II) [Kawamura et al., 1978; Romero et al., 1994; Poulsen et al., 1993]. The plant anthranilate synthase has two subunits, the enzyme complex does not contain other functionalities. The gene for these subunits has been isolated from *Arabidobsis thaliana* [Niyogi and Fink, 1992; Niyoki et al., 1993]. Poulsen et al. [1993] were the first to purify and characterize anthranilate synthase (EC 4.1.3.27). They used cell cultures of *Catharanthus roseus* as a source.

In this study we used transgenic *Escherichia coli* (M15) which overexpressed the anthranilate synthase gene (EC 4.1.3.27) from *C. roseus* [Bongaerts, 1998]. *Cannabis sativa* and *Humulus lupulus* extracts and isolated compounds were tested for their inhibition of AS using the only known inhibitor, tryptophan [Robinson and Levy, 1976] as a control.

6.2 Materials and Methods

6.2.1 Plant extracts and compounds

Cannabis sativa and *Humulus lupulus* chloroform-methanol ($CHCl_3$-MeOH, 1:1) extracts are described in Chapter 3. The supercritical carbon dioxide extract of *H. lupulus* flowers was received from Botanix (Paddock Wood, Kent, UK).

A total of seven pure cannabinoid compounds, Δ^9–tetrahydrocannabinol (Δ^9–THC), tetrahydrocannabinolic acid (THCA), cannabidiol (CBD), cannabidiolic acid (CBDA), cannabigerol (CBG), cannabigerolic acid (CBGA) and cannabinol (CBN) were obtained from the *C. sativa* flower extract by centrifugal partition chromatography (CPC type LLB-M Sanki Engineering Limited Kyoto, Japan) according to a previous method [Hazekamp et al., 2004]. The bitter acids from *H. lupulus* supercritical carbon dioxide extracts were separated by CPC (type LLN, Sanki Engineering Limited Kyoto, Japan) following the method of Hermans-Lokkerbol et al. [1994]. The α-acids (humulone, cohumulone, adhumulone), β-acids, iso-α-acids and rest fractions of α-acids [patent pending, Wilson et al., 2006] were also used for this assay.

6.2.2 Enzyme preparation

Anthranilate synthase (EC 4.1.3.27) from transgenic *Escherichia coli* (M15) with Clone 4 (plasmid pQE-30-AS_4) was used in this study. Bacterial cell stock suspension from -80 °C was grown in LB broth (Difco, Le Pont de Claix, France) with antibiotics overnight and then streaked on LB agar plates containing antibiotics. The antibiotics used in this experiment were 25 mg/mL Kanamycin and 100 mg/mL Ampicillin (Duchefa, Haarlem, The Netherlands). These plates were incubated overnight before inoculating a single colony in 2 mL of LB broth with antibiotics for 16 hours. Bacterial cell suspensions were then mixed in 500 mL of LB broth containing antibiotics and incubated for 1 hour. One mM isopropyl-*β*-D-thiogalactopyranoside (IPTG, Eurogentec, Seraing, Belgium) was added to this culture 6 hours before incubation for the induction of gene expression. All incubations were done in a 37 °C incubator shaker (New Brunswick Scientific, New Jersey, USA) at 250 rpm. Cell suspensions were transferred to 50 mL tubes and centrifuged at 4 °C at 3,000 rpm for 20 minutes to precipitate the cells. The supernatants were discarded before mixing the cells with lysis buffer. The mixtures were centrifuged at 4 °C at 3,000 rpm for 20 minutes after swayed for 1 hour. Crude enzymes from the supernatants were transferred to Eppendorf tubes and put directly in liquid nitrogen and stored at -80 °C.

6.2.3 Incubation mixture

The incubation mixtures contained 250 mM $MgCl_2 \cdot (6\ H_2O)$ (Merck, Darmstadt, Germany), 0.1 M L-Glutamine (Merck Darmstadt, Germany), 0.1 M Tris-hydrochloric acid (pH 7.5) (Invitrogen, Scotland, UK), crude enzyme, Inhibitor and 10 mM Chorismate (Sigma, St. Louis, USA). The negative control did not contain an inhibitor, while the positive control contained L-tryptophan (Merck, Darmstadt, Germany) as an inhibitor. The mixtures were incubated in a water bath at 37 °C for 6 hours. The reaction was stopped by the addition of 1M phosphoric acid (H_3PO_4) (Merck, Darmstadt, Germany) before detecting the product of AS activity. These assays were done in triplicate.

6.2.4 Enzyme activity detection

HPLC with fluorescence detector (Shimadzu, Japan) was used to detect the AS at 340 nm excitation wavelength and 400 nm emission wavelength. The separations were done using a C_{18} reverse phase column (4.6 X 250 mm, 5 µM particle diameter, Vydac, USA). The mobile phase was a mixture of water-MeOH (4:1) with 50 mM H_3PO_4 that was adjusted with 6 M sodium hydroxide (NaOH) (Merck, Darmstadt, Germany) to the final pH 2.6-2.8. The flow rate was 1 mL/minute and the retention time of AS was 15 minutes. This method was adapted from Poulsen et al. [1991]. A twofold dilution series of AS (Fluka, Steinheim, Germany) from 0.078 to 5 µM were used as standard reference. From the peak areas of anthranilate the percentage of AS inhibition and the IC_{50} values were determined. All data were analyzed by SPSS 12.0 statistic analysis software (Chicago, USA) using one way analysis of variance (ANOVA) and least-significance difference (LSD), at 95% confidence.

6.3 Results and Discussion

Cannabis and hop flower $CHCl_3$-MeOH (1:1) extracts (2 mg/mL) inhibited anthranilate synthase enzyme activity. Cannabis flower extracts gave the highest inhibition followed by hop extracts and supercritical carbon dioxide extracts (Figure 6.1).

The seven cannabinoids (0.1 mM) were also tested for inhibition of AS. CBGA showed the highest percentage of enzyme inhibition followed by THCA. Weak inhibition was found for Δ^9-THC and CBN, no activity was found on CBD or CBDA (Figure 6.2). At a higher concentration of the seven cannabinoids (1.0 mM), CBGA again showed the highest percentage of enzyme inhibition, followed by THCA and CBDA. Weak inhibition was found for CBG, but no activity was found for Δ^9-THC, CBN or CBD (Figure 6.3).

The α-acids (cohumulone, humulone, adhumulone) and the β-acids, including two unknown contaminants of α-acids and iso-α-acids (1.0 mM), were tested for inhibition of AS. Adhumulone, β-acids and humulone showed the highest enzyme inhibition. The contamination of the α-acids also showed inhibition (Figure 6.4). At the lower test concentration of α-acids (0.1 mM), humulone and adhumulone still showed inhibition of anthranilate synthase but no inhibition was observed with cohumulone (data not shown).

The iso-α-acids (0.1 mM) were also tested for inhibition of AS. Iso-*trans*-adhumulone alone showed the highest percentage of enzyme inhibition, followed by the iso-mixture. Weak inhibition was found for iso-*cis*-cohumulone, iso-*cis*-adhumulone and iso-*trans*-cohumulone, no activity was found for iso-*cis*-humulone or iso-*trans*-humulone (data not shown).

For the AS enzyme feedback inhibitor, L-tryptophan, and the most active inhibitors, THCA and CBGA, the IC_{50} values were determined by twofold dilution to the concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 mM. The IC_{50} values of L-tryptophan, THCA and CBGA are 63.1, 313.8 and 398.2 µM, respectively (Figure 6.5).

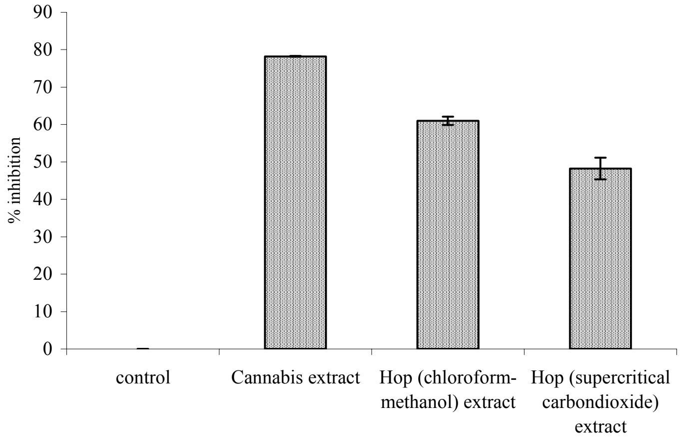

Figure 6.1 Percentage of anthranilate synthase enzyme inhibition from 2 mg/mL cannabis and hop flower extracts. Data shown as mean (n=3) with standard deviation as error bars; significant difference *($p<0.05$)*.

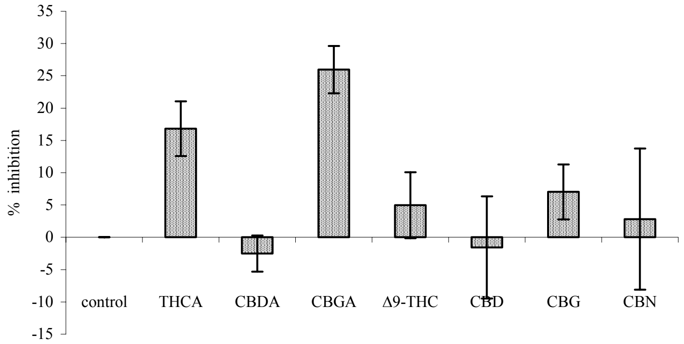

Figure 6.2 Percentage of anthranilate synthase enzyme inhibition by 0.1 mM cannabinoid. Data shown as mean (n=3) with standard deviation as error bars; significant difference (*$p<0.05$*).

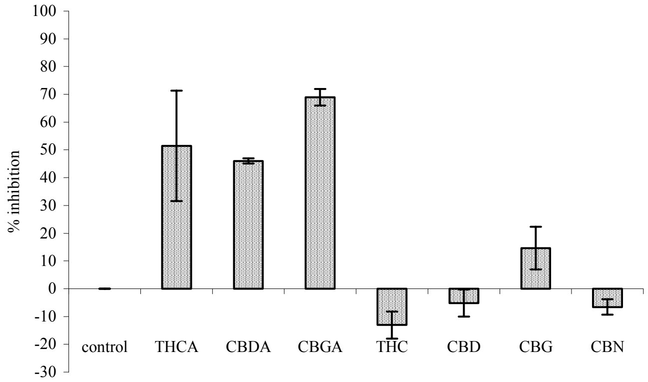

Figure 6.3 Percentage of anthranilate synthase enzyme inhibition by 1.0 mM cannabinoid. Data shown as mean (n=3) with standard deviation as error bars; significant difference (*$p<0.05$*).

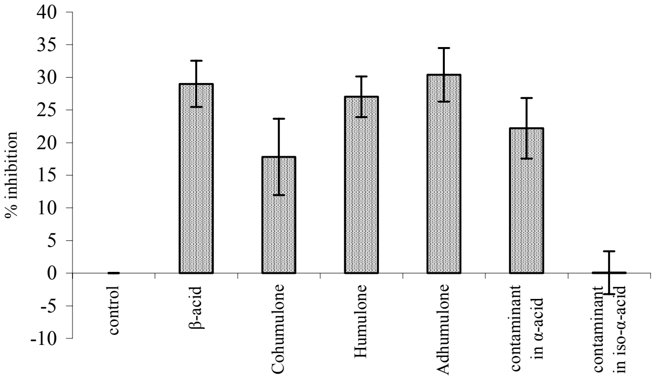

Figure 6.4 Percentage of anthranilate synthase enzyme inhibition by 1.0 mM *α*-acid (adhumulone, humulone or cohumulone), *β*-acids and two contamination compounds of *α*-acids and iso-*α*-acids. Data shown as mean (n=3) with standard deviation as error bars; significant difference (*$p<0.05$*).

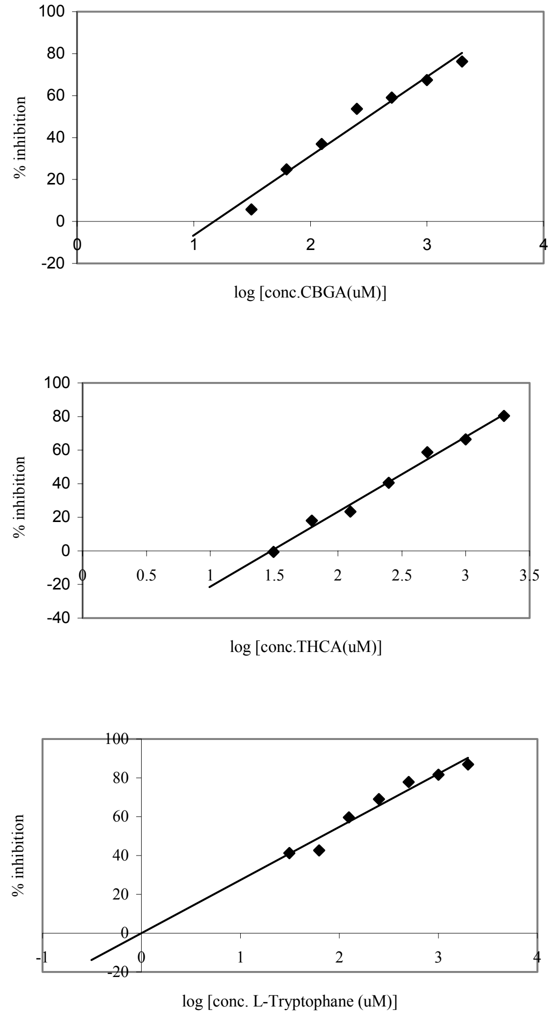

Figure 6.5 Percentage of anthranilate synthase enzyme inhibition by CBGA, THCA and L-tryptophan at the concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 mM. Linear equations of CBGA, THCA and L-tryptophan are $y = 37.828x - 44.446$ ($r^2 = 0.967$), $y = 44.546x - 65.825$ ($r^2 = 0.9891$) and $y = 27.364x$ ($r^2 = 0.958$), respectively.

6.4 Conclusion

Anthranilate synthase is an interesting target enzyme for antimicrobial activity due to its presence in microorganisms for the synthesis of the essential amino acid tryptophan. In the tests of the seven cannabinoids and hop α- and β-acids, CBGA showed the highest inhibition followed by THCA. For the bitter acids, the highest AS inhibition was found for adhumulone, followed by the β-acids and humulone. Although some inhibitory compounds were found, their activity was not as strong as tryptophan. This study shows that AS can be used as a target enzyme to investigate the mode of action of plant compounds in microorganisms.

Chapter 7

Anti-wood rot activity

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Abstract

Cannabis sativa, *Humulus lupulus* and the tropical hardwoods *Tectona grandis*, *Xylia xylocarpa, Shorea obtusa, Shorea albida* and *Hopea odorata* extracts were tested for anti-wood rot activity by paper disc diffusion assay. *Tectona grandis* and *H. lupulus* extract inhibited more wood rot strains than the other plant extracts. Deoxylapachol isolated from *T. grandis* extract inhibited the brown rot fungi *Gloeophyllum sepiarium* CBS 353.74 and *Gloeophyllum trabeum* CBS 318.50 and the white rot fungi *Phlebia brevispora* CBS 509.92 and *Merulius tremellosus* CBS 280.73. Fraction 87 (hemitectol + tectol) from the *T. grandis* extract showed a high percentage of cellulase inhibition, compared to other compounds isolated from *T. grandis* and *H. lupulus*. Humulone isolated from *H. lupulus* inhibited the brown rot fungi *G. sepiarium* CBS 317.50 and CBS 353.74, *G. trabeum* CBS 318.50 and CBS 335.49, and *Serpular lacrymans* CBS 520.91 and CBS 751.79, but showed weak cellulase inhibition.

7.1 Introduction

Wood rot fungi is a problem of wood construction in residences, museums, children's play grounds, and other things. The wood rot fungi consist of two major groups: the brown rot and the white rot fungi both belong to the division of Basidiomycetes. Dry rot is a form of brown rot caused by certain fungi that are able to transport water over long distances, thus wood appears dry when it is infected by, for example, *Serpular lacrymans* [Osiewacz, 2002].

The major wood preservative used for more than 50 years is Chromate Copper Arsenate (CCA). But since 2004 the European Union and the US Environmental Protection Agency (EPA) no longer allows pressure-treated wood containing CCA to be used for residential applications. Several new chemical formulations such as ACQ, Tanalith-E, Wolmanit CX-8 and copper dimethyldithiocarbamate (CDDC) have been developed and are currently used for building construction [Yildiz, et al., 2004]. The combination of organic biocides (for example, propiconazole) with antioxidants and metal chelators was developed for environmentally benign wood preservation [Schultz and Nicholas, 2002]. Natural products are a possible approach for developing new anti-wood rot compounds for wood preservation. For example, Nakayama et al. [2001] recently reported that the resin from the guayule plant (*Parthenium argentatum*, Gray) had insect and microbial resistance properties.

Brown rot fungi produce endoglucanases and hemicellulases for the degradation of cellulose and hemicellulose, respectively. Ligninolytic enzymes such as lignin peroxidase, manganese peroxidase, H_2O_2-generating enzymes and laccase are produced by white rot fungi. White rot fungi can degrade cellulose, hemicellulose and lignin. The lignocellulose-degrading enzymatic system is important for substrate colonization and carbon acquisition by wood rot fungi [Osiewacz, 2002; Valášková and Baldrian, 2005].

There are two basic approaches to the assay of inhibition of cellulase activity. The first is based on measuring the individual activities of the cellulase enzyme [Shultz, et al. 1995; Sharrock, 1988; Mawadza, et al., 2000; Geiger, et al., 1998], the second is based on measuring the activity of the total enzyme complex towards an appropriate substrate, typically filter paper, powdered crystalline cellulose, carboxymethyl cellulose (CMC), trinitrophenyl-carboxymethyl cellulose (TNP-CMC) or cellulose-azure [Sharrock, 1988; Semenov, et al.,1996; Cohen, et al., 2005; Criquet, 2002; Nolte, 1990; Dhillon, et al. 1996; Lai, et al., 2006].

In this study the inhibition of white and brown rot fungi was studied using the paper-disc diffusion assay and the agar-plate dilution assay, for screening of anti-wood rot compounds from plants. In order to know the mode of action of possible anti-wood rot compounds, cellulase was selected as a target enzyme. Cellulose-azure was used as a substrate for total cellulase assay measuring by UV spectrometry the release of colorant. To develop cheap natural anti-wood rot compounds sources which are abundant and easy accessible are needed. Therefore, well known agricultural plants and tropical hardwoods were chosen. Cannabaceae plants were tested because of their antimicrobial activity and some tropical hardwoods known to be resistant against wood rot.

7.2 Materials and Methods

7.2.1 Plant extraction

Cannabis sativa L., *Humulus lupulus* L. and the tropical hardwoods *Tectona grandis* L.f., *Xylia xylocarpa* Roxb., *Shorea obtusa* Wall. ex Blume, *Shorea albida* Symington and *Hopea odorata* Roxb. were extracted and fractionated as described in Chapter 3 supercritical carbon dioxide extract of *H. lupulus* flowers was received from Botanix (Paddock Wood, Kent, UK). *Cannabis sativa* extract was fractionated with *n*-hexane-90% methanol (*n*-hexane-90% MeOH). *Tectona grandis* extract was fractionated with *n*-hexane-90% MeOH and chloroform-*n*-buthanol ($CHCl_3$-*n*-BuOH). *X. xylocarpa* extract was fractionated with $CHCl_3$- *n*-BuOH.

A total of seven pure cannabinoid compounds, Δ^9–tetrahydrocannabinol (Δ^9–THC), tetrahydrocannabinolic acid (THCA), cannabidiol (CBD), cannabidiolic acid (CBDA), cannabigerol (CBG), cannabigerolic acid (CBGA) and cannabinol (CBN) were obtained from cannabis flower extract according to Hazekamp et al.[2004]. Cannflavin A+B and Cannflavin B were obtained from cannabis flower extract [Choi et al., 2004]. The hop α-acids (humulone, cohumulone and adhumulone) and β-acids were isolated from *H. lupulus* supercritical carbon dioxide extract following the method of Hermans-Lokkerbol et al.[1994]. Hop iso-α-acids, CIM-α-CD complex and TIM-α-CD complex (CIM = cis-iso-α-acid mixture and TIM = trans-iso-α-acid mixture) were isolated by our laboratory [Wilson, et al., 2004]. Deoxylapachol, tectoquinone and fraction 87 (hemitectol + tectol) were isolated from *T. grandis* sawdust using a centrifugal partition chromatography (CPC, type LLN, Sanki engineering limited Kyoto, Japan) as described in Chapter 4. The reference compounds 1,4-naphthoquinone, 1,4-naphthohydroquinone (Fluka, Switzerland), anthraquinone and 2-hydroxymethylanthraquinone (Sigma-Aldrich, USA), were used as controls to test anti-wood rot activity from quinones.

7.2.2 Paper-disc diffusion assay

Seven species of brown rot fungi, *Gloeophyllum trabeum* CBS 318.50 and CBS 335.49, *Gloeophyllum sepiarium* CBS 317.50 and CBS 353.74, *Serpular lacrymans* CBS 520.91 and CBS 751.79 and *Piptoporus betulinus* CBS 378.51 and 6 species of white rot fungi, *Bjerkandera* *adusta* CBS 595.78 and CBS 230.93, *Trametes versicolor* CBS 410.66 and CBS 114372, *Phlebia brevispora* CBS 509.92 and *Merulius tremellosus* CBS 280.73, were used to test the activity by paper-disc diffusion assay. Fungi were grown on malt extract agar (MEA, Fluka, Spain) plates until the mycelium covered the surface of the agar plate completely. Cork borers were used to cut out mycelium with a diameter of 5 mm which was subsequently pressed on the center of the test Petri dishes containing MEA. The dried plant extracts and compounds were dissolved in EtOH or MeOH [Nieva Moreno et al., 1999] to a final concentration of 100 and 10 mg/mL, respectively. Either EtOH or MeOH was used as a negative control, and 10 mg/mL of Pentachlorophenol (PCP) 98% (Sigma-Aldrich, Steinheim, Germany) was used as a positive control. Sterile filter paper discs (Whatman No.42, Maidstone, England), 5 mm in diameter, were impregnated with 0.2 mg of plant extract or compound solution dried and pressed on a fungus-inoculated plate. The inhibition zones were evaluated after incubating plates in the dark at room temperature for 7-18 days (depending on the fungal species). The assays were performed in four replicates.

7.2.3 Agar plate dilution assay

Plant extracts were dissolved in dimethylsulfoxide (DMSO) to obtain a stock solution with a concentration of 400 mg/mL. MEA media was autoclaved and cooled to 60-65 ºC before divided into 100 mL per treatment, for 14 treatments. Plant extract was added by twofold dilution to the concentrations of 0.97, 1.95, 3.90, 7.80, 15.60, 31.20, 62.50, 125, 250, $5\text{x}10^2$, $1\text{x}10^3$, $2\ \text{x}10^3$ and $4\ \text{x}10^3$ µg/mL. PCP, at the same concentrations, was used as the positive control and DMSO was used as the negative control. Plant extracts were gently mixed with 100 mL of media using a magnetic stirrer and 2 mL media was loaded to the wells of a 12 well plate and left to solidify. The wood rot mycelium grown on agar plates was cut using a cork borer (diameter of 5 mm) and subsequently pressed onto the center of the media in a well. The plates were incubated in the dark at room temperature for 7-18 days. The growth of fungi on each plant extract agar well was checked and Minimal Inhibitory Concentration (MIC) values were observed. The pieces of fungi which did not grow in the media containing plant extracts or PCP were transferred to MEA media without plant extract or PCP. The Minimal Fungicidal Concentration (MFC) value was determined on the basis of no regrowth.

7.2.4 Inhibition of the cellulase enzyme

Cellulose-azure (product number C1052, Sigma-Aldrich, Steinheim, Germany) was used as the substrate in this study. Before being used in the assay, cellulose-azure was washed by milli-Q water to remove loosely attached dye, and was then dried in a freeze-dryer. A commercially available enzyme from *A. niger* (EC3.2.1.4 Cellulase, product number: C-1184, Sigma-Aldrich, Steinheim, Germany) with an estimated activity of 0.45 units/mg was used. Cellulose-azure solution (1.0 mg/mL) was prepared in acetate buffer at pH 5.0 and gently stirred with a magnetic stirrer. The plant extracts and compounds were used as cellulase inhibitors by dissolving in DMSO (20% in acetate buffer) to the concentration of 2 mg/mL. Ammonium-hexachloropalladate (IV) (Sigma-Aldrich, Steinheim, Germany) [Shultzet al., 1995] was used as a positive control, while DMSO was used as a negative control. The incubation mixture had the final concentrations of 0.2 mg/mL inhibitor, 0.8 mg/mL cellulose-azure, 0.3 units/mL cellulase, and the total volume was made to 1.5 mL with addition of acetate buffer. The mixtures were incubated in the dark for 2 h at 38 ºC in a water bath. The sample was filtered using a syringe filter (pore size 0.45 µM, type Spartan RC 30, Sigma-Aldrich, Steinheim, Germany) prior to measurement with spectrophotometer at 595 nm. The percentages of cellulase inhibition were analyzed by SPSS 12.0 statistic analysis software (Chicago, USA) using one way analysis of variance (ANOVA) and least-significance difference (LSD), at 95% confidence.

7.3 Results

7.3.1 Anti-wood rot growth

Cannabis sativa flower $CHCl_3$-MeOH (1:1) extract, *n*-hexane fraction and 90% MeOH fraction inhibited *G. sepiarium* CBS 317.50 and *G. trabeum* CBS 318.50. Testing the pure cannabinoid compounds showed that 10 mg/mL THC, THCA, CBD, CBDA, CBG, CBGA and CBN inhibited *G. sepiarium* CBS 317.50 while 10 mg/mL THCA, CBD, CBG and CBN inhibited *G. trabeum* CBS 318.50 only in the first week after incubation. CBG (1 mg/mL) inhibited *G. sepiarium* CBS 317.50 while THC, THCA, CBGA, CBD, CBDA and CBN (1 mg/mL) did not show any inhibition. Cannflavin (A+B) and cannflavin B (both 10 mg/mL) inhibited *G. sepiarium* CBS 317.50, and only cannflavin B inhibited *G. trabeum* CBS 318.50.

Humulus lupulus $CHCl_3$-MeOH (1:1) and supercritical carbon dioxide extracts inhibited the brown rot fungi, *G. sepiarium* CBS 317.50 and CBS 353.74, *G. trabeum* CBS 318.50 and CBS 335.49, *S. lacrymans* CBS 520.91 and CBS 751.79, but did not inhibit white rot fungi. After testing pure hop bitter acids, it was found that at the concentration of 10 mg/mL humulone inhibited *G. sepiarium* CBS 317.50 and CBS 353.74, *G. trabeum* CBS 318.50 and CBS 335.49 and *S. lacrymans* CBS 520.91 and CBS 751.79. Adhumulone inhibited *G. sepiarium* CBS 353.74, *G. trabeum* CBS 335.49 and *S. lacrymans* CBS 751.79 while cohumulone did not show any inhibition. The *β*-acids inhibited *S. lacrymans* CBS 520.91 and CBS 751.79, while *P.*

betulinus CBS 378.51, *G. sepiarium* CBS 317.50 and CBS 353.74, *G. trabeum* CBS 318.50 and CBS 335.49 were only inhibited in the first week after inoculation.

Tectona grandis $CHCl_3$-MeOH (1:1) and 80% MeOH extract inhibited all brown rot fungal strains in this assay (*P. betulinus* CBS 378.51, *G. sepiarium* CBS 317.50 and CBS 353.74, *G. trabeum* CBS 318.50 and CBS 335.49, *S. lacrymans* CBS 520.91 and CBS 751.79) and inhibited the white rot fungi (*B. adusta* CBS 230.93, *P. brevispora* CBS 509.92 and *M. tremellosus* CBS 280.73). The *n*-hexane fraction showed inhibition of *G. sepiarium* CBS 317.50 and CBS 353.74, *G. trabeum* CBS 318.50 and *M. tremellosus* CBS 280.73, while the 80% MeOH fraction showed inhibition on *M. tremellosus* CBS 280.73. Both fractions inhibited *P. brevispora* CBS 509.92 but only within the first week after inoculation. The $CHCl_3$ fraction showed stronger inhibition of *G. sepiarium* CBS 317.50 and CBS 353.74, *G. trabeum* CBS 318.50, *P. brevispora* CBS 509.92 and *M. tremellosus* CBS 280.73 than the *n*-BuOH fraction that inhibited only the first week after inoculation. Deoxylapachol inhibited *G. sepiarium* CBS 353.74, *G. trabeum* CBS 318.50, *M. tremellosus* CBS 280.73 and *P. brevispora* CBS 509.92, while tectoquinone and Fraction 87 (hemitectol + tectol) did not inhibit any of the test organisms. The reference compounds 1,4-naphthoquinone and 1,4-naphthohydroquinone inhibited *G. trabeum* CBS 318.50, *M. tremellosus* CBS 280.73 and *P. brevispora* CBS 509.92, while anthraquinone and 2-hydroxymethylanthraquinone did not inhibit any strain.

Xylia xylocarpa $CHCl_3$-MeOH (1:1) and 80% MeOH extracts inhibited the brown rot fungi *G. sepiarium* CBS 317.50 and CBS 353.74, *G. trabeum* CBS 318.50 and CBS 335.49, and the white rot fungi *P. brevispora* CBS 509.92, *M. tremellosus* CBS 280.73 and *T. versicolor* CBS 410.66. *S. lacrymans* CBS 520.91 and CBS 751.79 were inhibited by this plant only during the first two weeks after inoculation. The $CHCl_3$ fraction inhibited the brown rot fungi *G. sepiarium* CBS 317.50 and CBS 353.74, *G. trabeum* CBS 318.50 and the white rot fungi *P. brevispora* CBS 509.92 and *M. tremellosus* CBS 280.73, while *n*-BuOH fraction only inhibited *G. sepiarium* CBS 353.74. *Shorea obtusa* and *S. albida* $CHCl_3$-MeOH (1:1) extract inhibited the brown rot fungi *G. sepiarium* CBS 353.74 and *G. trabeum* CBS 318.50, but did not show any white rot fungi inhibition. *Hopea odorata* $CHCl_3$-MeOH extract inhibited brown rot fungi, *G. sepiarium* CBS 353.74 and *G. trabeum* CBS 318.50 and white rot fungi, *B. adusta* CBS 230.93, *T. versicolor* CBS 114372, *P. brevispora* CBS 509.92 and *M. tremellosus* CBS 280.73.

MIC and MFC values from the agar plate dilution assay were used to evaluate plant extracts and compounds which showed strong inhibition on the wood rot fungi. These might be suitable for the development of anti-wood rot compounds. The results are shown in Table 7.1.

Shorea and *Hopea* did not inhibit wood rot fungi at the test concentrations 0.97 to 4.00 x 10^3 µg/mL.

Table 7.1 MIC and MFC values of plant extracts for anti-wood rot activity

Wood rot species	MICs (µg/mL)				
	C. sativa	*H. lupulus*	*T. grandis*	*X. xylocarpa*	PCP
Brown rot fungi					
Piptoporus betulinus CBS 378.51	ni	ni	ni	ni	0.97
Gloeophyllum trabeum CBS 318.50	$4x10^3$	$4x10^3$	$1x10^3$	ni	1.95
Gloeophyllum trabeum CBS 335.49	-	-	-	-	-
Gloeophyllum sepiarium CBS 317.50	$2x10^3$	$5x10^2$	$2x10^3$	ni	3.90
Gloeophyllum sepiarium CBS 353.74	ni	ni	$4x10^3$	$2x10^3$	3.90
Serpula lacrymans CBS 520.91	ni	31.20	ni	ni	1.95
Serpula lacrymans CBS 751.79	ni	$2x10^3$	ni	ni	1.95
White rot fungi					
Bjerkandera adusta CBS 595.78	-	-	-	-	-
Bjerkandera adusta CBS 230.93	ni	ni	ni	ni	7.80
Trametes versicolor CBS 410.66	-	-	-	-	-
Trametes versicolor CBS 114372	ni	ni	ni	ni	31.20
Phlebia brevispora CBS 509.92	ni	ni	$1x10^3$	ni	7.80
Merulius tremellosus CBS 280.73	ni	ni	$2x10^3$	ni	7.80
Wood rot species	MFCs (µg/mL)				
	C. sativa	*H. lupulus*	*T. grandis*	*X. xylocarpa*	PCP
Brown rot fungi					
Piptoporus betulinus CBS 378.51	-	-	-	-	1.95
Gloeophyllum trabeum CBS 318.50	-	-	-	-	1.95
Gloeophyllum trabeum CBS 335.49	-	-	-	-	-
Gloeophyllum sepiarium CBS 317.50	$2x10^3$	$1x10^3$	-	-	-
Gloeophyllum sepiarium CBS 353.74	-	-	-	-	-
Serpula lacrymans CBS 520.91	-	31.20	-	-	1.95
Serpula lacrymans CBS 751.79	-	$2x10^3$	-	-	1.95
White rot fungi					
Bjerkandera adusta CBS 595.78	-	-	-	-	-
Bjerkandera adusta CBS 230.93	-	-	-	-	15.60
Trametes versicolor CBS 410.66	-	-	-	-	-
Trametes versicolor CBS 114372	-	-	-	-	31.20
Phlebia brevispora CBS 509.92	-	-	-	-	31.20
Merulius tremellosus CBS 280.73	-	-	-	-	31.20

ni, no inhibition at the highest tested concentration; -,not tested

7.3.2 Inhibition of cellulase

The inhibition of cellulase by plant extracts and compounds was evaluated using cellulose-azure as a substrate. *Humulus lupulus* and the five tropical sawdust hardwood extracts (2 mg/mL) inhibited cellulase activity. *Hopea odorata* extract gave the highest inhibition, followed by *T. grandis*, *X. xylocarpa* and *H. lupulus* (supercritical carbon dioxide extract) (Figure 7.1). The hop α-acids (humulone, cohumulone and adhumulone), β-acids, iso-α-acids, CIM-*α*-CD complex and TIM-*α*-CD complex showed weak cellulase inhibition (Figure 7.2). Deoxylapachol, tectoquinone and Fraction 87 (hemitectol + tectol) from *T. grandis* extract were also tested for inhibition of cellulase. Fraction 87 (hemitectol + tectol) showed the highest percentage of inhibition, followed by deoxylapachol (Figure 7.3).

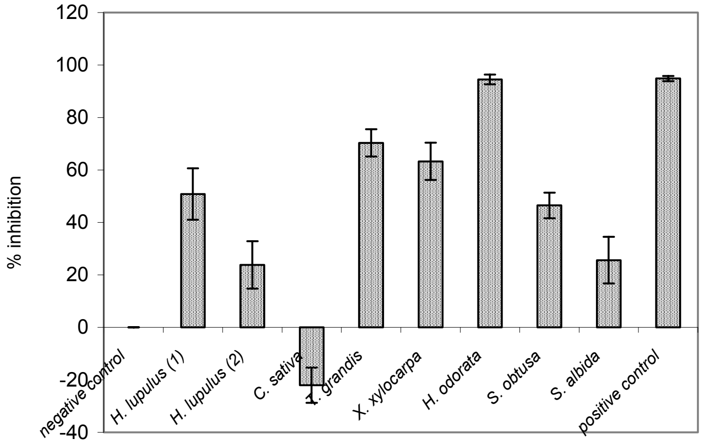

Figure 7.1 Inhibition of cellulase by plant $CHCl_3$-MeOH (1:1) extracts (0.2 mg/mL). *Humulus lupulus* (1), supercritical carbon dioxide extract; *Humulus lupulus* (2), $CHCl_3$-MeOH (1:1) extract. Positive control is ammonium-hexachloropalladate (IV) (0.2 mg/mL); (n=4, $p< 0.05$).

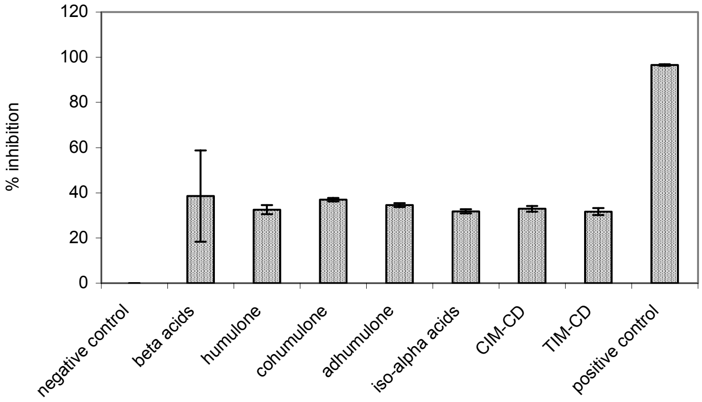

Figure 7.2 Inhibition of cellulase by hop compounds. CIM-CD, cis-iso- α-acid-mix-CD; TIM-CD, trans-iso-α-acid-mix-CD; ammonium-hexachloropalladate (IV) (0.2 mg/mL); (n=4, $p< 0.05$).

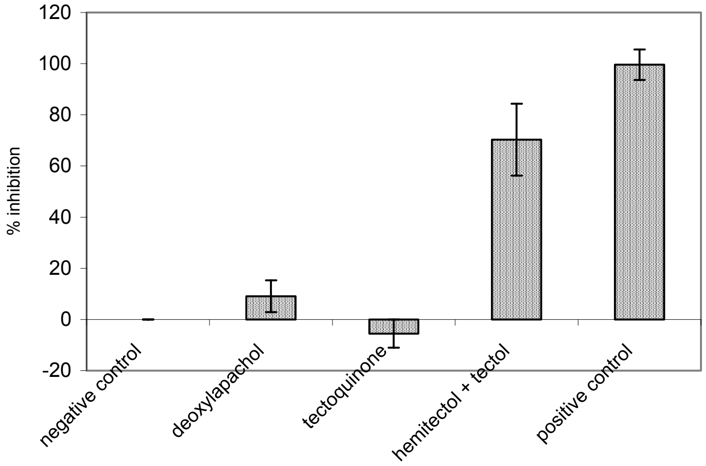

Figure 7.3 Inhibition of cellulase by *Tectona grandis* compounds (0.2 mg/mL); (n=4, $p< 0.05$).

7.4 Discussion

Tectona grandis sawdust extract inhibited more strains of wood rot fungi than any of the other plant extracts in this experiment. This plant inhibited all seven strains of brown rot fungi and three strains of white rot fungi. After isolation of the active compounds from *T. grandis*, it was found that deoxylapachol inhibited two brown rot fungi, *G. sepiarium* CBS 353.74, *G. trabeum* CBS 318.50, and two white rot fungi, *P. brevispora* CBS 509.92, *M. tremellosus* CBS 280.73. No inhibition of any other wood rot fungi was found by the other isolated *T. grandis* compounds. Fraction 87 (hemitectol + tectol), which was isolated from *T. grandis*, showed the highest inhibition of cellulase activity compared to other isolated compounds. The reference quinone compounds were also tested for the inhibition of wood rot fungi. 1,4-naphthoquinone and 1,4-naphthohydroquinone inhibited the same wood rot fungi as deoxylapachol. Anthraquinone and 2-hydroxymethylanthraquinone did not inhibit any of the fungal strains. Neither anthraquinones isolated from *T. grandis* extract nor the reference compounds inhibited cellulase activity. Apparently the broad activity of the total extract is due to a combination of activities of the compounds present.

Of the Cannabaceae plants, *H. lupulus* flower extract inhibited six out of the seven brown rot fungal strains, but did not inhibit white rot fungi. The major compounds found in *H. lupulus* supercritical carbon dioxide extract are α-acids and β-acids, which inhibited the same brown rot fungal strains as the crude extract. The individual α-acids were isolated. Humulone and adhumulone inhibited brown rot fungi while cohumulone did not show activity. *Cannabis sativa* extract inhibited some brown rot fungi. After the isolation of major compounds from *Cannabis sativa* extract we found that the seven isolated cannabinoids inhibited the same brown rot fungal strains which were inhibited by the crude extract. Cannflavin (A+B) and Cannflavin B, which are minor compounds found in this plant extract, inhibited *G. sepiarium* CBS 317.50 and *G. trabeum* CBS 318.50 as well.

The results indicate similar activities for isolated compounds as for the crude plant extracts. Concerning the possible mode of action, the results show that cellulase inhibition might be involved as a target, but not for all extracts. It is possible that the plant extracts inhibit through other fungal mechanisms. For application in wood protection we can use either active crude extracts or isolated compounds, but as the activities are similar for extracts and pure compounds it may be more cost-effective to use the extract. The extracts also contain many compounds which may inhibit wood rot fungi through different modes of action. For example, in this study, deoxylapachol is the most active compound found in *T. grandis* extract. The *T. grandis* extract has approximately 8 % of this compound. In the paper disc diffusion assay we used a 10 % less concentrated extract, and the results are the same. This means that either pure deoxylapachol or *T. grandis* extract can be used to inhibit wood rot fungi.

Chapter 8

Future perspectives in biodiversity exploration

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Thailand has extensive forestry, agri/horticulture and processing industries that produce raw materials and waste materials. It is interesting to add value to resources like sawdust, fruit peels, seeds, and bagasse of sugarcane. There are many possible applications of natural components from such resources, as medicines, food supplements, functional food, food additives, feed additives in animal farms, crop protectants in agri/horticulture, antibiotics in fish and prawn tanks, wood impregnation and household products. Table 8.1 summarizes possible uses of plant products and some of their associated risks. For applications in agri/horticulture and livestock, a mixture of active compounds or the crude extracts can be used to reduce costs. On the other hand, for applications in medicine this is not a possibility because they require extensive studies of toxicity and quality control, including tests for contamination with e.g. pesticides or heavy metals to assure safety in human use.

Table 8.1 The use of plant products and the consideration of contamination and toxicity in some applications.

Type of applications	Possible material used			Risks	
	Plant materials	Crude extracts	Pure compounds	Toxicity	Pesticide and heavy metal contamination
1. Medicines	+	+	+++	+++	+++
2. Food	++	++	++	+++	+++
3. Feed additives in animal farms	+++	++	+	+++	++
4. Fish and prawn tanks	+	+++	+	+	+
5. Agri/horticulture	+	+++	+	+	+
6. Wood impregnation and household products	-	++	+	+	+

+++, very important; ++, medium importance; +, important; –, not important.

The plant sources discussed above represent economically efficient sources to search for new bioactive compounds with various applications, for example, finding active compounds from fruit peels with the ability to inhibit pathogenic microorganisms in fish and prawn tanks, could increase profits without needing to increase fruit production. Selection of these plant materials may be based on local knowledge, chemotaxonomy or previous research. The activity should be proven by bioassays such as antifungal, antibacterial, antiviral or anti-nematode activity. TLC, HPLC, GC, IR, MS and NMR spectrometry can be used to identify and elucidate active compounds To know more about the interaction between compounds and living cells, investigations on their mechanism of action by advanced screening assays can be used to measure specific effects on cell walls and membranes, nucleic acids or enzymes. The safety of natural products is also important. Thus, the toxicity and possible contamination with pesticides and heavy metals should be evaluated in products having interesting activities. In the case of applications in fish and prawn tanks or feed additive in animal farms, residues such as toxic compounds, pesticides or heavy metals accumulating in animal tissues, can be harmful to the consumers. But, due to the dilution of the material in this sort of applications, risks are smaller than in cases of plant material directly used for human consumption.

Finding novel products from existing crops leads to increased economic value for these crops. From the point of view of environmental conservation there may be additional advantages, such as creating a sustainable production of materials and the use of biodegradation products. The transformation of wastes from agriculture and horticulture into new products may also solve the environmental problems of disposing of these wastes.

After screening for activities, identification of active compounds and their mode of action, including checking toxicity and monitoring for pesticide and heavy metal contamination, the next step is to optimize the extraction method in order to get a high yield of the bioactive compounds. For example the use of water, EtOH, MeOH, dichloromethane, ethyl acetate or supercritical CO_2, has to be considered in terms of yields and costs. Costs include the effect on the environment, and consequently, use of nontoxic solvents is preferred. Possibilities to improve the efficiency of extraction are method of grinding the material, use of ultrasonicator or microwaves, increasing the temperature or including additive solvents. The optimized extraction system should be suitable for large scale production. Air drying or freeze drying can be used to dry the crude extract. Metabolic profiling (with TLC, GC, HPLC or NMR) can be used to evaluate the extraction products. In the case of pure compounds, an efficient low-cost separation and isolation of those compounds should be developed, for example using a large-scale CPC (Centrifugal Partition Chromatography) apparatus.

For quality control of antimicrobial active/crude extracts, autobiography assays, paper disc diffusion assays (for non-polar fractions), well diffusion assays (for polar fractions) or broth dilution assays can be used. The end product can be developed into different formulations, for example a dry powder or a liquid with hydrophobic or hydrophilic character.

In large-scale production, the prospective fields of application and the type of plant product used (plant materials, crude extracts or pure compounds) are important parameters for choosing the processing methods. For crude extracts or pure compounds as end product, the (dry) plant materials need to be ground to a fine powder for an efficient extraction. Concerning quality control, the purity of the isolated compounds can be detected by TLC, HPLC, GC, MS or NMR spectrometry. In the case of crude extracts, a reproducible chemical profile needs to be established. Contamination with pesticides, mycotoxins and heavy metals also needs to be controlled. For crude extracts the active compounds and the ratios at which they are active, must be identified to be able to standardize the final formulation. For pure compounds the standardization of the final product is much easier. In Figure 8.1 a general flow scheme is presented for laboratory scale development of products based on the research presented in this thesis. Industrial processes are described in Figure 8.2.

Figure 8.1 Standard laboratory protocol for product development of bioactive compounds

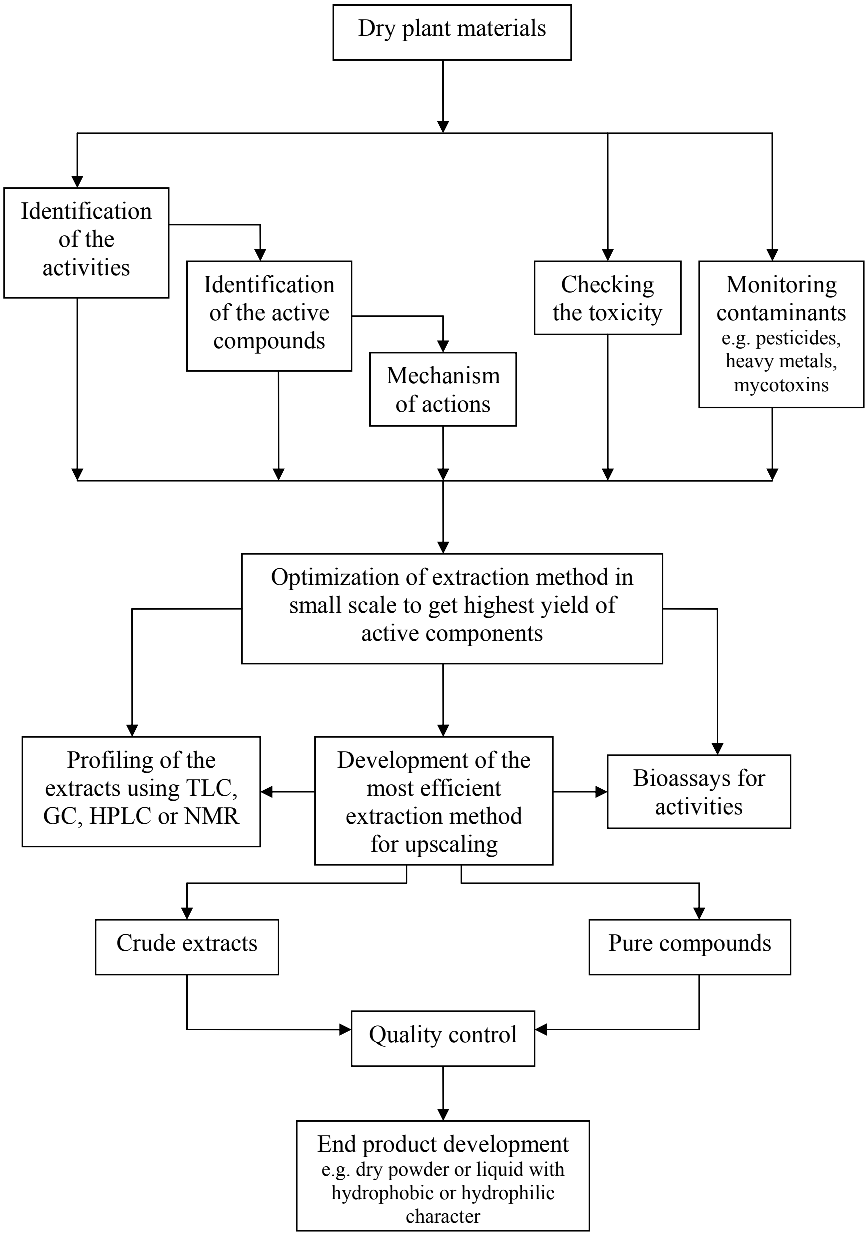
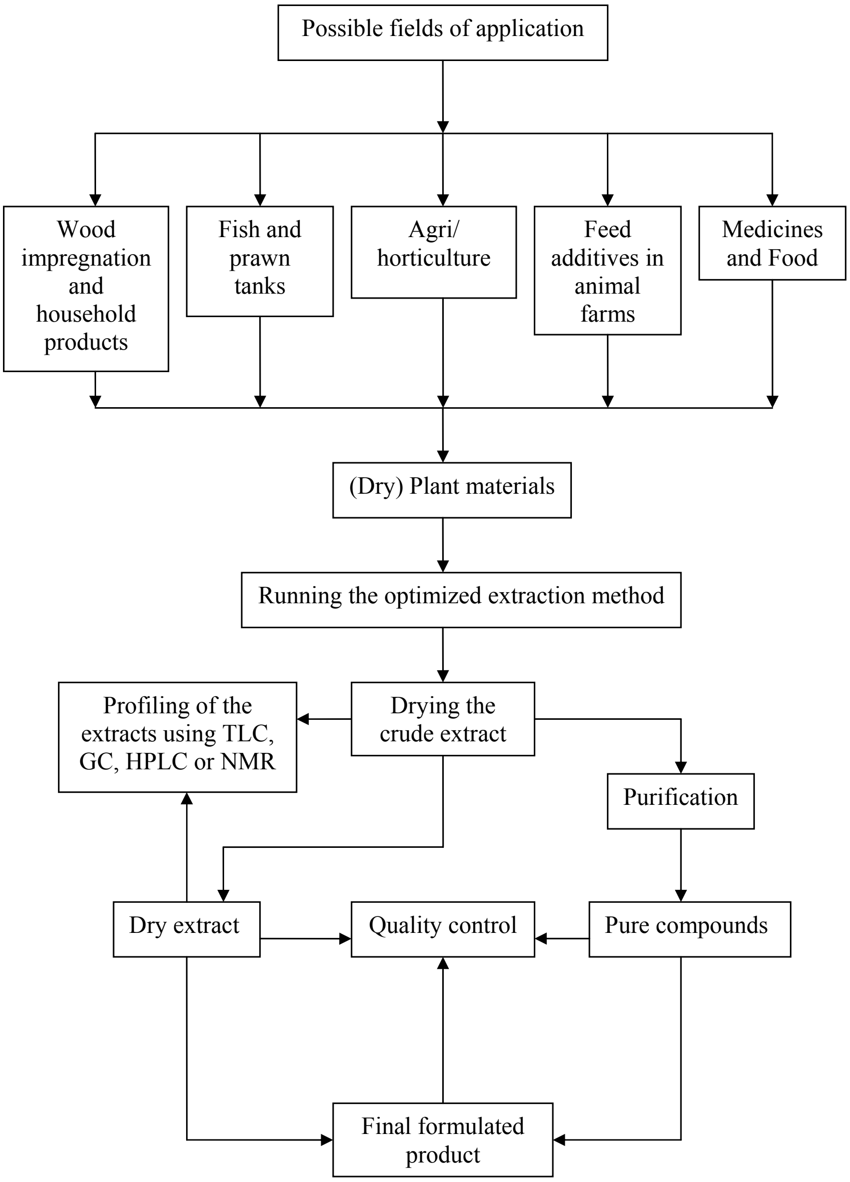


Figure 8.2 Large scale production of bioactive products



Summary

Microorganisms resistant to most antibiotics are rapidly spreading. Consequently there is an urgent need for novel antibiotics. Most antibiotics have been developed from microorganisms. Plants represent an interesting source for finding novel antimicrobial compounds. They are well protected against microorganisms, due to the presence of antimicrobial compounds or the induction of phytoalexin biosynthesis after infection. This thesis focus particularly on some abundantly available plant sources, with the idea that plants already in agricultural processing industry might be the source of antimicrobial active compounds which would add extra value to these crops. The research presented in this thesis focused on antifungal activity of such sources, as plants are known to be quite resistant to most fungi. Therefore, specific focus was given to anti-wood rot activity after a general screening of antimicrobial activity.

Chapter 1 is a general introduction which discusses antimicrobials used in human medicine, food, agriculture and household products as well as antibiotics used in livestock. The overconsumption of antibiotics is causing the development of resistance in microorganisms, therefore novel antibiotics are needed. Medicinal plants are considered as an interesting source for such novel antimicrobial compounds.

Chapter 2 is a review of the most common modes of action of antimicrobial agents, such as interactions with bacterial membranes, cell wall synthesis, DNA replication and repair, ribosome binding and metabolic enzymes, which can be used as targets to develop new drugs. The general screening methods such as diffusion assays, dilution assays, bioautographic assays, and more advanced assays, such as assays on molecular targets for screening antimicrobial activities are also described.

In **Chapter 3** we report antimicrobial activities for the extracts of *Cannabis sativa* and *Humulus lupulus* flowers as well as for sawdust of tropical hardwoods (*Tectona grandis*, *Xylia xylocarpa, Shorea obtusa, Shorea albida* and *Hopea odorata*). *Cannabis sativa* and *H. lupulus* have been previously been reported to have pharmacological and also antimicrobial effects. The waste which is left after isolation of their already economically used products is of interest for antimicrobial screening. Tropical hardwood sawdust is an interesting source for the screening of antimicrobial activity because it could create profit from easily accessible waste materials of the timber industry. Moreover, hardwoods are known to be resistant to termites and fungi. It was found that *Cannabis sativa* extract and fractions inhibited growth of *Bacillus subtilis* and *Escherichia coli* in the paper disc diffusion assay. Strongest inhibition was found against *B. subtilis*, in a fraction derived from *C. sativa* flower chloroform-methanol ($CHCl_3$-MeOH, 1:1)

extract. This fraction was compared with reference cannabinoids in the contact bioautographic assay (biogram assay), and the cannabinoid acids, tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA) have activity. *Humulus lupulus* flower ($CHCl_3$-MeOH, 1:1) extract showed inhibition of *Aspergillus niger* in a broth dilution assay with a MIC (Minimal Inhibitory Concentration) of 100 ppm. Sawdust from the tropical hardwoods *T. grandis*, *X. xylocarpa, S. obtusa, S. albida* and *H. odorata* ($CHCl_3$-MeOH, 1:1) extracts were tested for inhibition of *A. niger* in a broth dilution assay. Only *T. grandis* extract caused clear inhibition with a MIC of 25 ppm.

After the result showed the inhibition of *A. niger* by *T. grandis* extract, the active compounds from this plant sample were isolated (**Chapter 4)**. The compounds deoxylapachol**,** tectoquinone, 2-hydroxymethylanthraquinone, hemitectol (2,2-dimethyl-2*H*-benzo[*h*]chromen-6-ol), tectol and 3'-OH-deoxyisolapachol (2-[(1*E*)-3-hydroxy-3-methylbut-1-enyl]naphthoquinone) were isolated from the *T. grandis* sawdust ($CHCl_3$-MeOH, 1:1) extract. Centrifugal partition chromatography (CPC) was used to separate those compounds using *n*-hexane-methanol-water (50:47.5:2.5) as a solvent system. All compounds except tectol showed antifungal activity in a biogram assay.

In order to learn more about the possible mode of action of the active compounds, the effects of *Humulus lupulus* and *Tectona grandis* extracts on two transgenic strains of *A. niger* were studied (**Chapter 5**). The transgenic strains are good cell wall damage model, showing induction of the 1,3-α-ᴅ-glucan synthase gene by coupling it to a green fluorescent protein (GFP) marker encoding sequence. Induction of the gene encoding the glucan synthase is detected as fluorescence in the fungal cells. The results show that *T. grandis* extract, fraction 87 (hemitectol + tectol) and deoxylapachol, which were derived from this plant extract, induce fungal cell wall stress.

Chapter 6 reports another mode of action, Anthranilate synthase (AS) as an interesting target enzyme for the discovery of new antimicrobial compounds. AS is a key enzyme in the biosynthesis of the amino acid tryptophan. This enzyme is present in microorganisms, plants and some parasites but not in mammals. An HPLC (high performance liquid chromatography) assay was used to measure inhibition of a plant AS that was produced by a transgenic *E. coli* strain. *Cannabis sativa* flower extracts showed the strongest inhibition of AS, as compared with two kinds of *H. lupulus* flower extracts. Among cannabinoids, CBGA showed the highest inhibition, followed by THCA. Also, hop bitter acids inhibited AS and the strongest inhibition was shown by adhumulone, followed by *β*-acids and humulone. Iso-*trans*-adhumulone showed the highest inhibition compared with other iso-*α*-acids and iso-*cis*-adhumulone.

After these active compounds were identified, their effect on wood rot fungi was also tested. In **Chapter 7** the activities of extracts of *C. sativa*, *H. lupulus* and the tropical hardwoods, *T. grandis*, *X. xylocarpa, S. obtusa, S. albida* and *H. odorata* are described for anti-wood rot fungi by paper-disc diffusion and agar plate dilution assays. *Tectona grandis* and *H. lupulus* extracts inhibited more wood rot strains than the other plant extracts. Deoxylapachol isolated from *T. grandis* extract inhibited the brown rot fungi, *Gloeophyllum sepiarium* CBS 353.74 and *Gloeophyllum trabeum* CBS 318.50 and the white rot fungi, *Phlebia brevispora* CBS 509.92 and *Merulius tremellosus* CBS 280.73. A possible mode of action on wood rot was studied by choosing cellulase as a possible target enzyme for antifungal compounds. Fraction 87 (hemitectol + tectol) from *T. grandis* extract showed strong cellulase inhibition compared to other compounds isolated from *T. grandis* and *H. lupulus*. Humulone isolated from *H. lupulus* inhibited the brown rot fungi *G. sepiarium* CBS 317.50 and CBS 353.74, *G. trabeum* CBS 318.50 and CBS 335.49 and *S. lacrymans* CBS 520.91 and CBS 751.79, but showed a low percentage of cellulase inhibition.

The discovery of novel antimicrobial compounds required tools to determine the activity by general screening assays for growth inhibition, but also to determine the target of the antimicrobial compounds in the microorganisms. Although structure elucidation can be done with low milligram quantities, for extensive biological testing larger amounts are needed. CPC proved to be an excellent tool for up scaling. Of the active compounds isolated, hemitectol has strong activity against *A. niger* and strong cellulase inhibition, but this compound is not stable. Hop α-acids and *β*-acids are active in anthranilate synthase inhibition but they are also easily degradable. The known compound deoxylapachol has good antimicrobial activities such as induction of fungal cell wall stress. A similar compound, lapachol, has been reported to have antitumor activity. The cytotoxicity of deoxylapachol should be further studied. The compounds found in this thesis may serve as leads for (semi)synthesis of novel antimicrobial compounds. For applications in wood rot protection or other purposes such as cotton-coating or food processing, it is interesting to incorporate such active compounds into a polymer, or to bond them chemically to various other materials.

This work shows the proof of concept for the hypothesis that raw and waste materials from common industrial and agri/horticultural processes can serve as sources for critically new active compounds. The next step would be to develop a standardized protocol for a screening program for many kinds of materials. Considering the fact that from the small number of samples screened here some interesting compounds were discovered, one may expect that from a well organized screening a number of hits can be obtained. **Chapter 8** describes the possibility of screening the raw or waste materials from the agri/horticulture processing industry for developing new products such as medicines, food additives, feed additives for animal farms and crop protectants. They can also be used for the development of antibiotics for fish and prawn tanks, antifungal compounds for wood impregnation and household products. The approach to such applications is presented in two schemes, one at the laboratories scale and another at the industry scale.

Samenvatting

Micro-organismen resistent tegen de meeste antibiotica komen steeds vaker voor. Als gevolg hiervan is er een grote behoefte aan nieuwe antibiotica. De meeste antibiotica zijn ontwikkeld uit micro-organismen. Planten zijn een interessante bron voor nieuwe antimicrobiële stoffen. Ze zijn goed beschermd tegen micro-organismen, doordat ze antimicrobiële stoffen aanmaken of door de aanmaak van fytoalexinen na een infectie. In dit proefschrift concentreren we ons in het bijzonder op een aantal veel voorkomende plantaardige bronnen met het idee dat planten die al gebruikt worden in agro-industriële processen, een bron van antimicrobiële stoffen kunnen zijn en hiermee in feite een meerwaarde geven aan deze gewassen. Het onderzoek dat in dit proefschrift is gepresenteerd is geconcentreerd op anti-schimmel activiteit van zulke gewassen sinds deze planten bekend staan om hun resistentie zijn tegen de meeste schimmels. Er wordt specifiek gekeken naar anti-houtrot activiteit na een eerste algemene screening op antimicrobiële activiteit.

Hoofdstuk 1 is een algemene introductie die het gebruik van antibiotica in humane medicijnen, voeding, landbouw en huishouden alsmede het gebruik van antibiotica in de veestapel ter discussie stelt. De huidige overconsumptie van antibiotica leidt tot ontstaan van meer resistente micro-organismen. Medicinale planten worden gezien als een bron voor nieuwe antibiotica.

Hoofdstuk 2 is een overzicht van de meest bekende mechanismen achter de antimicrobiële stoffen zoals interacties met onder andere de bacteriële membranen, cel wand synthese, vermenigvuldiging en reparatie van DNA, ribosoombinding en enzymen van het microbiële metabolisme die gebruikt kunnen worden als target in de ontwikkeling van nieuwe medicijnen. De algemene screening methodes zoals de diffusion assay, dilution assay en bioautographic assay en andere meer geavanceerde assays zoals assays met microbiële cellen en assays zich richten op moleculaire targets om op antimicrobiële activiteiten te screenen, worden eveneens beschreven.

In **hoofdstuk 3** worden de antimicrobiële activiteiten beschreven van extracten van *Cannabis sativa* en *Humulus lupulus* bloemen evenals die van zaagsel van tropische hardhoutbomen (*Tectona grandis, Xylia xlyocarpa, Shorea obtusa, Shorea albida* en *Hopea odorata*). Van *C. sativa* en *H. lupulus* is al beschreven dat ze farmacologische en ook antimicrobiële activiteiten bezitten. De reststromen in de industrie na extractie van dit soort planten is een interessante bron om te screenen op antimicrobiële activiteit. Het screenen van zaagsel van tropisch hardhout is een andere interessante bron om op antimicrobiële activiteit te screenen, omdat hier gemakkelijk winst gemaakt kan worden van een eenvoudig toegankelijke bron. Bovendien is van hardhout bekend dat het resistent is tegen termieten en schimmels. Uit onze screening bleek dat *C. sativa* extract en fracties hiervan de groei van *Bacillus subtilis* en *Escherichia coli* remmen in de zg. paper disc diffusion assay. De sterkste remming werd gevonden bij *B. subtilis*. Deze remming werd gevonden in een fractie uit een chloroform-methanol ($CHCl_3$-MeOH, 1:1) extract van de *C. sativa* bloem. Deze fractie werd vergeleken met referentie cannabinoïden in de bioautographic assay (biogram assay) en de cannabinoïd zuren tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA) en cannabigerolic acid (CBGA) bleken activiteit te hebben. Het $CHCl_3$-MeOH (1:1) extract van de bloemen van *H. lupulus* bleek de groei van *Aspergillus niger* in broth dilution te remmen bij een MIC (Minimaal inhiberende concentratie) waarde van 100 ppm. Zaagsel van tropische hardhout bomen, *T. grandis, X. xlyocarpa, S. obtusa, S. albida* en *H. odorata* ($CHCl_3$-MeOH, 1:1) extracten werden getest op een remmende activiteit voor de groei van *A. niger* in de broth dilution assay. Alleen *T. grandis* bleek een duidelijk remmende werking te hebben op de groei van *A. niger* (MIC = 25 ppm).

Nadat de inhiberende activiteit van *T. grandis* op *A. niger* was aangetoond, werden vervolgens de actieve stoffen uit deze bron geïsoleerd (**Hoofdstuk 4**). De stoffen deoxylapachol, tectoquinone, 2-hydroxymethylanthraquinone, hemitectol (2,2-dimethyl-2*H*-benzo[*h*]chromen-6-ol), tectol en 3'-OH-deoxyisoapachol (2[(1E)-3-hydroxy-3-methylbut-1-enyl]naphthoquinone) werden geïsoleerd uit het $CHCl_3$-MeOH (1:1) extract van zaagsel van *T. grandis*. Deze stoffen werden gescheiden door middel van Centrifugal Partitioning Chromatography (CPC) met een oplosmiddelsystem bestaande uit *n*-hexane-MeOH-H_2O (50:47.5:2.5). Al deze stoffen, met uitzondering van tectol, vertoonden een anti-schimmel activiteit in de biogram assay.

Om meer te weten te komen over de mogelijk mechanismen van de actieve stoffen werden de extracten van *H. lupulus* en *T. grandis* getest op transgene varianten van *A. niger* (**hoofdstuk 5**). Een aantal transgene varianten vormen een goed model om de schade aan de celwand te meten, doordat ze bij schade, inductie vertonen van het *green fluorescent protein* (GFP) gelabeld 1,3-α-D-glucan synthase. De inductie van het gen met het daaraan gekoppelde GFP gen kan in de schimmelcellen worden gedetecteerd door middel van fluorescentie. De resultaten uit deze test laten zien dat *T. grandis* extract, fractie 87 (hemitectol + tectol) en deoxylapachol, die geisoleerd zijn uit dit plantenextract, in staat zijn de celwand van de schimmel onder druk te zetten.

Hoofdstuk 6 beschrijft anthranilate synthase (AS) als een interessante target in de zoektocht naar nieuwe antimicrobiële stoffen. AS is een sleutelenzym in de biosyntheseroute van tryptofaan. Dit enzym komt voor in micro-organismen, planten en sommige parasieten, maar niet in zoogdieren. Een HPLC (High Performance Liquid Chromatography) assay werd gebruikt om de mate van inhibitie van plantaardig AS te meten, geproduceerd in een transgene variant van *E. coli*. *Cannabis sativa* bloemen extracten hadden de sterkste inhibitie van AS vergeleken met extracten van twee soorten *H. lupulus* bloemen extracten. Onder de cannabinoïden vertoonde CBGA de sterkste inhibitie, gevolgd door THCA. Ook de hop bitterzuren inhibeerden AS sterk, met name adhumulone vertoonde een sterkste inhibitie, gevolgd door de β-zuren en humulone. Iso-*trans*-adhumulone vertoonde de sterkste inhibitie vergeleken met andere iso-α-zuren en iso-*cis*-adhumulone.

Nadat de actieve stoffen waren geïdentificeerd, werden ze ook getest op hun activiteit tegen houtrot schimmels. In **Hoofdstuk 7** worden de houtrot schimmel remmende werkingen beschreven van extracten van *C. sativa* en *H. lupulus* evenals die van zaagsel van tropische hardhoutbomen (*Tectona grandis, Xylia xlyocarpa, Shorea obtusa, Shorea albida* en *Hopea odorata*) in de paper-disc diffusion assay en de agar plate dilution assays. *Tectona grandis* en *H. lupulus* extracten remde meer houtrot varianten vergeleken met de andere extracten. Deoxylapachol geïsoleerd uit *T. grandis* remde de groei van *brown rot fungi*, *Gloephyllum sepiarium* CBS 353.74 en *Gloephyllum trabeum* CBS 318.50 en de *white rot fungi*, *Phlebia brevispora* CBS 509.52 en *Merulius tremellosus* CBS 280.73. Een mogelijk mechanisme achter de inhiberende activiteit op houtrot door cellulase te gebruiken als een target enzym voor het vinden van schimmel remmende stoffen. Fractie 87 (hemitectol + tectol) van het *T. grandis* extract vertoonde een sterke inhibitie van het cellulase enzym vergeleken met andere uit *T. grandis* en *H. lulupus* geïsoleerde stoffen. Humulone uit *H. lupulus* inhibeerde *brown rot fungi*, *G. sepiarium* 317.50 CBS en CBS 353.74, *G. trabeum* CBS 318 en CBS 335.49 en *S. lacrymans* CBS 520.91 and CBS 751.79, maar vertoonde een laag percentage cellulase inhibitie.

Het ontdekken van nieuwe antimicrobiële stoffen vereist niet alleen goede assays om de activiteit te bepalen tijdens het screenen, maar eveneens de assays om het mechanisme achter de activiteit te bepalen en daarmee de target van de stoffen in kwestie. Daar waar de structuuropheldering van deze stoffen gedaan kan worden met hoeveelheden in de orde van milligrammen, zijn er grotere hoeveelheden nodig voor uitgebreide biologische testen. CPC heeft bewezen een uitstekend middel te zijn voor opschaling van dit soort stoffen. Van de geteste actieve stoffen bleek hemitectol een sterk remmende activiteit te hebben op *A. niger* en cellulase, maar hemitectol is geen stabiele stof. α-zuren en β-zuren remmen anthranilate synthase, maar zijn eveneens makkelijk afbreekbaar. De bekende stof deoxyapachol heeft sterke antimicrobiële activiteiten zoals het onder druk zetten van de schimmel celwand en van een vergelijkbare stof, lapachol, is beschreven dat het een antitumor werking heeft. De cytotoxiciteit moet nog verder worden onderzocht. De in dit onderzoek gevonden stoffen zouden kunnen dienen als leads voor (semi-)synthese van nieuwe antimicrobiële stoffen. Voor de toepassing in houtrotbescherming of andere toepassingen zoals bijvoorbeeld katoen-coatings of levensmiddelproductie is het interessant om een polymeer van de actieve stoffen te maken of ze chemisch te binden aan bepaalde materialen.

Dit werk is een *proof of concept* voor de hypothese dat eenvoudige ruwe grondstoffen en reststromen uit talrijke industrieën en land- en tuin-bouw kunnen dienen als bronnen voor interessante en belangrijke bio-actieve stoffen. De volgende stap is de ontwikkeling van een gestandaardiseerd protocol voor een screeningsprogramma voor verschillende soorten materiaal. Gezien het feit dat er in een klein aantal gescreende samples hier al een aantal interessante stoffen zijn ontdekt, kan men verwachten dat er uit een goed opgezette screening een aantal interessante *hits* zullen voortkomen.

Hoofdstuk 8 beschrijft de mogelijkheid om te screenen op ruwe grondstoffen en reststromen van de industrie en land- en tuin-bouw om nieuwe producten te ontwikkelen zoals medicijnen, voedsel- en veevoer additieven en gewasbeschermers. Ze kunnen ook worden gebruikt in de ontwikkeling van antibiotica voor vis-, en garnalenkwekerijen, anti-schimmel stoffen voor houtimpregnatie en huishoudelijke producten. De aanpak van zulk soort toepassingen wordt toegelicht in twee schema’s, een op laboratoriumschaal en een andere op industriële schaal.

บทสรุป

ปัจจุบันจุลินทรีย์ที่ต้านทานยาปฏิชีวณะได้ขยายวงกว้างขึ้นอย่างรวดเร็ว ด้วยเหตุนี้จึงมีความต้องการยาปฏิชีวณะชนิดใหม่อย่างเร่งด่วน อย่างไรก็ตามยาปฏิชีวณะพัฒนาขึ้นจากจุลินทรีย์ ในขณะที่พืชเป็นทรัพยากรที่ได้รับความสนใจเนื่องจากมีคุณสมบัติในการป้องกันจุลินทรีย์หลายชนิด โดยจะเห็นได้จากสารยับยั้งจุลินทรีย์ที่ค้นพบในพืชหรือสารที่พืชสังเคราะห์ขึ้นมาเมื่อถูกจุลินทรีย์เข้าทำลาย (Phytoalexin) ดังนั้นจึงชี้ให้เห็นว่าพืชเป็นทรัพยากรที่น่าสนใจในการค้นคว้าหาสารยับยั้งจุลินทรีย์ชนิดใหม่

วิทยานิพนธ์ฉบับนี้มุ่งเน้นในการศึกษาพืชบางชนิดที่สามารถหาได้ง่าย ด้วยความคิดที่ว่าพืชดังกล่าวใช้เพื่อวัตถุประสงค์ในการผลิต ผลผลิตทางอุตสาหกรรมการเกษตร และด้วยเหตุนี้จึงเป็นการเพิ่มมูลค่าให้แก่พืชผลที่เก็บเกี่ยวได้ งานวิจัยฉบับนี้เป็นการศึกษากิจกรรมการต่อต้านเชื้อราของสารสกัดจากพืช เนื่องจากเป็นที่ทราบกันดีว่าพืชมีคุณสมบัติในการต่อต้านเชื้อราหลายชนิด และได้มุ่งเน้นศึกษาสารสกัดจากพืชที่มีคุณสมบัติยับยั้งเชื้อราทำลายไม้ (wood rot fungi) โดยเริ่มต้นจากการคัดเลือกสารสกัดจากพืชบางชนิดที่มีฤทธิ์ยับยั้งจุลินทรีย์ทั่วไปบางชนิด

บทที่ 1 คือ บทนำ ซึ่งกล่าวถึงสารยับยั้งจุลินทรีย์ที่ใช้ในอุตสาหกรรมอาหาร เกษตรกรรม ผลิตภัณฑ์ที่ใช้ในบ้านเรือน และยาปฏิชีวณะที่ใช้ในทางปศุสัตว์ ซึ่งการบริโภคยาปฏิชีวณะเป็นสาเหตุให้จุลินทรีย์เกิดการพัฒนาการต่อต้านยาปฏิชีวณะ ดังนั้นจึงมีความต้องการสารยับยั้งจุลินทรีย์ชนิดใหม่ โดยพืชสมุนไพรจัดเป็นทรัพยากรที่ได้รับความสนใจเพื่อใช้ในวัตถุประสงค์ดังกล่าว

บทที่ 2 กล่าวถึงกลไกโดยทั่วไปของของสารยับยั้งจุลินทรีย์ที่ทำอันตรายต่อเซลล์จุลินทรีย์ เช่น มีผลกระทบต่อเยื่อหุ้มเซลล์ ผนังเซลล์ การสังเคราะห์และการซ่อมแซมดีเอ็นเอ (DNA) การยึดเกาะกับไรโบโซม และมีผลต่อเอนไซม์ที่ผลิตขึ้นโดยจุลินทรีย์ ซึ่งสิ่งที่กล่าวมานี้สามารถใช้เป็นเป้าหมายของการค้นหาสารยับยั้งจุลินทรีย์เพื่อการพัฒนายาชนิดใหม่ อย่างไรก็ตามในบทนี้ได้อธิบายวิธีการคัดเลือกสารยับยั้งจุลินทรีย์โดยทั่วไป ยกตัวอย่างเช่น วิธีการแพร่กระจายของสาร (diffusion assays) วิธีการเจือจางความ

เข้มข้นของสาร (dilution assays) วิธีการไบโอออโต้กราฟฟิก (bioautographic assays) และนอกจากนี้ยังมีวิธีการที่ซับซ้อนมากขึ้น เช่น การทดสอบกับโมเลกุลเป้าหมายในเซลล์จุลินทรีย์

บทที่ 3 แสดงให้เห็นการยับยั้งจุลินทรีย์ของสารสกัดจากดอกกัญชา (*Cannabis sativa*) ดอกฮอพ (*Humulus lupulus*) และขี้เลื่อยไม้เนื้อแข็งบางชนิด ได้แก่ ไม้สัก (*Tectona grandis*) ไม้แดง (*Xylia xylocapa*) ไม้เต็ง (*Shorea obtusa*) ไม้อาลันบาตู (*Shorea albida*) และ ไม้ตะเคียนทอง (*Hopea odorata*) อย่างไรก็ตามมีรายงานการใช้สารสกัดจากดอกกัญชาและฮอพในทางเภสัชกรรมและสารสกัดจากพืชดังกล่าวมีผลยับยั้งจุลินทรีย์ ดังนั้นด้วยแนวคิดที่ต้องการศึกษาสารหรือวัสดุส่วนที่เหลือใช้จากกระบวนการผลิตทางอุตสาหกรรมเกษตร จึงเป็นสิ่งที่น่าสนใจในการค้นหาสารยับยั้งจุลินทรีย์เพื่อเพิ่มมูลค่าให้แก่วัตถุดิบดังกล่าว ขี้เลื่อยไม้เนื้อแข็งในเขตร้อนเป็นหนึ่งในแหล่งทรัพยากรที่น่าสนใจในการตรวจสอบหาสารยับยั้งจุลินทรีย์ เนื่องจากสามารถสร้างผลกำไรจากวัสดุเหลือใช้ที่หาได้ง่ายจากอุตสาหกรรมแปรรูปไม้ อย่างไรก็ตามเป็นที่ทราบกันดีว่าไม้เนื้อแข็งบางชนิดมีความทนทานต่อการเข้าทำลายของปลวกและเชื้อราทำลายไม้

ผลงานวิจัยในบทนี้ พบว่า สารสกัดจากดอกกัญชามีฤทธิ์ยับยั้งแบคทีเรีย *Bacillus subtilis* และ *Escherichia coli* ด้วยวิธีการแพร่กระจายของสาร ทั้งนี้สารสกัดดังกล่าวยับยั้ง *B. subtilis* ได้ดีกว่า *E. coli* ผลการยับยั้งสูงสุดของสารสกัดจากดอกกัญชาพบในสารสกัดที่สกัดด้วยคลอโรฟอร์ม–เมธานอล อัตราส่วน 1:1 ดังนั้นจึงนำสารสกัดส่วนนี้มาเปรียบเทียบกับสารในกลุ่มคานาบินอย (Cannabinoids) โดยวิธีไบโอแกรม (biogram assay) จึงพบว่าสารที่อยู่ในสารสกัดดังกล่าว ได้แก่ tetrahydrocannabinolic acid (THCA) cannabidiolic acid (CBDA) และ cannabigerolic acid (CBGA) มีฤทธิ์ยับยั้งแบคทีเรีย ในขณะที่สารสกัดจากดอกฮอพมีฤทธิ์ยับยั้งเชื้อรา *Aspergillus niger* ที่ระดับความเข้มข้นในการยับยั้งต่ำสุด (MIC) เท่ากับ 100 ไมโครกรัม/มิลลิลิตร โดยใช้วิธีการเจือจางความเข้มข้นของสารในการทดสอบ ส่วนสารสกัดจากขี้เลื่อยไม้เนื้อแข็งชนิดต่างๆ ทั้ง 5 ชนิดดังที่กล่าวข้างต้น ซึ่งใช้คลอโรฟอร์ม–เมธานอลอัตราส่วน 1:1 ในการสกัดนั้น พบว่าสารสกัดจากขี้เลื่อยไม้สักยับยั้งเชื้อรา *A. niger* ที่ระดับความเข้มข้นต่ำสุดในการยับยั้งเท่ากับ 25 ไมโครกรัม/มิลลิลิตร หลังจากที่พบผลการยับยั้งเชื้อรา *A. niger* ในสารสกัดจากขี้เลื่อยไม้

สักจึงได้แยกสารบริสุทธิ์ที่ออกฤทธิ์ในสารสกัดดังกล่าวซึ่งวิธีการแยกและตรวจสอบโครงสร้างสารบริสุทธิ์ที่ออกฤทธิ์ได้อธิบายอยู่ใน**บทที่ 4** โดยสารที่แยกได้จากสารสกัดจากขี้เลื่อยไม้สักซึ่งใช้คลอโรฟอร์ม–เมธานอล อัตราส่วน 1:1 ในการสกัด ได้แก่ deoxylapachol, tectoquinone, 2-hydroxymethylanthraquinone, hemitectol (2,2-dimethyl-2*H*-benzo[*h*]chromen-6-ol), tectol และ 3'-OH-deoxyisolapachol (2-[(1*E*)-3-hydroxy-3-methylbut-1-enyl]naphthoquinone) ซึ่งเครื่องมือที่ใช้แยกสารเหล่านี้คือซีพีซี (centrifugal partition chromatography) โดยการใช้ เฮกเซน–เมธานอล–น้ำ อัตราส่วน 50:47.5:2.5 ในการแยกสาร สารทั้งหมดที่แยกได้ข้างต้นมีฤทธิ์ยับยั้งเชื้อราโดยวิธีไบโอแกรม

เพื่อศึกษากลไกการยับยั้งจุลินทรีย์จึงได้ทดสอบผลของสารสกัดจากดอกฮอพและขี้เลื่อยไม้สักต่อการยับยั้งเชื้อรา *Aspergillus niger* ที่ผ่านการตัดต่อพันธุกรรมเพื่อใช้เป็นต้นแบบในการศึกษาการถูกทำลายของผนังเซลล์เชื้อรา (**บทที่ 5**) โดยเชื้อราดังกล่าวได้ใส่จีเอฟพีมาร์คเกอร์ (GFP marker) ไปที่ยีนส์กลูแคนซินเทส (1,3-α-D-glucan synthase) ซึ่งผลการชักนำให้เกิดการแสดงออกของยีนส์กลูแคนซินเทสจะแสดงให้เห็นเป็นสีเขียวฟลูออเรสเซนซึ่งสามารถสังเกตเห็นได้เมื่อมองผ่านกล้องจุลทรรศน์ ผลการศึกษาในครั้งนี้แสดงให้เห็นว่าสารสกัดจากขี้เลื่อยไม้สักและสารที่แยกได้จากสารสกัดดังกล่าว ได้แก่ deoxylapachol และ แฟลกชั่น 87 (hemitectol + tectol) มีผลทำให้ผนังเซลล์เชื้อราได้รับความกดดันซึ่งอาจมีผลให้ถูกทำลายได้ต่อไป

ใน**บทที่ 6** แสดงผลของการยับยั้งจุลินทรีย์ผ่านอีกกลไกหนึ่ง ได้แก่ การยับยั้งเอนไซม์แอนทรานิเลท ซินเทส (anthranilate synthase) ซึ่งเอนไซม์นี้ถือเป็นเอนไซม์ที่น่าสนใจในการใช้เป็นเอนไซม์เป้าหมายเพื่อค้นหาสารยับยั้งจุลินทรีย์ชนิดใหม่ เอนไซม์แอนทรานิเลท ซินเทส เป็นเอนไซม์ที่สำคัญในการสังเคราะห์กรดอะมิโนทริปโตเฟน (tryptophan) เอนไซม์ดังกล่าวพบในจุลินทรีย์ พืช และ ปาราสิท แต่ไม่พบในมนุษย์และสัตว์เลี้ยงลูกด้วยนม การวัดค่าการยับยั้งเอนไซม์ แอนทรานิเลท ซินเทส โดยสารสกัดจากพืชใช้วิธี เอชพีแอลซี (high performance liquid chromatography) โดยวัดผลผลิตที่ได้จากการทำงานของเอนไซม์แอนทรานิเลท ซินเทส ซึ่งผลิตจากแบคทีเรีย *E. coli* ที่ผ่านการตัดต่อพันธุกรรม ผลการทดลองแสดงให้เห็นว่าสารสกัดจากดอกกัญชายับยั้งการทำงานของเอนไซม์ แอนทรานิเลท ซินเทส ได้สูงที่สุดเมื่อเปรียบเทียบกับสาร

สกัดจากดอกฮอพ ในจำนวน Canabinoids ที่ทดสอบ CBGA มีฤทธิ์ยับยั้งการทำงานของเอนไซม์แอนทรานิเลท ซินเทส สูงที่สุดรองลงมาคือ THCA อย่างไรก็ตาม hop bitter acids ยับยั้งการทำงานของเอนไซม์ แอนทรานิเลท ซินเทส โดย adhumulone มีฤทธิ์ยับยั้งสูงสุด รองลงมาคือ β-acids และ humulone ส่วนผลการทดสอบกับสารกึ่งสังเคราะห์ที่ได้จาก hop-α-acids พบว่า Iso-*trans*-adhumulone แสดงการยับยั้งเอนไซม์สูงสุดเมื่อเปรียบเทียบกับ iso-α-acids และ iso-*cis*-adhumulone

จากการที่ได้ศึกษากลไกการยับยั้งจุลินทรีย์โดยศึกษาผลการเกิดความกดดันที่ผนังเซลล์เชื้อราและการยับยั้งเอนไซม์แอนทรานิเลท ซินเทส แล้วนั้น จึงได้ศึกษาผลของสารออกฤทธิ์ดังกล่าวต่อการยับยั้งเชื้อราทำลายไม้ใน**บทที่ 7** กิจกรรมการยับยั้งเชื้อราทำลายไม้โดยสารสกัดจากดอกกัญชา (*C. sativa*) และดอกฮอพ (*H. lupulus*) รวมทั้งขี้เลื่อยไม้เนื้อแข็ง ได้แก่ ไม้สัก (*T. grandis*) ไม้แดง (*X. xylocapa*) ไม้เต็ง (*S. obtusa*) ไม้อาลันบาตู (*S. albida*) และ ไม้ตะเคียนทอง (*H. odorata*) ได้ทดสอบโดยวิธีการแพร่กระจายของสารโดยใช้กระดาษกรอง (paper-disc diffusion assay) และวิธีการเจือจางความเข้มข้นของสารโดยใช้อาหารแข็ง (agar plate dilution assay) ผลการทดสอบปรากฏว่าสารสกัดจากขี้เลื่อยไม้สักและสารสกัดจากดอกฮอพ ยับยั้งเชื้อรากลุ่มที่ทำลายไม้ได้จำนวนมากสายพันธุ์กว่าการใช้สารสกัดจากพืชชนิดอื่นๆ สาร deoxylapachol ที่แยกได้จากขี้เลื่อยไม้สักมีฤทธิ์ยับยั้งเชื้อราทำลายไม้ในกลุ่ม brown rot fungi ได้แก่ *Gloeophyllum sepiarium* CBS 353.74 และ *Gloeophyllum trabeum* CBS 318.50 และเชื้อราทำลายไม้ในกลุ่ม white rot fungi ได้แก่ *Phlebia brevispora* CBS 509.92 และ *Merulius tremellosus* CBS 280.73 จากนั้นจึงศึกษากลไกการยับยั้งเชื้อราทำลายไม้โดยเลือกศึกษาการยับยั้งเอนไซม์เซลลูเลส (cellulase) ซึ่งเป็นเอนไซม์ที่เชื้อรากลุ่มนี้ผลิตขึ้นเพื่อย่อยสลายไม้ ผลการทดลองพบว่าแฟรกชั่น 87 (hemitectol + tectol) ที่แยกได้จากขี้เลื่อยไม้สักมีฤทธิ์ยับยั้งเอนไซม์เซลลูเลสได้สูงที่สุด เมื่อเปรียบเทียบกับสารอื่น ๆ ที่แยกได้จากขี้เลื่อยไม้สักและดอกฮอพ ส่วนสาร humulone ที่แยกได้จากดอกฮอพมีฤทธิ์ยับยั้งเชื้อราทำลายไม้กลุ่ม brown rot fungi ได้แก่ *G. sepiarium* CBS 317.50 และ CBS 353.74, *G. trabeum* CBS 318.50 และ

CBS 335.49 และ *S. lacrymans* CBS 520.91 และ CBS 751.79 แต่มีฤทธิ์ยับยั้งเอนไซม์เซลลูเลสในระดับต่ำ

การค้นพบสารยับยั้งจุลินทรีย์ชนิดใหม่ต้องการทั้งวิธีทดสอบการยับยั้งจุลินทรีย์โดยทั่วไปและวิธีการที่จำเพาะเจาะจง ในการเข้าทำลายโมเลกุลเป้าหมายในเซลล์จุลินทรีย์หรือทำลายผนังเซลล์และเยื่อหุ้มเซลล์ และแม้ว่าการพิสูจน์โครงสร้างของสารภายหลังตรวจพบฤทธิ์ยับยั้งจุลินทรีย์จะสามารถทำได้โดยใช้สารปริมาณไม่มาก แต่เพื่อการทดสอบฤทธิ์ทางชีวภาพจึงจำเป็นต้องใช้สารปริมาณมากขึ้น งานวิจัยฉบับนี้พิสูจน์ให้เห็นว่าเครื่องมือซีพีซีสามารถใช้แยกสารเพื่อตอบสนองความต้องการนี้ได้ ในจำนวนสารที่แยกได้ทั้งหมด hemitectol จากขี้เลื่อยไม้สักมีฤทธิ์ยับยั้งเชื้อรา *A. niger* ในระดับสูงและมีฤทธิ์สูงในการยับยั้งเอนไซม์เซลลูเลสแต่สารนี้กลับไม่เสถียร α-acids และ *β*-acids จากสารสกัดจากดอกฮอพเป็นสารที่มีฤทธิ์ยับยั้งเอนไซม์แอนทรานิเลท ซินเทส แต่เป็นสารที่สลายตัวได้ง่าย สาร deoxylapachol ที่แยกได้จากขี้เลื่อยไม้สักมีฤทธิ์ยับยั้งจุลินทรีย์ได้ดีโดยเฉพาะอย่างยิ่งมีฤทธิ์ชักนำให้ผนังเซลล์เชื้อราเกิดความกดดัน อย่างไรก็ตามมีรายงานว่าสารที่มีโครงสร้างคล้ายสารนี้ซึ่งได้แก่ lapachol มีฤทธิ์ยับยั้งมะเร็ง ดังนั้นจึงน่าจะมีการศึกษาความเป็นพิษของสารดังกล่าวต่อเซลล์มนุษย์และสัตว์เลี้ยงลูกด้วยนม สารที่ค้นพบในพืชดังกล่าวข้างต้นนี้อาจนำไปสู่การวิจัย เพื่อให้ได้สารกึ่งสังเคราะห์ซึ่งมีพื้นฐานมาจากสารสกัดจากพืชที่ออกฤทธิ์ยับยั้ง จุลินทรีย์ ส่วนในการประยุกต์ใช้สารสกัดจากธรรมชาติหรือสารกึ่งสังเคราะห์ที่มีพื้นฐานมาจากสารสกัดจากธรรมชาติเพื่อการป้องกันเชื้อราทำลายไม้ ป้องกันเชื้อราขึ้นใยผ้าคอตตอน หรือการยับยั้งจุลินทรีย์ในผลิตภัณฑ์อาหารสำเร็จรูป อาจทำได้โดยการเชื่อมต่อสารออกฤทธิ์ดังกล่าวกับโพลีเมอร์หรือวัสดุต่าง ๆ เพื่อให้เกิดความคงทนถาวรในการออกฤทธิ์ยับยั้งจุลินทรีย์

งานวิจัยฉบับนี้พิสูจน์ให้เห็นถึงความน่าสนใจในการใช้วัตถุดิบหรือวัสดุเหลือใช้จากอุตสาหกรรมแปรรูปผลิตผลทางการเกษตร เพื่อให้เกิดประโยชน์ในรูปแบบของผลผลิตใหม่ เช่น การผลิตสารยับยั้งเชื้อราจากขี้เลื่อยไม้เนื้อแข็ง ขั้นตอนต่อไปจึงน่าจะเป็นการพัฒนาวิธีการมาตรฐานสำหรับการคัดเลือกสารออกฤทธิ์ที่ได้จากวัสดุเหลือใช้ในอุตสาหกรรมการเกษตร เพื่อพัฒนาผลิตภัณฑ์ใหม่ๆ เช่น ยา อาหารเสริม อาหารเพื่อสุขภาพ

สารป้องกันเชื้อโรคที่ปนเปื้อนในอาหาร และสารยับยั้งเชื้อโรคในอาหารสัตว์หรือในแปลงเกษตรกรรม นอกจากนี้ยังรวมไปถึงสารยับยั้งจุลินทรีย์ก่อโรคในบ่อปลาและบ่อกุ้ง และสารป้องกันเชื้อราขึ้นไม้หรือวัสดุเครื่องใช้ในบ้าน ดังจะเห็นได้จากแผนผังโครงการซึ่งแสดงอยู่ใน**บทที่ 8**

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Curriculum vitae

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