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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 produced 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 adsorption 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* resistant 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 fever, 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.