Antimicrobial compounds as side products from the agricultural processing industry

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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-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.

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.