Phytochemical and Biosynthetic Studies of Lignans, with a Focus on Indonesian Medicinal Plants
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Summary and concluding remarks
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In this thesis phytochemical and biosynthetic studies of lignans are described. The focus is on the Indonesian medicinal plants *Phyllanthus niruri* and *Piper cubeba* and on two *Linum* species, *Linum flavum* and *L. leontii*, native to European countries.

Both Indonesian plants are used in *jamu*. *Jamu* is the Indonesian traditional herbal medicine, practised for many centuries in the Indonesian community to maintain good health and to treat diseases. The manufacturing of *jamu* is shifting more and more from household scale to the bigger industries. As the economical and clinical value of *jamu* nowadays increases in Indonesia, there is a need for further scientific proof and well conducted research. *Jamu* has to be developed in order to assure its efficacy and safety.

Chapter 2 reviews the research carried out on *jamu* and *jamu* plants, covering a broad range of aspects including phytochemistry, pharmacology, toxicology and clinical studies. In addition, ethical issues such intellectual property right (IPR), benefit sharing, and conservation are addressed.

*Phyllanthus niruri* is an important medicinal plant for *jamu*. All parts of the plant are used, among others to treat gonorrhea, syphilis, nephralgia, diarrhea, fever, and tetanus. The leaves serve to treat epilepsy, malaria, constipation, hypertension, and menstrual disorders. Lignans seem to be an important group of secondary metabolites, responsible for the biological activity of *P. niruri*. We initiated cell cultures of *P. niruri* that were able to produce lignans. The lignan profiles of cell suspensions, callus cultures, aerial plant parts, roots and seeds were compared and significant differences, both qualitatively and quantitatively, were found (chapter 3). Two compounds that were not found in *P. niruri* before were isolated from the cell suspension cultures: a new dihydrocubebin-dimethylether and urinatetralin, a new lignan from *P. niruri*, but reported earlier from *P. urinaria*. Feeding 0.5 mM of ferulic acid or 0.5 mM of caffeic acid, being early precursors of lignan biosynthesis, resulted in an increase up to 0.7 mg g\(^{-1}\) DW of the dihydrocubebin-dimethylether (control value 0.1 mg g\(^{-1}\) DW) and up to 0.3 mg g\(^{-1}\) DW of urinatetralin (control value 0.2 mg g\(^{-1}\) DW) in the suspension cultures.

Lignans are also found in another important medicinal plant for *jamu*, *Piper cubeba*. The berries of this plant are used to treat gonorrhea, dysentery, syphilis, abdominal pain, diarrhea, enteritis and asthma. The lignan profile of berries (the particular plant part used in *jamu*), leaves and stalks were investigated and compared using gas chromatography (GC), gas chromatography coupled to mass spectrometry (GC-MS), and high pressure liquid chromatography (HPLC) (chapter 4). Thirteen lignans were identified in the berries, fifteen in the leaves and five in the stalks.

Our further phytochemical investigation of *P. cubeba* berries and leaves focused on the essential oil composition (chapter 5). The essential oil of the berries is commonly used as a constituent of cosmetics as well as for medicinal purposes. Antimicrobial, antiherp simplex, antifungal, cardiovascular, and gastroprotective are mentioned in the latter context. Hydrodistillation of the berries of *P. cubeba* yielded 11.8% (w/w) and the leaves 0.9% (v/w) oil. In total 105 components could be identified (GC, GC-MS) in the berries, dealing with 63.1% of the oil. In the leaves, 63 components could be identified, corresponding with 78.0% of the oil. The total amount of monoterpens was comparable in both oils (17.2% and 17.0%, for berries and leaves, respectively). The main monoterpenes in the berries and leaves oil were α-thujene, α-pinene, sabinene, and limonene. In the oxygenated monoterpe fractions *trans*-sabinene hydrate was the main component. α-Copaene, β-elemene, *E*-caryophyllene, and caryophyllene were the main sesquiterpenes in the berries oil. *E*-caryophyllene, and γ-cadinene were the main sesquiterpenes in the leaves oil.

Based on the similarity of the lignan and essential oil profiles we conclude that, in addition to the berries, also the leaves may be used for medicinal purposes. This knowledge can be used for the further development of (rationally designed) phytomedicines from *P. cubeba*.  

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Studies on the lignan biosynthesis were performed with the European plant, *Linum flavum*. *L. flavum* cell cultures accumulate a high amount of coniferin (12% on a dry weight basis). Cell suspension cultures of leaves from this plant were treated with glucosyltransferase inhibitors in order to enhance the production of the lignan 6-methoxypodophyllotoxin (6-MPT) and to reduce the coniferin production (chapter 6). These two compounds originate from the common precursor coniferyl alcohol by different biosynthetic branches. Enzymatic transformation of this substrate by coniferyl alcohol glucosyltransferase (CAGT) yields coniferin. It was hypothesized that by inhibiting this step more precursor would be available for the biosynthetic branch leading to the formation of lignans. Na₂EDTA inhibited the production of coniferin up to 88% and on the other hand enhanced the 6-MPT production up to 0.6 mg g⁻¹ DW, 3.2-fold more than in untreated cultures. The inhibition of the coniferin production related to an inhibition of the CAGT activity, as shown in cell homogenates incubated with coniferyl alcohol. This indicates that Na₂EDTA inhibits CAGT and therefore reduces the production of coniferin. The mechanism of inhibition is not clear as yet. To further study the role of CAGT in the biosynthesis of lignans, we partially purified this enzyme from cell suspension cultures of *L. flavum*. A complete purification of CAGT, however, appeared to be impossible because of its unstable nature.

We tried to clone the CAGT from cell suspension cultures of *L. flavum* in *Escherichia coli* (chapter 7). The total RNA isolation revealed that the quality of RNA was sufficient to synthesize the cDNA. A forward and a reverse degenerated primer were designed based on the most conserved region of 100 glucosyltransferases from various species. These glucosyltransferases were aligned using MegAline software from DNASTAR Inc. The conserved region, called the PSPG box, is highly characteristic and present in all GTs involved in natural product biosynthesis. This domain may also define the active site of GTs of animals and microorganisms. The PSPG box is considered to represent the nucleotide diphosphate binding site. Several GT-encoding genes are suitable candidates to be inserted into a variety of plants with the aim of improving food, crop quality as well understanding the biosynthesis of valuable natural products for medicine.

Three different potential GTs were cloned from cell suspension cultures of *L. flavum* in *E. coli*. The gene sequence of three different products was elucidated. Two of them were complete sequences (ORF): CAGT A and CAGT B. We were able to find the conserved region (PSPG box) for the third one. Alignment of CAGT A and B with the sequences from the plant GTs obtained from databases showed 50% and 40% homology, respectively. The conserved region (PSPG box) of CAGT A and B showed 80% and 89% homology respectively. Phylogenetic analysis revealed that CAGT A and B belong to the same subfamily together with other phenylpropanoid glucosyltransferases. Although the expression of these GTs is not yet finished, it may be concluded that at least one of the two CAGT is involved in the biosynthetic step from coniferyl alcohol to coniferin in *L. flavum* cell suspension cultures.

In chapter 8, we describe the production of justicidin B, a cytotoxic aryltetraline lignan in cell and hairy root cultures of *Linum leonii*. The hairy root cultures were obtained by genetic transformation using the agropine-type strain *Agrobacterium rhizogenes* 15834. The products encoded by *rol A* and *rol C* genes were found to have a synergistic effect on root induction and to induce increased sensitivity to auxin. The transformation of these genes from *A. rhizogenes* into the hairy root was checked by PCR (polymerase chain reaction). Proof of transformation was given by the PCR products showing that *rol A* and *rol C* genes were present in the hairy roots of *L. leonii*. Genetically modified hairy roots produced 5-fold higher yields of justicidin B (10.8 mg g⁻¹ DW) compared to untreated callus. This suggests that this technique may be used to enhance the accumulation of justicidin B. In addition to the production, we investigated the cytotoxic effect of justicidin B in three chronic human myeloid leukemia-derived cell lines (LAMA-84, K-562 and SKW-3), that show a lower responsiveness to cytotoxic drugs due to a strong expression of the fusion oncoprotein BCR-ABL (a non-receptor tyrosine kinase). IC₅₀ values of justicidin B were 1.1, 6.1 and 1.5 µM for the chronic myeloid leukemia (LAMA-84), pre-B-cell...
lymphoma (K-562) and chronic lymphoid leukemia (SKW) cell lines respectively. These IC_{50} were comparable to the anticancer drug etoposide (a semi-synthetic lignan derivative).

We conclude that the phytochemical studies of the two selected Indonesian medicinal plants as well as the production of lignans in cell suspension cultures provide further scientific approach for the development of jamu. Furthermore the genetic engineering studies of two Linum species contribute to medicinal plant research with respect to a better understanding of the lignan biosynthesis. The biotechnology approach may also be applied to use medicinal plants as a source for drugs discovery.