Separation, identification and quantification of lignans in native plant samples and enhancement of lignan production in *Forsythia in vitro* cell cultures

PhD thesis

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Summary

Among the plant secondary metabolites, the lignans have got special interest recently, due to their various and remarkable pharmaceutically and medicinally important properties.

In this thesis, lignan identification and quantification methods were examined in intact plant samples with high amounts of lignan content in order to produce and extract them efficiently. In addition, we studied the opportunities of stimulation of *in vitro* lignan production in cell cultures of *Forsythia* species and cultivars for scale-up production by fermentation technology. The secondary metabolite content was measured with spectrophotometric and chromatographic methods and the cell differentiation was observed with spectrofluorometer and fluorescence- and electron microscopy.

We worked out optimal extraction methods and studied spectrophotometric methods feasible for sample pre-selection and tested the suitability of several chromatographic methods for lignan analysis.

We evaluated qualitative and quantitative differences in lignan contents among *Forsythia* species and cultivars in leaf and *in vitro* cell culture samples.

Forsythia callus and suspension cultures were established. In optimization experiments, we stimulated the lignan production of suspension cultures via hormone, sugar, macro- and micro element contents modifications of culture media.

We found correlation between the light-induced cell differentiation and the increase of lignan contents in *Forsythia* cell cultures. The illumination enhanced the lignan production in general but it is not suitable for selective stimulation of the biosynthesis of selected lignans separately.

We have demonstrated that *Arctium lappa, Centaurea scabiosa* and *Forsythia* species and cultivars are suitable for production of the studied lignans for the pharmaceutical industry. The fruit of *Arctium lappa* is a good source of arctiin while the leaf of *Forsythia* is suitable for arctigenin, pinoresinol and phylligenin extraction. The fruit of *Centaurea scabiosa* and *in vitro* cultures of *Forsythia* can be applied for matairesinoside production.

Introduction

Nowadays natural active ingredients have become widely prevalent in the therapy. Most of these molecules belong to the plant secondary metabolites. The significance of this group has increased due to their various medical utilization and the development of analytical methods.

The group has diverse biological activities especially of lignans from phenolic compounds. As a folk medicine it has been used owing to anticancer and laxative effect for centuries. Therefore several researchers have started to elaborate and understand the mechanism of action. A number of lignans (arctigenin, matairesinol and its glycosides pinoresinol and phillygenin) have come to the fore in research. These molecules exhibit antitumor effects against various types of cancer, as well as anti-HIV, anti-inflammatory, antioxidant, hepato- and neuroprotective properties.

These lignans occur in the highest quantities in *Arctium, Centaurea* and *Forsythia* species and cultivars. *Forsythia* genus is intensively examined and more and more data is published about its active components and its biosynthesis.

The *in vitro* technology is widely used for the production of secondary metabolites. As the conditions of production of bioactive components are controlled and optimized and the separation is easier and more efficient than the production derived from intact plant samples.

Aims

Lignans are significant plant secondary metabolites from pharmaceutical point of view but their efficient production and separation is not totally reported. Therefore our aim is to separate, identify and quantify lignans in native plant samples for efficient lignan extraction and production as well as enhance the lignan production in *Forsythia in vitro* cell culture for large-scale production by fermentation technology.

Our aims:

1. To develop efficient methodologies for analysis of lignans:
    a) To select suitable extraction method by comparison of 3 methods' and 4 solvents' combinations in leaf of *Forsythia* x *intermedia,*

b) To find a suitable spectrophotometric analysis for pre-selection of *Forsythia* species and cultivars by determination of phenolic content and antioxidant activity.

c) To develop exact analytical methods HPLC-UV, HPLC-ESPI-MS and GC-MS techniques in fruits of *Arctium lappa*, *Centaurea scabiosa* and leaf of *Forsythia.*

2. To identify and quantify lignans in *Forsythia* species and cultivars in order to select suitable cultivars.
3. To establish *Forsythia* callus and suspension cell cultures as well as to enhance lignan production of suspension:
   a) With modification of medium components especially amount of hormones, saccharose and macro, micro elements,
   b) With selection of suitable cultivars.
4. To analyze correlation between light induced cell differentiation and lignan content in *Forsythia* suspension culture with modification of time of exposure.

Methods

The fruits of *Arctium lappa* and leaves of *Forsythia* were obtained from Botanic Garden of Corvinus University. Fruits of *Centaurea scabiosa* were obtained from the French B and World Seed Company.

Callus tissues were induced from leaves of *Forsythia* x *intermedia* in order to analyze the metabolite production of cell cultures. Calli were maintained on Gamborg B5 solid medium (supplemented with 0.5 mg/l 2,4-D, 8 g l^{-1} agar, 30 g l^{-1} saccharose) in dark and room temperature. Calli were transferred into 250 ml Erlenmeyer flask containing Murashige & Skoog or B5 liquid medium. Suspension cultures were maintained in room temperature in dark and under light, agitated at 110 rpm with 2-week subculture period.

For enhancement of lignan production of suspension medium optimization were used. We modified the components of medium. We changed the amount of hormones (combination of 3 auxins, IAA, NAA, 2,4-D and a cytokinin, the kinetin) and increased amount of saccharose (2 and 3 times) and decreased the amount of macro and micro elements (1/2 and 1/3. After three subcultures they were placed into liquid media, and in

a flask. A version of MS medium supplemented with 2 mg/l naphthalene acetic acid and 0.2 mg/g kinetin was proved to be the best, providing high lignan content (3.9 mg arctigenin/g dried matter), in suspension culture maintained under light. Besides the medium's hormone content, the lightning (10-15 µmol/m2/sec) played a great role in the high lignan production.

For analysis of impact of light on cell differentiation and lignan biosynthesis we maintained the suspension cultures in dark and under natural light and artificial white light exposure. At the controlled light 3 length of exposure time (4 h exposure a day; 8 h a day or 12 h a day) were applied.

We examined the ultrastructural changes with transmission electron microscope. We observed the effect of light on cell cultures with spectrophotometric measurements of chlorophyll a and b content.

For selection of optimal extraction method we compared the frequently applied methods and solvents. We combined extraction techniques by reflux condenser, ultrasonic bath and supercritical fluid extractor applying 60 or 100 % (*v/v*) ethyl or methyl alcohol in leaf of *Forsythia* x *intermedia*.

We determined phenolic content with Folin-Ciocalteu reagent and antioxidant activity (FRAP method) in order to find a suitable spectrophotometric analysis for pre-selection of *Forsythia* species and cultivars.

We identified and quantified lignans by Gas Chromatography-Mass Spectrometry (GC-MS), High Performance Liquid Chromatography-Electrospray Ionization in Positive Ion mode-Mass Spectrometry (HPLC-ESPI-MS) and High Performance Liquid Chromatography with UV Detection (HPLC-UV) techniques in native and in vitro samples.

Results

Optimization of lignan extraction in *Forsythia* x *intermedia* species

In literature data about lignan separation of *Forsythia* species were heterogeneous and not comparable. Three methods of extractions, involving four different solvents were compared in order to select the most efficient one, giving the highest quantity of the main lignan constituents (arctiin and arctigenin).

In the comparison of 12 combined methods we observed 2 times differences between the most efficient method (SFE with 60 % (*v/v*) methyl alcohol co-solvent) and the least efficient (refluxation with 100 % (*v/v*) ethyl alcohol). The impact of solvent on separation' effectiveness was similar to method effect. In the examination of methods it is important to pay attention to cost, yield of useful information, number of samples and time because it can change the order of suitability.

Phenolic and lignan content and antioxidant activity in leaves of *Forsythia* species and cultivars.

For pre-selection of species and cultivars with high lignan content we applied spectrophotometric methods in order to reduce the number of samples before the time consuming chromatographic measurements. As spectrophotometric methods for lignan content determination is not reported we applied phenolic content and antioxidant capacity determination in order to examine the suitability for pre-selection

The total lignan and phenolic content and antioxidant capacity were diverse. Good correlation was demonstrated between phenolic and total lignan content contrary to the low correlation between antioxidant capacity and total lignan content. The presence of flavonoid, phenylpropanoid and phenolic acids could decrease the correlation due to their antioxidant capacity.

Identification and quantification of lignans

We identified and quantified lignans by GC-MS, HPLC-ESPI-MS and HPLC-UV techniques in *Arctium lappa*, *Centaurea scabiosa* and *Forsythia* species. Identification of lignans was performed on the basis of the retention time of authentic lignan (arctigenin, arctiin and matairesinol) and characteristics in mass spectrometry.

In the analysis of metabolite the butyrolactone and furofuran type lignans could be measured simultaneously by each technique. However the arctigenin and phylligenin cannot be separated totally by HPLC-UV.

In the first time we evaluated the differences among *Forsythia* species and cultivars. Arctigenin was the major lignan constituents in all *Forsythia* species and cultivars. But the proportion of arctigenin varied a lot (41%-92) compared to the total lignan content in species and cultivars, too.

According to biplot analysis, the cultivars were categorized into groups. Identification of pinoresinol and phylligenin was superfluous in *Forsythia* x *intermedia* cultivars, measurement of one of the selected lignan was sufficient. However the amount of arctigenin was independent of these lignans and provided distinct information about the categories therefore the quantification of arctigenin is necessary for categorization of cultivars.

Establishment of *in vitro* cultures of *Forsythia* species cultivars

In vitro plant cell cultures are a good source of metabolite production thanks to its several advantages. There are numerous cultivars of *Forsythia* species but the lignan content of their *in vitro* cell cultures is hardly reported. Therefore it is important that we paid attention to cultivars and maintained cell cultures in the same condition in order to compare the lignan content of the cultivars.

We established callus and suspension culture of 3 *Forsythia* species *(F. intermedia, F. ovata, F. suspensa*) and of 6 cultivars (*F. intermedia* Beatrix Farrand, Melissa, Minigold, Primulina and Week-End, and *F. ovata Robusta, Tetragold*) for lignan production. We established in the first time *in vitro* cell culture of *Forsythia ovata*.

Optimization of culture medium effects on lignan content of *in vitro cultures*

Metabolite accumulation of *in vitro* cell culture usually cannot reach the level of intact samples therefore enhancement of metabolite production is needed via optimization of culture medium.

Lignan accumulation of *in vitro* cell culture increased via modification of components of culture medium. Changes in hormones of medium (2 mg g-1 NAA and 0.2 mg g-1 kinetin), in amount of macro- and micro elements (one third amount) and in amount of saccharose (90 g l^{-1}) enhanced the lignan accumulation. Furthermore the combination of these modifications reduced the cultivation period of cell culture with high lignan content.

Impact of light on cell differentiation and lignan accumulation of *in vitro* cultures

According to the total chlorophyll content and 77 K fluorescence spectrum we evaluated that photosynthetic chlorophyll-protein complex can function in all suspension cultures maintained under light although their low total chlorophyll content.

The ultrastructural changes confirmed that high-level cell differentiation was required for lignan production of *in vitro* cultures in *Forsythia* species and cultivars.

In suspension culture of *Forsythia* x *intermedia* the application of culture medium specialized for metabolite production was not sufficient, light exposure was needed for lignan biosynthesis. Our experiment confirmed it because lignan content was not detected in cell cultures maintained in dark contrary to every suspension cultures cultivated under light.

Moderate correlation could be observed between type and period of light exposure and lignan accumulation. We detected the lignan content at maximum level at 4-week cultivation period and 8-hour light exposure a day. The light generally increased the amount of lignans but did not influence it selectively as the light had effect on first steps of lignan biosynthesis.

Differences in lignan content of *Forsythia* species and cultivars *in vitro* cultures

We investigated in the first time the differences in lignan content of *Forsythia* species and cultivars *in vitro* cultures. We evaluated similar differences among cultivars to the level of enhancement in the lignan production via culture medium optimization. Therefore it is important to emphasise the significance of cultivars at metabolite production, too.

Conclusions

We investigated the lignan content in intact plant samples and the lignan production in *Forsythia in vitro* cell culture to obtain high lignan production.

1. At the development of efficient methodologies for analysis of lignans We evaluated
    a) To select suitable extraction method for arctigenin and arctiin we highlighted the significance of several parameters of extraction

methods. SFE with 60 % (*v/v*) methyl alcohol co-solvent is appropriate for small number of samples while refluxation with 100 % (*v/v*) methyl alcohol is suitable for numerous samples.

b) To find a suitable spectrophotometric analysis for pre-selection of *Forsythia* species and cultivars with high lignan content in order to reduce number of samples. We evaluated that determination of phenolic content (Folin Ciocaletu reagent) is appropriate for pre-selection due to the good correlation between phenolic and total lignan. The antioxidant capacity (FRAP) is not suitable for pre-selection due to the very moderate correlation between antioxidant capacity and total lignan content.

c) The butyrolactone and furofuran type lignans could be measured simultaneously by each technique in *Arctium lappa*, *Centaurea scabiosa* and *Forsythia* samples. However the arctigenin and phylligenin cannot be separated totally by HPLC-UV. For further precision and reliability it is recommended to use all these chromatographic techniques parallel.

2. In the first time we evaluated the differences among *Forsythia* species and cultivars in lignan content and composition. According to biplot analysis, the cultivars were categorized into groups. Therefore it is an appropriate statistical analysis for selection of cultivars and emphasizes the significance of cultivars.
3. We evaluated that callus and suspension culture can be established from numerous *Forsythia* species and cultivars and it confirms and extends the previous data. Lignan content of in vitro cultures derived from leafs differs from lignan content of leaf. On the basis of lignan content of leaf conclusion cannot be drawn for lignan content of *in vitro* cultures derived from leafs. Therefore it is necessary to investigate the metabolite production of in vitro cultures. Although metabolite accumulation of *in vitro* cell culture usually cannot reach the level of intact samples, the application of *in vitro* cell culture possesses several advantages in metabolites production. The enhancement of metabolite production could be performed; constituents could be selectively

produced and general advantages of in vitro technology. The differentiation at cell and tissue level influences the metabolite production in leaves and in vitro cultures, too.

a) Metabolite accumulation of *in vitro* cell culture usually cannot reach the level of intact samples therefore enhancement of metabolite production is needed via optimization of culture medium. Lignan accumulation of *in vitro* cell culture increased via modification of components of culture medium (hormones, macro- and micro elements and saccharose). Furthermore the combination of these modifications reduced the cultivation period of cell culture with high lignan.

b) We investigated in the first time the differences in lignan content of *Forsythia* species and cultivars *in vitro* cultures. For the enhancement of lignan accumulation it could be a new tool.

4. Moderate correlation could be observed between light induced cell differentiation and lignan accumulation in *Forsythia* suspension cultures. In the detailed investigation the amount of chlorophyll and developmental stage of chlorophyll-protein complexes indicated the level of cell differentiation but did not correlate with lignan production. Light exposure stimulated the cell differentiation and could indirectly increase the lignan accumulation. The light generally increased the amount of lignans but did not influence it selectively.

We have demonstrated that *Arctium lappa*, *Centaurea scabiosa* and *Forsythia* species and cultivars are suitable for production of the studied lignans for the pharmaceutical industry. The fruit of *Arctium lappa* is a good source of arctiin while the leaf of *Forsythia* is suitable for arctigenin, pinoresinol and phylligenin extraction. The fruit of *Centaurea scabiosa* and *in vitro* cultures of *Forsythia* can be applied for matairesinoside production.

Publications

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