Lignans in *Phaleria macrocarpa* (Scheff.) Boerl. and in *Linum flavum* var. *compactum* L.

[Lignane in *Phaleria macrocarpa* (Scheff.) Boerl. und *Linum flavum* var. *compactum* L.]

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vorgelegt von
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Erklärung

Hiermit erkläre ich eidesstattlich, dass ich die vorliegende Dissertation mit dem Titel „Lignans in *Phaleria macrocarpa* (Scheff.) Boerl. and in *Linum flavum* var. *compactum* L. [Lignane in *Phaleria macrocarpa* (Scheff.) Boerl. und *Linum flavum* var. *compactum* L.]“ selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe.

Düsseldorf, den 22. Oktober 2007

Ahmad Saufi

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CONTENTS

1. INTRODUCTION

1.1. Plant secondary metabolites

The compounds produced by plants have been separated into primary and secondary metabolites. Primary metabolites, by definition, are molecules that are found in all plant cells and are necessary for the life of the plant. Examples of primary metabolites are simple sugars, amino acids, lipids, and nucleic acids. Secondary metabolites, by contrast, are restricted in their distribution, both within the plant and among the different species of plants (Raven, *et al.*, 1999). Plant secondary metabolites comprise all organic compounds that occur usually only in special, differentiated cells and are not necessary for the cells themselves but apparently useful for the plant as a whole (Taiz and Zeiger, 2006).

The distinction between both groups was drawn in 1891 by Kossel in order to designate secondary products by their proposed less significant function (Hadacek, 2002). However, in some cases the distinction between primary and secondary metabolism cannot be easily drawn (Mohr and Schopfer, 1994; Croteau, *et al.*, 2002). Lignin, the essential structural polymer of wood and second only to cellulose as the most abundant organic substance in plants, is considered a secondary metabolite rather than a primary metabolite. Therefore, from this point of view the boundary between primary and secondary metabolism is still blurred (Croteau, *et al.*, 2002).

In contrast to the formation of primary metabolites, the synthesis and accumulation of secondary metabolites occur during differentiation of specialized cells. They are produced at various sites within the cell and are stored primarily within vacuoles. Their production typically occurs in a specific organ, tissue, or cell type at specific stages of development (e.g., during flower, fruit, seed, or seedling development). Some secondary metabolites namely the phytoalexins, are antimicrobial compounds that are produced only after wounding or after attack by bacteria or fungi. The ability of the plant to form these metabolites thus follows a distinctive pattern in space as well as time, and is often controlled by environmental factors, e.g. light. Their concentration in a plant often varies greatly during a 24-hours period (Mohr and Schopfer, 1994; Raven, *et al.*, 1999).

In the absence of a valid distinction based on either structure or phytochemistry, a functional definition can be explained, that primary metabolites participate in nutrition and essential metabolic processes inside the plant, and secondary metabolites are influencing ecological interactions between the plant and its environment (Croteau, *et al.*, 2002).

A simple description of the relationships between primary and secondary metabolism is described in Fig. 1.1 where primary carbon metabolism supplies the precursors for most of the secondary carbon metabolism.

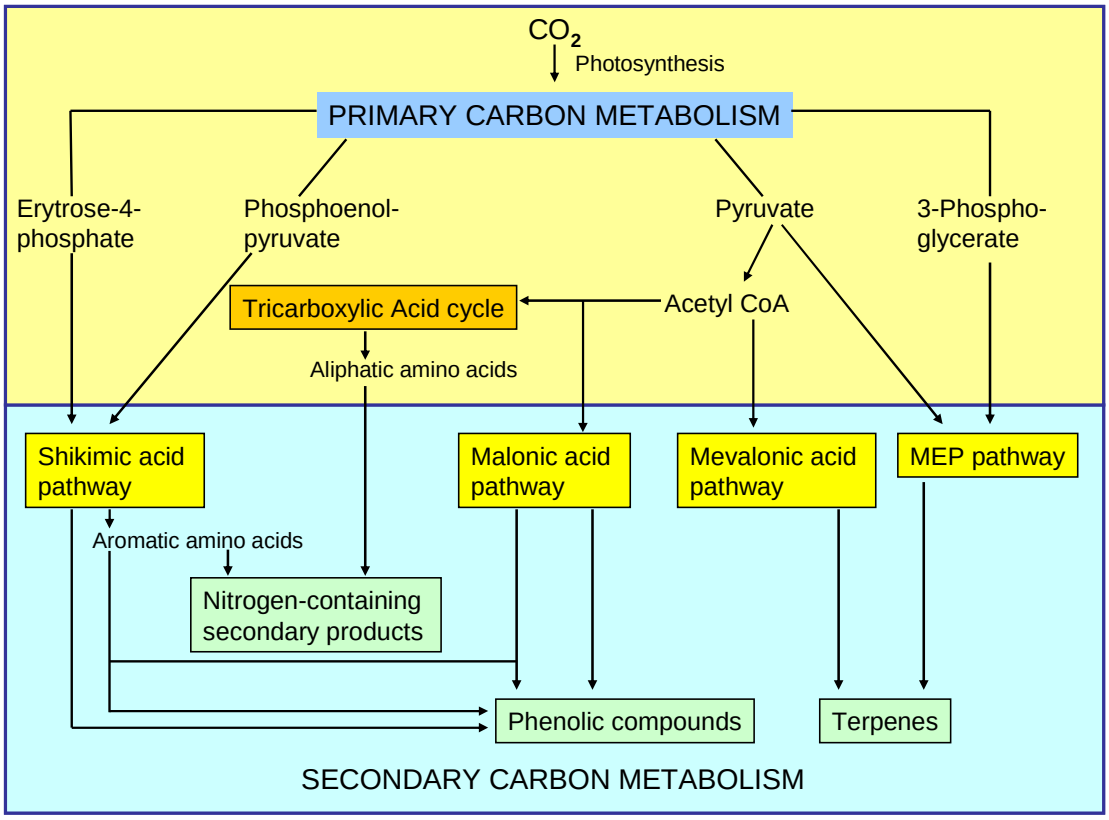


Figure1.1. The primary metabolism supplies the precursors for most of the secondary products (modified from Taiz and Zeiger, 2006)

Although there is no direct requirement for plant secondary metabolites in the cell, it would be incorrect to assume that these substances could be regarded as "luxury molecules". In general, the physiological significance of these substances becomes apparent at the level of the whole organism (Mohr and Schopfer, 1994). They may serve as plant defenses against predators, competitors, parasites and diseases; as agents of symbiosis between plants and microbes, nematodes, insects, and animals; as metal transporting agents; and as sexual hormones (Demain and Fang, 2000). Many serve as chemical signals that enable the plant to respond to environmental cues. Some provide protection from sun radiation, while others aid in pollen and seed dispersal (Raven, *et al.*, 1999). The information of secondary metabolites is therefore an integral activity of the differentiated plant. This also explains why, in higher plants, almost all species posses a specific pattern of secondary metabolites, whilst basic metabolism hardly differs (Mohr and Schopfer, 1994).

The modern chemistry of natural products generally defines secondary metabolites by a molecular weight of less than 1500 Daltons, thereby distinguishing them from high molecular weight polymers such as proteins or polysaccharides. Although the total number of structures designated as secondary metabolites exceeds 139000, nature only uses a few basic building blocks e.g. the acetate (C_2), isoprenoid (C_5) and the phenylpropanoid (C_9) unit (Verpoorte, 2000). Furthermore, plant secondary metabolites can be divided into three chemically distinct groups namely alkaloids, terpenoids and phenolic compounds (Raven, *et al.*, 1999; Croteau, *et al.*, 2002; Taiz and Zeiger, 2006). Some classes of secondary metabolites, their sources and their biological activity are resumed in Table 1.1.

Table 1.1 Classes of secondary metabolites, their building blocks, their sources and their biological activity

Class	**Building block**	**Sources**	**Biological activity**
alkaloids	acetate amino acids terpenoids cholesterol	plants (dicotyle), marine sponges, algae, fungi, bacteria (*Streptomyces* sp.),	nitrogen storage, detoxification, deterrent, allelochemical, poison
terpenoids	isoprene	plants, animal glands, fungi, intestinal bacteria	antimicrobial, neurotoxin, repellent
flavonoids	malonylCoA + cinnamoylCoA	gymnosperms, angiosperms, mosses, marine corals, ferns, algae, bacteria	antibiotic, coloration/dye
lignins	phenylpropanoids	gymnosperms, angiosperms, pteridophytes	structural support protective barrier
lignans	phenylpropanoids	gymnosperms, angiosperms, pteridophytes	antimicrobial insecticide germination inhibitor

The alkaloids are a large family of more than 15,000 nitrogen-containing secondary metabolites found in approximately 20% of the species of vascular plants. The nitrogen atom in these substances is usually part of a heterocyclic ring which contains both nitrogen and carbon atoms. Most alkaloids are alkaline, positively charged, and water soluble. Nicotine is an example of alkaloid which is derived from ornithine, an intermediate of arginine biosynthesis. Most alkaloids are now believed to function in defense against predator due to their general toxicity and deterrence capability (Hartmann, 1992).

The terpenoids or terpenes are substances derived from acetyl-CoA or glycolytic intermediates. All terpenes are built from the union of five-carbon elements which have the branched carbon skeleton of isopentane called isoprene units. Terpenes are biosynthesized from primary metabolites in two different ways: The mevalonic acid pathway and the methylerythritol phosphate pathway (Lichtenthaler, 1999). They are classified by their numbers of C_5 units. Ten-carbon terpenes, which contain two C_5 units, are called monoterpenes. A three-C_5 unit is called sesquiterpene, and a 20-carbon terpene is called diterpene. Giberellins, an important group of plant hormones, are diterpenes, whereas brassinosteroids are built from triterpenes.

Besides alkaloids and terpenoids, plants also produce a large variety of secondary metabolites which contain a phenol group. These substances are classified as phenolic compounds. Plant phenolic compounds are a chemically heterogenous group of nearly 10,000 individual compounds. They are biosynthesized by two different routes, namely the shikimic acid pathway and the malonic acid pathway. The shikimic acid pathway occurs in the biosynthesis of most plant phenolics, whereas the malonic acid pathway participates in the biosynthesis of phenolic compounds in fungi and bacteria.

1.2. The phenylpropanoid metabolism

Most plant phenolics including lignins and lignans are derived from the phenylpropanoid pathway (Fig. 1.2) and fulfill a very broad range of physiological roles in plants (Croteau, *et al.*, 2002). All phenylpropanoids (C_6C_3) are derived from *p*-coumaric acid and most from cinnamic acid. Thus, the formation of cinnamic acid from phenylalanine by the enzyme phenylalanine ammonia-lyase is referred to as the branch-point between primary metabolism (shikimic acid pathway) and secondary metabolism (phenylpropanoid pathway).

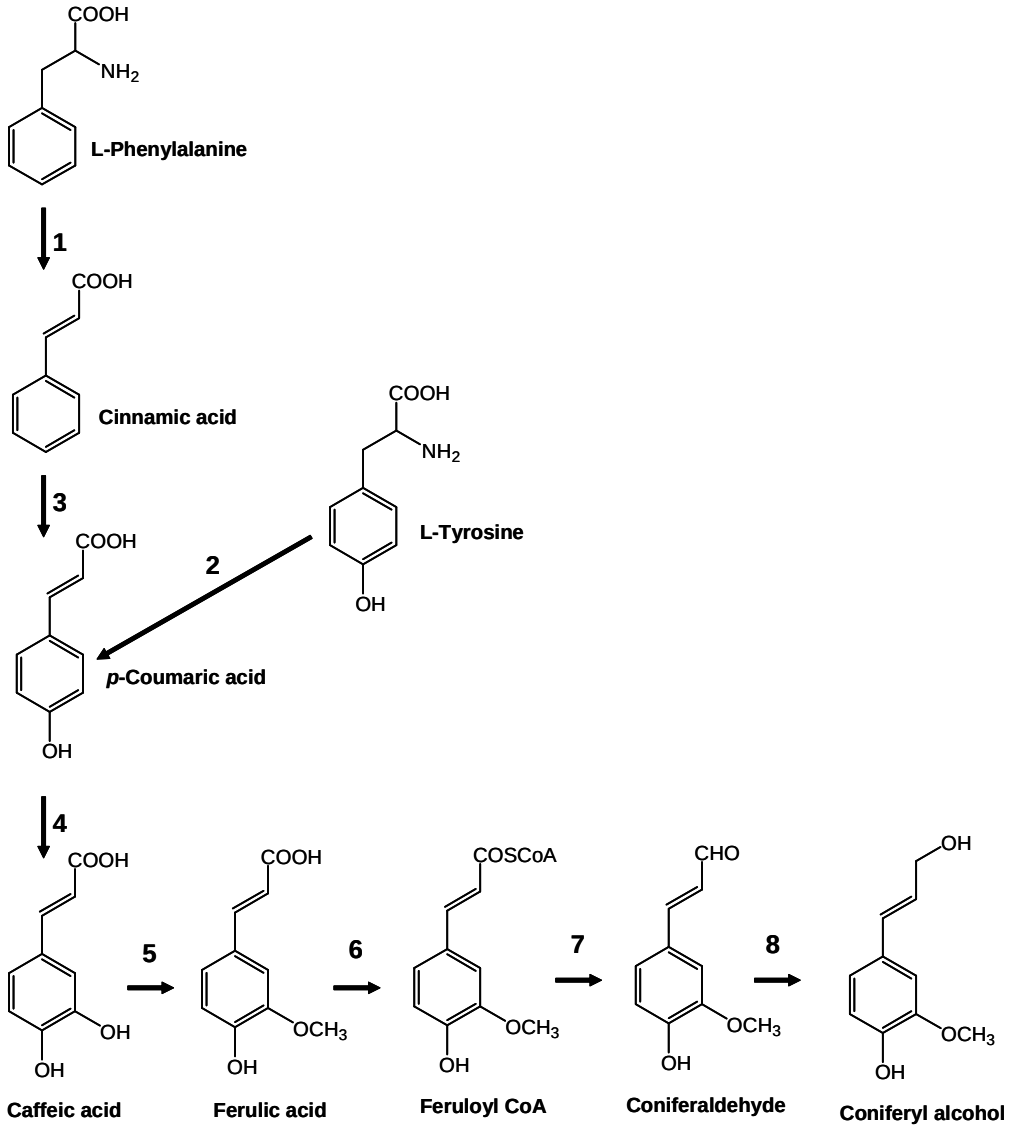


Figure 1.2. Main elements of the phenylpropanoid pathway leading to the formation of coniferyl alcohol. **1**=phenylalanine ammonia-lyase; **2**=tyrosine ammonia-lyase (mainly in grasses); **3**=cinnamate-4-hydroxylase; **4**=hydroxylase; **5**=caffeic acid *O*-methyltransferase; **6**=CoA ligases; **7**=cinnamoyl-CoA oxidoreductase; **8**=cinnamyl alcohol dehydrogenase (modified from Lewis, *et al.*, 1998).

Initially the deamination of L-phenylalanine by the enzyme phenylalanine ammonia-lyase yields cinnamic acid. Further, cinnamic acid could be transformed by cinnamate-4-hydroxylase into *p*-coumaric acid. The deamination of L-tyrosine by the enzyme tyrosine ammonia-lyase yields also *p*-coumaric acid. The next steps leading to

the formation of coniferyl alcohol comprise of four types of enzymatic reactions: aromatic hydroxylation, *O*-methylations, CoA ligations, and NADPH-dependent reductions (Lewis, *et al.*, 1998).

1.3. Lignans

The term lignan was first coined by Haworth in 1936 (Ayres and Loike, 1990; Moss, 2000; Croteau, *et al.*, 2002) for the structures where two units of phenylpropanoid are β,β' (or 8,8')-linked. However, they are called neolignans, when the two C_6C_3 molecules are coupled in other ways (e.g. 8,1' or 8,3'-linked dimers) or they are called oxyneolignans where the two C_6C_3 molecules are linked by an ether oxygen atom and not directly bonded together with the parent structure (Moss, 2000).

1.3.1. Biosynthesis of lignans

The biosynthetic pathway of lignans from coniferyl alcohol to matairesinol is presumed to be similar to the biosynthesis of matairesinol in *Forsythia x intermedia* (Xia Z.-Q. *et al.*, 2001). A so-called dirigent protein is involved in the dimerisation of two molecules of coniferyl alcohol to give enantiomeric pure (+)-pinoresinol (Fig. 1.3). In order to confer regio- and stereospecificity in a 8,8'-linked lignan formation Lewis and Davin have found a 78-kDa glycoprotein being involved (Davin, *et al.*, 1997; Davin and Lewis, 2000). Although this dirigent protein lacks any intrinsic oxidative properties, in vitro experiments containing the protein, coniferyl alcohol and an oxidant, resulted in optical pure (+)-pinoresinol. Subsequent cyclisation steps and the modifications of pinoresinol may lead to the formation of podophyllotoxin or related lignans in plant species like e.g. in *Linum flavum*.

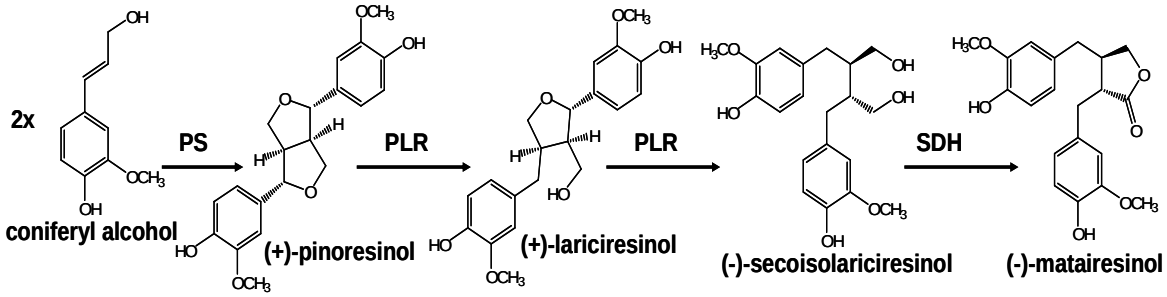
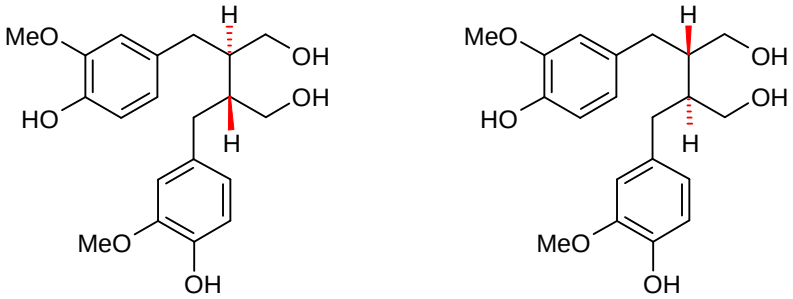


Figure 1.3. Biosynthesis of matairesinol in *Forsythia intermedia*. **PS**=pinoresinol-synthase; **PLR**=pinoresinol-lariciresinol reductase; **SDH**=secoisolariciresinol dehydrogenase

(+)-Pinoresinol is transformed to (-)-secoisolariciresinol (Fig. 1.4) via (+)-lariciresinol (Umezawa, 1990, Katayama, *et al.*, 1993). The enzyme pinoresinol-lariciresinol reductase (PLR) catalyses the two successive steps of reduction depending on the presence of NADPH as a cofactor. The hydride transfer from NADPH to the lignans is highly stereospecific. The formation of (-)-secoisolariciresinol and the following conversion of (-)-secoisolariciresinol into (-)-matairesinol were first elucidated in *F. intermedia* (Umezawa, *et al.*, 1991).



(-)-secoisolariciresinol (+)-secoisolariciresinol

Figure 1.4 Stereochemistry of secoisolariciresinol

Umezawa and co-workers (1991) showed that (-)-secoisolariciresinol could be converted into (-)-matairesinol by using cell-free extracts of *F. intermedia*, whereas the (+)-enantiomer was not metabolized further. At that time, it was unknown whether this NAD-dependent enantiospecific conversion involved either a bifunctional enzyme or whether two enzymes were required.

Later on, Xia and co-workers (2001) purified a ~32 kDa protein from *F. intermedia* which capable in converting (-)-secoisolariciresinol into (-)-matairesinol. They cloned the encoding gene and established the heterologously expressed recombinant SDH in *E. coli* which was able to enantiospecifically convert (-)-secoisolariciresinol into (-)-matairesinol. Subsequently, they isolated also (-)-lactol as an intermediate as well as (-)-matairesinol as the end-product during the recombinant protein assays using (-)-secoisolariciresinol as the substrate in the presence of NAD. This result proved that (-)-matairesinol formation utilized an enantiospecific bifunctional NAD-dependent secoisolariciresinol reductase (SDH) and not two distinct enzymes.

The same steps have been confirmed to occur in *L. flavum* (Xia, *et al.*, 2000). The dehydrogenation which is NADP-dependent is stereospecific as well and leads to an accumulation of (-)-matairesinol. Matairesinol is regarded as a key intermediate leading to two separate groups of lignans, those with a 3', 4', 5'-trimethoxy (e.g. podophyllotoxin) and those with a 4'-hydroxy-3',5'-dimethoxy substitution pattern (e.g. α-peltatin) in the pendant ring (Kamil and Dewick, 1986).

The occurence of different lignan stereoisomeres increases the diversity of lignans in the plant kingdom. Up to now, the enantiomeric purity in lignan biosynthesis is determined within these first steps, but on different levels. In cell cultures of *Linum album* and *Linum usitatissimum,* pinoresinol accumulates as a mixture of both enantiomers. Secoisolariciresinol (Fig. 1.4) is the first enantiomeric pure lignan, in its (+)-form in *Linum usitatissimum* and in its (-)-form in *Linum album* (von Heimendahl, *et al.*, 2005). On the other side the enantiomeric purity within the family of Thymelaeaceae is not reached before matairesinol (Umezawa, *et al*., 1997).

1.3.2. Stereochemistry of lignans

Most natural lignans or neolignans are optically active. In order to indicate the absolute or relative configuration, and to readily compare related structures, it is convenient to use a standard orientation of the molecule (Moss, 2000). Stereochemistry associated with a ring system which includes position 8 is shown by means of α (indicating below the plane) or ß (indicating above the plane) for a bridgehead hydrogen (or substituent) or for a substituent attached to the ring system. As an example, in podophyllotoxin (Fig. 1.5), it is assumed that absolute configuration is implied by the configuration symbols.

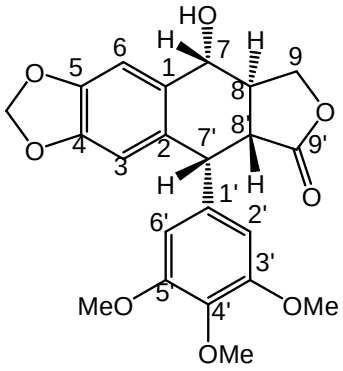


Figure 1.5 The lignan (7α,7'α,8α,8'ß)-7-hydroxy-3',4',5'-trimethoxy-4,5-methylenedioxy-2,7'-cyclolignano-9',9-lactone, trivial name: podophyllotoxin (adapted from Moss, 2000).

If α or ß are not applicable, then R or S should be used. With bridged bicyclical systems the largest ring is considered to be the plane, and the stereochemistry of the shortest bridge is indicated by α or ß. If there are two bridges of equal length it is recommended to use R or S for each bridgehead configuration. Also, the stereochemistry due to substituents on a bridge should be indicated by R or S.

Optical isomerism is an example of stereoisomerism. It occurs when substances have the same molecular and structural formula, but one cannot be superimposed into the other. Optical isomers can occur when there is an asymmetric carbon atom. An asymmetric carbon atom is one which is bonded to four different groups. The groups can be something hideously complex, or something comfortably simple likes a hydrogen or chlorine atom. They can not be interconverted without breaking bonds and have mostly identical physical properties and can often only be told apart by what we call “optical activity”. This is the observation that in a pair of enantiomers one will rotate plane polarized light clockwise, and the other an equal amount anticlockwise. The type of this isomerism can be found in thalidomide (Fig. 1.6).

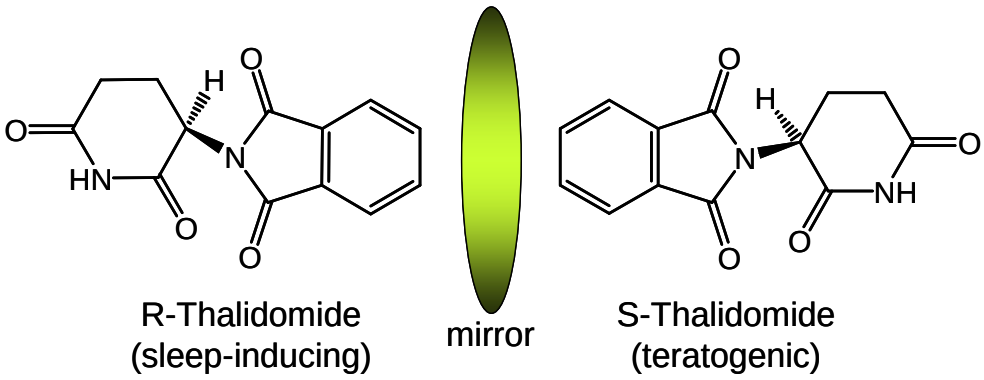


Figure 1.6 Stereoisomer of thalidomide

Thalidomide was distinguished by its highly sedative, sleep-inducing effect. It appeared to be particularly well tolerated and was not habit-forming. Despite all these advantages distinguishing it from other sleeping pills, it led to a tragedy that was unpredictable at that time. Administration of this sleeping pill caused deformities in newborn babies (Grunenthal, 2007). Laboratory tests after the thalidomide disaster showed that in some animals the S enantiomer was teratogenic but the R isomer was an effective sedative. It is now known that even when a stereo selective sample of thalidomide (only one of the optical isomers) is created, if administered pH in the body,

can cause racemizing. It means that both enantiomers are formed in a roughly equal mix in the blood. So, even if in this case a drug of only the 'R' isomer had been created, the disaster would not have been averted.

1.3.3. Functions of lignans

The physiological role of lignans is either the defense against various pathogens or they are antioxidants in flowers, seeds, seed coats, stems, nuts, bark, leaves, and roots. In Chinese traditional medicine lignans are used for treatment of viral hepatitis and protection of the liver.

Beside the ecological and toxic effects of lignans concerning their ability to interfere mitosis, there is an increasing interest in plant-derived and non-toxic lignans such as matairesinol, secoisolariciresinol and pinoresinol, which are found in e.g. *Linum usitatissimum L*. (Meagher, *et al.*, 1999).

Secoisolariciresinol and matairesinol are common constituents of various plants, including *Forsythia intermedia*, flax, certain vegetables, and grains (e.g., green beans and rye). These lignans have important nutritional functions in health protection. During digestion, intestinal bacteria convert secoisolariciresinol and matairesinol to enterodiol and enterolactone, respectively (Setchell, *et al.*, 1980; Axelson, *et al.*, 1982; Borriello, *et al.*, 1985; Ingram, *et al.*, 1997). The mammalian lignans enterolactone and enterodiol have some structural similarity to endogenous estrogens and therefore are included in the family of phytoestrogens (i.e. estrogenic compounds of plant origin). These “mammalian” lignans undergo enterohepatic circulation, in which they are conjugated in the liver, excreted in the bile, deconjugated in the intestine by bacterial enzymes, absorbed across the intestinal mucosa, and returned to the liver in the portal circulation (Croteau, *et al.*, 2002).

Enterolactone can be measured in human serum. Vanharanta and co-workers (2002) described that high serum enterolactone concentrations are associated with the decrease of *in vivo* lipid peroxidation (measured as plasma F2-isoprostane concentration) in men. Due to the biphenolic structure of enterolactone, it could act as an antioxidant and through this contribute to cardiovascular health.

There are published reports of an inverse relationship between dietary lignans such as matairesinol and breast cancer incidence (Ingram, *et al.*, 1997). Furthermore, Bylund and co-workers (2005) suggest that dietary hydroxymatairesinol inhibits the growth of the human prostate cancer xenografts in athymic male mice.

One of the famous naturally occuring aryltetralin lactone lignans is podophyllotoxin (Fig. 1.5). Since the discovery of its potent cytotoxicity, podophyllotoxin received much attention in the field of natural product chemistry. In order to avoid several side-effects of podophyllotoxin, like unacceptable gastrointestinal toxicity, modifications of the basic podophyllotoxin skeleton resulted in the clinical introduction. Derivatization produces the potent and widely used anticancer drugs etoposide, teniposide and etopophos®. These semisynthetic and clinically used derivatives show a completely different mechanism of cytotoxicity than the podophyllotoxin itself.

Podophyllotoxin inhibits the continuation of the mitosis by binding the microtubules and causing the arrest of mitosis in metaphase, the pharmaceutical drugs etoposide, teniposide and etopophos® are DNA-topoisomerase II inhibitors. Topoisomerase II is an enzyme that opens the double-stranded DNA and seals it again after unwinding. Topoisomerase II is necessary in the processes of DNA replication and repair. Etoposide and the other derivatives of podophyllotoxin, however, stabilize the complex between the DNA and the topoisomerase in such a way that connection of the two DNA strands is impossible. During the mitosis, they are more sensitive for this mechanism.

A total chemical synthesis of podophyllotoxin is not commercially practical due to the presence of four chiral centers and a high degree of oxygenation of this compound. Therefore, the preferred source of podophyllotoxin nowadays is the herbaceous plant *Podophyllum hexandrum* which is common in the subalpine forests of the Himalayan mountains. However, due to the intensive and uncontrolled harvesting of the wild grown plants led this plant to the status of an endangered species (Forster, 1993).

The North American species *P. peltatum* also shows podophyllotoxin production and therefore has emerged as an alternative. Recent studies reported that in addition to the rhizomes, the leaf blades of *P. peltatum* store lignans as glucopyranosides (Moraes, *et al.*, 1998). Since leaves are renewable organs and *P. peltatum* grows in large colonies in eastern North America, the species became more attractive for further research. However, *P. peltatum* contains lower amounts of podophyllotoxin than *P. hexandrum.* Podophyllotoxin has also been reported to exist in other plant families, such as Cupressaceae (Hartwell, *et al.*, 1953), Polygalaceae (Hoffmann, *et al.*, 1977) and Linaceae (Weiss, *et al.*, 1975).

1.4. Pinoresinol-lariciresinol reductase

Pinoresinol-lariciresinol reductase is an enzyme which reduces pinoresinol to give lariciresinol and also reduces lariciresinol into secoisolariciresinol. This bifunctional pinoresinol-lariciresinol reductase (PLR) was unambiguously demonstrated by purification (~3,000-fold) of two *F. intermedia* PLR isoforms to apparent homogeneity, with each (~34.9 kDa monomer) being subjected to detailed kinetic analysis and giving essentially the same catalytic properties, i.e., K_m for (+)-pinoresinol, 27 $\pm$ 1.5 and 23 $\pm$ 1.3μM; K_m for (+)-lariciresinol 121 $\pm$ 5.0 and 123 + 6.0 μM; V_{max} (+)-pinoresinol, 16.2 $\pm$ 0.4 and 17.3 + 0.5 μmol h^{-1} mg^{-1} protein and for (+)-lariciresinol, 25.2 $\pm$ 0.7 and 29.9 $\pm$ 0.7 μmol h^{-1} mg^{-1} protein, respectively (Dinkova-Kostova *et al*., 1996). Isolation of a cDNA encoding PLR from *F. intermedia* (PLR-Fi1) and its heterologous expression showed the same enantiospecificity as in crude extracts (Dinkova-Kostova *et al*., 1996).

Katayama and co-workers (1992) had demonstated that when the (+)- and (-)-enantiomers of pinoresinol were individually incubated with the *F. intermedia* cell-free extracts, in the presence of NADPH, preferential conversion was observed into (+)-lariciresinol. Incubation with ($\pm$)-lariciresinols also established that only the (+)-antipode was converted into (-)-secoisolariciresinol (Katayama, *et al.*, 1993). These data revealed the existence of a bifunctional enantiospecific pinoresinol-lariciresinol reductase in the readily soluble protein extract of *F. intermedia* which utilized (+)-pinoresinol and (+)-lariciresinol. Furthermore, the reduction of (+)-pinoresinol into (+)-lariciresinol and then into (-)-secoisolariciresinol was also demonstrated to occur via abstraction of the 4-pro-R hydrogen from NADPH by using either specifically labeled 4*R*- and 4*S*-^{3}H/^{2}H-NADPH (Chu, *et al*., 1993; Dinkova-Kostova, *et al*., 1996).

The presence of two distinct classes of PLRs in *Thuja plicata* has been shown by Fujita and co-workers (1999). Recombinant PLR-Tp1, a PLR from the first class catalyses the reduction of (-)-pinoresinol into (+)-secoisolariciresinol, whereas PLR-Tp2 a PLR from the second class catalyses the formation of (-)-secoisolariciresinol, analogous to the reactions performed by PLR-Fi1.

The same enantiospecificity as for PLR-Fi1 is reported by von Heimendahl and co-workers (2005) for a recombinant PLR from a cell suspension culture of *Linum album* (PLR-La1). It reduces (+)-pinoresinol into (-)-secoisolariciresinol via (+)-lariciresinol. Moreover, von Heimendahl and co-workers (2005) cloned a cDNA encoding a PLR from a cell suspension culture of *L. usitatissimum* (PLR-Lu1). The recombinant protein converts (-)-pinoresinol into (+)-secoisolariciresinol.

Hemmati and co-workers (2007) cloned a PLR from a cell suspension culture of *L. perenne* Himmelszelt. The recombinant PLR-Lp1 completely used up the racemic pinoresinol before the resulting lariciresinol was completely converted into secoisolariciresinol. Therefore, PLR-Lp1 seems to work more like a pinoresinol reductase than a PLR. This recombinant protein shows preference for (+)-pinoresinol (R,R configuration at C-atoms 8,8') in the first reaction step, but preference for (-)-lariciresinol (S,S configuration at C-atoms 8,8') in the second reaction step.

1.5. *Phaleria macrocarpa* (Scheff.) Boerl.

Phaleria macrocarpa (Scheff.) Boerl. (Fig. 1.7) is a shrub or small tree up to five meters (Winarto, 2004; Harmanto, 2005) but its height could also reach up to 18 meters (Hou, 1960). It features a many branched crown, a large straight root (one meter long) exuding sap, a brownish green bark and white wood. It has green tapering leaves, 7-10 cm long and 3-5 cm wide. Its flowers form a compound of 2-4 flowers, little white trumpet-like fragrant flowers. The fruit occurs in various sizes, changing color from green to maroon. The pit is round, white and very poisonous. It grows in areas of 10 – 1,200 m above sea level, and best in areas of 1.000 m above sea level with its productive age is between 10-20 years.

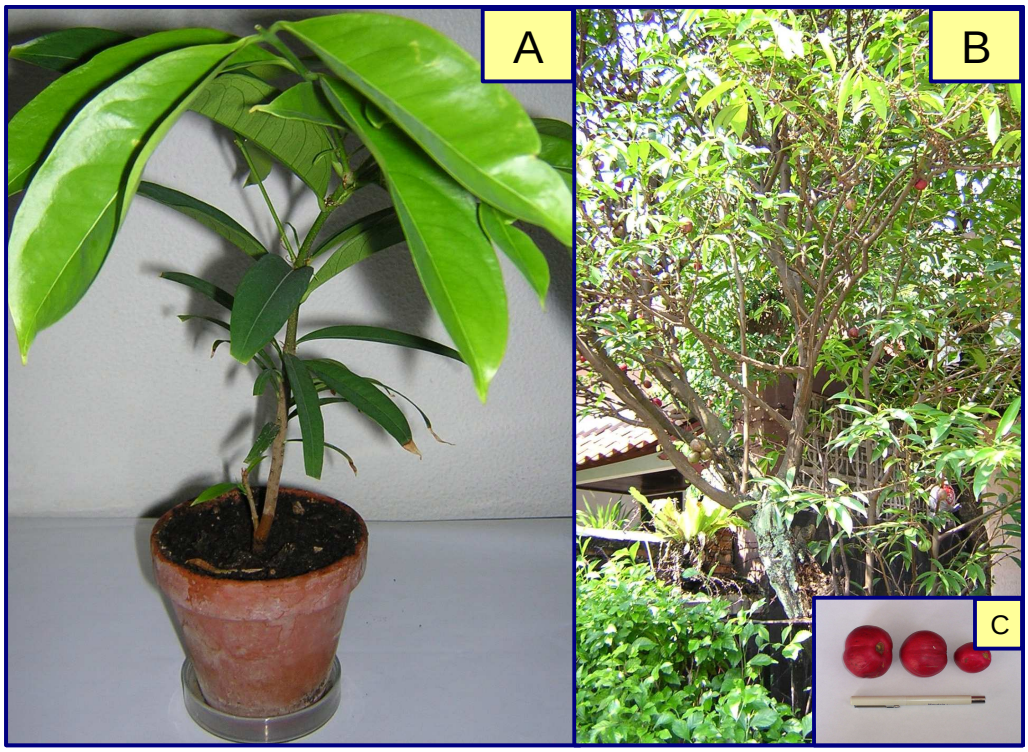


Figure 1.7. *Phaleria macrocarpa* (Scheff.) Boerl. (**A**=young plant; **B**=tree; **C**=fruits).

According to the Angiosperm Phylogeny Group (APG, 2003), *P. macrocarpa* (Scheff.) Boerl. is a member of the Superkingdom Eukaryota, Kingdom Viridiplantae, Phylum Streptophyta, Class Eudicotyledons, Subclass Eurosids II, Order Malvales, Family of Thymelaeaceae (Juss), and Genus *Phaleria.*

Phaleria macrocarpa (Fig. 1.7) was first described as *Drimyspermum macrocarpum* by Scheffer based on fruiting specimens collected by Teysmann (with the herbarium collection #7786, L, Bo) near Doré, in western New Guinea. These young fruits are ellipsoid or slightly obovoid (15 by 12 mm) and narrowed stipe-like towards the base. This shape is not uncommon and can also be observed in other herbarium collections e.g. *bb25746, BW 5468* from Hollandia and *NGF 7298* from Morobe distric. The vegetative and morphological characters of the type agree with those of *P. calantha* and *P. wichmannii.* This plant is found in primary and secondary forests, from the lowland up to 550 m in Malaysia and in western New Guinea (Hou, 1960).

For centuries, the people of Indonesia have used the fruit and leaves of the tree Mahkota Dewa (literally God's Crown), *Phaleria macrocarpa* (Scheff.) Boerl., to counter diabetes, liver diseases, vascular problems, cancer, and high blood pressure. Recent research proof that *P. macrocarpa* contains plant secondary metabolites that combat not only cancers and fevers such as malaria, but also the so called life-style diseases (Harmanto, 2005). Parts of *P. macrocarpa* that are used for medicinal treatment are the stem, leaves and fruit. Empirically, *P. macrocarpa* proved capable of controlling cancer, impotency, dysentery, hemorrhoid, diabetes mellitus, allergies, liver and heart diseases, kidney disorders, blood diseases, arthritis, rheumatism, high blood pressure, stroke, migraine, various skin diseases, acne and the level of cholesterol. It is also capable of controlling disorders in animals. This plant contains antihistamine, antioxidant and anti-cancer substances (Harmanto, 2005).

Kusmardiyani and co-workers (2004) isolated a white yellowish and odorless crystalline compound from the ethanolic extract of *P. macrocarpa* leaves. The isolate was presumed as a benzophenone glucoside based on its ultraviolet, infrared, mass, ^{1}H and ^{13}C NMR spectra. Another benzophenoic glucoside known as 4,5-dihydroxy,4'-methoxybenzophenone-3-O-ß-D-glucoside or Phalerin (Fig. 1.8A) was also isolated from methanolic extract of the leaves of *P. macrocarpa*. Phalerin was cytotoxic to myeloma cell line (NS-1) having IC50 of 83µg/ml or $1.9x10^{-1}$ mM (Mae, *et al.*, 2005).

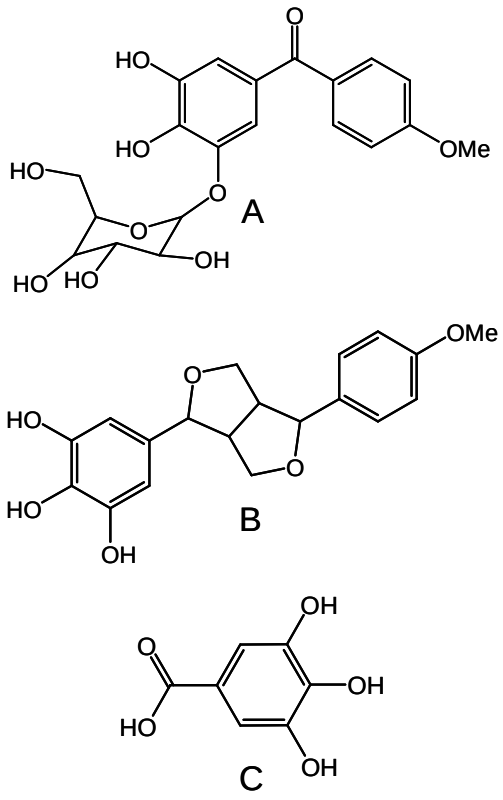


Figure 1.8. The isolated compounds from P. macrocarpa (**A**=Phalerin; **B**=5-[4(4-methoxy-phenyl)-tetrahydrofuro[3,4-c] furan-1-yl]-benzene-1,2,3-triol; **C**=Gallic acid).

A lignan similar to syringaresinol was isolated by Lisdawati (2002) from the ethyl acetate fraction of *P. macrocarpa*‘s mesocarp. The isolate was elucidated by using ultraviolet-visible (UV-Vis) spectra data, Fourier transform infra red (FTIR) spectrometric, liquid chromatography-mass spectrometry (LC-MS), and the proton nuclear magnetic resonance spectral data ^{1}H-RMI and combination of 2D ^{1}H,^{1}H-COSY, TOCSY and NOESY RMI. The spectral evidence show molecule structures of the isolate was $C_{19}H_{20}O_6$: *5-[4(4-Methoxy-phenyl)-tetrahydrofuro[3,4-c] furan-1-yl]-benzene-1,2,3-triol* (Fig. 1.8B). Moreover, *P. macrocarpa* also contains gallic acid (Fig. 1.8C) which can show a significant inhibition of cell proliferation in a series of cancer cells and induction of apoptosis in esophageal cancer cell but not in non-cancerous cell (Faried, *et al.*, 2006).

1.6. The genus *Linum*

An aryltetralin lignan, podophyllotoxin (Fig. 1.5), has been isolated from herbaceous perennial *Podophyllum* species and has long been reputed to have anti tumor activity. This lignan is exploited commercially as a source of a semisynthetic anticancer drug, etoposide, which is being applied successfully in cancer chemotherapy (Canel, *et al.*, 2000). However, some *Podophyllum* species are endangered due to over-collection. Further, much attention has been focused on the availability of podophyllotoxin in various plants. Some *Linum* species especially those belonging to the section Syllinum including *Linum flavum* and *Linum album* have been known to produce podophyllotoxin and related lignans. In addition, suspension, root, and hairy root cultures of *Linum* species produce significant amounts of 6-methoxypodophyllotoxin (in former time also called 5-methoxypodophyllotoxin) and related lignans. Thus, *Linum* plants are attractive as plant systems for elucidating the biosynthetic mechanisms of podophyllotoxin congeners (Mikame, *et al*., 2002).

The genus *Linum* consists of about 300 species and is therefore probably the greatest genus containing species from which one can isolate lignans. The taxonomy of this genus was based only on morphological data. This genus has received considerable attention from botanists during the last three centuries. The wide range of diversity within the genus continues to challenge its systematic treatment by botanists. Several proposals exist for dividing the genus into sections, and the status of many species remains to be clarified.

Within the genus *Linum*, the chromosome number varies between 2n = 16 to 2n = 80, with most of the species having either 2n = 18 or 2n = 30 chromosomes. However, the phylogenetic relationships within the genus are not yet clarified and all proposed systems are artificial. The first proposal for a natural system for arranging the species was published in 1837 by Reichenbach. A thorough overview of the genus was presented by Winkler in 1931 (Muir and Westcott, 2003).

According to the Angiosperm Phylogeny Group (APG, 2003), the genus *Linum* is a member of the Superkingdom Eukaryota, Kingdom Viridiplantae, Phylum Streptophyta, Class Eudicotyledons, Subclass Eurosids I, Order Malpighiales, Family of Linaceae (flax) and Genus *Linum.* Within the flax family, the genus *Linum* belongs to the tribe Linoideae H. Winkl. There are four other genera within this Linoideae tribe: (1) *Reinwardtia* (Dumort) occurring in India and South-East Asia; (2) *Tirpitzia* (Hallier) occurring in South China; (3) *Hesperolinon* (A. Gray) occurring in the western USA; and (4) *Radiola* (Dillen.) Roth. occurring in Europe and the USA.

Several of the *Linum* species are shrubs and occur in tropical areas, while perennial and annual species are found in temperate areas of the world. Three geographical centers of distribution are known: (1) the Mediterranean area; (2) southern North America and all of Mexico; and (3) South America. However, several other species also occur in the temperate areas of Europe, Asia and the Americas (Hickey, 1988).

Although some botanists had different ways of classification, Winkler's proposal for grouping still appears to be most useful, because its structure is clear and based on traits which are suitable for taxonomic distinctions. Chemotaxonomic investigations of the fatty oil composition in the seeds support this morphological grouping. The genus *Linum* can be subdivided into the following six sections namely Linum, Dasylinum, Linastrum, Cathartolinum, Syllinum, and Cliococca.

Linum usitatissimum L. belongs to the section Linum. Some characteristics of section Linum: Flowers are usually large; fruiting pedicels are elongate; petals are free with blue, pink, or white color; sepals are without obvious longitudinal veins; stigmas are longer than wide, clavate or linear. Leaves are alternate without stipular glands and glabrous. This section could be perennials, biennials or annual.

Morphological characteristics of section Syllinum are quite similar to section Linum, but petals are connected before opening of the flowers. The colors of petals are yellow or white. Stem with wings decurrent from leaf bases. Leaves have glands at their base. Perennial species of this section are distributed in the eastern Mediterranean area. An important species of this section is *Linum flavum* L. (Fig. 1.9). which has bright yellow flowers and is widely cultivated as an ornamental plant (Muir and Westcott, 2003).

The group of Kadereit (University of Mainz) developed recently a phylogenetic tree based on molecular biological data (ITS sequences). Recently, only about 29 species of about 300 of the genus *Linum* have been investigated concerning their lignan content. That means only parts of the phylogenetic tree were investigated up to date (McDill, *et al*., 2005).

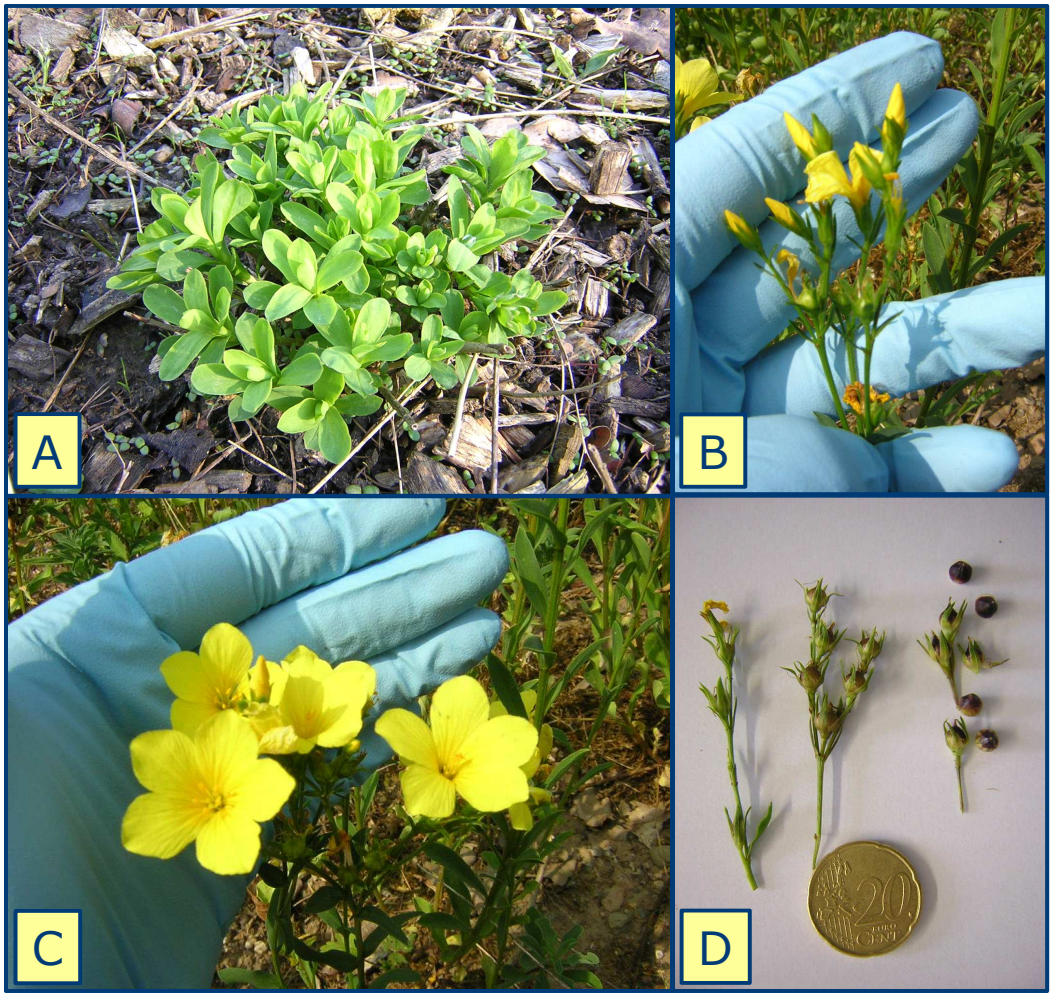


Figure 1.9. *Linum flavum* var. *compactum* L. (**A**=young plant, **B**=flowerbuds; **C**=flowers; **D**=capsules).

1.7. Plant cell cultures

According to a modern definition, plant cell culture is described as the culture of cells of higher plants by the proliferation of organ fragments or the explants and maintained them in a disorganized state or "callus", by means of growth factors. It is referred as a callus culture if the culture is made on a solid agar medium and it is referred as a suspension culture if it is made in liquid medium (Pe' and Selva, 1996).

Cell suspension cultures consist of small aggregates of cells which are dispersed in a stirred liquid medium and are mainly used for plant cell line selection and for the production of secondary metabolites. This type of culture is obtained by placing small fragments of callus tissue in stirred liquid medium. The callus used as inoculum for a liquid culture must be composed by cells in active division and must be fragile, in order to allow a better dispersal of cells in the medium (Pe' and Selva, 1996).

For many years the production of fine chemicals and drugs with *in vitro* cultured plant cell systems has been a subject of study. Plant cell culture provides several advantages over whole-plant cultivation for the production of secondary metabolites and their biosynthetic studies.

Chawla (2003) described that the development of plant cell suspension culture as an alternative source of secondary metabolites has been encouraged by a number of factors, including: (a) independence from various environmental factors including climate, pests and microbial diseases, geographical and seasonal constrains, (b) any cell of a plant could be multiplied in order to yield specific metabolites, (c) a consistent product quality could be assured with the use of characterized cell lines, (d) cell growth could be automatically controlled and metabolic processes could be regulated rationally, all contributing to the improvement of productivity and the reduction of labour and costs, (e) production of substances in chemically-controlled environment facilitates subsequent processing and product recovery steps, (f) culture of cells may prove suitable in cases where plants are difficult or expensive to grow in the field due to their long life cycles, e.g. *Papaver bracteatum*; the source of the baine takes two to three seasons to reach maturity, (g) new routes of synthesis can be recovered from mutant cell lines, and (h) some cell cultures have the capacity for biotransformation of specific substrates to more valuable products by means of single or multiple step enzyme activity.

Currently, only a few plant metabolites have been produced via cell cultures on industrial scale e.g. paclitaxel. The yield of paclitaxel was up to 74 mg/l in a 500-litre bioreactor (Son, *et al.*, 2000). The production of paclitaxel has been scaled up and presently bioreactors of up to 70,000 liters are being successfully employed by Phyton Biotech to supply an important part of the yearly demand of Bristol-Myers-Squibb for paclitaxel (Wink, *et al.*, 2005).

Despite of the advantages, there are still many problems, including insufficient knowledge of biosynthetic routes and the low yield of the expected secondary metabolites in cell cultures in comparison to naturally growing plants. The reason for the lower concentrations might be due to the connection between secondary metabolites of plants and the differentiation process of organs like roots, stems, and leaves (Phyton, 2002).

1.8. Objectives of the research

Lewis and coworkers were the first scientists who investigated the first steps of lignan biosynthesis on a biochemical as well as on a molecular biological level (Davin and Lewis, 2003). Recently, it seems that the enantiomeric purity in lignan biosynthesis is determined within these first steps, but on different levels. The coupling of two coniferyl alcohol monomers in *Forsythia* x *intermedia* leads to enantiomeric pure (+)-pinoresinol (Davin, *et al.*, 1990), whereas in cell cultures of *Linum album* and *Linum usitatissimum* pinoresinol accumulates as a mixture of both enantiomers. In cell cultures of *Linum* species, secoisolariciresinol is the first enantiomeric pure lignan, in its (+)-form in *Linum usitatissimum* and in its (-)-form in *Linum album* (von Heimendahl, *et al.*, 2005).

In contrast to the formation of (-)-matairesinol in *Forsythia* species, the opposite lignan enantiomers can be found in other species, especially of the Thymeleaceae familiy (Umezawa, 2003). The enantiomeric purity in most species of the Thymelaeaceae is not reached before matairesinol (Umezawa, *et al.*, 1997). Since *Phaleria macrocarpa* (Scheff.) Boerl. is a member of Thymelaeaceae and still little is known about the lignans in this plant, it is interesting to investigate the occurrence of lignans and their chiral composition from different organs as well as from *in vitro* culture of this plant species. Moreover, a cDNA encoding the enzyme pinoresinol–lariciresinol reductase should be cloned in *E. coli* as a starting point to understand the biosynthetic pathways of lignans on the molecular level.

The genus *Linum* is the greatest genus containing species from which one can isolate lignans and many species of this genus are easy to cultivate in our botanical garden. Studies conducted by Alfermann and co-workers showed that lignans of all structural types shown in Fig 1.3 could be isolated from different species of the genus *Linum* (Fuss, 2003). Moreover, six known lignans namely 6-methoxypodophyllotoxin, α-peltatin, ß-peltatin, pinoresinol, lariciresinol, secoisolariciresinol were isolated from *Linum flavum* var. *compactum* by Mikame and co-workers (2002). In order to have a better view about the lignan formation in this plan, it is also necessary to investigate the occurrence of different lignans in different organs of *Linum flavum* var. *compactum* L. during different developmental stages.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

Plant materials of *Phaleria macrocarpa* (Scheff.) Boerl. were collected on January and July 2006 from "Pembibitan Ibu Yati", a local nursery in Mataram city, Indonesia. The plant was about three years old. Those materials were dried in the shadow.

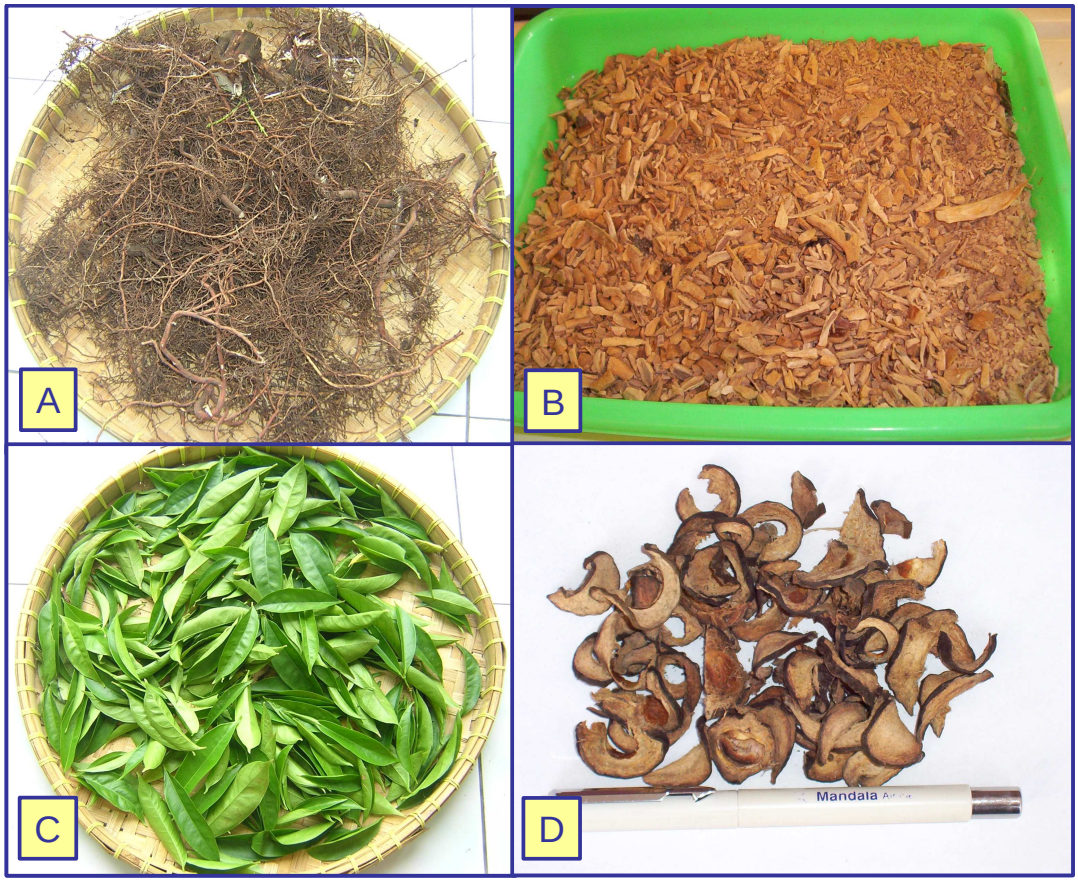


Figure 2.1. Plant materials of *P. macrocarpa* (**A**= root; **B**=wood; **C**=leaves; **D**=fruit)

The other plant materials consist of *Linum flavum* var. *compactum*. The seeds of *Linum flavum* var. *compactum* were purchased from Jelitto Staudensamen GmbH Schwarmstedt, Germany. They were germinated on June 17th, 2003 and planted in the botanical garden of Heinrich-Heine-Universität Düsseldorf on April 1st, 2004.

The plant materials were harvested during May - July 2005 and May - July 2006 (Tab. 2.1). The plant organs were root, stem, and leaves (vegetative organs) as well as flower-buds, flower, and capsule (generative organs). The vegetative organs were divided into three different developmental phases namely the phase before the formation of flower or growth phase (GP), flowering phase (FP), and the phase during the formation of capsules (CP). Meanwhile, the generative organs were divided into five different developmental phases namely flower buds (FB), flower (F), capsules in early phase (CE), capsules in middle phase (CM), and capsules in late phase (CL) (Fig. 2.2).

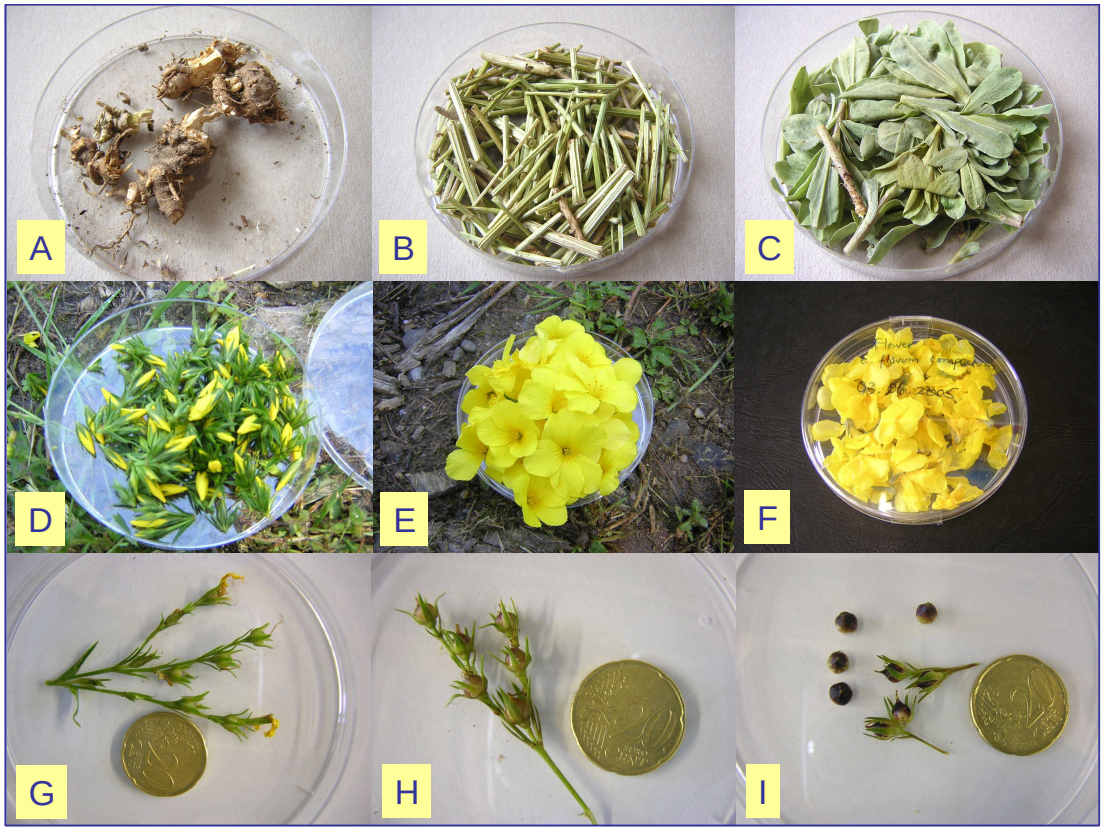


Figure 2.2. *Linum flavum* var *compactum* L. (**A**=dried roots; **B**= dried stem; **C**=dried leaves; **D**=fresh harvested flowerbuds; **E**= fresh harvested flowers; **F**=dried flowers; **G**= capsul "early" stage; **H**=capsule "middle" stage; **I**= capsule "late" stage).

Table 2.1. Date of harvesting of different organs of *L. flavum* var. *compactum* L. in different developmental phases

No.	Plant material	harvested in	
		2005	2006
1.	Root during growth	-	29.05.2006
2.	Stem during growth	12.05.2005	29.05.2006
3.	Leaves during growth	12.05.2005	29.05.2006
4.	Root during flowering	-	26.06.2006
5.	Stem during flowering	03.06.2005	26.06.2006
6.	Leaves during flowering	03.06.2005	26.06.2006
7.	Root during capsuling	-	18.07.2006
8.	Stem during capsuling	17.06.2005	18.07.2006
9.	Leaves during capsuling	17.06.2005	18.07.2006
10.	Flowerbuds	03.06.2005	12.06.2006
11.	Flower	03.06.2005	26.06.2006
12.	Capsul “early” stage	17.06.2005	05.07.2006
13.	Capsul “middle” stage	23.06.2005	18.07.2006
14.	Capsul “late” stage	-	23.07.2006

2.1.2. Bacteria

2.1.2.1. *E. coli* DH5α

E. coli DH5α is a host for Blue/White screening by utilizing the activity of β-galactosidase. As this strain does not carry *lacI*^{q}, basically IPTG is not needed. Therefore, DH5α allows easy selection of recombinant DNA with X-Gal when constructing gene library or subcloning recombinant plasmid. The genotype is: F^{-}, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk^{-}, mk^{+}), *phoA*, *supE*44, λ^{-}, *thi*-1, *gyrA*96, *relA*1.

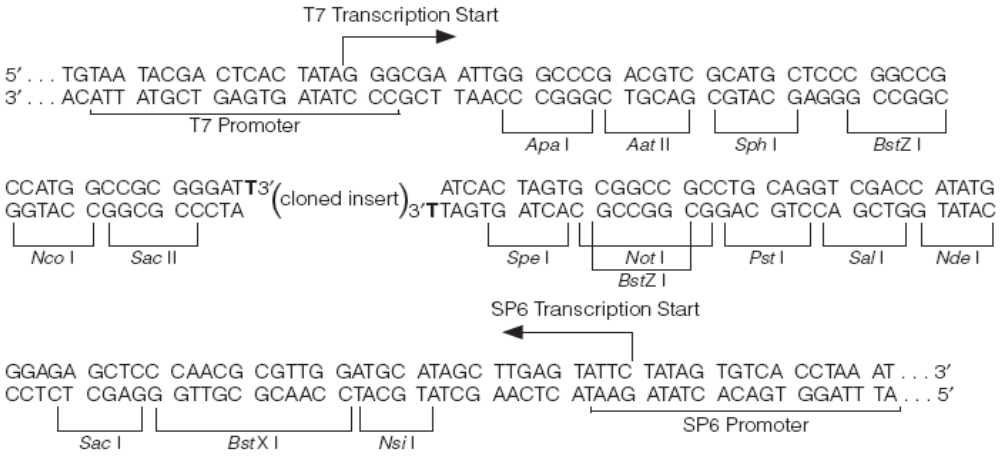
2.1.2.2. *E. coli* Rosetta™ 2(DE3)

The Rosetta™ 2(DE3) strain is designed to alleviate codon bias when expressing heterologous proteins in *E. coli*. When the mRNA of heterologous genes is overexpressed in *E. coli*, differences in codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the population. It has been well established that insufficient tRNA pools can lead to translational stalling, premature translation termination, translation frameshifting, and amino acid misincorporation. The genotype of *E. coli* Rosetta™ 2(DE3) is: F^{-} *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3).

2.1.3. Plasmids

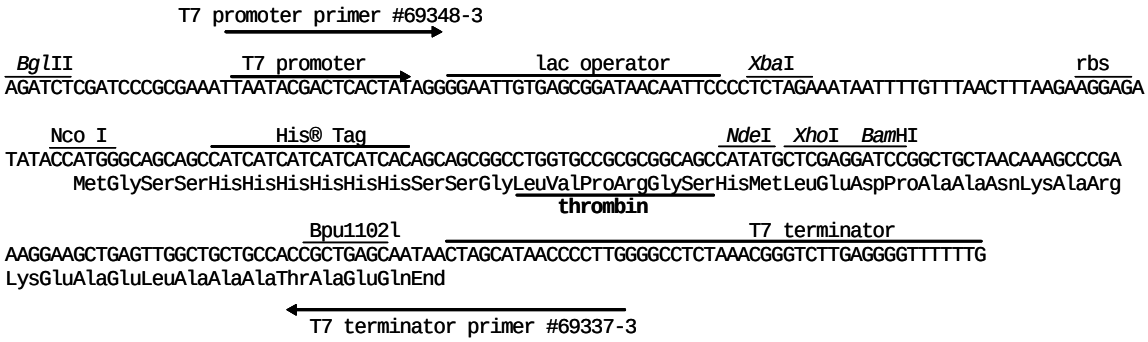
2.1.3.1. The pGEM®-T Vector

The pGEM®-T is a system for the cloning of PCR products. The high-copy-number pGEM®-T contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme ß-galactosidase. The multiple cloning sequences of pGEM®-T are as follow:



2.1.3.2. The pET-15b Vector

The pET-15b vector carries an N-terminal His•Tag® sequence followed by a thrombin site and three cloning sites. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.



2.1.4. Solvents and chemicals

All solvents and chemicals were of reagent or HPLC grade unless otherwise specified. The following material was purchased from the following companies:

Acetone	Mallinckrodt Baker B.V., Deventer, Holland
Acetonitrile, HPLC grade	Fisher Scientific, Loughborough, UK
Ammonium nitrate	Merck KgaA, Darmstadt
Bovine serum albumin	SERVA GmbH & Co., Heidelberg
Calcium chloride dihydrate	Merck KgaA, Darmstadt
Chloroform	Mallinckrodt Baker B.V., Deventer, Holland
Cobalt(II) chloride	Ferak, Berlin
Coniferyl alcohol	Fluka Chemie GmbH, Buchs, Switzerland
Coomassie Brilliant Blue G-250	Merck KgaA, Darmstadt
Diethyl ether	KnF Laborchemie Handels GmbH, Lohmar
DTT	Carl Roth GmbH, Karlsruhe
Dipotassium hydrogen phosphate	Riedel-de Haën, Seelze
Ethanol, HPLC grade	Riedel-de Haën, Seelze
Ethanol p.a	Merck KgaA, Darmstadt
Ethyl acetate	Fisher Scientific, Loughborough, UK
β-glucosidase	SERVA GmbH & Co., Heidelberg
glycine	Carl Roth GmbH, Karlsruhe
n-hexane	Merck KgaA, Darmstadt
hydrochloric acid (36-38 %)	Mallinckrodt Baker B.V., Deventer, Holland
Hydroxymatairesinol (100 %)	given by Prof. Sjöholm, Abo-Academy Finland
Iron(III) chloride hexahydrate	Merck KgaA, Darmstadt
Iron(III) sulfate heptahydrate	Merck KgaA, Darmstadt
Lariciresinol	given by Prof. Lewis, Washington State University
Magnesium sulfate heptahydrate	Merck KgaA, Darmstadt

Manganese (II) sulphate-1-hydrate	Riedel-de Haën, Seelze
Matairesinol (98 %)	given by Prof. Sjöholm, Abo-Academy Finland
Methylene chloride	Fluka Chemie GmbH, Buchs, Switzerland
Methanol	Merck KgaA, Darmstadt
6MPTOX	given by Prof. van Uden, University of Groningen
myo-inositol	Merck KgaA, Darmstadt
NAA	Merck KgaA, Darmstadt
$NADP^+$	Biomol, Hamburg
NADPH	Biomol, Hamburg
Nicotinic acid	Merck KgaA, Darmstadt
α-peltatin	McChesney, University of Mississippi
β-peltatin	McChesney, University of Mississippi
Phosphoric acid	Janssen Chimica, Beerse, Belgium
Polyclar AT	Carl Roth GmbH, Karlsruhe
Potassium dihydrogen phosphate	Mallinckrodt Baker B.V., Deventer, Holland
Potassium hydroxide (pellets)	Merck KgaA, Darmstadt
Potassium iodide	Merck KgaA, Darmstadt
Potassium nitrate	Merck KgaA, Darmstadt
Podophyllotoxin	Carl Roth GmbH, Karlsruhe
Secoisolariciresinol	given by Prof. Metzler, University of Karlsruhe
Silica gel	Merck KgaA, Darmstadt
Sodium molybdate-2-hydrate	Ferak, Berlin
Sodium sulfate	Grüssing GmbH, Filsum
Sucrose	Kölner Raffinade Zucker, Cologne
Thiamine chloride hydrochloride	Merck KgaA, Darmstadt
Titriplex III (EDTA)	Merck KgaA, Darmstadt
Tris (TRIZMA BASE)	Sigma-Aldrich Chemie GmbH, Steinheim
Zincsulphate heptahydrate	Janssen Chimica, Beerse, Belgium

2.1.5. Buffers, reagens, and media

Buffer / Medium	Components	Concentration
Bradford reagent	Coomassie®Brillantblue G250 Ethanol p.A. o-Phosphoric acid	100 mg/l 50 ml/l 100 ml/l
Kalium-phosphate (KPi) buffer	KH_2PO_4 K_2HPO_4 pH 7.1	100 mM 100 mM
Wash buffer (for His_6-Tag protein purification)	NaH_2PO_4 NaCl Imidazol pH 8.0	50 mM 300 mM 16 mM
Extraction buffer (for His_6-Tag protein purification)	Imidazol Tris NaCl β-Mercaptoethanol pH 7.1	5 mM 20 mM 100 mM 0.25 mM
Elution buffer (for His_6-Tag protein purification)	NaH_2PO_4 NaCl Imidazol pH 8.0	50 mM 300 mM 250 mM
Tris-EDTA buffer	Tris EDTA DTT pH 7.2	20 mM 2 mM 5 mM
DNA running buffer	Sucrose Bromphenolblue In 1 x TAE	50 % (w/v) 0.25 % (w/v)
50 x TAE buffer	Tris EDTA Acetic acid	2 M 0.25 M 1 M
LB-Medium	NaCl Bacto®-Trypton Yeast extract for solid medium: + Agar pH 7.0	10 g/l 10 g/l 5 g/l 15 % (w/v)
PBS	NaCl KCl Na_2HPO_4 KH_2PO_4	140 mM 2.7 mM 10 mM 1.8 mM
Elution buffer (GST)	Glutathione Tris pH 8.0	10 mM 50 mM

2.1.6. Enzymes

Apa I	10 U/µl, MBI Fermentas
Bam HI	10 U/µl, MBI Fermentas
NdeI	10 U/µl, MBI Fermentas
Pst I	10 U/µl, Roche Mannheim
Xba I	10 U/µl, MBI Fermentas
Xho I	10 U/µl, Boehringer
Taq-/Tgo-DNA Polymerase	3.5 U/µl, Roche Mannheim
T4 DNA Ligase	1 U/µl, Invitrogen LIFE TECHNOLOGIES
Lysozyme	Fluka Chemie GmbH, Buchs, Switzerland
RNase A (Ribonuclease A)	Serva GmbH & Co, Heidelberg

2.2. Instruments

Autoclave	Webeco Typ No. V, Bad Schwartau
Centrifuges	Sorvall RC-5B, Kendro Laborating Products GmbH; Rotor Fiber Lite F21-8x50 Piramoon Technologies Inc.
	Eppendorf table centrifuge 5415C, Hamburg
	Eppendorf Centrifuge 5804 R
	Eppendorf Centrifuge 5415 D
Conductometer	Conductometer LF 91 with battery, KLE 1/T, WTW Weilheim
Freeze dryer	Alpha 1-4, Christ, Osterode
	Vaccumpump: Pfeiffer, Balzers Gruppe, Vaduz

HPLC	Thermoquest, Thermo Seperation® Products, Egelsbach
	equipped with:
	Degaser of solvent
	Injector: Spectra SYSTEM AS 1000
	Detector: Spectra SYSTEM UV 6000LP
	Pump: Spectra SYSTEM P2000
	LDC / Milton Roy, Gelnhausen
HPLC columns	GROM-Sil 120 ODS-5 ST (250 x 4 mm, 5 µm) with precolumn (20 x 4 mm, 5µm), GROM, Herrenberg-Kayh
	Hypersil hypurity ™Elite C18 (250 x 4.6 mm, 5 µm) with precolumn (50 x 4.6 mm, 5 µm)
	CHIRALCEL OD-H (250 x 4.6 mm, 5 µm), DAICEL CHEMICAL INDUSTRIES, LTD
HPLC-MS	HP1100, Agilent
	equipped with:
	MS system: Finnigan LCQ^{Deca}, Thermoquest
	Ion source: ESI and APCI, Thermoquest
	Pump: Edwards 30, BOC
	Injector: G 1313 A ALS 1100, Agilent
	Detector: G 1315 B DAD 1100, Agilent
	Column: Eurospher 100-C18, [5 µm; 227 mm x 2 mm], Knauer
	Program: Xcalibur, version 1.3
pH-electrode	Mettler Toledo, Urdorf, Switzerland
pH-meter	WTW pH 523, Knick, Weilheim
Photospectrometer	Uvikon 930, Kontron Instruments
Refractometer	Krüss, Hamburg
Rotavapor	Rotavapor RE 111, Waterbath 461, Büchi, Switzerland

Shakers	G53, New Brunswick Scientific Co., New Jersey/USA
Speedvac	Univapo 150 H, Martinsried
Ultrasonic bath	Bandelin SONOREX SUPER RK 103 H, Berlin
Ultra Turrax	IKA, Janke & Kunkel, Staufen
Vortex	Vortex Genie 2^{TM} Bender & Habein, Zürich, Switzerland
Water system	E-PURE: Reinstwasseranlage, Wilhelm Werner GmbH, Leverkusen
Waterbaths	Thermomix M, B. Braun, Melsungen
	Thermomix MM, B. Braun, Melsungen
	Shakingwaterbath, Julabo SW 2, Seelbach

2.3. Methods

2.3.1. Initiation of *in vitro* cultures of *Phaleria macrocarpa*

Sterile seedlings of *Phaleria macrocarpa* (Scheff.) Boerl. were initiated by seed propagation on a 10% modified MS medium (Murashige and Skoog, 1962) without phytohormones (Table 2.1). Seeds were sterilized using ethanol (96%) for one minute and sodium hypochlorite (1%) for 9 minute, and then rinsed three times with sterile distilled water. The initial tissue of callus culture was derived from explants of sterile seedlings. Explants were induced to form callus on a solid modified MS medium containing 1.25 mg/l 6-benzylaminopurine (BAP) and 2.0 mg/l 2,4-dichlorophenoxy acetic acid / 2,4-D (Murashige and Skoog, 1962). Callus cultures were subcultured every 4 weeks.

Table 2.2. MS Mediums (Murashige und Skoog, 1962)

	Substances	**Final Concentration**
Macro elements	KNO_3	1,900 mg/l
	NH_4NO_3	1650 mg/l
	$CaCl_2$ - 2 H_2O	440 mg/l
	$MgSO_4$ - 7 H_2O	370 mg/l
	KH_2PO_4	170 mg/l
Microelements	Na_2 –EDTA	37.3 mg/l
	$FeSO_4$ - 7 H_2O	27.8 mg/l
	$MnSO_4$ - H_2O	16.9 mg/l
	$ZnSO_4$ - 7 H_2O	10.6 mg/l
	H_3BO_3	6.2 mg/l
	KI	0.83 mg/l
	Na_2MoO_4 - 2 H_2O	0.25 mg/l
	$CuSO_4$ - 5 H_2O	0.025 mg/l
	$CoCl_2$ - 6 H_2O	0.025 mg/l
Vitamin	Nicotinic Acid	0.5 mg/l
	Pyridoxol HCl	0.5 mg/l
	Thiamin HCl	0.1 mg/l
Phytohormone	Naphtylacetic acid	0.4 mg/l
Organic Supplements	myo-Inositol	100 mg/l
	Sucrose	30 g/l
	Glycine	2 mg/l

Cell suspension cultures of *P. macrocarpa* (Scheff.) Boerl. were initiated from callus cultures. Suspension cultures were grown in a 50-ml liquid modified MS-culture medium containing 1.25 mg/l 6-benzylaminopurine (BAP) and 2.0 mg/l 2,4-dichlorophenoxy acetic acid / 2,4-D in 300-ml Erlenmeyer flasks. The pH was adjusted to 5.8. The suspension cultures were shaking on a horizontal rotary shaker (5 cm elongation, 120 rpm), serially propagated every 14 days with an inoculation density of 5g/flask, and kept at 26 °C and dark conditions. For the characterization of cell suspension cultures, parallel flasks of the cultures were inoculated and cultivated as above. Samples were taken during a cultivation period of 21 days, the accumulation of fresh weight, dry weight, change of pH, conductivity, and sugar content of cell-free medium were measured. The pH was determined by a pH electrode (Mettler Toledo, Urdorf, Switzerland; pH-meter: WTW pH 523, Weilheim), whereas the conductivity was measured with a conductometer (LF 91, WTW, Weilheim). The sugar content was refractometrically measured. The refractometer (Krüss, Hamburg) determined all refracting substances which correlated with the sugar content.

2.3.2. Lignan extraction

Lignan extraction was modified according to Wichers and co-workers (1990). About 0.2g of powdered freeze-dried cells were suspended in 2 ml methanol and incubated in an ultrasonic bath for 2 times 30 seconds with 30 seconds break on ice. The pH was adjusted into 5.0 by the addition of diluted phosphoric acid shortly after the addition of 8 ml ultra pure water. After adding 1 mg ß-glucosidase (from almonds, ≥1000 u/mg, Roth, Germany) and incubation at 35 ^{o}C for 1 hour, the samples were extracted 3 times with the 8 ml ethyl acetate in each step. The combined ethyl acetate phases were dried and the residue resolved in an appropriate volume of methanol for HPLC analysis.

The extraction method of Westcott and Muir (2000) was also applied in this experiment to determine the lignan content in seeds of *Linum flavum* var. *compactum* L. The powdered seeds (0.2g) were extracted with 10 ml methanol/water (70:30 v/v) and incubated 60 °C for 3 hours. The mixture was hydrolyzed with 0.5 ml of 0.5 N NaOH (base hydrolysis) for 3 hours and shake every 30 minutes. After that, the mixture was neutralized with 0.5 ml of 0.5 N acetic acid.

2.3.3. HPLC analysis

High performance liquid chromatography (HPLC) is the most common and widely applied method for quantitative and qualitative analysis of lignans.

2.3.3.1. Reversed phase column HPLC

Reversed phase column HPLC operates on the basis of hydrophilicity and lipophilicity. Compounds stick to packing material in a high aqueous mobile phase and are eluted from the column with high organic mobile phase. The stationary phase consists of silica based packing with n-alkyl chains as ligands covalently bound. The more hydrophobic the matrix on each ligand, the greater is the tendency of the column to retain hydrophobic moieties. Thus, hydrophilic compounds are washed out more quickly than hydrophobic compounds. By using solvent gradients, the character of separation can be changed during the run.

For the detection of separated or partially separated lignans UV-spectrophotometry can be used while lignans generally have a maximal absorbance around 290 nm. The separated compounds appear in the detector as peaks that rise and fall when the detector signal is sent to a computer. Those peaks can be used either to quantification, with standard calibration, the amount of each material present, or to control the collection of purified material.

In this experiment, the identification and quantification of the lignans in *P. macrocarpa* was conducted by comparing the retention time and UV spectra in HPLC with authentic standards of secoisolariciresinol, 7-hydroxysecoisolariciresinol, lariciresinol, α-peltatin, pinoresinol, matairesinol, podophyllotoxin, ß-peltatin, 6-methoxypodophyllotoxin, and justicidin B.

In preparation for the analysis cell extracts were centrifuged at 13.000 rpm for 5 min and diluted in appropriate volumes of methanol. For lignan analysis of the sample, reversed phase chromatography was carried out with a GROM-Sil 120 ODS-5 ST column (250 x 4mm, 5 µm; precolumn 20 x 4 mm, 5 µm). The solvents employed here were H_2O with 0.01% phosphoric acid (solvent A) and acetonitrile (solvent B). A solvent gradient program was developed to record a most extensive range of already known and unknown lignans.

Table 2.3. HPLC gradient program (alle_lignane_neu.met):

Time [min]	Solvent A [%]	Solvent B [%]	Flow [ml/min]
0	75	25	0.8
25	62	38	0.8
43	57	43	1.0
46	45	55	1.0
54	30	70	1.0
56	75	25	0.8
60	75	25	0.8

2.3.3.2. Identification of lignans by LC-MS

The Liquid Chromatography-Mass Spectrometry (LC-MS) is a combination of High Pressure Liquid Chromatography (HPLC) and Mass Spectrometry (MS) system. In this method, the sample was injected to the HPLC system for the fractionation and the fractions were flow into the ionization chamber in the MS system. In HPLC system, ultra pure water (containing 0.1% formic acid) and methanol were used as mobile phase with the flow rate 0.4 ml/minute according to a gradient described in Table 2.3.

Table 2.4. Gradient elution of mobile phase in LC-MS

Time [min]	Water [%]	Methanol [%]	Flow [ml/min]
0	90	10	0.4
2	90	10	0.4
35	0	100	0.4
45	0	100	0.4
47	90	10	0.4
60	90	10	0.4

Mass spectrometer is an analytical instrument to determine the molecular weight of a compound. Mass spectrometer is divided principally into three parts namely ionization source, analyzer and detector, which should be maintained under high vacuum condition to keep the ions travel through the instrument without any hindrance from air molecules.

Once a sample is injected into the ionization source, the molecules are ionized. In this system, ionization process is conducted by Electro Spray Impact (ESI) and carried out at atmospheric pressure by spraying sample solution out of a small needle (capillary), to which a strong electric field is applied. This process produce highly charged droplets, and the solvent is evaporated leaving the highly charged molecules

(sample ions) in the gas phase. The sample ions pass through sampling cone into an intermediate vacuum region and through a small aperture into the analyzer of the mass spectrometer which is held under high vacuum. In the analyzer, the ions are separated according to their mass (m) to charge (z) ratio. Further, the separated ions flow into the detector and the signals are transmitted to the data system where the mass spectra are recorded.

Mass spectrometry is useful for quantification of atoms or molecules and also for determination of chemical and structural information of molecules. The output of mass spectrometer shows a plot of relative intensity *versus* the mass-to-charge ratio (*m*/*z*).

2.3.3.3. Chiral HPLC

In order to determine enantiomeric compositions, chiral HPLC analysis was used. Chiral HPLC columns are made by immobilizing single enantiomers onto the stationary phase. Resolution relies on the formation of transient diastereoisomers on the surface of the column. The compound which forms the most stable diastereoisomer will be mostly retained, whereas the opposite enantiomer will form a less stable diastereoisomer and will be firstly eluted. Three points of interaction are needed to achieve chiral recognition and to achieve discrimination between enantiomers. The forces that lead to this interaction are weak, therefore a careful optimization to maximize selectivity by adjustment of the mobile phase and temperature are required.

The enantiomeric composition of pinoresinol and lariciresinol was analyzed by using Chiralcel OD-H; 250 x 4.6 mm, 5 μm. Ethanol (A) and n-hexane (B) were used as solvents at a flow rate of 0.5 ml/min. The gradient of mobile phase was done according to von Heimendahl and co-workers (2005) (Table 2.4). Meanwhile, the separation of the enantiomers of matairesinol was conducted according to Umezawa and co-workers (1990 and 1994) by using 15% ethanol and 85% n-hexane added with 1% acetic acid as solvents. The flow rate was 0.5 ml/min and the detection wavelength was 280 nm. Identification of the substances was achieved by comparison of retention time and UV-spectra to authentic standards.

Table 2.5. HPLC gradient (chiral_pino.met, chiral_lari.met, chiral_matai.met)

Compounds	Time [min]	Ethanol [%]	n-Hexane [%]	Flow [ml/min]
Pinoresinol	0	50	50	0.5
	45	60	40	0.5
Lariciresinol	0	25	75	0.5
	30	25	75	0.5
Matairesinol	0	15	85	0.5
	70	15	85	0.5

2.3.4. Synthesis and purification of pinoresinol

A solution of coniferyl alcohol (1 mmol) in acetone (7 ml) was added to an aqueous solution of iron (III) chloride hexahydrate (2.6 mmol, 24 ml) and stirred at room temperature for 10 minutes. The mixture was extracted three times with diethyl ether (30 ml). The ethereal solution was washed with water (20 ml) to remove iron (III) chloride and dried over sodium sulfate ($NaSO_4$) for approximately 1 hour. By filtrating the sodium sulfate was taken away. The filtrate was evaporated to dryness using a rotavapor. The residue was taken up in methylene chloride (5 ml). To verify whether pinoresinol was synthesized, an aliquot diluted in methanol (1:10) was analyzed by reversed-phase column.

For purification column chromatography was performed with silica gel (230-240 mesh). A column (19 cm x 2.5 cm inner diameter) was packed with a slurry of silica gel (30 g) dissolved in the eluting solvent methylene chloride/diethyl ether (4:1) and washes with 1 l of the same eluent. After careful addition of the sample to the column, fractions (50 ml) were collected and concentrated by evaporation.

Residues were subjected to reverse-phase HPLC analysis. Fractions which yielded amounts of pinoresinol above 94% purity at 230 nm were unified and taken for enzyme assay. Subsequently a gradient of water (A) and acetonitrile (B) was used in order to determine the purity of the synthesized pinoresinol.

Table 2.6. HPLC gradient program (PINOneu.met)

Time [min]	Solvent A [%]	Solvent B [%]	Flow [ml/min]
0	70	30	0.8
30	45	55	1.0
33	55	45	1.0
38	30	70	1.0
40	70	30	0.8

2.3.5. Preparation of protein extracts from cell suspension cultures

A 14 days old cell suspension culture of *P. macrocarpa* was separated from the medium. After addition of 1 ml extraction buffer (20 mM Tris/HCl, 2 mM EDTA, pH 7.1; 5 mM DTT), 0.2 g polyclar 10 per 1 g fresh weight, the cells were homogenized (4 x 30 s) by using an Ultraturrax (Janke and Kunkel, Freiburg im Breisgau, Germany) with intermediate cooling on ice. The supernatant of a centrifugation (4 °C, 40,000G, and 20 min) was used to precipitate the protein between 0% and 60% ammonium sulfate. The protein was sedimented (4 °C, 40,000G, and 20 min), redissolved in extraction buffer and desalted by gel filtration on PD10 columns using the same buffer. Protein concentrations were determined as mentioned in section 2.3.7.

2.3.6. Cloning of the cDNA encoding PLR-like proteins

Standard methods such as DNA restriction, ligation, and transformation are conducted according to Sambrook and co-workers (1989).

2.3.6.1. Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) for RT-PCR was prepared from reverse transcription of 2 µg RNA using an AMV cDNA Synthesis Kit according to the manufacturer's protocol. Degenerated primers used to clone a first fragment of a PLR cDNA from *P. macrocarpa* were designed based on a multiple sequence alignment (ClustalW, version 1.82) of the following amino acid sequences: PLR-Fi1 (GenBank accession number U81158), PLR-Tp1 (GenBank accession number AF242503), PLR-Tp2 (GenBank accession number AF242504), PLR-Tp3 (GenBank accession number AF242505), PLR-Tp4 (GenBank accession number AF242506), PLR-Th1 (GenBank accession number AF242501), PLR-Th2 (GenBank accession number AF242502), PCBER-Fi-2 (GenBank accession number AF242492), PCBER-Pt1 (GenBank accession number AF242490), IFR-Mt1 (GenBank accession number AF277052), IFR-Ms1 (GenBank accession number U17436) and IFR-Ps1 (GenBank accession number S72472). The sequences of the resulting primers are LPLR4F: 5'-CCITCIGARTTYGGIATGGAYCC-3' and LPLR6R: 5'-GTRTAYTTIACYTCIGGGTA-3'. RT-PCR was performed as follows: 2 µl of the synthesized cDNA, 25 pmol of each degenerated primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 µl Taq DNA Polymerase per 50 µl reactions. PCR conditions were 94 °C, 3 min; denaturation 94 °C 30 s; annealing 46 °C 30s; 72 °C 1 min in 34 cycles; final extension 72 °C 3 min. The resulting amplicon was ligated into pGEM-T and transformed into *E. coli* DH5α and sequenced.

2.3.6.2. Rapid Amplification of cDNA Ends (RACE) experiment

Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and unknown sequences at either the 3' or the 5' -end of the mRNA. This methodology of amplification with single-sided specificity has been described as “one-sided” PCR or “anchored” PCR. In general, PCR amplification of relatively few target molecules in a complex mixture requires two sequence-specific primers that flank the region of sequence to be amplified. However, to amplify and characterize regions of unknown sequences, this requirement imposes a severe limitation. This 3' and 5' RACE methodologies offer possible solutions to the problem.

The 3' RACE takes advantage of the natural poly(A) tail in mRNA as a generic priming site for PCR amplification. In this procedure, mRNAs are converted into cDNA using reverse transcriptase (RT) and an oligo-dT adapter primer. Specific cDNA is then directly amplified by PCR using a gene-specific primer (GSP) that anneals to a region of known exon sequences and an adapter primer that targets the poly(A) tail region. This permits the capture of unknown 3'-mRNA sequences that lie between the exon and the poly(A) tail. Meanwhile, 5' RACE, or “anchored” PCR, is a technique that facilitates the isolation and characterization of 5' ends from low-copy messages.

First strand cDNA synthesis is primed using a gene-specific antisense oligo-nucleotide (GSP1). This permits cDNA conversion of specific mRNA, or related families of mRNAs, and maximizes the potential for complete extension to the 5' -end of the message. Following cDNA synthesis, the first strand product is purified from unincorporated dNTPs and GSP1. An enzyme called TdT (Terminal deoxynucleotidyl transferase) is used to add homopolymeric tails to the 3' ends of the cDNA. Tailed cDNA is then amplified by PCR using a mixture of three primers: a nested gene-specific primer (GSP2), which anneals 3' to GSP1; and a combination of a complement-ary homopolymer-containing anchor primer and corresponding adapter primer which permit amplification from the homopolymeric tail. This allows amplification of unknown sequences between the GSP2 and the 5'-end of the mRNA.

RACE experiments were used for rapid amplification of the 5’ and 3’ end of the obtained cDNA fragment. To acquire the 5’ terminus of the cDNA, 5 µg of the sample RNA was mixed with 2.5 pmol gene specific primer as follows: PmPLR-GSP5-1: 5’-GGAAGCTTCTTCTGCC-3’. cDNA was synthesized and purified using the 5’-RACE system version 2.0 (Invitrogene) according to the manufacturer’s instruction. Five microliters of the dC-tailed cDNA was next used as the template in a 50 µl PCR

reaction by using HiFi-Taq polymerase according to the manufacturer with the gene specific primer PmPLR-GSP5-2: 5'-GATCTAGAGGATCCCTGAGAAGCCAAGTC-CATGCC-3' and the adaptor primer: PLR-AAP-5': 5'-GGAATTCGAGCTCG-GTACCACGGGIIGGGIIGGGIIG-3' 0.3 nM each. PCR conditions were 94 °C, 3 min; denaturation 94 °C 30 s; annealing 60 °C 30s; 72 °C 1 min in 34 cycles; final extension 72 °C 3 min. A nested PCR was performed with PmPLR-GSP5-3: 5'-GATCTAGA-GGATCCTTGCTCCCAGGTTT-CGACCAG-3' and PLR-AUAP: 5'-CCGGAATTC-GAGCTCGGTACCAC-3' as gene specific primers. The reverse transcription for the 3'-end of the cDNA was the same as 5'RACE with the poly(dT) primer PLR-AAP-3': 5'-GGAATTCGAGCTCGGTA-CCACTTTTTTTTTTTTTTTT-3'. The cDNA was amplified with the PmPLR-GSP3-2 primer: 5'-GGTCTAGAGGA-TCCTTGCCG-GTTTGGCTCAGATGG-3' and PLRAUAP as mentioned above. The nested PCR was performed with the gene specific primers PmPLR-GSP3-3: 5'-GATCTAGAGGA-TCCTTGCTCCCAGGTTTCGACCAG-3' and PLRAUAP. The resulted cDNA consists of 816 base-pairs which will be over-expressed in the expression vector pET15b.

2.3.6.3. Cloning of the cDNA of *P. macrocarpa* into an expression vector

The obtained cDNA (PM1) was amplified by RT-PCR with the primers PmPLRORF-F: 5'-GGAATTCCATATGTTGCAAGACTTCAAACGGCAA-3' and PmPLRORF-R: 5'-GGCTCGAGTCAGAGGAAGATTTTCAAGTAATCG-3', intro-ducing an NdeI restriction site at the start codon and an XhoI site behind the stop codon. The resulting PCR product was ligated into pGEM-T, then cloned into *E. coli* DH5α and fully sequenced. The ORF from the plasmid was ligated via NdeI, XhoI (MBI Fermentas) into the expression vector pET15b.

2.3.6.4. Heterologous expression of protein

The expression vector pET15b was transformed into *E. coli* Rosetta2 (DE3) cells. Protein overexpression and purification by metal chelate chromatography was performed as described by von Heimendahl and co-workers (2005). All protein concentrations were determined according to Bradford (Bradford, 1976) with BSA as standard.

2.3.7. Quantification of protein concentration

The Bradford assay is used for determining protein content of crude enzyme extracts. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to a protein occurs. For the preparation of Bradford reagent, Coomassie Brilliant Blue G-250 (100 mg) was dissolved in ethanol (50 ml). After having added 85 % (w/v) phosphoric acid (100 ml), the resulting solution was diluted to a final volume of 1 liter. When the dye had completely dissolved, the solution was filtered twice.

Dye reagent (1 ml) was pipetted into a plastic cuvette, mixed with protein solution (5 µl) and incubated for 15 min at room temperature. The absorbance at 595 nm was measured against a reagent blank, containing Bradford dye (1 ml) and buffer (5 µl), using a spectrophotometer. When the sample absorbance was outside a certain range (0.1 – 0.8), the assay was repeated with appropriate dilutions. Each sample was done in triplicate. Standard was prepared by dissolving bovine serum album (1 mg) in distilled water (1 ml) and treated in the same manner as the samples.

2.3.8. Enzyme assay

Enzyme activity was assayed by monitoring the formation of lariciresinol and secoisolariciresinol according to von Heimendahl and co-workers (2005). The assay mixtures (500 µl) consisted of 10 µl (±)-pinoresinol (8 mM in methanol), the crude enzyme extract (100 µl) and 340 µl KP_i buffer (0.1 M, pH 7.1) were preincubated for 15 min at 30 °C. The enzyme reaction was initiated by adding 50 µl NADPH (50 mM). After 1 h incubation at 30 °C, the assay mixture was extracted with ethyl acetate (500 µl). After centrifugation (13.000 rpm, 5 min), the ethyl acetate solubles were removed and the extraction procedure was repeated twice. The ethyl acetate phases were combined, evaporated to dryness in vacuum, redissolved in methanol (100 µl) and subjected to HPLC analysis. Controls were performed by stopping the reaction with ethyl acetate (500 µl) immediately after the addition of NADPH. The protein eluted from PD10 column was assayed by the same way. Separation was conducted on a C18 column GROM-Sil (Grom, Rottenburg, Germany). The elution system was water containing 0.01% phosphoric acid (A) and acetonitrile (B). Products were separated by using the following gradient: 25% B for the first 25 min, 25–43% B in 7 min, 43–75% in 3 min, hold 75% for 1 min, 75–25% in 3 min, hold 25% for 4 min at a flow rate of 1 ml/min at 25 °C.

3. RESULTS

Phaleria macrocarpa (Scheff.) Boerl. is a native tropical plant of Indonesia, consequently, it is essential to initiate the *in vitro* culture of this plant in order to provide an adequate amount of research material. Since this plant is a member of Thymelaeaceae family which mostly accumulate lignans with S,S-configuration instead of the usually occurring R,R-configuration at C-atoms 8,8' and since little known about the lignan content in this plant, it is interesting to investigate the occurrence of lignans and their chiral composition from different organs as well as from *in vitro* culture of this plant. Additionally, a cDNA encoding the enzyme pinoresinol–lariciresinol reductase (PLR) from this plant was investigated as a starting point to understand the biosynthetic pathway of lignans on the molecular level.

3.1. Initiation of *in vitro* cultures of *P. macrocarpa*

In vitro cultures of *P. macrocarpa* were initiated in order to provide a sufficient amount of research material (Fig. 3.1). The seeds germinated 20 days after sowing. The cotyledons were open on the 30th day after sowing. The seedlings formed roots, shoots and leaves and were ready to be used as the source of explants on the 75th day of cultivation.

In order to initiate callus formation, roots of the sterile seedling were planted on a solid modified MS medium containing 1.25 mg/l 6-benzylaminopurine (BAP) and 2.0 mg/l 2,4-dichlorophenoxy acetic acid / 2,4-D (Murashige and Skoog, 1962). Callus cultures (Fig. 3.1 G) were subcultured every 4 weeks. Cell suspension cultures were initiated by transfer of callus tissues into liquid MS medium (as above, but without agar; 50 ml in 300 ml Erlenmeyer flasks) and incubated on a rotary shaker (120 rpm). Cell suspension cultures were subcultured in the same medium every 14 days and cultures were maintained at 25 °C under permanent darkness. The cell suspension cultures physically formed two types of cell aggregates, fine cell and big cell aggregates with a diameter of about one cm (Fig. 3.1 H). However, only the suspension culture consisting of the fine aggregates was used for further experiments.

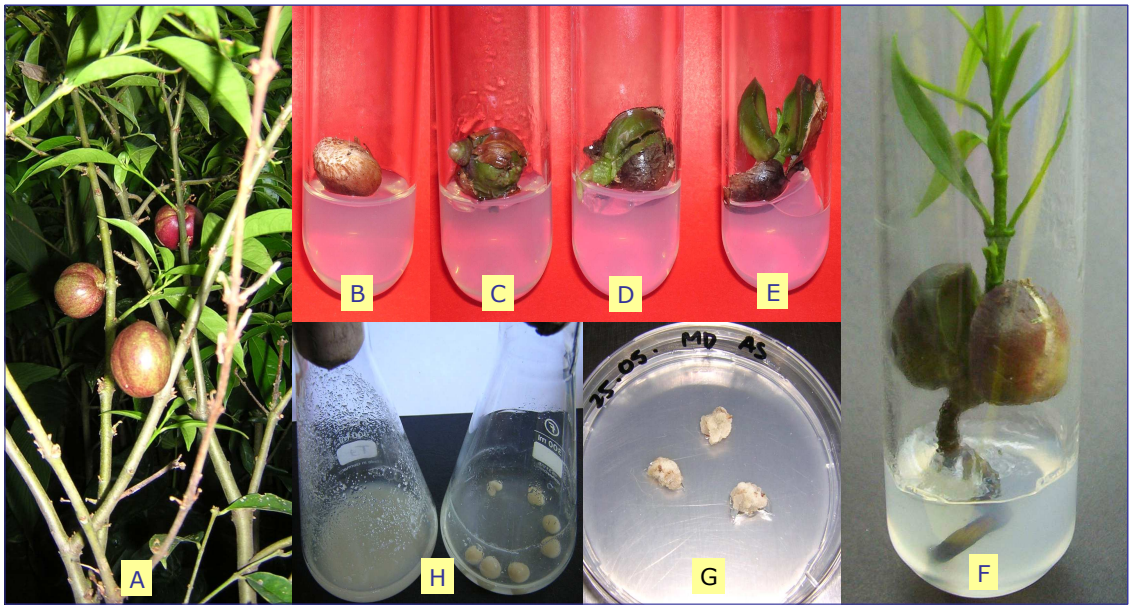


Figure 3.1. Initiation of in vitro cultures of *P. macrocarpa.* **A=** a 3 years old tree of *P. macrocarpa*; **B-F=** germination of *P. macrocarpa's* seed on MS-media under sterile conditions. (**B=** on the day of sowing; **C**=20 days after sowing; **D**=25 days; **E**=30 days; and **F**=75 days after sowing); **G**= callus culture; **H**= cell suspension cultures.

The cell suspension cultures were maintained in fresh liquid MS medium for two months (four subcultivations) before characterization. The maximum biomass based on fresh weight (FW) of the cell suspension culture of *P. macrocarpa* was achieved on day 11 of cultivation period with 12.25 g/flask, as well as its dry weight (DW) accumulation with 0.72 g/flask (Fig.3.2).

Based on the fresh and dry weight accumulation, the growth phase occurred between the days 3 and 11. It was supported by a slightly increase of pH of the medium from day 3 until day 11 (Fig. 3.3). The stationary phase occurred between day 11 and 14 followed by a decrease phase. The conductivity (mS/cm) of the medium decreased from 5.5 on day 0 and reached a minimum at day 11. The sugar content reached its minimum at day 21 (Fig. 3.3).

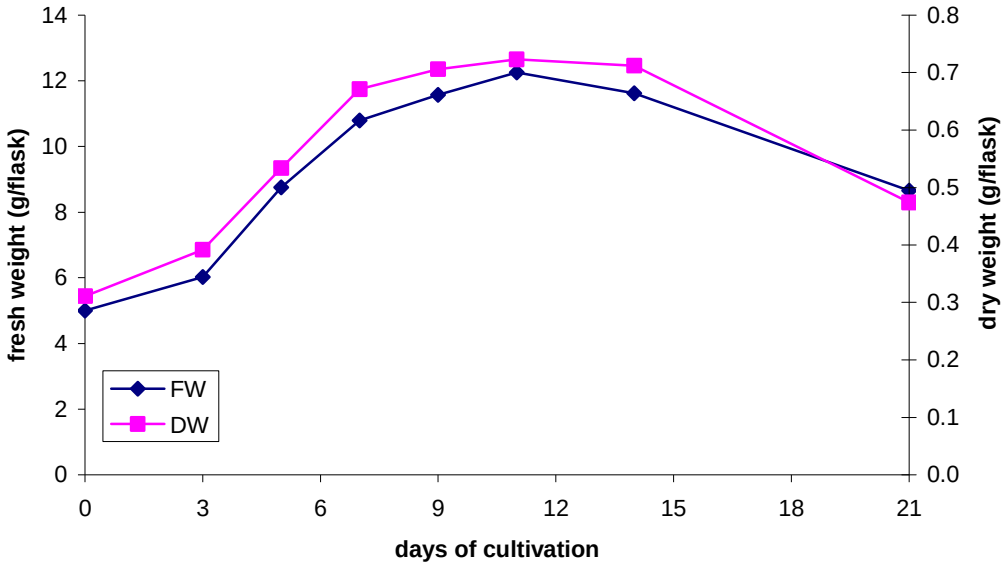


Figure 3.2. Accumulation of fresh weight (FW) and dry weight (DW) during a cultivation period of the cell suspension culture of *P. macrocarpa*

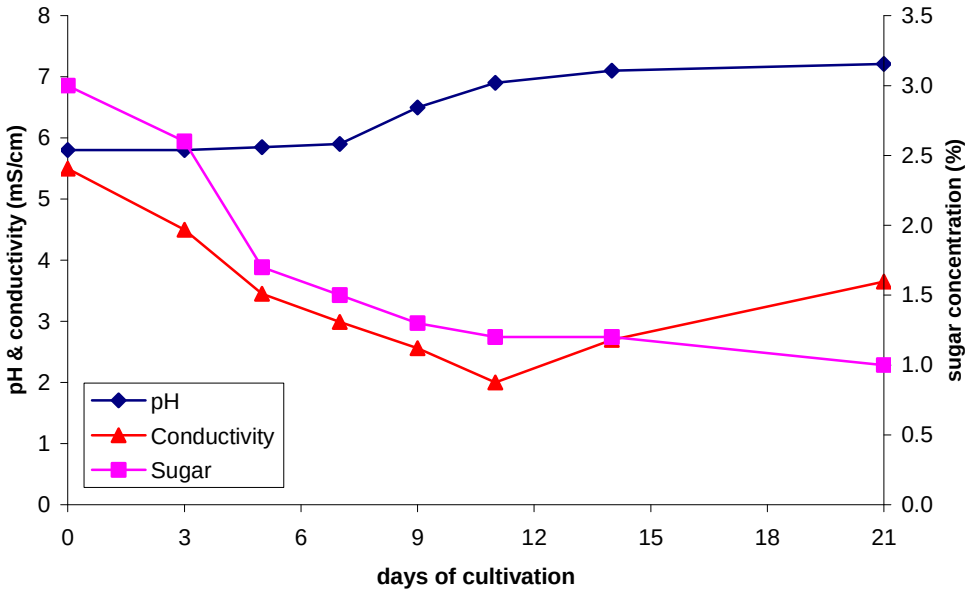


Figure 3.3. Change of pH, conductivity and sugar concentration during a cultivation period of the cell suspension culture of *P. macrocarpa*

3.2. Extraction and identification of lignans

3.2.1. Lignans in *Phaleria macrocarpa*

3.2.1.1. Identification by using RP-HPLC

Identification and quantification of the lignans in *P. macrocarpa* was conducted by comparing the retention time and UV spectra in HPLC with authentic standards (chapter 2.3.3.1). Chromatograms of wood extract, callus extract and cell suspension extract as well as the ultra violet spectra of lignans from wood extract of *P. macrocarpa* were shown in Fig. 3.4 – Fig. 3.7. Pinoresinol, lariciresinol and matairesinol could be detected in the different organs of *P. macrocarpa*. The wood and the root were especially rich in pinoresinol, but contain also lariciresinol and matairesinol. The seeds contain smaller amounts of pinoresinol as main lignan and lariciresinol. Matairesinol could be detected in callus cultures but not in the seeds. Lariciresinol was the only lignan which could be detected in the bark, leaf and fruit flesh. However, no lignans could be detected in the cell suspension culture (Tab. 3.1).

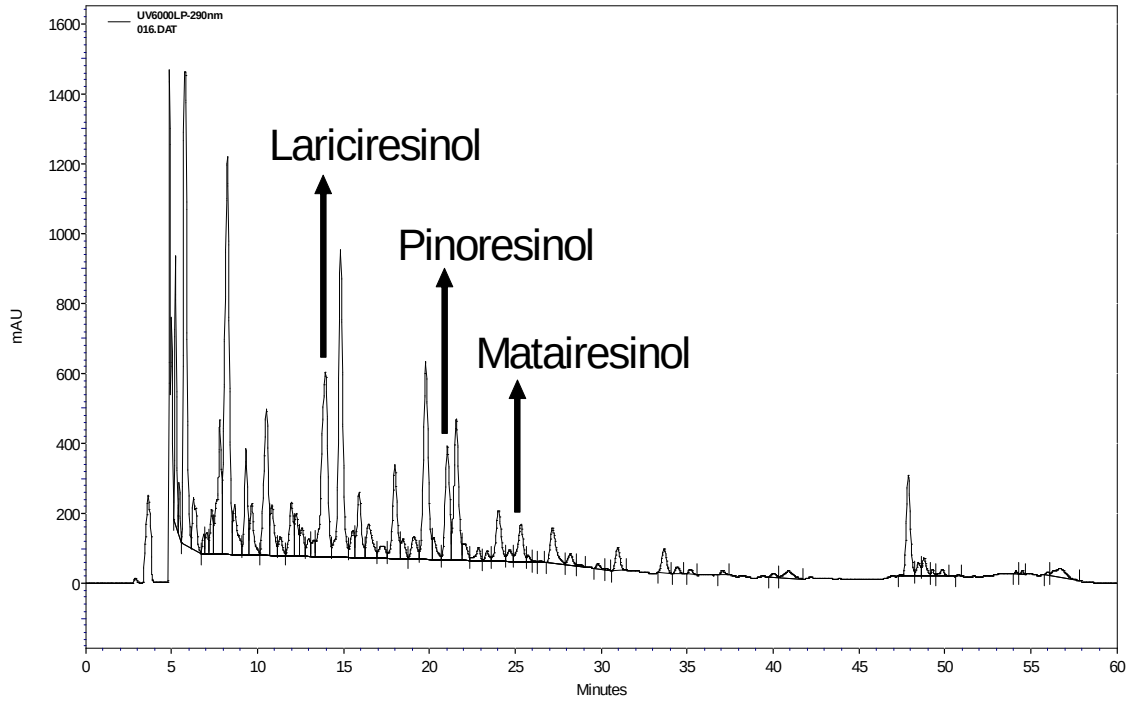


Figure 3.4. RP-HPLC chromatogram of wood extract of *P. macrocarpa*

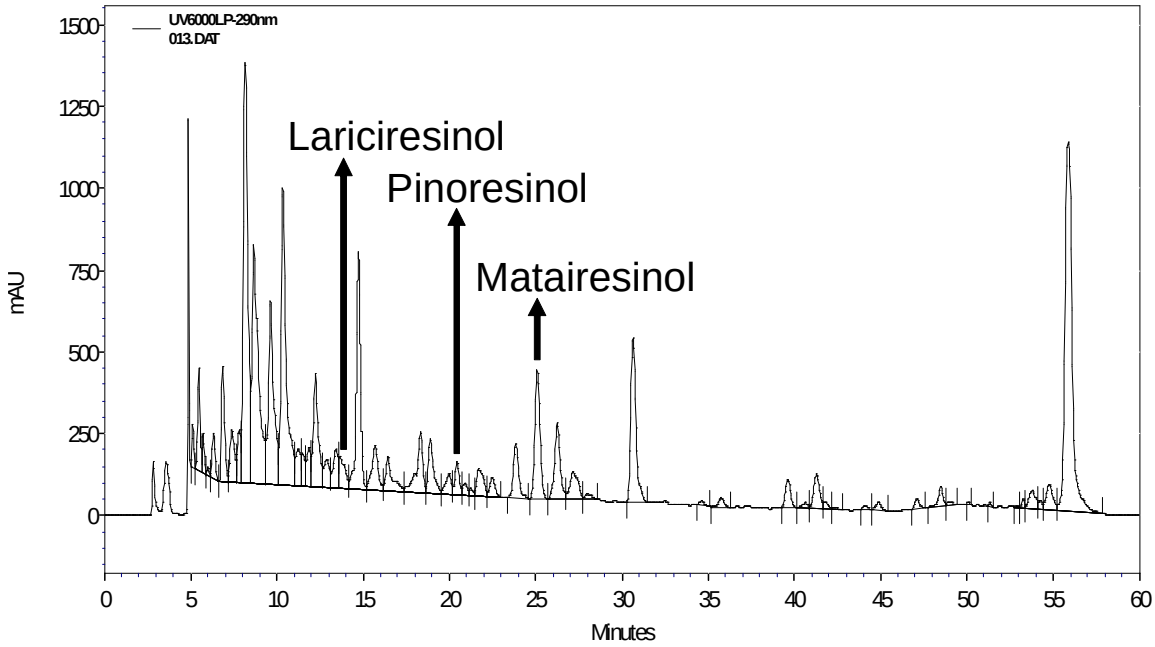


Figure 3.5. RP-HPLC chromatogram of callus extract of *P. macrocarpa*

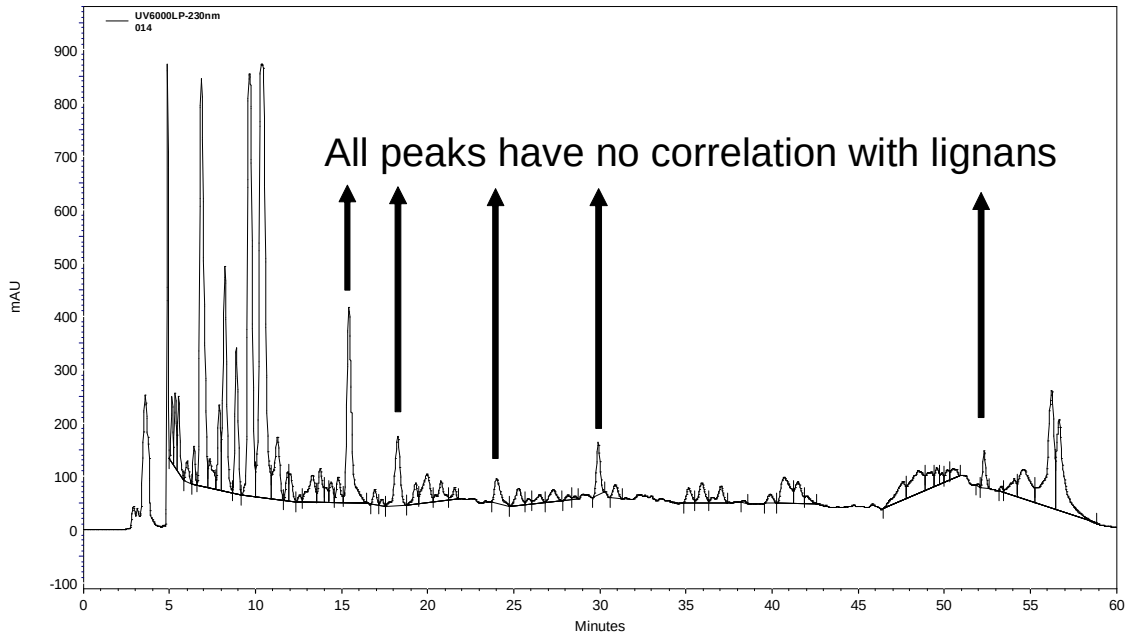


Figure 3.6. RP-HPLC chromatogram of cell suspension extract of *P. macrocarpa*

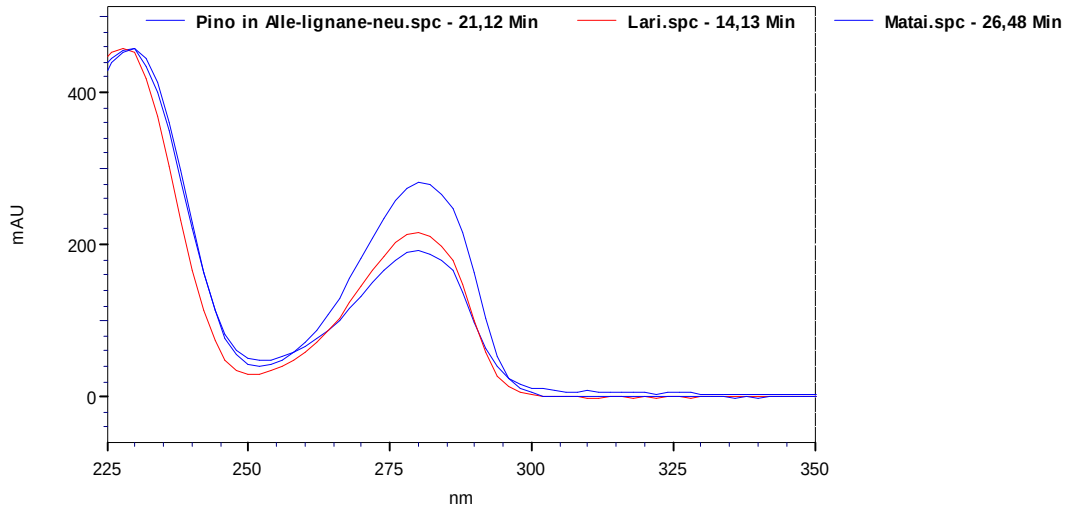


Figure 3.7. Ultra violet spectra of lignans from wood extract of *P. macrocarpa*

Table 3.1. Lignans in *Phaleria macrocarpa* (Schef.) Boerl.

Plant Material	Lignan content (mg / g dry weight)		
	Pinoresinol	**Lariciresinol**	**Matairesinol**
Root	3.404	1.533	1.563
Bark	-	0.883	-
Wood	2.242	1.768	0.801
Leaf	-	0.495	-
Fruit	-	0.637	-
Seed	0.803	0.261	-
Callus	0.084	trace amount	0.220
Suspension culture	-	-	-

(-) = not detected

3.2.1.2. Identification of lignans by using HPLC-MS

Pinoresinol, lariciresinol, and matairesinol collected from reversed phase HPLC were purified by extraction with ethyl acetate and analysed by using HPLC-MS (Fig. 3.8).

Signal peaks of pinoresinol, lariciresinol, and matairesinol collected from wood extract of *P. macrocarpa* appeared at m/z 357$[M-H]^-$, 359$[M-H]^-$, and 357$[M-H]^-$, respectively, in the negative mode of ESI-MS which correspond to the molecular weight of 358, 360, and 358, respectively.

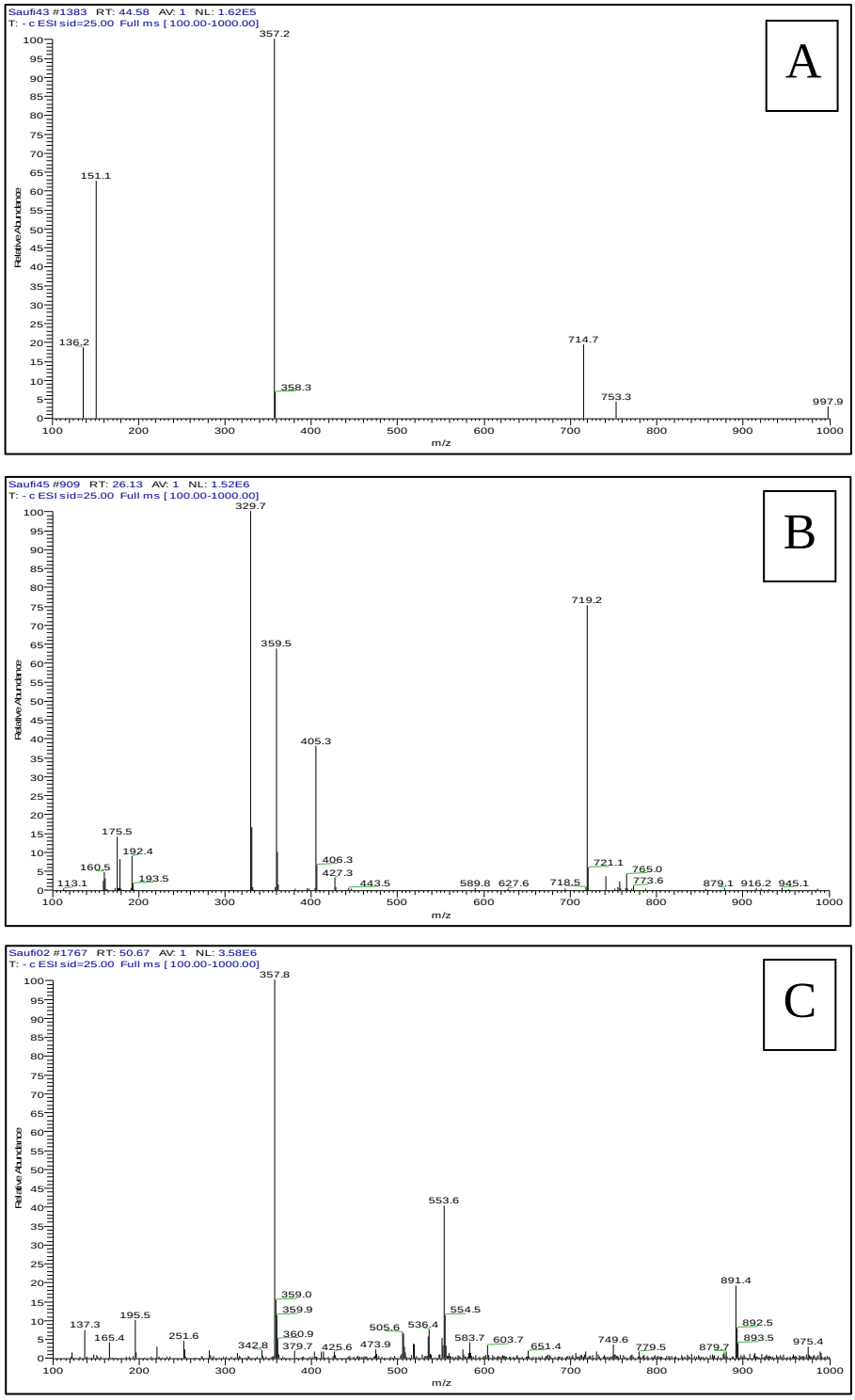


Figure 3.8. Mass spectra of pinoresinol (A), lariciresinol (B), and matairesinol (C) isolated from the wood of *P. macrocarpa*

3.2.1.3. The enantiomeric composition of lignans from *P. macrocarpa*

Pinoresinol, lariciresinol, and matairesinol were analysed again by chiral column HPLC (Chiralcel OD-H) in order to determine their enantiomeric composition (Fig. 3.9). Pinoresinol and lariciresinol were mixtures of both enantiomers, whereas matairesinol accumulates as pure (+)-enantiomer (Tab. 3.2).

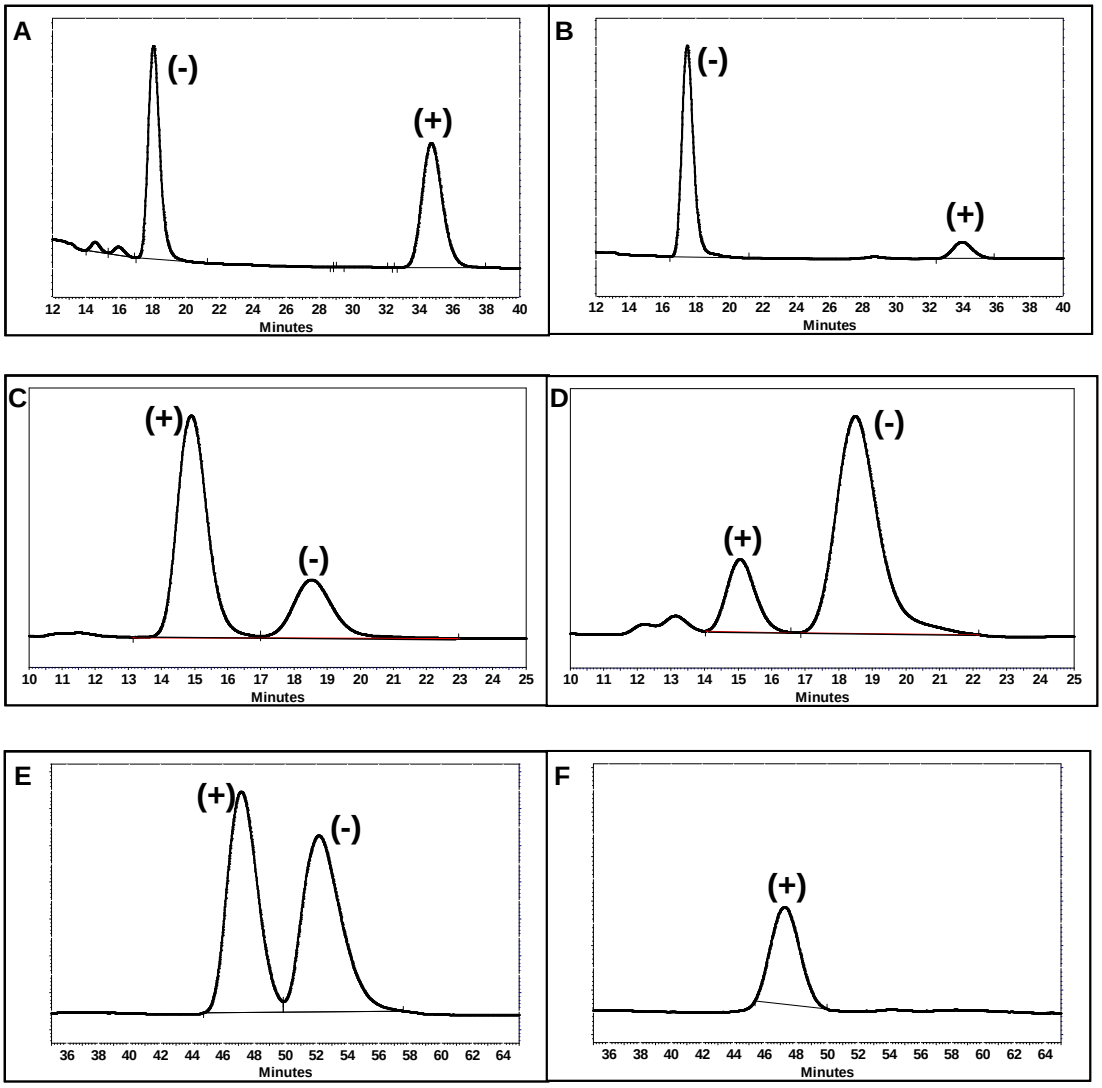


Figure 3.9. Chiral column HPLC analysis (**A**=racemic pinoresinol; **B**=pinoresinol isolated from wood extract of *P. macrocarpa*; **C**=mixture of (±)-lariciresinol; **D**=lariciresinol isolated from wood extract of *P. macrocarpa*; **E**=racemic matairesinol; **F**=matairesinol isolated from wood extract of *P. macrocarpa*).

Table 3.2. Enantiomeric composition of lignans in the wood of *P. macrocarpa* (Schef.) Boerl.

Lignans	(+)-enantiomer (%)	(-)-enantiomer (%)	% enantiomeric-excess
Pinoresinol	10.5 ± 1.7	89.5 ± 1.7	79 ± 3.5
Lariciresinol	22.5 ± 3.1	77.5 ± 3.1	55 ± 6.2
Matairesinol	100	0	100

mean +/- standard deviation from 4 chiral column analyses

3.2.2. Lignans in *Linum flavum* var. *compactum* L.

Linum flavum var. *compactum* L. (Linaceae) is easy to cultivate in our botanical garden. In addition, six known lignans namely 6-methoxypodophyllotoxin, α-peltatin, ß-peltatin, pinoresinol, lariciresinol, secoisolariciresinol were previously identified in this species by Mikame and co-workers (2002). Hence, it is remarkable to investigate the variety of lignan in different organs of this species during different developmental stages as an assessment to the lignan's diversity in *P. macrocarpa* (Schef.) Boerl. Identification and quantification of lignans in *L. flavum* var. *compactum* L. (Fig. 3.10 and Fig. 3.11) were conducted by using the same method applied to *P. macrocarpa* (section 2.3.2).

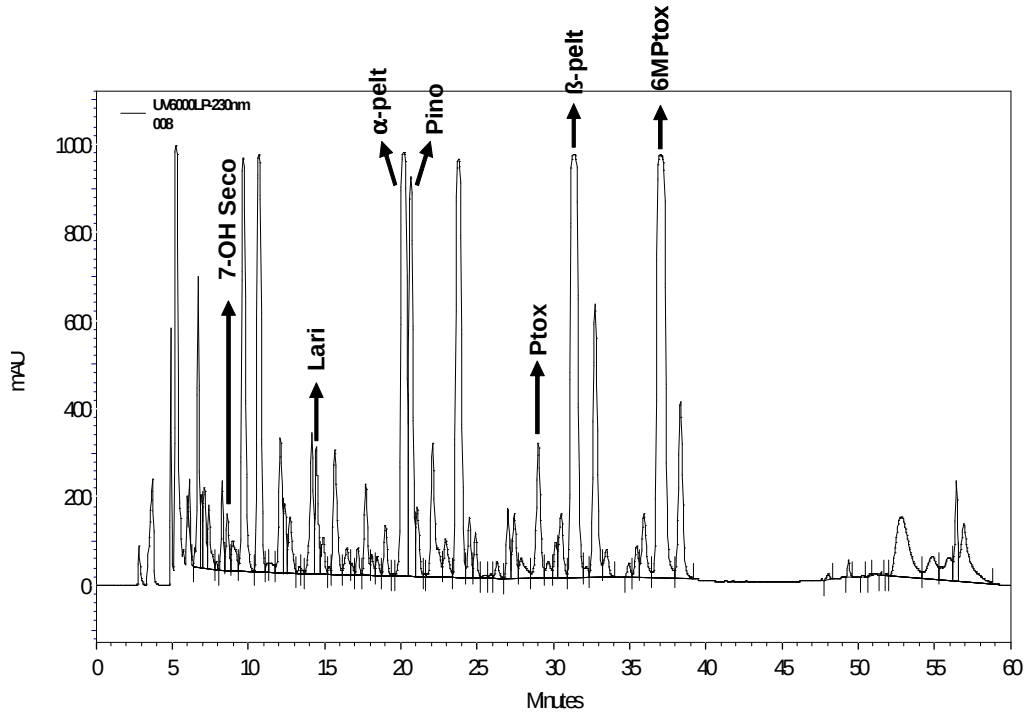


Figure 3.10. Chromatogram of flowerbud extract of *L. flavum* (harvested in 2005)

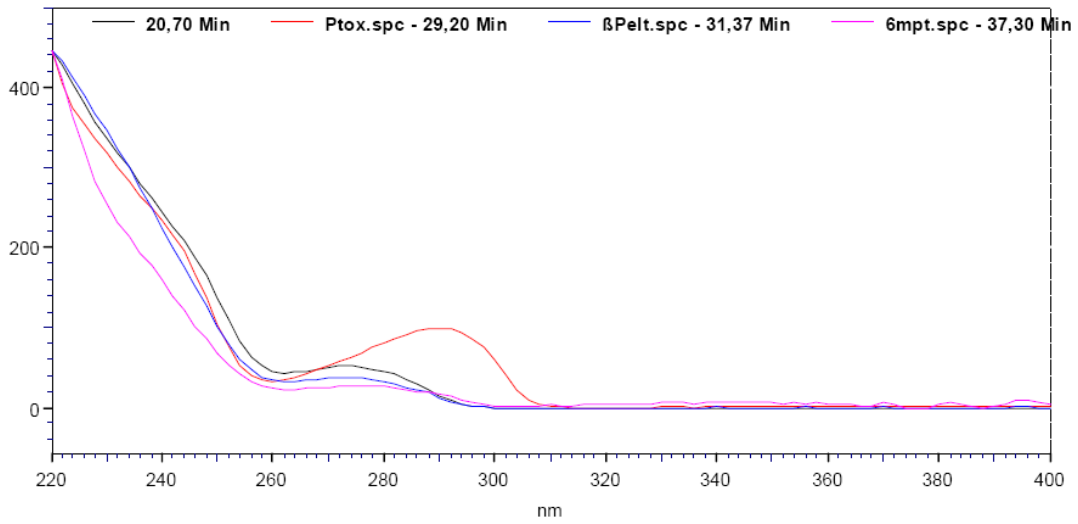


Figure 3.11. Ultra violet spectra of lignans from the root extract of *L. flavum* during flowering stage

The lignan content from different parts of *L. flavum* var. *compactum* L. as well as in *L. perenne* var *Himmelszelt* and *L. usitatissimum* var. Lirina harvested in 2005 are shown in Figure 3.12 – 3.14.

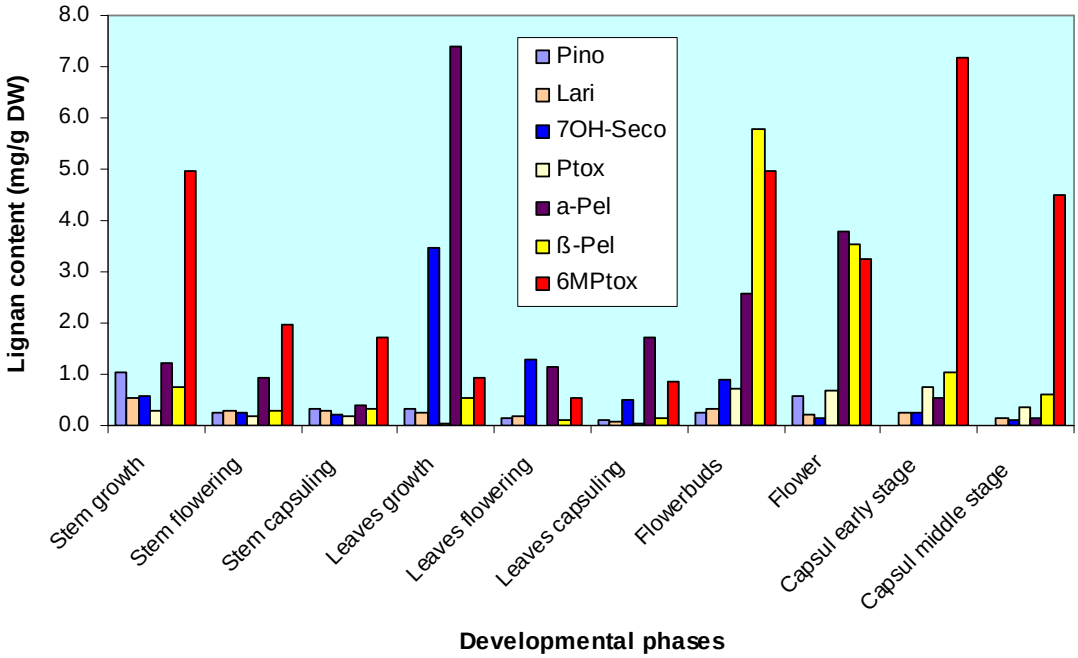


Figure 3.12. Lignan content in *L. flavum* var. *compactum* (harvested in 2005)

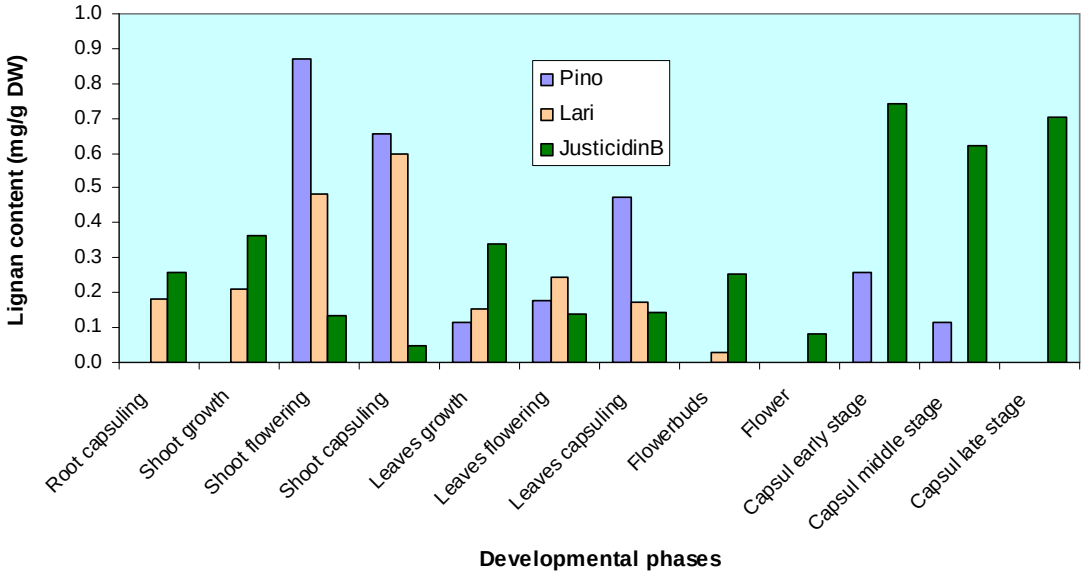


Figure 3.13. Lignan content in *L. perenne* var. *Himmelszelt* (harvested in 2005)

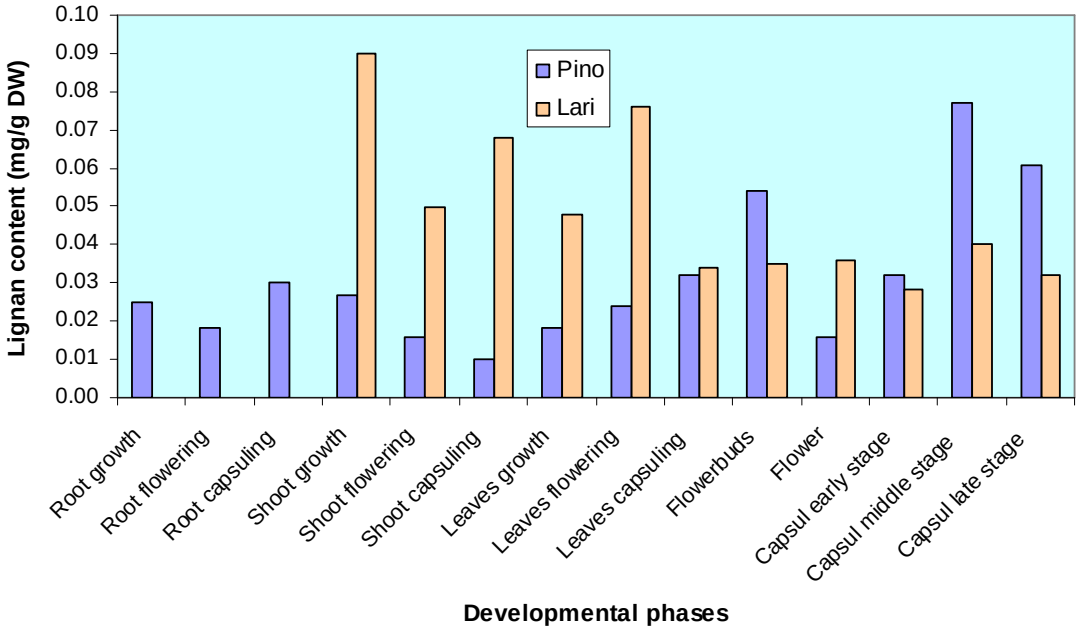


Figure 3.14. Lignan content in *L. usitatissimum* var. Lirina (harvested in 2005)

In general, the diversity of lignans found in *L. flavum* var. *compactum* L. harvested in 2005 was higher than in *L. perenne* var. *Himmelszelt* and *L. usitatissimum* var. *Lirina.* Therefore, it was more attractive to investigate the lignan diversity of *L. flavum* var. *compactum* L.

In order to have a better view about the lignan formation in this plant, it was also necessary to investigate the occurrence of different lignans in different organs of *L. flavum* var. *compactum* L. during different developmental stages which were harvested in 2005 and 2006 (section 2.1.1; Tab. 2.1and Fig. 2.2).

The lignans pinoresinol (Pino), lariciresinol (Lari), 7-hydroxysecoisolariciresinol (7-OH Seco), podophyllotoxin (Ptox), α-peltatin (α-pelt), ß-peltatin (ß-pelt), and 6-methoxypodophyllotoxin (6MPtox) were detected in the different organs of *L. flavum* by HPLC in comparison to authentic standards (section 2.3.3.1.).

The roots of *L. flavum* accumulated 6-methoxypodophyllotoxin as the main lignan (Fig. 3.15 and Fig. 3.16). The highest level of 6-methoxypodophyllotoxin was shown in the roots during flowering with 6.7 mg/g dry weight (DW). The root during growth accumulated also high levels of the other lignans such as α-peltatin and 7-hydroxysecoisolariciresinol with about 2.0 and 1.6 mg/g DW, respectively. In comparison to the lignan content in the root during flowering and root during capsule formation, the lignan content in root during growth phases was slightly higher. The content of pinoresinol, lariciresinol, podophyllotoxin and ß-peltatin in root remained low during the three different developmental phases.

All three developmental phases showed that the stem of *L. flavum* also accumulated 6-methoxypodophyllotoxin as the main lignan (Fig. 3.15 and Fig. 3.16). The highest level was found in stem during flowering phase with about 4.3 mg/g DW. However, the amount of 6-methoxypodophyllotoxin was still lower than the content in root organ (5.2 – 6.7 mg/g dry weight). The other lignan, which also appear in high levels, was α-peltatin. This α-peltatin accumulated at large amount in the stem growth phase (GP) and in stem flowering phase (FP) with 2.7 – 2.9 mg/g DW. The amount of α-peltatin in stem during these two developmental phases was much higher than in stem during the capsuling phase (CP). Pinoresinol and 7-hydroxysecoisolariciresinol were also found in moderate levels in the stem during the growth phase (1.4 mg/g DW and 1.4 mg/g DW, respectively) and in the stem during the flowering phase (1.5 mg/g DW and 1.2 mg/g DW, respectively). The amount of both lignans in stem during growth and flowering phases was much higher than in stem during capsuling phase (0.2 mg/g DW and 0.5 mg/g DW, respectively). The content of the other lignans such as lariciresinol, podo-

phyllotoxin and ß-peltatin in stem organ during three developmental phases were on a low level (0.6 mg/g DW, 0.3 mg/g DW, and 0.7 mg/g DW, respectively).

In contrast to root and stem, the leaves of *L. flavum* accumulated α-peltatin as the main lignan (Fig. 3.15 and Fig. 3.16). The highest level was shown in leaves during the growth phase with about 4.2 mg/g DW. The leaves during growth phase accumulated an intermediate level of 6-methoxypodophyllotoxin and 7-hydroxysecoisolariciresinol (2.0 mg/g DW and 1.2 mg/g DW, respectively). The amount of lariciresinol was slightly increased in the leaves flowering phase but decreased again in the leaf during capsuling phase. The content of pinoresinol, podophyllotoxin, and ß-peltatin were on a low level in leaves of *L. flavum*.

The flowerbuds and flowers accumulated mainly ß-peltatin, 6-methoxypodophyllotoxin, and α-peltatin (Fig. 3.15 and Fig. 3.16). The content of those lignans in flowerbuds were as much as 7.5, 4.9, and 3.9 mg/g DW, respectively, whereas the content of ß-peltatin, 6-methoxypodophyllotoxin, and a-peltatin in flower were 3.2, 1.9, and 3.7 mg/g DW, respectively. The amount of α-peltatin in flower buds was quite similar as in flowers, however the amount of ß-peltatin and 6-methoxypodophyllotoxin in flower buds were double as in flowers. The content of pinoresinol, lariciresinol, 7-hydroxysecoisolariciresinol, and podophyllotoxin in flowerbuds were 0.9, 0.5, 0.5, and 0.6 mg/g DW, respectively, whereas the content of pinoresinol, lariciresinol, 7-hydroxysecoisolariciresinol, and podophyllotoxin in flower of *L. flavum* were 0.8, 0.3, 0.1, and 0.7 mg/g DW, respectively.

The capsules of *L. flavum* accumulated 6-methoxypodophyllotoxin as the main lignan (Fig. 3.15 and Fig. 3.16). The highest amount of 6-methoxypodophyllotoxin was observed in the capsule early phase with 5.9 mg/g DW. This amount however was still lower than the amount of 6-methoxypodophyllotoxin in the root of *L. flavum* (6.7 mg/g DW). The content of pinoresinol, lariciresinol, 7-hydroxysecoisolariciresinol, podophyllotoxin, α-peltatin, and ß-peltatin in capsules of *L. flavum* were remaining low.

Based on those figures, the lignan 6-methoxypodophyllotoxin were found in all parts of *L. flavum* L. var. *compactum* with the highest amount in root and capsule early stage. The lignans pinoresinol, lariciresinol, 7-hydroxy-secoisolariciresinol, podophyllotoxin were found in all parts of *L. flavum* L. var. *compactum* in low amount. There was no difference of lignans content between the harvesting year 2006 and 2005, except that the amount of α-peltatin in leaves “growth phase” which harvested in 2005 was about twice higher than in leaves “growth phase” harvested in 2006.

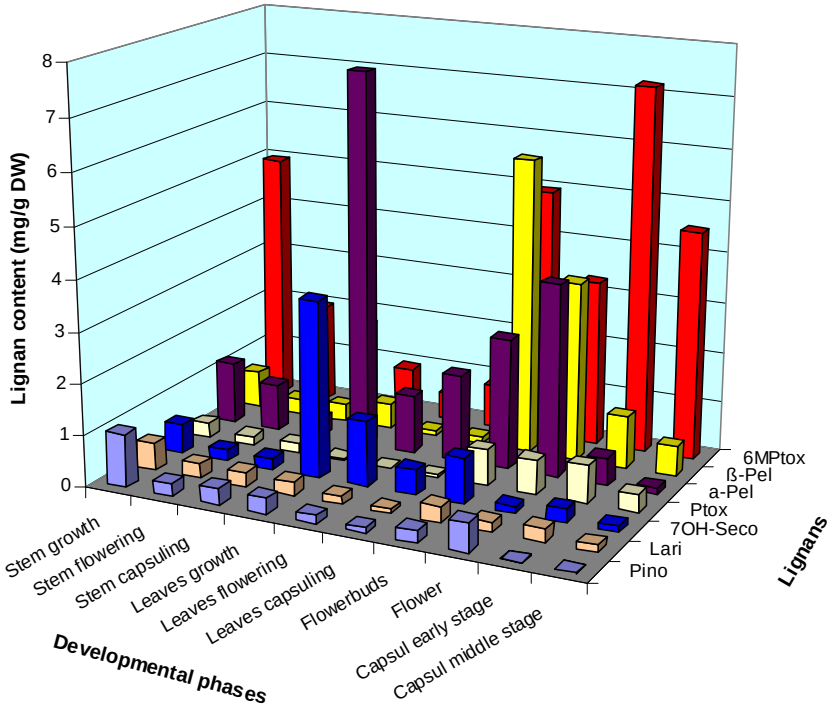


Figure 3.15. Lignan content in *L. flavum* var. *compactum* (harvested in 2005)

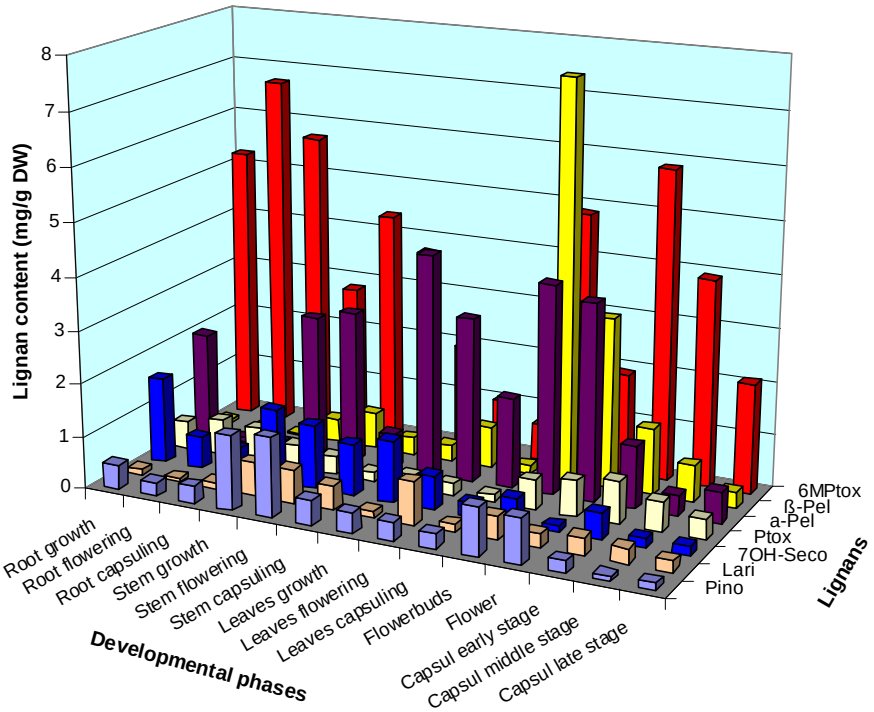


Figure 3.16. Lignan content in *L. flavum* var. *compactum* (harvested in 2006)

The comparison between two extraction methods (Fig. 3.17) of the seed of *L. flavum* (Jelitto Staudensamen GmbH Schwarmstedt, Germany) shown that the extraction method of Westcott and Muir (2000) was more effective to pull out α-peltatin, ß-peltatin, and 6-methoxypodophyllotoxin in comparison to the extraction method of Wichers (1990) that only pulled out 6-methoxypodophyllotoxin. On contrary, the amount of 6-methoxypodophyllotoxin was found higher by using the extraction method of Wichers (1990) compared to the extraction method of Westcott and Muir (2000).

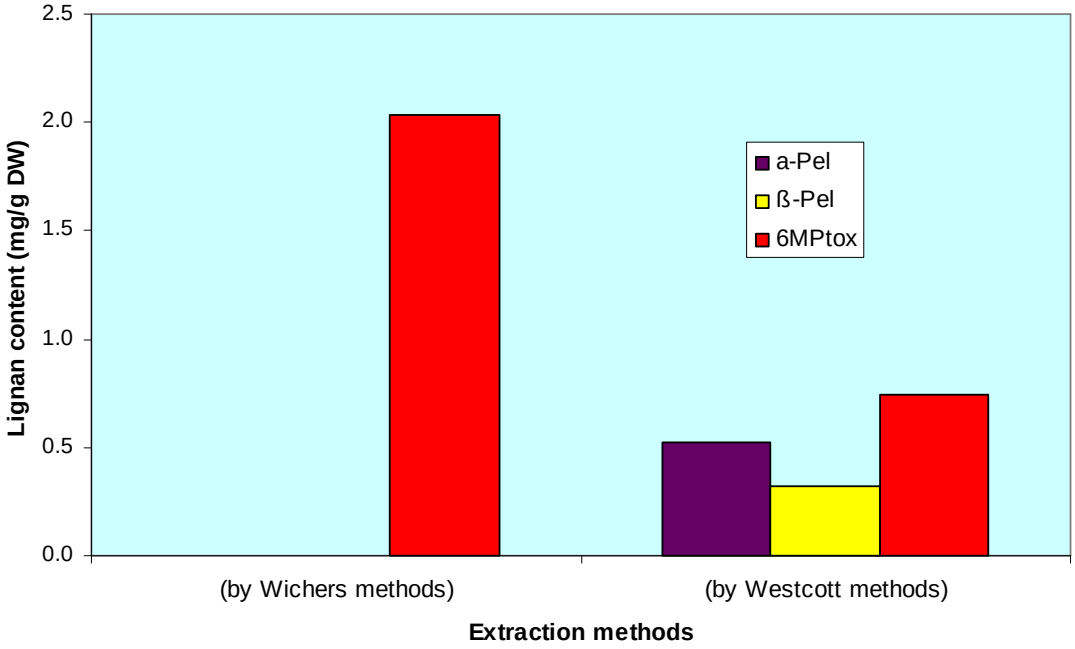


Figure 3.17. Comparison of lignan content in seed of *L. flavum* var. *compactum* extracted according to Wichers and Westcott

3.3. PLR-like proteins from *P. macrocarpa* (Scheff.) Boerl.

3.3.1. PLR activity in cell suspension cultures of *P. macrocarpa*

A 14 days old cell suspension culture of *P. macrocarpa* was separated from the medium. The cell free extract of this cell suspension culture was used for the determination of enzyme activities according to von Heimendahl and co-workers. (2005).

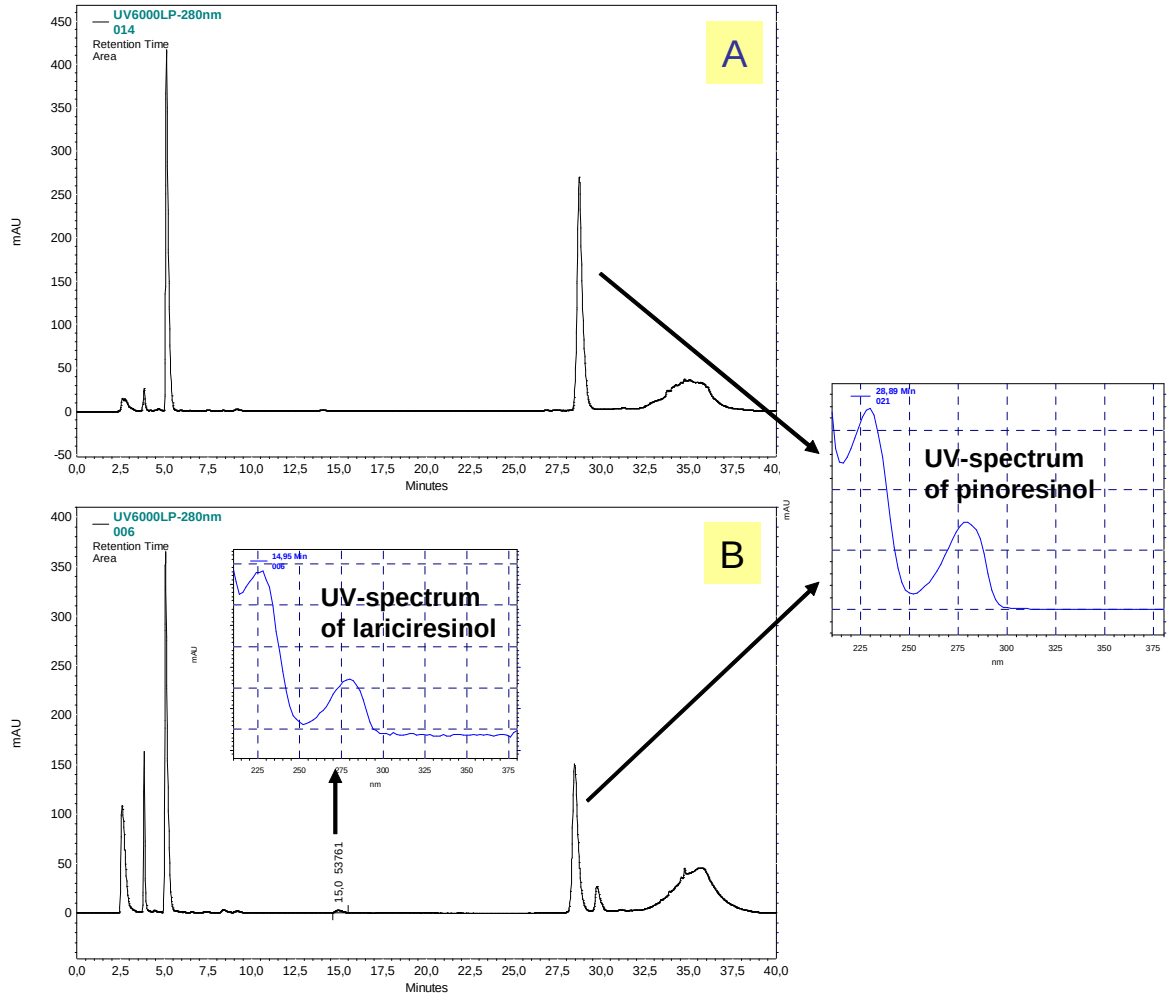


Figure 3.18. Chromatogram of an enzyme assay with the cell-free extract of *P. macrocarpa*'s cell suspension culture (**A**=by using **cooked** cell free extract resulting no peak correlated with lariciresinol; **B**=by using **fresh** cell free extract resulting a peak correlated with lariciresinol).

The assay mixtures (500 µl) consisted of 340 µl KPi buffer (0.1 M, pH 7.1), 8 mM racemic pinoresinol (see section 2.3.4), 50 µl NADPH (50 mM) and 100 µl cell free extract of *P. macrocarpa*. Protein, buffer and pinoresinol were preincubated for 15 min

at 30 °C. The enzyme reaction was initiated by addition of NADPH and terminated by addition of 500 µl ethyl acetate after 3 hours. Controls were performed by stopping the reaction with ethyl acetate (500 µl) immediately after the addition of NADPH. The assays were extracted with ethyl acetate (3 x 500 µl in total). The combined ethyl acetate phases were dried under vacuum. The residue was dissolved in 100 µl methanol and subjected to HPLC analysis.

According to Fig 3.18, there was a small peak with the similar retention time and UV-spectrum of lariciresinol standard. It implies that pinoresinol was converted into a small amount of lariciresinol by the cell-free extract of cell suspension culture of *P. macrocarpa*. The conversion of pinoresinol into lariciresinol therefore showed the occurrence of the enzyme pinoresinol-lariciresinol reductase in the cell-free extract of *P. macrocarpa*‘s cell suspension culture.

3.3.2. Isolation of RNA, cDNA synthesis and cloning of a partial cDNA sequence of *P. macrocarpa*

The cDNA for RT-PCR was prepared from reverse transcription of 2 µg RNA using an AMV cDNA synthesis kit according to the manufacturer’s protocol. The resulting amplicon (Fig. 3.19) was ligated into pGEM-T and transformed into *E. coli* DH5α and sequenced.

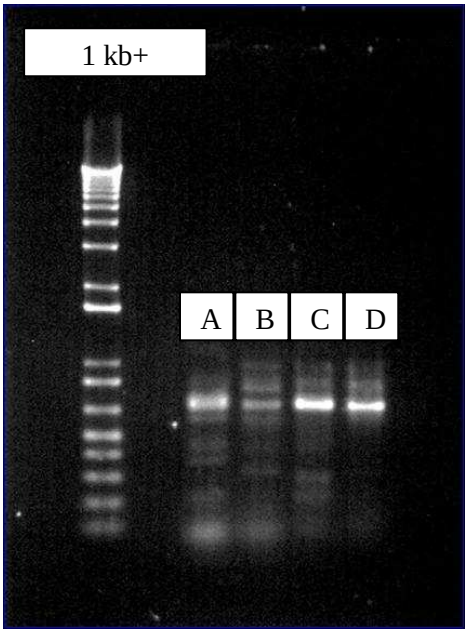


Figure 3.19. Agarose gel electrophoresis of the PCR products resulting bands of 650 bp (A and B: bands of *L. flavum*; C and D: bands of *P. macrocarpa*)

The RT-PCR of *P. macrocarpa* yielded a partial sequence (pOV4K4) encoding for approximately 200 amino acids with highest similarities to PLRs. The sequence of pOV4K4 was used as a template to design primers for 5'-RACE and 3'-RACE primers.

3.3.3. RACE experiment

The RACE experiments were used for rapid amplification of the 5' and 3' end of the obtained cDNA fragment. The 5'-RACE experiment yielded two partial sequences (pOV9K2 and pOV13K3) with similarities to PLRs, meanwhile, the 3'-RACE experiment yielded a partial sequence (pOV6K3) with similarities to PLRs. The results of 5'-RACE and 3'-RACE have the overlap to each other. For instance, pOV9K2 has an overlap sequence with pOV4K4, as well as pOV13K3 with pOV6K3. Therefore, the connection of those overlapped sequences yielded two protein sequences, namely PM1 and PM2 (Fig. 3.20).

PM1

```
ATGTTGCAAGATTTCAAACGGCAAGGAGCTCATCTCGTGGAGGCCTCCTTTGAGGACCAC
CAGAGCCTGGTTGACGCCGTTGAGAAGGCCGACGTCGTCATCTCCGCCATGTCCGGTGTG
CACTTCCGATCTCACAACCTTCTCCTCCAGCTGAAGCTCGTCGACGCCATCAAGGAAGCT
GGAAATGTTAAGGTTGGAAATGTTAAGCGATTCTTGCCATCGGAATTTGGGATGGATCCG
TCGCGGATGGGTCATGCACTGGAGCCGGGAAGAGTGTCGTTCGACGACAAGATGAAAGTG
AGAAAAGCTATCGAAGAAGCGCAAATCCCTTTCACTTACGTCGCCGCCAACTGCTTCGCT
GGTTATTTTGTTGTCGGTTTGGCTCAGATGGGAAGGATCACTCCTCCCAAGGACAAAGTT
TACCTCTACGCTGGTGGAGATATCAAAGTGGCCTTCACGGATGAGGATGACGTGGCAACC
TATGCGATCAAGACAATAGACGATCCTCGAACACTAAACAAGACTCTCTACATTAGGCCT
CCAGACAACACTCTGACGCAAAGACAACTGGTCGAAACCTGGGAGCAAGTTTCTCGCAAC
AAACTAGACAAGATCACTCTCGCCGCCGATGACTTTTTGGCCTCCATGAAAGGCATGGAC
TTGGCTTCTCAGGTCGGGGTAGGACATTTCTACCACATATTCTATGAAGGATGCCTCACC
AATTTTGAAATAGGCCAAGGGGCAGAAGAAGCTTCCCAGCTTTATCCGGAGGTCCAATAC
ACTCGGATGCACGATTACTTGAAAATCTTCCTCTGA
```

PM2

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GCACACGAGCTTCGAGATACCGCTCCATCAGACCCTGTCAAAGCAAAACTCATCGACTCC
TTCAAAAACCTCGGAGTTCAGATGTTGCAAGGAGACCTCCGCGATCGGGAGAGTTGGGTG
AAGGCAATTAAGCAGGCTGATGTTGTAATTTCCGCTGTTGGGATCCCTCAGGTCGCTGAT
CAGACCAATATCATCGCCGCCATTAAAGAAGCTGGAAATGTTAAGAAATTCTACCCTTCA
GAGTTTGGGCCTGATTGCGACAGGATCAATGCTGTCGAGCCAGCAGCAACGTTAATAGCA
TCGAAGGCTGTGATTCGGCGCGAGATAGAGGCCCTAGGGATCCCTTACACTTATGTATGC
TCCAATGGATTTGCTGGTTACTTTGCCCGCAGTTTACTTCAGATTGGAGCTACCTCTCCT
CCCAGAGACAAAGTCATTATCCTTGGCAATGGGAATACGAAAGTCGTTTCCAACAAGGAG
GAAGACATTGCAACCTACACAATCCGGAGTATTGATGATCCCAGAACCTTGAACAAGAGT
GTGTACATTAAACCCCCCAAGTGTACACTCTCTTCCAATGAGCTTGTTGCAACGTGGGAG
AAGAAGATTTGTAAGACGCTCGAGAAAGCCTATGTTCCAGAGGACGAAATTCTCAAACAG
ATTGAAGAGTCTCCTCATCCTCGCAATCTTCTCCTGGCAATATACCATGCAGTCATTGTG
AGAGGTGCCACGAGCTTCGAGATTGACTCGTCTTTCAGTGTTGAGGCTTCGGAGATATAC
CCTGATGTTGAGTACACTTCTGTGGAAGAGTACCTTGGCTTTCTTGTCTGA
```

Figure 3.20. Two protein sequences (PM1 and PM2) resulted from RACE experiments.

A multiple sequence alignment by using ClustalW version 1.83 (www.ebi.ac.uk/-emboss/) was conducted to identify the relationship between already known PLRs with the proteins from *P. macrocarpa* (Fig. 3.21, Fig. 3.22).

```
Lp1_PLR_EF050530      -MKPCSVLVVGGTGYIGKRIVSASLYLGHDTYVLKRPGTGLDIEKLQLLLSFKKRGAHLV 59
Lu1_PLR_AJ849359      -MGRCRVLVVGGTGYIGKRIVKASIEHGHDTYVLKRPETGLDIEKFQLLLSFKKQGAHLV 59
PM1                   ----------|----------|----------|----------MLQDFKRQGAHLV 13
Fi1_PLR_U81158        -MGKSKVLIIGGTGYLGRRLVKASLAQGHETYILHRPEIGVDIDKVEMLISFKMQGAHLV 59
Th1_PLR_AF242501      ----------|----------|----------|----------MLLSFKQAGARLL 13
Th2_PLR_AF242502      ---MSRVLIVGGTGYIGRKFVKASLALGHPTFVLSRPEVGFDIEKVHMLLSFKQAGARLL 57
Tp4_PLR_AF242506      MDKKSRVLIVGGTGFIGKRIVKASLALGHPTYVLFRPEALSYIDKVQMLISFKQLGAKLL 60
PM2                   ----------|----------|----------AHELRDTAPSDPVKAKLIDSFKNLGVQML 29
                                                                :: .**  *.:::

Lp1_PLR_EF050530      EASFSDHDSLVRAVRLVDVVICTMSGVHFRSHNILLQLKLVEAIKEAG-----NVKRFIP 114
Lu1_PLR_AJ849359      EASFSDHESLVRAVKLVDVVICTVSGAHSRS--LLLQLKLVEAIKEAG-----NVKRFIP 112
PM1                   EASFEDHQSLVDAVEKADVVISAMSGVHFRSHNLLLQLKLVDAIKEAGNVKVGNVKRFLP 73
Fi1_PLR_U81158        SGSFKDFNSLVEAVKLVDVVISAISGVHIRSHQILLQLKLVEAIKEAG-----NVKRFLP 114
Th1_PLR_AF242501      EGSFEDFQSLVAALKQVDVVISAVAGNHFRN-LILQQLKLVEAIKEAG-----NIKRFLP 67
Th2_PLR_AF242502      EGSFEDFQSLVAALKQVDVVISAVAGNHFRN-LILQQLKLVEAIKEAR-----NIKRFLP 111
Tp4_PLR_AF242506      EASLDDHQGLVDVVKQVDVVISAVSGGLVRH-HILDQLKLVEAIKEAG-----NIKRFLP 114
PM2                   QGDLRDRESWVKAIKQADVVISAVGIPQVAD-----QTNIIAAIKEAG-----NVKKFYP 79
                      ...: * :. * .:. .****.::.           * ::: *****       *:*:* *

Lp1_PLR_EF050530      SEFGMDPAR-MGQAMEPGRETFDQKMVVRKAIEEANIPHTYISANCFAGYFVGNLSQLG- 172
Lu1_PLR_AJ849359      SEFGMDPAR-MGDALEPGRETFDLKMVVRKAIEDANIPHTYISANCFGGYFVGNLSQLG- 170
PM1                   SEFGMDPSR-MGHALEPGRVSFDDKMKVRKAIEEAQIPFTYVAANCFAGYFVVGLAQMG- 131
Fi1_PLR_U81158        SEFGMDPAKFMDTAMEPGKVTLDEKMVVRKAIEKAGIPFTYVSANCFAGYFLGGLCQFG- 173
Th1_PLR_AF242501      SEFGMEPDL-MEHALEPGNAVFIDKRKVRRAIEAAGIPYTYVSSNIFAGYLAGGLAQIG- 125
Th2_PLR_AF242502      SEFGMDPDL-MEHALEPGNAVFIDKRKVRRAIEAAGIPYTYVSSNIFAGYLAGGLAQIG- 169
Tp4_PLR_AF242506      SEFGMDPDV-VEDPLEPGNITFIDKRKVRRAIEAATIPYTYVSSNMFAGFFAGSLAQLQD 173
PM2                   SEFGPDCDR--INAVEPAATLIASKAVIRREIEALGIPYTYVCSNGFAGYFARSLLQIG- 136
                      **** :       .:**.   :  *  :*: **   **.**:.:* *.*::  .* *:

Lp1_PLR_EF050530      --TLTPPSDKVIIYGDGNVKVVYVDEDDVAKYTIKAIEDDRTVNKTVYLRPPENMMSQRE 230
Lu1_PLR_AJ849359      --PLTPPSDKVTIYGDGNVKVVYMDEDDVATYTIMTIEDDRTLNKTMYLRPPENVITHRQ 228
PM1                   --RITPPKDKVYLYAGGDIKVAFTDEDDVATYAIKTIDDPRTLNKTLYIRPPDNTLTQRQ 189
Fi1_PLR_U81158        --KILPSRDFVIIHGDGNKKAIYNNEDDIATYAIKTINDPRTLNKTIYISPPKNILSQRE 231
Th1_PLR_AF242501      --RLMPPRDEVVIYGDGNVKAVWVDEDDVGIYTLKTIDDPRTLNKTVYIRPLKNILSQKE 183
Th2_PLR_AF242502      --RLMPPRDEVVIYGDGNVKAVWVDEDDVGIYTLKTIDDPRTLNKTVYIRPLKNILSQKE 227
Tp4_PLR_AF242506      APRMMPARDKVLIYGDGNVKGVYVDEDDAGIYIVKSIDDPRTLNKTVYIRPPPMNILSQKE 233
PM2                   --ATSPPRDKVIILGNGNTKVVSNKEEDIATYTIRSIDDPRTLNKSVYIKPPKCTLSSNE 194
                          *. * * : ..*: *    .*:* . * : :*:* **:**::*: *    :: .:

Lp1_PLR_EF050530      LVAVWEKLSGNQLEKIELPPQDFLALMEGTTVAEQAGIGHFYHIFYEGCLTNFEINAENG 290
Lu1_PLR_AJ849359      LVETWEKLSGNQLQKTELSSQDFLALMEGKDVAEQVVIGHLYHIYYEGCLTNFDIDAAQD 288
PM1                   LVETWEQVSRNKLDKITLAADDFLASMKGMDLASQVGVGHFYHIFYEGCLTNFEIG--QG 247
Fi1_PLR_U81158        VVQTWEKLIGKELQKITLSKEDFLASVKELEYAQQVGLSHYHDVNYQGCLTSFEIG---D 288
Th1_PLR_AF242501      LVAKWEKLSGKCLKKTYISAEDFLAGIEDQPYEHQVGISHFYQMFYSGDLYNFEIG--PD 241
Th2_PLR_AF242502      LVAKWEKLSGKFLKKTYISAEDFLAGIEDQPYEHQVGISHFYQMFYSGDLYNFEIG--PD 285
Tp4_PLR_AF242506      VVEIWERLSGLSLEKIYVSEDQLLN-MKDKSYVEKMARCHLYHFFIKGDLYNFEIG--PN 290
PM2                   LVATWEKKICKTLEKAYVPEDEILKQIEESPHPRNLLLAIYHAVIVRG-ATSFEIDS-SF 252
                      :*  **:     *.*  :. :::*  ::      :      : .   *   .*:*.

Lp1_PLR_EF050530      EEEASRLYPEVEYTRVHDYLKIYL 314
Lu1_PLR_AJ849359      QVEASSLYPEVEYIRMKDYLMIYL 312
PM1                   AEEASQLYPEVQYTRMHDYLKIFL 271
Fi1_PLR_U81158        EEEASKLYPEVKYTSVEEYLKRYV 312
Th1_PLR_AF242501      GREATVLYPEVQYTTMDSYLKRYL 265
Th2_PLR_AF242502      GREATMLYPEVQYTTMDSYLKRYL 309
Tp4_PLR_AF242506      ATEGTKLYPEVKYTTMDSYMERYL 314
PM2                   SVEASEIYPDVEYTSVEEYLGFLV 276
                        *.: :**:*:*  :..*:   :
```

Figure 3.21. A multiple amino acid sequences of already known PLRs with proteins from *P. macrocarpa* (PM1 and PM2). The NADPH-binding domain (^{11}GXXGXXG^{17}) is marked in box.

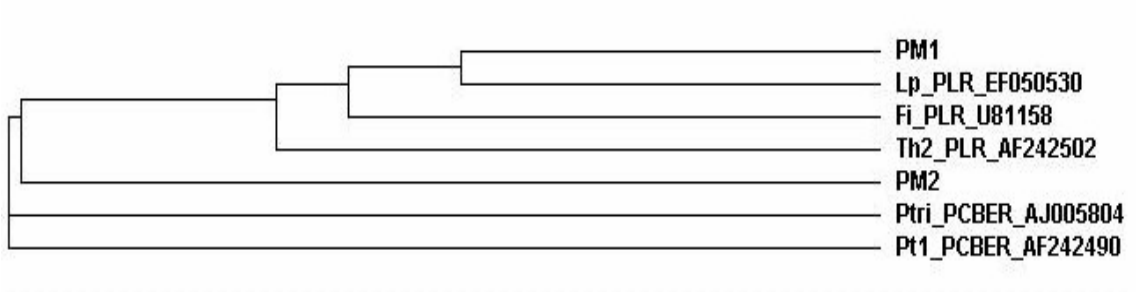

Figure 3.22. A dendrogram showing the relationship between proteins from *P. macrocarpa* (PM1 and PM2) and known PLRs and PCBERs

A Blastx search revealed highest sequence similarities of PM1 to PLRs from *Linum perenne* (EF050530, 67 % identity, 80 % similarity), *Forsythia intermedia* (U81158, 61 % identity, 79 % similarity) and *Tsuga heterophylla* (AF242502, 58 % identity, 72 % similarity). The sequence of PM1 was less similar to phenylcoumaran benzylic ether reductases (PCBERs) like the ones from *Pinus taeda* (AF242490, 45 % identity, 63 % similarity) and *Populus trichocarpa* (AJ005804, 39 % identity, 59 % similarity). PM2 showed highest sequence similarity to PCBERs like the ones from *Populus trichocarpa* (AJ005804, 63 % identity, 79 % similarity) and *Pinus taeda* (AF242490, 59 % identity, 75 % similarity) whereas it was less similar to PLRs from e.g. *Forsythia intermedia* (U81158, 47 % identity, 64 % similarity) and *Tsuga heterophylla* (AF242502, 45 % identity, 63 % similarity). These results indicate that PM1 is most probably a PLR whereas PM2 can be a PCBER.

The Lp1_PLR (PLR from *Linum perenne* var. *Himmelszelt*) and the Lu1_PLR (PLR from *Linum usitatissimum*) consist of 314 and 312 amino acids, respectively, whereas the PM1 and PM2 consist of 271 and 276 amino acids, respectively. Moreover, the already known PLRs have conserved ^{11}GXXGXXG^{17} [NAD(P)H-binding motif] sequences in their “N-terminal”, whereas the amino acids sequences of PM1 and PM2 were lack about 40 amino acids in their “N-terminal”.

The SWISS-MODEL was used to describe the tertiary structure of the protein PM1 and PM2 (Fig. 3.23 and 3.24). SWISS-MODEL is a fully automated protein structure homology-modeling server, accessible via the ExPASy web server, or from the program DeepView (Swiss Pdb-Viewer). Visualizations of the SWISS-MODEL were conducted by using the PyMOL which is a user-sponsored molecular visualization system on an open-source foundation (PyMOL software, DeLano Scientific LLC, South San Fransisco, California, USA).

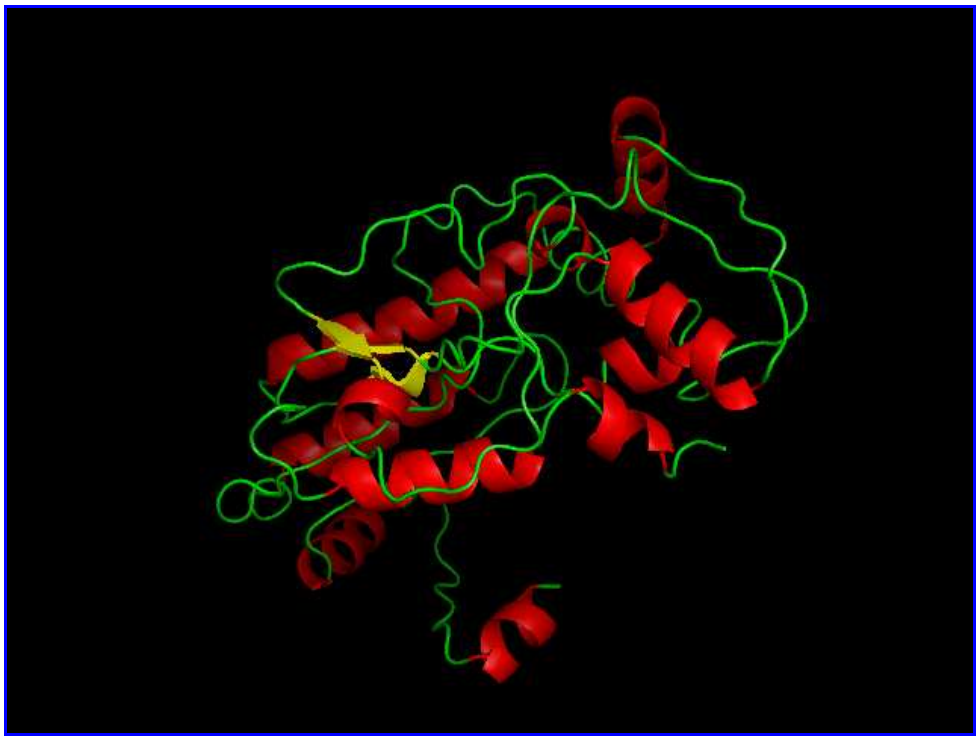

Figure 3.23. The SWISS MODEL of PM1

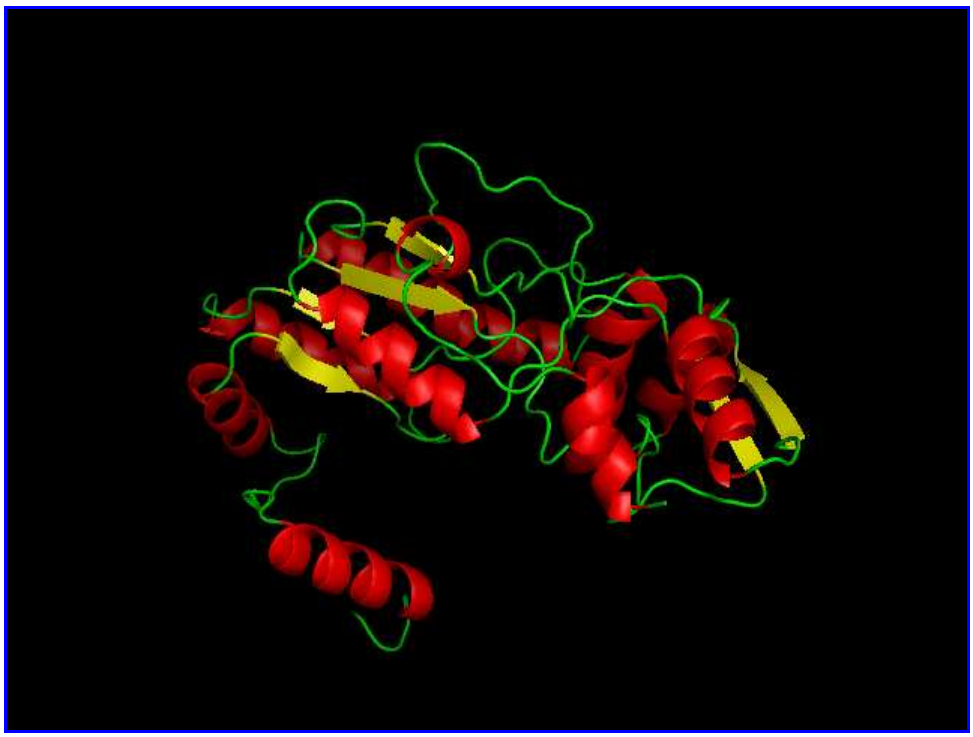

Figure 3.24. The SWISS MODEL of PM2

According to the SWISS-MODEL, the PM1 consist of 11 α-helixes, 3 ß-sheets, and 15 loops. Meanwhile, the PM2 consist of 11 α-helixes, 6 ß-sheets, and 18 loops.

3.3.4. Heterologous expression of PM1

The PM1 was amplified by PCR and the resulted PCR product was ligated into the expression vector pET15b (pOVETK19) for overexpression (chapter 2.3.6.3). The pOVET19 was transformed into *E. coli* Rosetta2 (DE3) cells. Protein overexpression and purification by metal chelate chromatography was performed as described by von Heimendahl and co-workers (2005). According to the SDS PAGE, the size of overexpressed protein was about 35 kilo Daltons (Fig. 3.25) and accumulated mostly in the pellet.

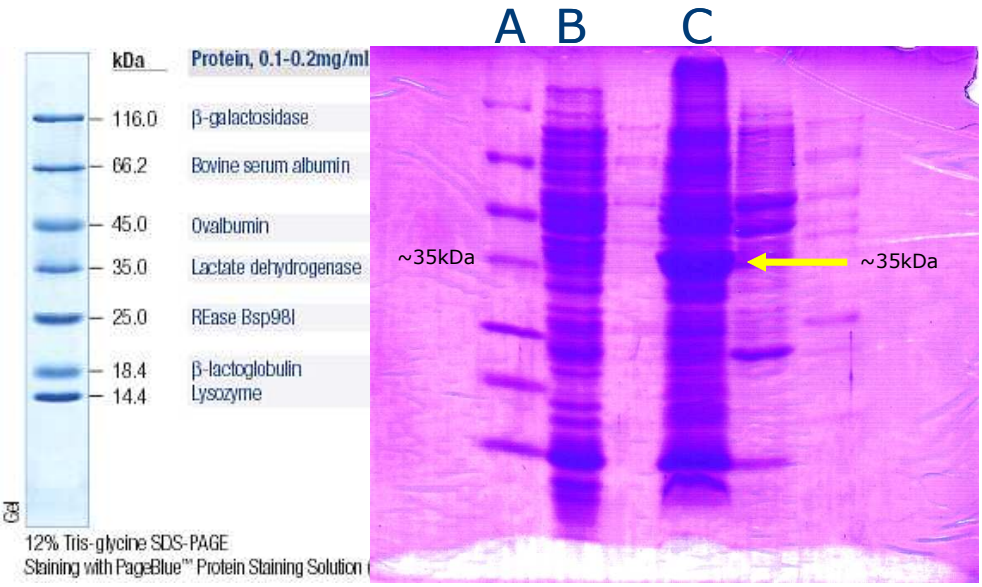

Figure 3.25. The SDS PAGE of proteins from *P. macrocarpa* (**A**=marker; **B**=crude protein; **C**=pellet)

3.4. PLR-like proteins from *L. flavum* var. *compactum*

RNA was extracted from young leaves as well as from flowers of *Linum flavum*. The cDNA was synthesized by using AMV kit according to the manufacturer's protocol (section 2.3.6.1). The resulting amplicon was ligated into pGEM-T, transformed into *E. coli* DH5α and sequenced (Fig. 3.26).

```
p0V3K2          NNNNGGGGCAGTCAGCTCCGGCCGCCTGGCCGCGGGATTGTGTATTTGACTTCGGGGTAC 60
p0V3K3          NNNNCCGACAGTCAGCTCCGGCCGCCTGGCCGCGGGATTGTGTATTTGACTTCGGGGTAC 60
p0V3K5          -NNAAGGCGAGCCAGCTCCGGCCGCATGGCCGCGGGATTGTGTATTTGACTTCGGGGTAC 59
p0V3K1          NNNAAAGCGAATCAGCTCCGGCCGCATGGCCGCGGGATTGTGTATTTGACTTCGGGGTAC 60
p0V3K4          NAAAAAGCGATTCAGCTCCGGCCGCATGGCCGCGGGATTGTGTACTTGACCTCGGGGTAC 60
p0V11K3         -NNNGGGCGATCCAGCTCCGGCCGCATGGCCGCGGGATTGTGTACTTGACCTCGGGGTAC 59
                      *  *  ************* ****************** ***** *********

p0V3K2          CTGCGATGCTATGTTGTCGGTAACAACAAGTCTTGGAATGGAATGTTCTCAGAAATTCTA 120
p0V3K3          CTGTGATGCTATGTTGTCGGTAACAACAAGTCTTGGAATGGAATGTTCTCAGAAATTCTA 120
p0V3K5          CTGTGATGCTATGTTGTCGGTAACAACAAGTCTTGGAATGGAATGTTCTCAGAAATTCTA 119
p0V3K1          CTGTGATGCTATGTTGTCGGTAACAACAAGTCTTGGAATGGAATGTTCTCAGAAATTCTA 120
p0V3K4          CTGTGATGCTATGTTGTCGGTAACAACAAGTCTTGGAATGGAATGTTCTCAGAAATTCTA 120
p0V11K3         CTGTGATGCTATGTTGTCGGTAACAACAAGTCTTGGAATGGAATGTTCTCAGAAATTCTA 119
                *** ************************************************************

p0V3K2          CCCTTCAGAGTTTGGGCCTGATTGCGACAGGATCAATGCTGTCGAGCCAGCAGCAACGTT 180
p0V3K3          CCCTTCAGAGTTTGGGCCTGATTGCGACAGGATCAATGCTGTCGAGCCAGCAGCAACGTT 180
p0V3K5          CCCTTCAGAGTTTGGGCCTGATTGCGACAGGATCAATGCTGTCGAGCCAGCAGCAACGTT 179
p0V3K1          CCCTTCAGAGTTTGGGCCTGATTGCGACAGGATCAATGCTGTCGAGCCAGCAGCAACGTT 180
p0V3K4          CCCTTCAGAGTTTGGGCCTGATTGCGACAGGATCAATGCTGTCGAGCCAGCAGCAACGTT 180
p0V11K3         CCCTTCAGAGTTTGGGCCTGATTGCGACAGGATCAATGCTGTCGAGCCAGCAGCAACGTT 179
                ************************************************************

p0V3K2          AATAGCATCGAAGGCTGTGATTCGGCGCGAGATAGAGGCCCTAGGGATCCCTTACACTTA 240
p0V3K3          AATAGCATCGAAGGCTGTGATTCGGCGCGAGATAGAGGCCCTAGGGATCCCTTACACTTA 240
p0V3K5          AATAGCATCGAAGGCTGTGATTCGGCGCGAGATAGAGGCCCTAGGGATCCCTTACACTTA 239
p0V3K1          AATAGCATCGAAGGCTGTGATTCGGCGCGAGATAGAGGCCCTAGGGATCCCTTACACTTA 240
p0V3K4          AATAGCATCGAAGGCTGTGATTCGGCGCGAGATAGAGGCCCTAGGGATCCCTTACACTTA 240
p0V11K3         AATAGCATCGAAGGCTGTGATTCGGCGCGAGATAGAGGCCCTAGGGATCCCTTACACTTA 239
                ************************************************************

p0V3K2          TGTATGCTCCAATGGATTTGCTGGTTACTTTGCCCGCAGTTTACTTCAGATTGGAGCTAC 300
p0V3K3          TGTATGCTCCAATGGATTTGCTGGTTACTTTGCCCGCAGTTTACTTCAGATTGGAGCTAC 300
p0V3K5          TGTATGCTCCAATGGATTTGCTGGTTACTTTGCCCGCAGTTTACTTCAGATTGGAGCTAC 299
p0V3K1          TGTATGCTCCAATGGATTTGCTGGTTACTTTGCCCGCAGTTTACTTCAGATTGGAGCTAC 300
p0V3K4          TGTATGCTCCAATGGATTTGCTGGTTACTTTGCCCGCAGTTTACTTCAGATTGGAGCTAC 300
p0V11K3         TGTATGCTCCAATGGATTTGCTGGTTACTTTGCCCGCAGTTTACTTCAGATTGGAGCTAC 299
                ************************************************************

p0V3K2          CTCTCCTCCCAGAGACAAAGTCATTATCCTTGGCAATGGGAATACGAAAGTCGTTTCCAA 360
p0V3K3          CTCTCCTCCCAGAGACAAAGTCATTATCCTTGGCAATGGGAATACGAAAGTCGTTTCCAA 360
p0V3K5          CTCTCCTCCCAGAGACAAAGTCATTATCCTTGGCAATGGGAATACGAAAGTCGTTTCCAA 359
p0V3K1          CTCTCCTCCCAGAGACAAAGTCATTATCCTTGGCAATGGGAATACGAAAGTCGTTTCCAA 360
p0V3K4          CTCTCCTCCCAGAGACAAAGTCATTATCCTTGGCAATGGGAATACGAAAG---------- 350
p0V11K3         CTCTCCTCCCAGAGACAAAGTCATTATCCTTGGCAATGGGAATACGAAAG---------- 349
                ************************************************

p0V3K2          CAAGGAGGAAGACATTGCAACTTACACAATCCGGAGTATTGATGATCCCAGAACCTTGAA 420
p0V3K3          CAAGGAGGAAGACATTGCAACTTACACAATCCGGAGTATTGATGATCCCAGAACCTTGAA 420
p0V3K5          CAAGGAGGAAGACATTGCAACTTACACAATCCGGAGTATTGATGATCCCAGAACCTTGAA 419
p0V3K1          CAAGGAGGAAGACATTGCAACTTACACAATCCGGAGTATTGATGATCCCAGAACCTTGAA 420
p0V3K4          ----GAGGAAGACATTGCAACTTACACAATCCGGAGTATTGATGATCCCAGAACCTTGAA 406
p0V11K3         ----GAGGAAGACATTGCAACTTACACAATCCGGAGTATTGATGATCCCAGAACCTTGAA 405
                    ********************************************************

p0V3K2          CAAGAGTGTGTACATTAAACCCCCCAAGTGTACACTCTCTTCCAATGAGCTTGTTGCAAT 480
p0V3K3          CAAGAGTGTGTACATTAAACCCCCCAAGTGTACACTCTCTTCCAATGAGCTTGTTGCAAT 480
p0V3K5          CAAGAGTGTGTACATTAAACCCCCCAAGTGTACACTCTCTTCCAATGAGCTTGTTGCAAT 479
p0V3K1          CAAGAGTGTGTACATTAAACCCCCCAAGTGTACACTCTCTTCCAATGAGCTTGTTGCAAT 480
p0V3K4          CAAGAGTGTGTACATTAAACCCCCCAAGTGTACACTCTCTTCCAATGAGCTTGTTGCAAT 466
p0V11K3         CAAGAGTGTGTACATTAAACCCCCCAAGTGTACACTCTCTTCCAATGAGCTTGTTGCAAT 465
                ************************************************************

p0V3K2          GTGGGAGAAGAGGATTTGTAAGACGCTTGAGAAGGCCTATGTTCCAGAGGACGAAATTCT 540
p0V3K3          GTGGGAGAAGAAGATTTGTAAGACGCTTGAGAAAGCCTATGTTCCAGAGGACGAAATTCT 540
p0V3K5          GTGGGAGAAGAAGATTTGTAAGACGCTTGAGAAAGCCTATGTTCCAGAGGACGAAATTCT 539
p0V3K1          GTGGGAGAAGAAGATTTGTAAGACGCTTGAGAAAGCCTATGTCCCAGAGGACGAAATTCT 540
p0V3K4          GTGGGAGAAGAAGATTTGTAAGACGCTTGAGAAAGCCTATGTTCCAGAGGACGAAATTCT 526
p0V11K3         GTGGGAGAAGAAGATTTGTAAGACGCTTGAGAAAGCCTATGTTCCAGAGGACGAAATTCT 525
                *********** ******************** ******** ****************
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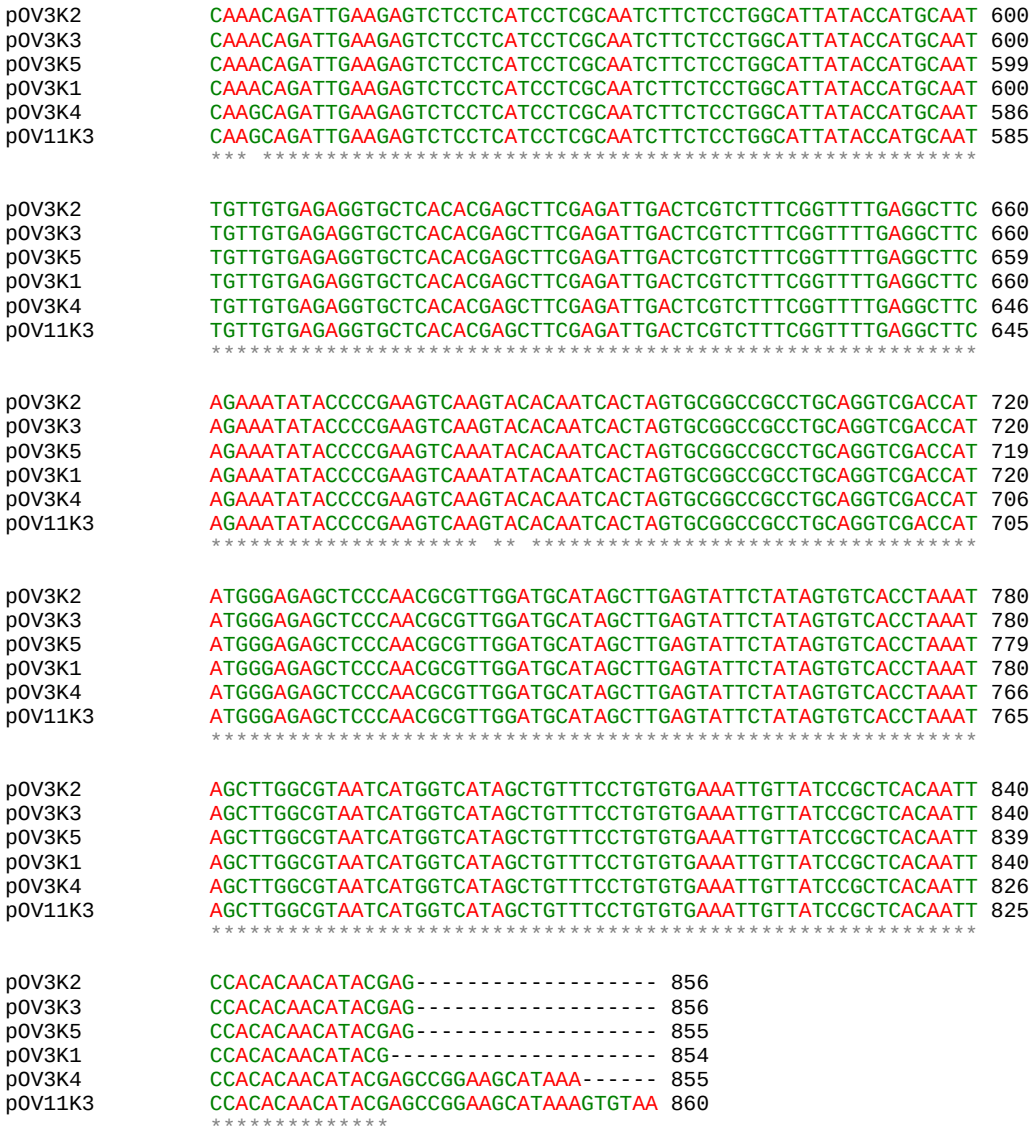

Figure 3.26. Multiple sequence alignment of cDNA from *L. flavum* var. *compactum* L.

According to the BLAST search, the whole partial-sequences (pOV3K1-pOV3K5 and pOV11K3) were similar (one clone) and had highest similarities to phenylcoumaran benzylic ether reductases. They were less similar to PLRs e.g. from *Tsuga heterophylla* AF242502 (Tab. 3.3).

Table 3.3. BLAST search result cDNA from *L. flavum* var. *compactum* L.

	% identity	% similarity
Populus trichocarpa PCBER (AJ005804)	62	79
Pinus taeda PCBER (AF081678)	62	79
Pinus strobus PCBER (DQ491575)	60	76
Tsuga heterophylla PLR (AF242502)	48	65

4. DISCUSSION

4.1. *In vitro* cultures of *Phaleria macrocarpa* (Scheff.) Boerl.

The *in vitro* cultivation of *P. macrocarpa* was successfully established. Callus cultures were subcultured in the modified MS medium containing 1.25 mg/l 6-benzyl-aminopurine (BAP) and 2.0 mg/l 2,4-dichlorophenoxy acetic acid / 2,4-D (Table 2.1) every 4 weeks whereas the cell suspension cultures were subcultured in the same liquid medium every 14 days.

Cell suspension culture was preferred while the existing microbial fermentation technology can be easily adapted to plant cell production. Plant cells are totipotent; therefore, cells in culture can theoretically produce the same metabolites as the whole plant. In this experiment, however, only the callus of *P. macrocarpa* could produce the lignan matairesinol, whereas the cell suspension culture of *P. macrocarpa* lost its ability to accumulate lignans.

The product profiles of callus or cell suspension cultures can differ from those of the parent plants. It is therefore not surprising that the production and yield of metabolites in cell culture will depend on a number of factors. Differences between the products of cell suspension culture and the whole plant may be the result of phenomena frequently observed in callus and cell suspension cultures such as the lack of differentiation and cell culture-induced variation.

The differences between RP-HPLC chromatograms of wood, callus, and cell suspension culture of *P. macrocarpa* (Fig. 3.5 – 3.7) are in good agreement with the explanation of Jalal and Collin (1978). They reported that callus cultures of *Theobroma cacao* contained markedly lower levels of certain polyphenols than the parent plants, and most of the polyphenols found in the callus culture were not detected in the plant at all. Furthermore, Saito (2007) reported that the hairy root culture of *Ophiorrhiza pumila* (Rubiaceae) could produce a high level of camptothecin and antraquinones whereas the cell suspension culture of that plant could not accumulate these secondary metabolites.

Some secondary metabolites accumulate entirely in specific structures, showing so-called morphological differentiation. For examples: Essential oils are found in glandular hairs of *Mentha piperita*, latex in laticifiers of *Papaver somniferum*, and tobacco alkaloids are primarily synthesized in roots. Thus, accumulation of such products occurs only if specific structures are present in the cultures. The yields in most callus and liquid cells suspension cultures are low. This could be due to the lack of tissue and organ differentiation (Chawla, 2004). Morphological and cellular differentiation is often necessary for the expression of many plant secondary metabolites. The lack of morpho-

logical differentiation in callus cells prevents formation of these metabolites. The lack of differentiation in callus cells may also disrupt regular metabolic pathways and may result in the accumulation of precursors of desired compounds (Collin and Watts, 1984).

Matairesinol was only detected in root, wood, and callus of *P. macrocarpa* but not in the cell suspension culture. This phenomenon could be a type of a so called “cell culture-induced variation” that frequently is seen among cell suspension cultures initiated from the same cell line. Large clone-to-clone variability in growth characteristics and secondary metabolite production are often observed in callus and cell suspension cultures (Hall and Yeoman, 1987). This variation may originate in the preexisting heterogeneity in parts of the plants, from which the culture was derived, or the cell culture process itself may introduce the changes. It is well known that increasing numbers of subculture could also increase the likelihood of variation, so the number of subcultures in micro propagation protocols should be kept to a minimum. Regular reinitiating of clones from new explants might reduce variability over time.

Rapidly-growing plant cell suspension cultures, initiated and maintained by repeated subculturing on the same medium, are the result of an unconscious but systematic selection for those cells in a heterogeneous population with the best growth rate. Thus, culture medium, which is suitable for growth of cells, may not be suitable for production of secondary metabolite by the cell (Chawla, 2003).

The type and concentration of growth regulators in cell suspension is probably one of the most important factors influencing their potential for secondary product synthesis. In this experiment, cell suspension cultures were grown in a liquid modified MS-culture medium (Murashige and Skoog, 1962) containing 1.25 mg/l 6-benzylamino-purine (BAP) and 2.0 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D). According to Chawla (2004), 2,4-D can stimulate both cell division and cell expansion, but it is known to introduce variation and it can also bring about a dramatic suppression of secondary metabolite synthesis. This could be a reason that the cell suspension culture of *P. macrocarpa* lost its ability to produce matairesinol.

In contrast, Wichers and co-workers (1990) demonstrated that cell cultures of *L. flavum* could produce amounts of 6-methoxypodophyllotoxin that are comparable to the concentration in fully differentiated plants. The production of 6-methoxypodophyllo-toxin depends on the hormonal balance of the growth medium. The use of 2,4-dichlorophenoxyacetic acid (2,4-D) as the growth regulator was favorable for 6-methoxypodophyllotoxin production when compared to naphthylacetic acid (NAA).

4.2. Extraction and identification of lignans in *Phaleria macrocarpa* (Scheff.) Boerl.

In this study, pinoresinol, lariciresinol and matairesinol could be detected in the different organs of *P. macrocarpa* by HPLC in comparison to authentic standards. The wood and the root were especially rich in pinoresinol, but contained lariciresinol and matairesinol as well. The seeds contained smaller amounts of pinoresinol and lariciresinol. Matairesinol could not be detected in the seeds. Lariciresinol was the only lignan which could be detected in the bark, leaf and fruit flesh.

The fragmentation patterns of the lignans collected from wood extract of *P. macrocarpa* were identical to the one of authentic standards in HPLC-mass spectrometry. Pinoresinol, lariciresinol, and matairesinol appeared at m/z 357[M-H]$^-$, 359[M-H]$^-$, and 357[M-H]$^-$ respectively in the negative mode of ESI-MS which correspond to the molecular weight of 358, 360, and 358 respectively.

Pinoresinol and lariciresinol isolated from *P. macrocarpa* were mixtures of both enantiomers. Moreover, the protein fraction collected from a 14 days old cell suspendsion culture of *P. macrocarpa* catalyzed the formation of lariciresinol from racemic pinoresinol which indicate the occurrence of the PLR in the cell suspension culture of *P. macrocarpa.* Since pinoresinol and lariciresinol were mixtures of both enantiomers, it is postulated that *P. macrocarpa* has two pinoresinol-lariciresinol reductase (PLR) isoforms that reduce the opposite enantiomers of pinoresinol, as in the case of the *Arctium lappa* enzymes and *Thuja plicata* recombinant PLR isozymes.

The accumulation of pure (+)-matairesinol in *P. macrocarpa* indicated that only (+)-secoisolariciresinol could be converted into (+)-matairesinol. Therefore it is also postulated that *P. macrocarpa* has only one NAD-dependent secoisolariciresinol dehydrogenase (SDH) which converted (+)-secoisolariciresinol into (+)-matairesinol.

A lignan similar to syringaresinol was isolated from the mesocarp of *P. macrocarpa* and it was identified as *5-[4(4-Methoxy-phenyl)-tetrahydrofuro[3,4-c] furan-1-yl]-benzene-1,2,3-triol* by Lisdawati (2002). This lignan could be a precursor of matairesinol in this plant.

Syringaresinol, pinoresinol, daphnoretin, and wikstromol were identified from *Wikstroemia foetida* var. oahuensis and *Wikstroemia uva-ursi* which are members of the family Thymelaeaceae (Torrance, *et al.*, 1979). Beside that, Okunishi and co-workers (2002) initiated the cell suspension culture of *Daphne odora* which is also a member of family Thymelaeaceae that could produce pinoresinol, lariciresinol, secoisolariciresinol,

matairesinol, and wikstromol. The production of matairesinol in that cell suspension culture was much higher than that in *Daphne odora* stem tissues.

Furofuran and furan lignans have been isolated from *Wikstroemia, Daphne, Passerina,* and *Dirca* plants (Thymelaeaceae). Many of them have the opposite configuration at C8 and C8' to those of the (+)-pinoresinol and (+)-lariciresinol isolated from *Forsythia* plants as in the following examples: (-)-pinoresinol, (-)-lariciresinol and (-)-dihydrosesamin from *Daphne tangutica* and from *Dirca occidentalis*. In contrast, the isolation of dextrorotatory furofuran lignans, (+)-pinoresinol from *Wikstroemia viridiflora* and (+)-syringaresinol from *Passerina vulgaris* and *Dirca occidentalis,* have been reported, and even racemic furan lignans, (±)-lariciresinols and (±)-5-methoxy-lariciresinols, have been isolated from *Wikstroemia elliptica* (Umezawa, *et al.,* 1998).

The levorotatory dibenzylbutyrolactone lignans such as (-)-matairesinol, (-)-arctigenin, and (-)-nortrachelogenin [=(-)-wikstromol] have been isolated from many plants including *Forsythia spp., Arcticum lappa,* and *Trachelospermum asiaticum* var. intermedium. In contrast, several plants from the family of Thymelaeaceae produce dextrorotatory dibenzylbutyrolactone lignans such as (+)-matairesinol from *D. odora* and *D. genkwa* (Okunishi, *et al*., 2002) and (+)-arctigenin from *W. indica* (Umezawa, *et al.*, 1998).

The optical rotation of a furofuran lignan can vary even within plants of the same genus: (-)-pinoresinol with 74% e.e. was isolated from *W. sikokiana,* but the (+)-pinoresinol was obtained from *W. viridiflora.* A comparable difference in enantiomeric composition was also observed for a furan lignan isolated from *Wikstroemia* plants: (-)-lariciresinol with 39% e.e. was extracted from *W. sikokiana,* while racemic lariciresinol was obtained from *W. elliptica* (Umezawa, *et al.*, 1998). Furthermore, pinoresinol and lariciresinol isolated from *D. odora* and *D. genkwa* has their enantiomeric compositions ranged from 88% to 95% e.e. in favor of (–)-enantiomers (Okunishi, *et al*., 2002).

The predominant enantiomers of pinoresinol, lariciresinol, and matairesinol isolated from *P. macrocarpa* were opposite to those obtained from *Forsythia* plants. In additon, the optically pure dibenzylbutyrolactone lignan from *P. macrocarpa,* (+)-matairesinol, has the S,S configuration at C-atoms 8,8', which is opposite to (-)-matairesinol with R,R- configuration at C-atoms 8,8' isolated from *Forsythia.* Therefore, it is obvious that different stereochemical mechanisms are operating in plants that produce (or accumulate) this different series of enantiomeric lignans.

Pinoresinol and lariciresinol from the wood extract of *P. macrocarpa* were mixtures of both enantiomers with 79 ± 4% e.e. and 55 ± 6% e.e. for the (-)-enantiomers respectively. This enantiomeric purities are lower than those lignans isolated from *Forsythia* plants, and those of the lignans obtained from *in vitro* reactions with *Forsythia* enzymes with more than 97% e.e of (+)-pinoresinol and (+)-lariciresinol that almost optically pure. The findings indicated that the formation of these lignans in *P. macrocarpa* was less enantioselective than that in *Forsythia* plants. Catalysis by less enantioselective enzymes or contribution from two types of ezymes could account for the enantiomeric composition of the *P. macrocarpa* lignans.

Analysis of the enantiomeric compositions of the *P. macrocarpa* lignans has indicated that the stereochemical control mechanisms involved in lignan biosynthesis in *P. macrocarpa* are quite similar to *W. sikokiana* and different from those in *Arctium lappa* and *Forsythia spp.*

The isolation of dextrorotatory and optically pure matairesinol from *P. macrocarpa* is in good agreement with the previous isolation of the dextrorotatory dibenzylbutyrolactone lignans (matairesinol, arctigenin, and wikstromol) from other Thymelaeaceae plants. This result suggests that the occurrence of the dextrorotatory dibenzylbutyrolactone lignans is one of the special characteristic of *P. macrocarpa.* Therefore, the precise stereochemical mechanisms involved in formation of the optically pure dibenzylbutyrolactone lignans in *P. macrocarpa* will be an interesting subject of a future study with isolated enzymes.

4.3. Extraction and identification of lignans in *Linum flavum* var. *compactum* L.

This study was conducted to identify the lignan content in different organs of *L. flavum* var. *compactum* in different developmental phases. The plant organs were root, stem, leaves, flower-buds, flower, and capsules. The lignan content in vegetative organs (root, stem, leaves) was observed in three different developmental phases namely the phase before the formation of flower or growth phase, the flowering phase, and the phase during the formation of capsules. The lignan contents in generative organs were observed in five different developmental phases namely flowerbuds, flowers, capsules in early phase, capsules in middle phase, and capsules in late phase (see section 2.1.1; Tab. 2.1 and Fig 2.2).

Seven known lignans were found in all organs by using HPLC in comparison to authentic standards. The lignans are pinoresinol, lariciresinol, 7-hydroxysecoisolariciresinol, podophyllotoxin, α-peltatin, ß-peltatin and 6-methoxypodophyllotoxin. Moreover, the lignan 6-methoxypodophyllotoxin was found in all parts of *L. flavum* var. *compactum* with the highest amount were found in root and capsule early stage. The lignans pinoresinol, lariciresinol, 7-hydroxy-secoisolariciresinol, podophyllotoxin were found in all parts of *L. flavum* var. *compactum* in relatively low amount. There was no extreme difference of lignans content harvested in 2005 and 2006, except that the amount of α-peltatin in leaves "growth phase" harvested in 2005 was about twice as high as in leaves "growth phase" harvested in 2006.

Secondary metabolites are often transported from source cells to neighboring cells, or even further to other tissues or remote organs (Yazaki, 2005). Accumulation or secretion of secondary metabolites has to be highly regulated, for instance, flavonoids acting as UV protectant are specifically accumulated in epidermal cells (Schmitz-Hoerner and Weissenbock,, 2003), whereas insect attractants are emitted from flower petals (Kolosova, *et al.*, 2001).

Biosynthetic genes responsible for the formation of secondary metabolites may be highly expressed in such tissues where the metabolites are mainly accumulated, while translocation of these metabolites among plant organs often occurs as well (Shoji, *et al.*, 2000). As an illustration, biosynthetic genes for nicotine in *Nicotiana* species are mostly expressed in root tissues as the source organ; however nicotine is transported to the aerial part and accumulated in leaves as the sink organ (Shoji, *et al.*, 2000).

Many phenolic compounds including the lignans are detected as glycosylated form in plants (Broomhead and Dewick, 1990; Wichers *et al.*, 1991; van Uden *et al.*, 1997). Glucosidation plays a key role in detoxification of endogenous secondary metabolites in plants and these glucosides often accumulate in the vacuoles (Yazaki, 2006). Special transporter proteins are involved in the vacuolar translocation of such glycosides (e.g. MRP-type ATP Binding Cassette (ABC) transporters, Bartholomew, *et al.*, 2002).

The root-like tissue culture (Wichers, *et al.*, 1991), hairy roots culture (Oostdam, *et al.*, 1993), and shooty suspension culture (Konuklugil, *et al.*, 2001) of *L. flavum* could synthesize 6-methoxypodophyllotoxin. Therefore, it is most likely that 6-methoxypodophyllotoxin is produced and accumulated *in vivo* in roots and stems of this plant.

Jonasson and co-workers (1986) claimed that the fluctuation in the level of secondary metabolites might be caused by climatic variations. For instance, the carbon-nutrient balance could be altered by climatic variation that changes carbohydrate production. When carbohydrates are produced in excess of growth demands, excess photosynthates might be used for the production of phenolics or terpenes. Conversely, when photosynthesis is more limiting than nutrient absorption, carbohydrate concentration in plant tissues decline, as do phenolic or terpene levels.

According to Briskin (2002), the "carbon-nutrient balance" model was developed by Bryant and co-workers in 1983 to explain the effects of soil nutrient supply and light levels on secondary product metabolism. A central theme of this model is that the carbon/nitrogen (C/N) ratio of the plant under a given set of environmental conditions will have a strong bearing on the types and levels of secondary products generated by the plant. In this model, production of "carbon-based" secondary products (e.g., phenolics, terpenes, and other chemicals having only C, O, and H as part of their structure) would be directly proportional to the C/N ratio, whereas production of nitrogen-based secondary products (e.g., alkaloids, cyanogenic glycosides, nonprotein amino acids) would be inversely proportional to the C/N ratio. For example, under conditions of adequate light and low nitrogen supply, the C/N ratio of the plant would increase and this would lead to increased production of carbon-based secondary products. On the other hand, with conditions such as low light and adequate nitrogen that lead to a decrease in the plant C/N ratio, increased production of nitrogen-containing secondary products would be expected. Several studies have shown that soil fertilization tends to increase levels of nitrogen-based secondary products (Gershenzon, 1984) and decrease levels of carbon-based secondary products (Bryant, *et al.*, 1987).

On contrary, Zimmermann and co-workers (2007) examined the effects of two locations and enhanced nitrogen fertilization at one site on 12 linseed (*L. usitatissimum*) varieties. It could be shown that the variability of both lignans caused by the factor "cultivar" was much greater than either "location" or "fertilizer intensity". The ranking of the cultivars was very consistent, however, indicating that the genotypic share of the expression was greater than the phenotypic share.

The result of Zimmermann and co-workers (2007) is parallel with the result of this experiment, since the lignan content in *L. flavum* var. *compactum* harvested in different years were almost the same and there was no dramatic variation of the climate during harvesting in 2005 and 2006 (Tab. 4.1 – 4.3).

The average temperature in April, June, and August 2006 were quite similar to the average temperature in April, June, and August 2005. It was slightly warmer in May and July 2006 in comparison to the temperature in May and July 2005.

The average rainfall during June-July 2005 (51 – 75 mm) was slightly higher than the average rainfall during June-July 2006 (26 – 50 mm), whereas the average rainfall during May 2005 (76 – 100 mm) was lower than the average rainfall during May 2006 (126 – 150 mm). The average rainfall on April 2005 (51 – 75 mm) was similar with the average rainfall on April 2006.

The length of sunshine's during April - May 2005 and during April - May 2006 were similar (126-150 and 176-200 hours/month, respectively). The sunshine's duration during June and July 2005 were shorter than on June and July 2006. However, the sunshine's duration during August 2005 was longer than in August 2006.

Table 4.1. Temperature (°C) in Düsseldorf

Months	Years	
	2005	2006
April	10.1 – 12.0	8.1 – 10.0
May	12.1 – 14.0	14.1 – 16.0
June	16.1 – 18.0	16.1 – 18.0
July	18.1 – 20.0	22.1 – 24.0
August	16.1 – 18.0	16.1 – 18.0

(source: www.dwd.de/de/FundE/Klima/KLIS/daten/online/klimakarten/index.htm).

Table 4.2. Rainfall (mm) in Düsseldorf

Months	Years	
	2005	2006
April	51 - 75	51-75
May	76 - 100	126 - 150
June	51 - 75	26 - 50
July	51 - 75	26 - 50
August	101 - 125	126 - 150

(source: www.dwd.de/de/FundE/Klima/KLIS/daten/online/klimakarten/index.htm).

Table 4.3. Sunshine (hours per month) in Düsseldorf

Months	Years	
	2005	2006
April	126 - 150	126 - 150
May	176 - 200	176 - 200
June	226 - 250	251 - 275
July	201 - 225	325 - 350
August	151 - 175	101 - 125

(source: www.dwd.de/de/FundE/Klima/KLIS/daten/online/klimakarten/index.htm).

Linum flavum var. *compactum* has demonstrated its capability to produce and store relatively large amounts of cytotoxic aryltetralin lignans structurally related to podophyllotoxin. Lignans 6-methoxypodophyllotoxin, its glycosides and acetate are the main constituents of roots and stem/leaves of this plant with the roots containing up to 3.5% dry weight of these compounds. Moreover, almost 90% of the 6-methoxypodophyllotoxin was glycosilated (Broomhead and Dewick, 1990). Podophyllotoxin and ß-peltatin were also detected in the root of *L. flavum* var. *compactum*. However, both of them were not detected in aerial parts (stem/leaves) of this plant. Furthermore, Broomhead and Dewick (1990) observed the occurrence of α-peltatin in the stem/leaves of this plant.

Wichers and co-workers (1991) found that the roots of *L. flavum* contain over 48% of total podophyllotoxin derivatives which appear to be the major site of accumulation of these compounds since the roots comprise only 15% of total plant mass. The glycoside of 6-methoxypodophyllotoxin was found to a major extent in the leaves on a basis of absolute amounts (79%) and in roughly equal amounts in leaves and roots on a dry weight basis. Furthermore, podophyllotoxin and matairesinol were also detected in *in vitro* culture of *L. flavum.*

Konuklugil (1997) reported the presence of coniferyl alcohol, pinoresinol, podophyllotoxin, syringin, and coniferin in the ethanolic extract of *L. flavum.* Moreover, lariciresinol was also identified by GC-MS in this plant (Sicilia, *et al*., 2003). Furthermore, Mikame and co-workers (2002) found a new lignan called 7,6'-dihydroxybursehernin, along with six known lignans namely 6-methoxypodophyllotoxin, α-peltatin, ß-peltatin, pinoresinol, lariciresinol, and secoisolariciresinol in *L. flavum* var. *compactum*. However, cell and root cultures of *L. flavum* produce only 6-methoxypodophyllotoxin (Berlin *et al.*, 1986; van Uden, *et al.*, 1990; Wichers, *et al.*, 1990; Wichers, *et al.*, 1991).

According to Molog and co-workers (2001), cell-suspension cultures of *L. flavum* synthesized and accumulated aryltetraline lactone lignans with 6-methoxypodophyllotoxin as the main component. The biosynthesis of 6-methoxypodophyllotoxin occurs via deoxypodophyllotoxin, α-peltatin, and ß-peltatin-A methyl ether. The enzyme catalyzing the introduction of the hydroxyl group in position 6 was deoxypodophyllotoxin 6-hydroxylase (DOP6H). The enzyme was shown as a cytochrome P450-dependent monooxygenase by blue-light reversion of carbon monoxide inhibition and inhibition by cytochrome *c*. The DOP6H was a membrane-bound microsomal enzyme with a pH optimum of 7.6 and a temperature optimum of 26 °C (Molog, *et al*., 2001).

Little information was available concerning biosynthetic steps from matairesinol to podophyllotoxin in *L. flavum* var. *compactum*. Up to date, there are at least two theories regarding the biosynthesis of podophyllotoxin starting from matairesinol. The first theory was published by Dewick and co-workers which claimed that matairesinol, yatein, and deoxypodophyllotoxin could be converted into podophyllotoxin (Jackson and Dewick, 1984; Kamil and Dewick, 1986), whereas the second theory is based on the observation that the feeding of matairesinol to roots of *L. flavum* resulted in the formation of 7-hydroxymatairesinol which afterward will be converted into 6-methoxy-podophyllotoxin (Xia, *et al.*, 2000).

Van Uden and co-workers (1995) delivered evidence for the first theory by feeding deoxypodophyllotoxin to *L. flavum* cells, which resulted in the formation of 6-methoxy-podophyllotoxin and much smaller amounts of podophyllotoxin. Deoxypodophyllotoxin was regarded as a precursor for podophyllotoxin, β-peltatin and 6-methoxypodophyllo-toxin. The 4-hydroxylation of deoxypodophyllotoxin results in podophyllotoxin, where-as 6-hydroxylation leads to β-peltatin. The formation of 6-methoxypodophyllotoxin requires either 6-hydroxylation and methylation of podophyllotoxin or 4-hydroxylation and 6-methylation of β-peltatin. In *L. flavum* suspension cultures, deoxypodophyllo-toxin and β-peltatin were converted to 6-methoxypodophyllotoxin and their β-gluco-sides (van Uden *et al.*, 1997).

Cell suspension cultures of *L. flavum* are able to convert large amounts of deoxypodophyllotoxin into the glycoside of 6-methoxypodophyllotoxin (Koulman, *et al.*, 2003). Recent studies showed that the first step from deoxypodophyllotoxin towards 6-methoxypodophyllotoxin was carried out by deoxypodophyllotoxin 6-hydroxylase (Molog, *et al.*, 2001).

Xia and co-workers (2000) proved that (-)-matairesinol was hydroxylated into 7-hydroxymatairesinol and the feeding of synthetic labelled (^{13}C) 7- hydroxylmatairesinol to *Linum flavum* roots yielded labelled 6-methoxypodophyllotoxin (Xia, *et al.*, 2000).

In this experiment, the extraction method of Westcott and Muir (2000) was applied beside the extraction method of Wichers (1990) in order to determine the lignan content in seeds of *Linum flavum* var. *compactum*. The Westcott and Muir method was based on base-hydrolysis, whereas the Wichers method was based on enzymatic-hydrolysis. The results showed that base-hydrolysis was more effective to pull out α-peltatin, ß-peltatin, and 6-methoxypodophyllotoxin in comparison to the enzymatic-hydrolysis. However, the amount of 6-methoxypodophyllotoxin was found to be higher by using the enzymatic-hydrolysis.

Kartal and co-workers (2004) compared several extraction methods for the determination of podophyllotoxin and its derivates from *Linum* species. No statistical differences on the percentage of recovery were found between the methods. The glycosidase-method showed the best result with respect to the accuracy studies whereas the acetone-method has an advantage compared to the other methods due to its capability to calculate the aglycon, lignan glycoside and total lignan. Apparently, the glycosidase-method showed the proper result due to the fact that podophyllotoxin, 6-methoxypodophyllotoxin, and α- and β-peltatin are present as glucosides (Broomhead and Dewick, 1990; Wichers *et al.*, 1991; van Uden *et al.*, 1997).

4.4. Cloning of cDNA encoding PLR-like protein

The PM1 and PM2 consist of 271 and 276 amino acids, respectively, whereas the Lp1_PLR (PLR from *Linum perenne* var. Himmelszelt) and the Lu1_PLR (PLR from *Linum usitatissimum*) consist of 314 and 312 amino acids, respectively. Moreover, the amino acids sequences of PM1 and PM2 were lack about 40 amino acids in their "N-terminal".

According to the energy minimized model proposed by Min and co-workers (2003), the already known PLRs have conserved ^{11}GXXGXXG^{17} [NAD(P)H-binding motif] sequences in their "N-terminus". Moreover, there are three conserved positions in most PLRs namely Leucine^{164}, Glycine^{268}, Phenylalanine^{272} which are discussed to be responsible for the enantioselectivity of (+)-pinoresinol and Phenylalanine^{164}, Valine^{268}, Leucine^{272} for the preference of (-)-pinoresinol. Based on amino acids sequence alignment, the PM1 has Glycine in position 226 and Phenylalanine in position 121 and 230. Therefore, the PM1 has a high similarity with the model of Tp2_PLR [L164, G268, F272] and Tp1_PLR [F164, V268, L272] proposed by Min et al. (2003).

The RACE experiments and the cloning steps were successfully conducted. On contrary, the efforts to capture the full-length sequence of PLR from *P. macrocarpa* yielded only two partial sequences. It means that the unsuccessful efforts to capture the full-length sequence of PLR from *P. macrocarpa* could have occured due to the failure to accomplish the complete cDNA as the starting material in those molecular biology works.

All commonly used cDNA synthesis methods rely on the ability of reverse transcriptase (RT) to transcribe mRNA into single-stranded (ss) DNA in the first strand reaction. In some cases, RT terminates before transcribing the complete mRNA sequence. This is particularly true for long mRNAs, especially if the first strand

synthesis is primed with oligo(dT) primers only or if the mRNA contains abundant secondary structures. As a result, under-represented 5' ends of genes in cDNA populations tend to be 5–30 nucleotides shorter than the original mRNA (D'Allessio, 1988).

The secondary structure of an RNA molecule is the collection of base pairs that occur in its three dimensional structure. It is similar to an alignment of protein and nucleic acid sequences. Therefore, an RNA secondary structure is a simplification of a complex three dimensional folding of a biopolymer.

Several methods are used to predict the RNA structure. Once the primary structure is known, the next step would be to identify its function. The first step in achieving the RNA structure would be to predict its secondary structure. The secondary structure of RNA can expose biologically relevant features. There are two main approaches used to predict the secondary structure namely free energy estimation and multiple sequence alignments.

Most of the RNA prediction methods are based on free energy estimates. This method looks for the fold with the lowest free energy out of possible folds. The fold with more negative free energy is more stable, since it releases more stored energy. The free energy of a fold is the addition of free energy of all the motifs found in the structure. This works with single sequences. Meanwhile, the multiple sequence alignments use the alignments of many homologous sequences to predict the structure.

Most of the RNA folding algorithms can predict the secondary structure. However, these algorithms are not that successful in predicting tertiary structure. Tertiary structure of RNA is investigated by X-ray crystallography and NMR. Secondary structure predictions can be used along with NMR and X-ray crystallography in determining the three dimensional (3D) structure.

The secondary structure of RNA can be predicted by using an RNA secondary structure prediction tool such as GENE BEE (http://darwin.nmsu.edu/~molb470/fall-2003/Projects/tumban/RNA_secondary_structure_prediction.html). The GENE BEE can be used to predict the secondary structure of RNA of multiple alignments including the sequence of interest. If the sequence itself is used, the structure is predicted using energy model (which give the structure with minimum energy). When multiple sequences are given, the secondary structure predicted included the information on conservative positions within the sequences. The GENE BEE therefore can be used to predict the secondary structure of RNA from *P. macrocarpa.*

By submitting the sequences of PM1 and PM2 to the GENE BEE, the secondary structure of RNA from *P. macrocarpa* can be predicted. The RNA secondary structures prediction of *P. macrocarpa* are in good agreement with the description of D'Allessio (1988), that the fail to accomplish the cDNA could be due to the fail of reverse transcriptase to transcript the complex mRNA into a proper cDNA.

Up to date, there are no reports regarding the recombinant pinoresinol-lariciresinol reductase from *L. flavum*. Therefore it is interesting to characterize the pinoresinol-lariciresinol reductase from this plant. In this experiment, however, the RT-PCR of *L. flavum* yielded a partial-sequence with highest similarities to phenylcoumaran benzylic ether reductase (PCBER).

Xia and co-workers (2000) reported that optically pure (+)-lariciresinol and optically pure (-)-secoisolariciresinol were formed following incubation of racemic pinoresinol with a crude pinoresinol-lariciresinol reductase preparation from *L. flavum.* It was demonstrated that the biochemical pathway leading to formation of (-)-matairesinol in *L. flavum* parallels whit that occurring in *F. intermedia*. In contrast, it has been found that there was a great stereochemical diversity in the upstream steps of lignan biosynthesis. Mikame and co-workers (2002) proved that secoisolariciresinol isolated from *L. flavum* var. *compactum* was an optically pure (-)-enantiomer, whereas pinoresinol and lariciresinol are not, with 65% and 70% enantiomer excess (e.e.) in favor of (+)-enantiomers, respectively. Therfore, they postulated that *L. flavum* has two pinoresinol-lariciresinol reductase isoforms that reduce the opposite enantiomers of pinoresinol.

5. OUTLOOK

Callus and suspension cultures from *Phaleria macrocarpa* could be established. The differentiated plant is able to accumulate small amounts of lignans up to matairesinol. Lignan accumulation stops in suspension cultures after some subcultivations. Since matairesinol was detected in roots, the development of hairy root cultures could be promising in order to produce matairesinol and - perhaps - other lignans to study the biosynthesis in this plant family.

Secoisolariciresinol could not yet be detected in *P. macrocarpa*. In principle, its occurrence should be expected due to the identification of matairesinol. Chiral HPLC demonstrated that pinoresinol and lariciresinol contained besides the (-)-enantiomer substantial amounts of the opposite enantiomer. Hairy root cultures could serve as an interesting system to study, at which stage of biosynthesis an enantiomeric pure lignan is formed. This could be at the secoisolariciresinol or at the matairesinol stage. Hairy root cultures could serve as well for completing the ORF of PLR as well as to search for a secoisolariciresinol dehydrogenase (SDH) responsible for the step from secoisolariciresinol to matairesinol.

Seven known lignans (pinoresinol, lariciresinol, 7-hydroxysecoisolariciresinol, podophyllotoxin, α-peltatin, ß-peltatin and 6-methoxypodophyllotoxin) accumulated in all organs of *Linum flavum* var. *compactum*. Further research regarding their formation and translocation among plant organs is necessary to conduct in order to understand the source organs and the sink (target) organs.

Since Mikame and co-workers (2002) postulated that *L. flavum* has two PLR isoforms and since there are no reports regarding the recombinant PLR from *L. flavum* up to now, it is still interesting to characterize the PLR from this plant.

6. SUMMARY

Lignans are a class of secondary metabolites, which mostly are optically active. The lignans secoisolariciresinol and matairesinol are precursors of the mammalian lignans enterolactone and enterodiol. The latter are believed to be responsible for preventing the expansion of prostate and breast cancers.

Phaleria macrocarpa (Scheff.) Boerl., a member of the Thymelaeaceae, is traditionally used in Indonesia as medicinal plant against cancer, diabetes, hypertension, and cardiovascular diseases. The lignans pinoresinol, lariciresinol, and matairesinol were found in different organs of *P. macrocarpa*. Meanwhile, only matairesinol was detected in the initial callus and no lignans were observed in the cell suspension cultures of this plant. Pinoresinol and lariciresinol isolated from the wood extract consist of a mixture of both enantiomers, whereas matairesinol was found as pure (+)-enantiomer. These results support previous reports stated that the Thymelaeaceae family mostly accumulates lignans with S,S-configuration instead of the usually occurring R,R-configuration at their C-atoms 8,8'.

The protein fraction collected from a 14 days old cell suspension culture of *P. macrocarpa* catalyzed the formation of lariciresinol from racemic pinoresinol. This was an indicator of the occurrence of the pinoresinol-lariciresinol reductase (PLR) in the cell suspension culture of *P. macrocarpa*. Subsequently, a cDNA encoding a PLR of *P. macrocarpa* (PM1) was heterologously expressed in *E. coli*. This putative PLR shows highest similarities to the PLR of *Linum perenne* (67% identity and 80% similarity) on the amino acid level. However, this protein was not active since it has no NADPH binding site in its sequence. Another cDNA was also identified in *P. macrocarpa.* The sequence shows highest similarities to a phenylcoumaran benzylic ether reductase (PCBER) of *Populus trichocarpa* (63 % identity and 79 % similarity) on amino acid level.

The genus *Linum* is the greatest genus containing species from which one can isolate lignans. The lignans pinoresinol, lariciresinol, 7-hydroxysecoisolariciresinol, podophyllotoxin, α-peltatin, ß-peltatin and 6-methoxypodophyllotoxin were detected in different organs of *Linum flavum* var. *compactum* L. during plant development. Based on the analysis of lignan content, the lignan 6-methoxypodophyllotoxin was found in all organs of *L. flavum* var. *compactum* with the high amounts in the roots and capsule early stage. In contrast, the leaves of *L. flavum* var. *compactum* accumulated α-peltatin as the main lignan, whereas the flowerbuds and flowers accumulated mainly ß-peltatin, 6-methoxypodophyllotoxin, and α-peltatin. The lignans pinoresinol, lariciresinol, 7-hydroxysecoisolariciresinol, and podophyllotoxin were found in all parts of *L. flavum* var. *compactum* in relatively low amount. The RT-PCR of *L. flavum* var. *compactum* yielded a partial-sequence with highest similarities to PCBERs.

6. ZUSAMMENFASSUNG

Lignane liegen in Pflanzen gewöhnlich als chiral reine Verbindungen vor und entstehen durch Dimerisierung von zwei Molekülen des achiralen Coniferylakohols. Die Lignane Secoisolariciresinol und Matairesinol können von der menschlichen Darmflora z.B. in Enterodiol und Enterolacton umgewandelt werden. Diese sogenannten „Säugtier-Lignane“ hemmen das Wachstum von estrogenabhängigen Tumorarten wie z.B. Brust- und Prostatakrebst. Extrakte der Thymelaeaceae *Phaleria macrocarpa* (Scheff.) Boerl. werden in Indonesien gegen ganz unterschiedliche Krankheiten, z.B. Krebs, Diabetes, hohen Blutdruck und Herzkrankheiten angewendet. Thymelaeaceae enthalten gewöhnlich S,S-konfigurierte Lignane. In dieser Arbeit wurden daher verschiedene Organe bzw. *in vitro* Kulturen von *P. macrocarpa* auf Lignane untersucht.

Pinoresinol, Lariciresinol, und Matairesinol waren in verschiedenen Organen von *P. macrocarpa* vorhanden, Matairesinol nur im Kallus, und keine bekannten Lignane waren im aus dem Kallus angelegten Suspensionkulturen weiterhin nachweisbar. Pinoresinol und Lariciresinol, die aus dem Holz extrahiert wurden, liegen als Enantiomerengemische mit einem Überschuß von 79 bzw. 55% der (-)-Enantiomere vor. Matairesinol aus dem Holzexstrakt ist reines (+)-Enantiomer.

Eine mögliche PLR-cDNA wurde aus *P. macrocarpa* isoliert und in *E. coli* transformiert. Die putative PLR (PM1) aus *P. macrocarpa* weist zur PLR von *Linum perenne* 67% Übereinstimmung und 80% Ähnlichkeit auf. Die putative PLR (PM1) war jedoch nicht enzymatisch aktiv, möglicherweise, weil die die typische Sequenz für eine „NADPH-Bindung“ fehlte. Die enantiomere Zusammensetzung der Lignane von *P. macrocarpa* deutet darauf hin, dass die stereochemische Kontrolle im Lignanbiosyntheseweg von *P. macrocarpa* ähnlich wie in *Wikstroemia sikokiana* erfolgt. Die zweite cDNA hat zur PCBER von *Populus trichocarpa* 63% Übereinstimmung und 79% Ähnlichkeit.

Linum ist mit ca. 300 Arten eine der größten Gattungen des Pflanzenreichs. Der Gehalt an Lignanen in den verschiedenen Organen von *Linum flavum* var. *compactum* L. wurde über zwei Vegetationsperioden verfolgt.

6-Methoxypodophyllotoxin war das Hauptlignan der Wurzeln und Stengel, α-Peltatin das Hauptlignan der Blätter. Knospen und Blüten akkumulierten α- und ß-Peltatin sowie 6-Methoxypodophyllotoxin. 6-Methoxypodophyllotoxin war auch das Hauptlignan der Kapseln. Bisher konnte keine PLR aus *L. flavum* var. *compactum* L. isoliert werden.

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8. APPENDICES

8.1. List of abbreviations:

A	Adenine
ATP	Adenosine triphosphate
AU	Absorption unit
BLAST	Basic Local Alignment and Search Tool
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
cDNA	complementary DNA
CoA	Coenzyme A
dATP	Deoxyadenosine-5'-triphosphate
dCTP	Deoxycytidine-5'-triphosphate
dCTP	Deoxythymidine-5'-triphosphate
dGTP	Deoxyguanosine-5'-triphosphate
DIR	Dirigent Protein
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside-5'-triphosphate
DOP	Deoxypodophyllotoxin
DTT	Dithiothreitol
DW	Dry weight
E. coli	*Escherichia coli*
EDTA	Ethylene dinitrilo tetra acetate (Titriplex)
Fig.	Figure
FW	Fresh Weight
G	Guanine
gDNA	genomic DNA
h	hour
His	Histidine
HPLC	High performance liquid chromatography
kb	Kilo base
kDa	Kilo Dalton
Kpi-buffer	Kalium-phosphate buffer
Lari	Lariciresinol
LB-Medium	Luria-Berthani medium
Matai	Matairesinol
6-MPtox	6-Methoxypodophyllotoxin
MS-Medium	Murashige and Skoog medium (1962)
NAA	Naphtyl acetic acid
NAD	Nicotinamide-adenine-dinucleotide (oxidized Form)
NADPH	Nicotinamide-adenine-dinucleotide-phosphate (reduced Form)
nt	Nucleotid(e)
OD	optical density
7OH-Seco	7-hydroxy-secoisolariciresinol

ORF	open reading frame
p.A.	pro Analys (for analysis)
PCBER	Phenylcoumaran-Benzylic Ether-Reductase
PCR	Polymerase chain reaction
Pino	Pinoresinol
PLR	Pinoresinol-lariciresinol-reductase
ppm	parts per million
PS	Pinoresinol-Synthase
Ptox	Podophyllotoxin
RNA	Ribonucleic acid
RNase	Ribonuclease
RP-HPLC	Reversed-Phase-HPLC
RPM	Rotation per minute
RT	Room temperature
SDH	Secoisolariciresinol-Dehydrogenase
SDS	Sodiumdodecylsulfat
SDS-PAGE	SDS- Polyacrylamide-Gelelectrophorese
Seco	Secoisolariciresinol
T	Thymine
Tab.	Table
TAE	Tris-Acetate-EDTA
Taq-polymerase	Polymerase from *Termophilus aquaticus*
Tm	melting temperature
tRNA	Transfer-RNA
UTR	untranslatable region
v/v	volume per volume
w/v	weight per volume
X-GAL	5-Bromo-4-chloro-indolyl-ß-galactoside

8.2. List of figures

8.3. List of tables

8.4. List of publications

Saufi, A., Fuss, E., Alfermann, A.W., 2004. Lignans in seeds of *Linum* species. 4. Vortragstagung Sektion Pflanzliche Naturstoffe der Deutschen Botanischen Gesellschaft. Kaub am Rhein, Germany. March 16-18th, 2005.

Saufi, A., Fuss, E., Alfermann, A.W., 2005. Matairesinol from *Phaleria macrocarpa* (Scheff.) Boerl. Phytotherapy - The Role of an Ancient Tradition in Modern Times. Madeira, Portugal. November 1-5th, 2005. ISBN: 972-97794-7-3.

Saufi, A., Fuss, E., Alfermann, A.W., 2006. Lignans of *Phaleria macrocarpa* (Scheff.) Boerl. Future Trends in Phytochemistry: A Young Scientists Symposium. Olomouc, Czech Republic. June 28th-July 1st, 2006.

Saufi, A., Alfermann, A.W., Fuss, E., 2007. A putative pinoresinol-lariciresinol reductase from *Phaleria macrocarpa* (Scheff.) Boerl. Plants for Human Health in the Post-Genome Era. Helsinki, Finland. August 26-29th, 2007. ISBN 978-951-38-6322-7.

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