Anticancer Lignans - from Discovery to Biotechnology

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Abstract: Malignant diseases are the second mortality cause within the human population. Despite the serious progress in establishing and introduction of novel specifically targeted drugs the therapy of these diseases remains severe medical and social problem. Some of the most effective cancer treatments to date are natural products or compounds derived from plant products. Isolation of anticancer pharmaceuticals from plants is difficult due to their extremely low concentrations. The industry currently lacks sufficient methods for producing all of the desired plant-derived pharmaceutical molecules. Some substances can only be isolated from extremely rare plants. Plant cell cultures are an attractive alternative source to whole plant for the production of high-value secondary metabolites. The biotechnological method offers a quick and efficient method for producing these high-value medical compounds in cultivated cells.

Due to the pharmaceutical importance and the low content in the plants the present review focuses on discovery and alternative production systems for anticancer lignans - aryltetralin and arylnaphthalene lignans. The aim is to focus on recent progress of *in vitro* production of anticancer lignans, together with structure elucidation, the methods of increasing the levels of desired substances in plant cell and tissue cultures in general. Experience of different authors, working worldwide on plant biotechnology, has been discussed to show positive results in experiments.

Keywords: Anticancer aryltetralin and arylnaphthalene lignans, biotechnology, *in vitro* production, linum, podophyllotoxin and derivatives, podophillum.

1. INTRODUCTION

Cancer remains a significant unmet clinical need, causing millions deaths annually in the world. Some of the most effective cancer treatments to date are natural products or compounds derived from plant products. Plant-Made Pharmaceuticals is a category of therapeutic agents (pharmaceutical compounds) produced in live plants. A number of plant-derived compounds with diverse chemical structures have played significant roles in the development of several potent anticancer drugs. The plant-specific secondary products were long considered as a major limitation for an extensive use of plant-made pharmaceuticals in human therapy. To extend the research to human clinical studies, we needed to find a reliable supply of plant material, produced target compounds. Our goal here is to emphasise all the progress recently made towards humanization of secondary metabolites in plant *in vitro* cultures, and to illustrate that plant typical anticancer compounds progressively emerge as additional advantages for using this promising expression system.

2. THE NEED TO SCREEN THE PLANTS FOR ANTICANCER COMPOUNDS

The majority of high-value plant secondary metabolites are still isolated from wild or cultivated plant species. However, many of these plants are difficult to cultivate or are becoming endangered due to over-harvesting [1]. Furthermore, the chemical synthesis of plant-derived compounds is often not economically feasible due to their highly complex structures and the specific stereochemical requirements of the compounds. Seven from the most consuming anticancer drugs are with plant origin: Etoposide, Teniposide, Taxol, Vinblastine, Vincristine, Topotecan, and Irinotecan. They are some of the most vigour products in cancer therapy and still derive from plants since the chemical synthesis of the chiral molecules is not economic [2, 3].

Cancer comprises a large variety of malignant tumours that can affect nearly all organs of the body. Treatment includes surgery, radiotherapy and chemotherapy. Cancer cells usually divide much faster than 'normal' cells. Therefore, compounds that stop cell division (e.g. alkaloids, such as vinblastine, vincristine, paclitaxel, docetaxel, camptothecin, colchicine, demecolcine, or the lignan podophyllotoxin) or cytotoxic compounds (triterpene cicloartane saponins) are the two most commonly employed means of chemotherapy with plant natural products [4]. The increased interest in, and constantly expanding use of, the medicinal plants clearly raises questions about the alternative production of valuable biologically active compounds. Although wild plants in many countries are presently under-utilised, the need for sustainable raw material for plant derived pharmaceuticals is projected to grow. The natural variation within plants is very wide and fairly untapped. In most countries, the reserves of these natural resources are highly depleted or the collecting of herbs from their natural habitats is unprofitable.

3. FOCUS ON LIGNAN DISCOVERY

Lignans are a large group of dimeric phenylpropanoids defined as ββ'-dimers of phenylpropane (C6C3) units. This widely spread group of natural products possess a long and remarkable history of medicinal use in the ancient cultures of

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many peoples. The first unifying definition of lignans was made by R. D. Howarth in 1936, who described them as a group of plant phenols with a structure, determined by the union of two cinnamic acid residues or their biogenetic equivalents. According to IUPAC nomenclature, lignans are 8,8'-coupled dimmers of coniferyl alcohol or other cinnamyl alcohols [5].

The both aryltetralin lignans and arylnaphthalene lignans are lead compounds with the most important pharmacological properties and with a wide range of biological activities.

3.1. Aryltetralin Lignans

The supply of podophyllotoxin (PTOX) depends mainly on its extraction from roots and rhizomes of *Podophillum hexandrum* Royle (from Himalayas region) and *Podophillum peltatum* L. (North America), which contain 4% and 0.2% of the active substance on a dry mass basis, respectively. The supply of PTOX has become increasingly limited due to both intensive collection and lack of cultivation. *P. hexandrum* is listed in the appendix II of CITES (Convention for International Trade in Endangered Species) [4]. Those resources are, however limited, because of the intensive collection of the plants, lack of cultivation and the long juvenile phase and poor reproduction capacities of the plant [6].

Lignans occour in many plant species, but only in low concentrations. Podophyllotoxin and related compounds (Fig. **1**) are not only present in Podophyllaceae. Genera in which abundance of PTOX has been reproted are *Linum* (Linaceae) [7, 8, 9]. *Juniperus* (Cupressaceae), *Hyptis* (Lamiaceae), *Thymus* (Lamiaceae), *Teucrium* (Lamiaceae), *Nepeta* (Lamiaceae), *Dysosma* (Berberidaceae), *Diphylleia* (Berberidaceae), *Jeffersonia* (Berberidaceae) and *Thuja* (Cupressaceae) [10]. A detailed phytochemical analysis of the lignans in the Linaceae is done by international collaboration of dfferent groups including researchers from many laboratories: I. Ionkova (Sofia, Bulgaria), T.J. Schmidt (Münster, Germany), A.W. Alfermann, (Düsseldorf, Germany), B. Konuklugil (Ankara, Turkey) and their co-workers [7]. The recent discovery of PTOX-biosynthesizing fungus cultures is also particularly promising, since these are easier to genetically manipulate and scale up [11].

Species of the genus Linum contain biogenetically simple (furofurans, dibenzylbutans, dibenzylbutyrolactones) but also more complex lignans (aryltetralin-, arylnaphthalene lignans) with a wide range of biological activities. A great number of aryltetralin and arylnaphthalene lignans were identified from *in vivo* and *in vitro* species of the Linum genus in our research group - *L. bulgaricum* (Podp.) Petrova, *L. tauricum* Willd., *L. serbicum* (Podp.) Petrova, *L. linearifolium* (Lindem.) Petrova, *L. elegans* Sprun. ex Boiss. *L. flavum* L. *ssp. sparsiflorum* (Stoj.) Petrova *L.capitatum* Kit. ex Schult. var. *laxiflorum* (Stoj.) Petrova, *L. cariense* Boiss, *L. altaicum* Ledeb., *L. austriacum* ver *euxinum* Juz., *L. lewissi* Pursh., *L. campanulatum* L., *L. setaceum* Brot., *L. africanum*, *L. strictum* L., *L. leonii* F. W. Schulz., *L. narbonense* L., *L. thracicum* L. Recently we reported that the aerial parts and roots of several taxa of section Syllinum contain a wide variety of aryltetralin lignans [12, 13].

Lignans in different samples of Linum species, mainly occurring in Bulgaria, were analysed by HPLC-ESI/MS and HPLC-UV/DAD. The ESI/MS fragmentation pathways recently established for aryltetralin lignans are now extended to ester and glycoside derivatives. In total, ca 40 different lignans, mainly of the aryltetralin type, were identified. Their mass spectral data and distribution in the samples are reported in [12]. The occurrence of new 6 compounds is reported here for the first time. A variety of aryltetralin glycosides (all hexosides) could be detected in the crude dicholoromethane extracts. It was found that their fragmentation follows essentially the same rules as recently established for the aglycones [14] so that the spectra are very similar to those of the latter but supplemented by the characteristic changes induced by gycosidation. Thus, the structures of several of these compounds (except stereochemisty) could be established in a straightforward manner.

For example:

The aryltetralin lignan 6-methoxypodophyllotoxin, well known as a constituent of many Linum species of section Syllinum, was identified in considerable quantities in different plant species. Further common compounds readily identified on grounds of their characteristic ESI-MS spectra and retention data in comparison with authentic reference samples are 4'-demethyl-6-methoxypodophyllotoxin, podophyllotoxin, β-peltatin and α-peltatin as well as 5'-demethoxy-6-methoxypodophyllotoxin and 4'-demethylpodophyllotoxin (analytical conditions are described in [8, 12, 27]).

The major glycoside, detected in all samples showed an [M+NH4]+ ion at m/z 624 and otherwise identical mass spectrum as 6-methoxypodophyllotoxin (MPTOX) with a base peak [M+H-180 (C6H12O6)]+ at m/z 427 formed by loss of a complete sugar molecule (identical with [M+H-H2O]+ of the aglycone). The mass difference from MPTOX of 162 mass units clearly showed the presence of a hexose derivative, which, on grounds of previous reports on the occurrence of this compound in Linum species, was assumed to be 6-methoxypodophyllotoxin-β-D-glucoside.

The hexosides of the 7-hydoxy aryltetralins, generally show a base peak at [M+H-C6H12O6)]+ formed by loss of a complete sugar molecule from [M+H]+. The latter was usually of low intensity but complemented by quasimolecular ions [M+NH4]+ and [M+Na]+ proving the molecular masses. The further fragmentation is almost identical with that of the respective aglycones, including the characteristic formation of retro-Diels Alder fragments [A+H]+ [14], which is absent in the spectra of 7-deoxy aryltetralins.

The ESI/MS spectra of the 7-deoxy-6-hydroxy aryltetralin hexosides, as those of their aglycones, showed conspicuous differences from those of the 7-oxygenated compounds described above. Here, [M+H]+ is generally very intense. The major following fragment is derived from [M+H]+ by loss of the sugar moiety [M+H-162 (C6H10O5)]+. While the typical retro-Diels-Alder fragments characteristic of 7-oxygenated aryltetralins are missing, the [B+H]+ fragment at m/z 409 and the fragment related to the latter by loss of the sugar moiety ([B+H-C6H10O5]+ at m/z 247 are of considerable intensity in these compounds.

The aryltetralin type compounds are products of the most common biosynthetic pathway of lignans, starting from monolignol coupling to furofurans such as pinoresinol, reduction of the latter by pinresinol-lariciresinol reductase to secoisolarciciresinol, which is oxidized by secoisolariciresinol dehydrogenase and cyclisation of the resulting 9-hydroxy-9'carboxylic acid to the dibenzyl butyrolactone type lignan matairesinol [15]. Further cyclisation of dibenzylbutyrolactones is then assumed to lead to the cyclolignans of the aryltetralin and napthalene type, although the responsible enzymes are not known up to present.

The aryltetralin lactone podophyllotoxin is the most important lignan used in pharmacy. Therefore the identification of different sources of this rare natural lignan is required. The structure of podophyllotoxin (PTOX) can be elucidating using a combination of HPLC, LC-MS and NMR techniques. LC-MS is important in order to obtain information on molecular weight of the isolated compounds. LC-MS analysis led to good responses and produced protonated ([M+H]+), ammoniated ([M+NH4+]) and sodiated ([M+Na+]) species, typical for electrospray ionisation [16].

Specifically, the LC/(+)ESI-MS spectrum of PTOX (molecular weight 414) showed abundant ion peaks at m/z 415 ([M+H]+), 432 ([M+NH4+]) and 437 ([M+Na+]). Ions of [M+81]+ and [M+91]+ is also observed in the spectrum of PTOX, corresponding to the following clusters of the formate ion: [M+((NH4)2HCOO)+] and [M+(Na2HCOO)+]. The product ion scans in the fragmentation pattern of PTOX led to detection of ([M+H]+-18) ions, primarily derived from successive loss of water [M+H-H2O]+. From this data we have a suggestion for two potential lignans with a molecular mass of 414, characteristic for Section Syllinum of the genus Linum: podophyllotoxin and β-peltatin.

In a follow-up step, the structure of PTOX is assigned by one- and two-dimensional NMR spectra. 1H NMR spectrum of PTOX indicated an aryltetralin structure. The signal at δ 2.79 (1H, m) and δ 2.85 (1H, dd, J=4.5, 14.0 Hz) are easily assigned to H-8 and H-8' respectively, based on their chemical shift and multiplicity. 1H-1H-COSY spectra showed that a doublet at δ 4.78 was coupled to H-8 which allowed this signal to be unambiguously assigned to H-7. COSY spectra also assisted in the assignment of the other three alicyclic hydrogens: the diastereotopic protons at C-9 and H-7'. COSY connectivities revealed that the doublet of doublets at δ 4.10 was coupled to the multiplet at δ 4.61-4.64 and to H-8. Therefore the signal at δ 4.10 could be ascribed to one of the two H-9a or H-9b protons. The multiplicity and coupling-constant (J= 9.5 Hz) were consistent with the H-9b proton, which showed diaxial coupling with H-8. The integration of the signal intensity in the range of δ 4.61-4.64 showed the presence of two protons: the second diastereotopic proton and H-7'. Despite the complexity of the signal it is possible to assign the doublet at δ 4.61 to H-7' based on the coupling with H-8' (J=4.5 Hz). Finally, the residual aliphatic proton at δ 4.63 is conclusively assigned to H-9a. The doublet at δ 5.99 is typical for a methylenedioxy group commonly found in aryltetralin lignans. Another characteristic feature of podophyllotoxin and all of its derivatives is that the 2',6'-protons and the 3',5'-methoxy protons are observed as single resonance peaks. The equivalence of the 2'- and 6'- protons and the equivalence of the 3'- and 5'-methoxy protons result from a rapid 180° rotation of the E ring about the C-1',7' bond. On these ground the singlets at δ 6.38 and δ 3.79 are attributed to the 2', 6' aromatic and 3',5' methoxy protons. The signal at δ 3.85 (3H, s) is ascribed to the 4'-methoxy protons. The two aromatic protons at H-3 and H-6 gave rise to single peaks at δ 6.52 and 7.12, respectively. In conclusion PTOX possesses four aromatic protons which are consistent with the structure of podophyllotoxin, not β-peltatin.

The structure of podophyllotoxin can be confirmed by 13C-NMR spectrum which showed characteristic signals for alicyclic, methoxy, aromatic carbons and a C=O moiety. The exact assignment of the carbon nuclei of TOX is conducted by heteronuclear correlation techniques. HMQC enabled association of all alicyclic carbons with their directly bonded protons. Consequently, signals at 41.1, 44.4 and 45.6 ppm belong to C-8, C-7' and C-8' respectively. The alicyclic signals of C-9 and C-7 were downfield at 71.5 and 73.2 ppm due to the oxygen atoms directly bonded to them. The negative amplitude at 71.5 ppm in the DEPT spectrum allowed the assignment of this signal as C-9 and the peak at 73.2 ppm was attributed to C-7. DEPT spectra also helped to identify the carbon atom from the methylenedioxy bridge at 101.7 ppm. Two signals (56.6 and 61.0 ppm) are observed in the area of the methoxy carbons and the distinction between them is made on the basis of the integration intensity: OCH3 at C-3',5' (56.6 ppm) and OCH3 at C-4' (61.0 ppm). The identified CH connectivities between 106.5 ppm and δ 7.12 (H-6), 108.9 ppm and δ 6.38 (H-2',6'), 110.1 ppm and δ 6.52 (H-3), allowed assignment of the protonated aromatic carbons as follows: C-6 (106.5 ppm), C-2',6' (108.9 ppm) and C-3 (110.1 ppm). The assignments of the non-protonated non-oxygenated carbons can be obtained using HMBC data. HMBC correlations are identified between 131.5 ppm and H-6, 133.4 ppm and H-3, 135.6 ppm and 2',6'-H, which permitted assignment of the above signals to C-1, C-2 and C-1', respectively. Non-protonated oxygenated carbons C-4', C-4, C-5 and C-3',5' are also assigned on the basis of their HMBC correlations and signal intensities. Finally, the signal at 174.5 ppm is readily attributed to the C=O of the lactone moiety. 13C NMR of podophyllotoxin is shown in Table **1**.

Isolation and structure elucidation of lignans previously not known from Linum species, with a major interest in stereochemical diversification, are now in progress.

General approaches to the chemical synthesis of podophyllotoxin derivatives [17] and chemical synthesis of lignans have been proposed, however, an efficient commercially viable rout to the synthesis of podophyllotoxin is still to be sought. Chemical synthesis of the PTOX skeleton is possible [18, 19] but the presence of four chiral positions and the trans-γ-lactonic ring hinder commercial production. Four general approaches to the synthesis of PTOX derivatives have been developed, each with several variations and innovations: the oxo-ester route, the dihydroxy acid route, the tandem conjugate addition route or the use of a Diels–Alder reaction. Alternatives are being explored, usually starting with the preparation of four coplanar rings and the late introduction of the E ring, such as enzyme-catalyzed asymmetric and dearomatizing cyclization [17].

Table 1. ^{13}C Spectra of Podophyllotoxin (PTOX) 6-Methoxypodophyllotoxin (MPTOX) and 4'-Demethylpodophyllotoxin (DPTOX) (Instrumentation and Chromatography are Described in [8])

C-	1 δ (ppm)	2 δ (ppm)	3 δ (ppm)
1	133.4	134.9	131.7
2	131.5	133.1	131.2
3	110.1	104.6	110.1
4	148.0	149.7	147.9
5	148.1	141.8	148.1
6	106.5	125.2	106.5
7	73.2	72.1	73.1
8	41.1	39.2	41.0
9	71.5	70.7	71.5
1’	135.4	135.1	133.4
2'	108.9	108.3	108.5
3’	152.9	152.8	146.8
4'	137.8	137.3	134.5
5’	152.9	152.8	146.8
6’	108.9	108.3	108.9
7’	44.4	44.8	44.2
8’	45.6	45.3	45.7
9’	174.5	174.6	174.6
$-OCH_2O-$	101.7	101.6	101.7
$-OCH_3$ at 3' + 5'	56.6	56.4	56.8
$-OCH_3$ at 4'	61.0	61.0	61.0
OCH_3 at 6	n.a.	60.1	n.a.

n.a.: not applicable.

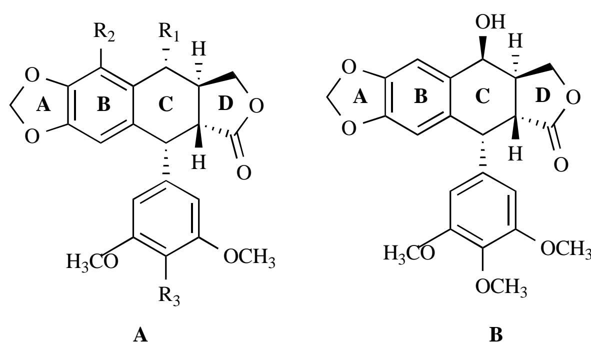


Fig. (1). Structures of aryltetralin lignans: Podophyllotoxin (R_1=OH, R_2=H, R_3=OCH_3), Deoxypodophyllotoxin (R_1=H, R_2=H, R_3=OCH_3), β-peltatin (R_1=H, R_2=OH, R_3=OCH_3) - **A**, Epipodophyllotoxin - **B**.

The commercial production of PTOX is based on an ethanol extraction process where warm ethanol is percolated through dried plant materials followed by purification. Other processes have been described based on rehydration of powdered tissues of *P. peltatum* prior to extraction with an organic solvent. This allows endogenous β-glucosidases to hydrolyze lignans [20].

3.2. Arylnaphthalene Lignans

Due to the presence of the anticancer agent podophyllotoxin in *Linum album* [21], there was an increasing interest in investigation of *Linum* species for lignans. Furthermore, it has been reported that some *Linum* species accumulate arylnaphthalene lignans such as justicidin B.

Justicidin B is an arylnaphthalene lignan which exerts cytotoxic, antiviral, fungicidal, antiprotozoal and antiplatelet properties. The potent bone resorption inhibitor justicidin B was used as a lead compound for design of new antirheumatic drugs. Several tumour types including sarcomas and breast, prostate, and lung carcinomas grow in or preferentially metastasize the skeleton where they proliferate, and induce significant bone remodelling, bone destruction, and cancer pain. Thus, justicidin B may have significant clinical utility as a lead compound in the management of bone cancer and osteoclastogenesis, due to its cytotoxic and bone resorption inhibitory properties. Justicidin B was first isolated from *Justicia* spp. (Acanthaceae) and *Haplophyllum* spp. (Rutaceae). Justicidin B has further been isolated from different *Phyllanthus* species (Euphorbiaceae). It was shown that cell cultures of *Linum austriacum* produce justicidin B, which is the first report on existence of arylnaphthalene lignans in a species of the Linaceae [22].

Since there is a growing interest in justicidin B due to its various pharmacological effects, the sustainable biotechnological supply of this valuable lignan would be a feasible alternative. In recent years, arylnaphthalene lignans such as justicidin B were reported from cell cultures of *L. austriacum* [22]. *L. austriacum* ssp. *euxinum*, *L. lewisii* and *L. altaicum* [9], *Linum campanulatum* [23], *L. narbonense*, *L. leonii* [24] and *L. glaucum* [25]. Justicidin B was also detected in aerial parts and roots of *L. linearifolium*, a species very rich in AT-type lignans [12].

The EI-MS of the justicidin B showed an ion at m/z 364 and mass fragmentation, which is consistent with the data for an arylnaphthalene lignan. Further NMR experiments were performed in order to distinguish between justicidin B and isojusticidin B (Fig. **2**) as these two isomers have slightly different MS fragmentation pattern. A closer look at the 1H NMR spectrum showed that the proton signals at δ 7.12 ppm and δ 7.05 ppm appeared as singlets, which is indicative only for 4,5-dimethoxy substitution. Therefore the resonance signals at δ 7.12 ppm and δ 7.05 ppm were assigned to H-6 and H-3 respectively, due to the shielding effect of the piperonyl group from the pendant ring. Thus the compound can be unambiguously identified as justicidin B [36].

Besides arylnaphthalenes, recently was reported on the presence of 7''-aryl-7,8-dihydronaphthalenes in *L. perenne*. This type of lignans was previously unknown to occur in the

genus Linum where it has, up to present, only been found in this species [26].

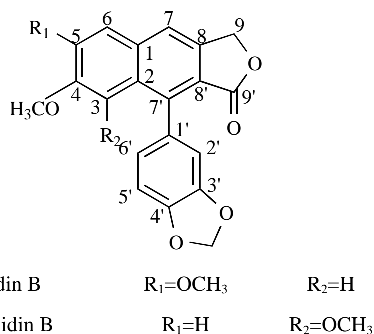


Fig. (2). Structures of arylnaphthalene lignans: Justicidin B and Isojusticidin B.

Most Linum taxa reported to contain lignans of the arylnaphthalene type are members of section Linum and it thus appears that arylnaphthalenes are typical for this section. So far, with respect to intact plants, the occurrence of arlynaphthalenes has only been reported from species of section Linum [9, 22, 24]. Quite noteworthy, the analyzed accession of Bulgarian endemic plant species *L. linearifolium*, as the sole representative, to the best of our knowledge, of section Syllinum studied so far, was found to contain small amounts of the arylnaphthalene lignan justicidin B, which was identified by direct comparison with authentic material previously isolated from *L. austriacum* [22]. *Linum usitatissimum* and *Linum bienne* are somewhat untypical representatives of section Linum that were found to contain dibenzylbutyrolactone- and furofuranolignans, respectively, while none of the aforementioned cyclolignans were hitherto detected in aerial parts of these species [14, 25, 27].

Our recent studies on the diversity of lignans in the genus Linum have recently presents the chemical data obtained for 54 accessions representing 41 different species. Sixty-four different lignans were detected. Their HPLC-ESI/MSMS, complemented by HPLC- UV/DAD data are reported [27].

Most representatives of groups from taxa of sections Syllinum, Linopsis and Cathartolinum accumulate aryltetralin (AT) lignans as major lignans (structures in Fig. **1**). The second group, comprising taxa of sections Linum and Dasylinum, contain arylnaphthalene (AN) (structures in Fig. **2**) and aryldihydronaphthalene (ADN) lignans as predominant metabolites. Some taxa of both groups contain dibenzylbutyrolactone (DBBL) lignans in varying amounts along with the mentioned AT and AN/ADN lignans. DBBL lignans are considered biogenetic precursors of cyclolignans of both mentioned types.

4. MEDICINAL USE AND MECHANISM OF ACTION

Lignans have a long history of medicinal use as the first records date back over 1000 years [28]. The roots of wild Chervil (*Anthriscus sylvestris* L. Apiaceae), containing several lignans, including deoxypodophyllotoxin, were used in a salve for treating cancer [29].

Another source from 400-600 years ago reveals the use of the resin, derived from an alcoholic extract of the roots and rhizomes of *Podophyllum perennials* as a catharctic and poison, both by the natives of the Himalayas and the American Penobscot Indians of Maine [30].

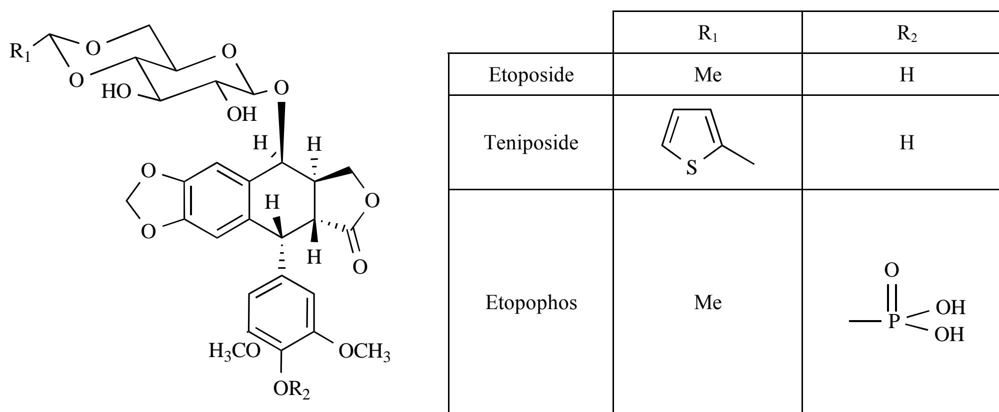
Throughout the years, lignan-containing plant products were used for a wide number of aliments in Chinese medicine – roots of *Kadsura coccinea* , Hance. ex Benth. (Schizandraceae) for treatment of rheumatoid arthritis, gastric and duodenal ulcers [31].

The plant is poisonous but when processed has medicinal properties. The rhizome of the plant contains a resin, known generally and commercially as Indian Podophyllum Resin, which can be processed to extract podophyllotoxin, or podophyllin, a neurotoxin. It has been historically used as an intestinal purgative and emetic, salve for infected and necrotic wounds, and inhibitor of tumor growth. The North American variant of this Asian plant contains a lower concentration of the toxin but has been more extensively studied. All the parts of the plant, excepting the fruit, are poisonous. Even the fruit, though not dangerously poisonous, can cause unpleasant indigestion. Podophyllum gets its name from the Greek words podos and phyllon, meaning foot shaped leaves. *Podophyllum* rhizomes have a long medicinal history among native North American tribes who used a rhizome powder as a laxative or an agent that expels worms (anthelmintic). A poultice of the powder was also used to treat warts and tumorous growths on the skin.

Podophyllotoxin is a plant-derived compound used to produce two cytostatic drugs, etoposide and teniposide. The substance has been primarily obtained from the American mayapple (*Podophyllum peltatum*). The Himalayan mayapple (*Podophyllum hexandrum* or *Podophyllum emodi*) contains this constituent in a much greater quantity, but is endangered in the wild. The substance they contain (podophyllotoxin or podophyllin) is used as a purgative and as a cytostatic. Posalfilin is a drug containing podophyllin and salicylic acid that is used to treat the plantar wart. Several podophyllotoxin preparations are on the market for dermatological use to treat genital warts. Since the total synthesis of podophyllotoxin is an expensive process, availability of the compound from natural renewable resources is an important issue for pharmaceutical companies that manufacture these drugs [32].

Antitumor activity will undoubtedly continue to be the most clinically relevant property of lignans [10]. Due to these biological activities, lignans, and especially cyclolignans, have been the objective of numerous studies focused to prepare better and safer anticancer drugs [4, 10, 12]. The lignan podophyllotoxin, occurring in *Podophyllum emodi* Wall. ex Royale and *Podophyllum peltatum* L., is the starting compound for the semi-synthesis of the anticancer drugs etoposide, teniposide, etopophos (Fig. **3**), which are used for the treatment of lung and testicular cancers and certain leukemias [33]. The mechanism by which podophyllotoxin blocks cell division is related to its inhibition of microtubule assembly in the mitotic apparatus [34]. In contrast to PTOX-like compounds, neither etoposide nor teniposide has any effect on tubulin assembly [35].

However, etoposide and teniposide were shown not to be inhibitors of microtubule assembly which suggested that



	R1	R2
Etoposide	Me	H
Teniposide	S	H
Etopophos	Me	O P OH OH

Fig. (3). Structures of Etoposide, Teniposide and Etopophos.

their antitumor properties were due to another mechanism of action. The cellular target of etoposide has been identified as DNA topoisomerase II, an essential nuclear enzyme required for regulating the topology of DNA. The drug acts on topoisomerase II by stabilizing the intermediate topoisomerase II-cleaved DNA covalent complexes (also referred to as cleavage complexes), converting the enzyme into a highly potent cellular toxin that triggers irreversible apoptotic processes [33].

Other podophyllotoxin derivatives has also been reported which retained or even improved the cytotoxic activity, but these were weak inhibitors of topoisomerase II *in vitro*; the data revealed that such analogs exhibit a different, as yet unknown, mechanism of action.

The drug etoposide (VePesid®) is the semisynthetic derivative of podophyllotoxin, and is approved by the U.S. Food and Drug Administration (FDA) for various types of cancer. Currently, extracts of the podophyllum plant are used also in topical medications for genital warts, HIV-related oral hairy leukoplakia, and some skin cancers. Preliminary research also shows that CPH 82, an oral form of Podophyllum emodi composed of two purified semisynthetic lignan glycosides, may be useful in treating rheumatoid arthritis. However, when used orally, podophyllum can be lethal and should be avoided.

In addition to the current data on cytotoxic, antiviral, fungicidal, antiprotozoal and antiplatelet properties of arylnaphthalene lignans such as justicidin B, we underwent further cytotoxicity examination of justicidin B on two chronic myeloid leukemia-derived LAMA-84 and K-562 cell lines, which show a lower responsiveness to cytotoxic drugs due to the strong expression of the fusion oncoprotein BCR-ABL (a non-receptor tyrosine kynase). The IC50 values of screened leukemic cell lines were determined [36]. As evident from the presented results both compounds caused concentration-dependent cytotoxic effects in the panel of human tumor cell lines under investigation. Justicidin B proved to be slightly less active in respect to relative potency. At the higher concentrations, however, it inhibited the proliferation of malignant cells at the same extend as the reference drug etoposide. The electrophoretic analysis of DNA, isolated from the cytosolic fraction of SKW-3 after 24 h treatment cells with 0.5 and 0.25 µM justicidin B evoked oligonucleosomal DNA fragmentation. The observed DNA laddering is a consequence of the action of specific nucleases which degrade the higher order chromatin structure during the apoptotic process. Therefore it is firmly established that the primary cytotoxic effect of justicidin B is mediated by activation of the programmed cell death pathways. The potent bone resorption inhibitor justicidin B was used as a lead compound for design of new antirheumatic drugs.

5. BIOTECHNOLOGICAL APPROACHES FOR THE PRODUCTION OF POTENTIAL ANTICANCER LEADS

In the coming decades, several new enabling technologies will be required to develop the next generation of advanced plant-based pharmaceuticals. With modern biotechnology, it has become possible to use plant cells for the production of specific pharmaceuticals. Using the right culture medium and appropriate phytohormones it is possible to establish *in vitro* cultures of almost every plant species. Starting from callus tissue, cell suspension cultures can be established that can even be grown in large bioreactors. Moreover, the biotechnological production of these plant products is more environmentally friendly way than is currently occurring.

The industry currently lacks sufficient methods for producing all of the desired plant-derived pharmaceutical molecules. Some substances can only be isolated from extremely rare plants. Since the natural supply is limited, several research groups have explored the possibility of employing plant cell or organ *in vitro* cultures for the biotechnological production of these compounds as alternative. The plant-specific secondary products were long considered as a major limitation for an extensive use of plant-made pharmaceuticals in human therapy.

This supply problem has stimulated considerable interest in the development of alternative strategies offering greater sustainable availability of the compound at affordable costs. Since 20 years of exploration of the total chemical synthesis of podophyllotoxin has not proved economically viable, extensive research has now been undertaken to find methods for stimulating the accumulation of 2,7′-cyclolignans using tissue cultures [7, 37].

The biotechnological approach offers a quick and efficient method for producing these high-value medical

Table 2. Podophyllotoxin and Related Lignans in Plant *In Vitro* Cultures (the Data are Citated in [4, 7, 10, 36, 39])

Species	*In vitro* culture	Lignans synthesized
Callitris drummondii	Callus, Suspension	PTOX
Daphne odora	Callus, Suspension	Matairesinol, Lariciresinol, Pinoresinol, Secoisolariciresinol, Wikstromol
Forsythia x intermedia	Callus, Suspension	Epipinoresinol
Forsythia x intermedia	Callus, Suspension	Matairesinol
Forsythia x intermedia	Suspension	Pinoresinol, Matairesinol
Forsythia spec.	Callus, Suspension	Matairesinol, Epipinoresinol, Phillyrin, Arctigenin
Haplophyllum patavinum	Callus	Justicidin B, Diphyllin, Tuberculation, Arctigenin
Ipomea cairica	Callus	Trachelogenin, Arctigenin
Ipomea cairica	Callus	Pinoresinol
Jamesoniella autumnalis	Gametophyte	8', 8,2'-Tricarboxy-5,4-dihydroxy-7'(5')-6'-pyranonyl-7',8'-dihydronaphtalene and its two monomethylesters
Juniperus chinensis	Callus	PTOX
Larix leptolepis	Callus	Pinoresinol; 2,3-dihydro-2-(4-hydroxy-3-metoxyphenyl)-3-hydroxymethyl-5-(ω-hydroxypropyl)-7-metoxybenzofuran, Lariciresinol, Secoisolariciresinol, Iso-lariciresinol
Linum album	Suspension	PTOX, 6MPTOX, DPTOX, Pinoresinol, Matairesinol, Lariciresinol, β-peltatin, α-peltatin
Linum altaicum	Cell cultures	Justicidin B Isojusticidin B
Linum austriacum	Callus, Suspension, Root, Hairy root	Justicidin B, Isojusticidin B
Linum austriacum ssp. euxinum	Cell cultures	Justicidin B, Iisojusticidin B
Linum africanum	Callus, Suspension	PTOX, DPTOX
Linum campanulatum	Callus, Suspension	Justicidin B
Linum cariense	Callus, Suspension	6MPTOX, 5'-demethoxy-6-methoxypodophyllo-toxin, and the corresponding 8'-epimers 6-methoxypicropodophyllin, 5'-demethoxy-6-methoxypicro-podophyllin
Linum flavum	Root cultures	6MPTOX
Linum flavum	Suspension, Embryogenic Suspension	6MPTOX
Linum flavum	Suspension, Root like tissue	6MPTOX, 5'-demethoxy-6-methoxy-PTOX
Linum flavum	Hairy roots	6MPTOX
Linum flavum	Hairy roots	Coniferin
Linum elegans	Callus, Suspension	6MPTOX, β-peltatin
Linum leonii	Callus,	Justicidin B
Linum leonii	Hairy roots	Justicidin B
Linum lewisii	Cell cultures	Justicidin B, Isojusticidin B
Linum linearifolium	Callus, Suspension, Shoots, Hairy roots	PTOX, 6MPTOX, Justicidin B
Linum mucronatum ssp. armenum	Shoot, Suspension	6-MPTOX, PTOX

(Table 2). Contd…..

Species	*In vitro* culture	Lignans synthesized
Linum narbonese	Callus	Justicidin B, Isojusticidin B
Linum nodiflorum	Suspension	6MPTOX
Linum nodiflorum	Suspension	6-MPTOX, DPTOX, PTOX
Linum nodiflorum	Suspension	6MPTOX, PTOX, DPTOX
Linum persicum	Callus, Cell cultures	PTOX, 6MPTOX, α- and β-peltatin
Linum tauricum	Callus, Suspension, Shoots, Hairy roots	6MPTOX 4'-demethyl-6MPTOX
Linum serbicum	Callus, Suspension,	6MPTOX, β-peltatin
Linum thracicum ssp. tracicum	Callus, Suspension	PTOX, 6MPTOX
Picea glechni	Suspension	Pinoresinol, Dihydrodehydrodiconiferil alcohol
Podophyllum hexandrum	Callus, Suspension	PTOX
Podophyllum hexandrum	Embryogenic callus	PTOX
Podophyllum peltatum	Callus	PTOX
Podophyllum peltatum	Embryogenic suspension	PTOX, DPTOX, 4'- DPTOX
Podophyllum species	Callus	PTOX
Sesamum indicum	Callus, Suspension, Hairy roots	Sesamin, Sesamolin

compounds in cultivated cells. In the future, the new production method may also offer alternatives to other highly expensive drugs. Biotechnological production in plant cell cultures is an attractive alternative but has so far had only limited commercial success (for example, paclitaxel or TaxolTM), due to a lack of understanding of the complex multistep biosynthetic events leading to the desired end-product. The strong and growing demand in today's marketplace for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression *in vitro*. However, it is not only commercial significance that drives the research initiatives. There is a series of distinct advantages for producing a valuable secondary product in plant cell culture, rather than *in vivo* in the whole crop plant. These include the following:

- Production can be more reliable, simpler, and more predictable
- Isolation of the phytochemical can be rapid and efficient, as compared to extraction from complex whole plants
- Compounds produced *in vitro* can directly parallel compounds in the whole plant
- Interfering compounds that occur in the field-grown plant can be avoided in cell cultures
- Cell cultures can yield a source of defined standard phytochemicals in large volumes

Recent biotechnological approaches, including the use of cell cultures, biotransformation, or metabolic engineering techniques to manipulate the biosynthetic pathway, represent an alternative for the production of podophyllotoxin are discussed in [38]. This review is focused only on the production of PTOX. Many other publications about the production of other lignans with also high value from the phytochemical, pharmacognostical and pharmacological point of view is now included in discussion. Both undifferentiated and differentiated cell cultures, mainly from *Linum, Podophyllum, Juniperus, Callitris, Anthriscus* and *Forsynthia* genera, have been reported.

Discoveries of cell cultures, capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants, is an important scientific direction of scientific research in Bulgaria [7, 39]. We demonstrated that the biosynthesis of PTOX and related lignans *in vitro* is possible. Something more, our group is currently applying these technologies to improve the production of podophyllotoxin in bioreactor culture of *Linum linearifolium* (Linaceae) as a renewable source of podophyllotoxin.

Accumulation of lignans in plant tissue and organ cultures has been discussed in many publications. In Table **2**, the accumulation of lignans in plant tissue and organ cultures has been summarized.

6. INCREASING THE YIELDS OF PODOPHYLLOTOXIN AND RELATED LIGNANS IN PLANT CELL CULTURES

Medium factors as phytohormones, carbon source, macro- and micronutritiens, light or dark conditions, oxygen supply and pH values have been reported to play decisive role in production of lignans from *in vitro* cultures, based on

the work of different authors. During the last years, experiments for optimization of growth conditions, physical and chemical elicitation, and selection are being carried out in the many working groups [7, 9, 15, 22]. A model for complex impact is being sought, based on achieved results and current research on cell and tissue cultures.

Cell cultures of different Linum species of section Syllinum are shown to produce considerable amounts of lignans, mainly MPTOX. Although the both PTOX and MPTOX have comparable cytotoxic activity, due to the different substitution in position 6, MPTOX is not used for the production of anticancer drugs [40].

Since PTOX is the preferred precursor for the semi-synthesis of anti-cancer drugs like etoposide and etopophos®, the accumulation of predominantly PTOX is especially interesting. The Bulgarian endemic species *L. linearifolium* is now beside *L. album* and *L. persicum* the third Linum species of section Syllinum with PTOX (ca 1.0% DW) as the main lignan [41]. The different methods and approaches have been applied to *in vitro* cultures producing PTOX, its derivatives and other lignans [7].

Results, summarized in Table **3**, show that a broad range of experiments have been carried out, resulting in enhancement of lignan production of *in vitro* cultures.

Results, summarized in Tables **2** and **3**, show that a broad range of experiments have been carried out, resulting in enhancement of lignan production of *in vitro* cultures. Cell cultures of different plant species are shown to produce considerable amounts of lignans. Although the both PTOX and MPTOX have comparable cytotoxic activity, due to the different substitution in position 6, MPTOX is not used for the production of anticancer drugs.

Although undifferentiated suspension cultures are very "convenient" for the development of secondary metabolites production, with their high growth rates and many possibilities to enhance it, mentioned above, there is one very significant drawback. Although the plant cell is considered to be totipotent, the biosynthesis of many second metabolites requires a certain level of differentiation of the tissues. The maintenance of a culture in a certain differentiated state is obtained through the phytohormone regime. Development of differentiated cultures as a general rule results in higher production of active substances. This approach however is not economically feasible for scale-up of production, as it encounters problems of *in vitro* cultivation and processing of great biomass and longer growth periods than undifferentiated cultures. For this reason it is not focused for *in vitro* cultures producing lignans.

An efficient alternative of differentiated cultures are the genetically transformed, hairy roots (HR) cultures. A certain type of "hairy roots" culture can be induced by means of transformation with a specific soil *Agrobacterium rhizogenes*, and can be further maintained without phytohormones in the medium. They show a logarithmic pattern of growth with doubling times and are characterized by a high degree of branching [42]. As a result of this transformation, bacterial genes, catalizing or interefring with the plant's phytohormone biosynthesis are integrated into its genome, resulting in the development of specific, transformed "hairy roots", independent of exogenous phytohormones. This growth is associated with the production of the characteristic secondary metabolites that resemble the parent plants. Only a few studies have been carried out for the induction of hairy roots in Linum plants, because of its sometime highly resistance to infection by *A. rhizogenes.* A systematic study using different strains of *A. rhizogenes* for the evaluation of transformation frequency, growth and ariltetralin lignan production in *Linum tauricum* hairy roots has been carried out in our research group. We have examined the possibility of generating high lignan-yielding hairy root cultures of *L. tauricum* using different strains of *A. rhizogenes*. Various bacterial strains exhibit different levels of virulence to this plant species. Induction of hairy roots occurred at a 32 % frequency for ATCC 15834 and a success rate of approximately 55% using *A. rhizogenes* strains TR 105. Wild type *A. rhizogenes* ATCC 15834 (harbouring pRi 15834) shows more resistance for hairy induction in *L. tauricum*, but production of ariltetralin lignans in this hairy root clones was highly, compared with hairy root clones, transformed with TR 105 and 10 to 12 times higher than in cell suspensions [43].

Stress factors as biotic [Chito-oligosaccharides (COS) in Juniperus chinensis] [7, 10] and abiotic elicitors - methyl jasmonate in *Forsythia x intermedia*, *Linum tauricum* [43] have been demonstrated to enhance production of lignans. The feeding of a cheaper precursor as phenylalanine, coniferin and coniferil alcohol in plant cell and tissue cultures of lignan producing plants, results in higher levels of production, as plant cells represent a "ready" and organized system for bioprocessing and synthesis of target compounds.

7. NEW STRATEGIES FOR PRODUCTION OF DESIRABLE LIGNANS

Elucidation in details of the biosynthetic pathway of PTOX, isolating all enzymes and genes, responsible for their encoding, would lead to new strategies for enhancement of the yields of this valuable substance by means of combination of biotechnological and biochemical methods. Certain enzymes of podophyllotoxin biosynthetic pathway have been isolated and characterized and podophyllotoxin and 6MPTOX have been established to be stored in the vacuole [44].

The biotransformation of aryltetralin lignans to arylnaphthalene lignans in presence of yeast is described. Podophyllotoxone, an oxidation product of podophyllotoxin on incubation with yeast from different sources produced dehydropodophyllotoxin, an important representative of arylnaphthalene lignans [45].

The conversion of PTOX into other lignans by plant cell cultures and/or bacterial cultures is not new. An elegant example of the use of this technology in the field of lignans is the successful stereoselective hydroxylation of deoxypodophyllotoxin by recombinant CYP3A4 in *Escherichia coli* to epipodophyllotoxin with high efficiency (90%) [46]. This novel system for the production of 2,7'-cyclolignans was recently demonstrated. Deoxypodophyllotoxin is stereoselectively converted into epipodophyllotoxin by recombinant

Table 3. Optimisation of Lignan *In Vitro* Production (the Data are Citated in [4, 7-10, 23, 24, 41])

Species, (*In vitro* culture)	Lignans synthesized	Varying factors with impact on the culture	Results of optimization
Callitris drummondii (Suspension)	PTOX-beta-D-glucoside	Illumination	0.02 % in dark 0.11 % in light
Forsythia x intermedia (Suspension)	Pinoresinol Matairesinol	Carbon source	2 % succrose - 0.001 % lignans 6 % succrose - 0.07 % pinoresinol; 0.1 % matairesinol
Ipomoea cairica (Callus)	Arctigenin, trace-Logenin	Phytohormones, Carbon source	4 mg/L 2,4-D, 3% maltose, pH 6.4 - 0.03 % lignans
Juniperus chinensis (Immobilized cells)	PTOX	Various calcium alginate concentrations	1.8 % Ca-alginate gel - 5-fold increased levels, compared to free cell suspension; maximum excretion of PTOX in the medium 3 % Ca-alginate gel - (0.21-0.025 $mg.g^{-1}$ dw) 4-5-fold increase 6 % Ca-alginate gel - small amount
Linum africanum (Suspension)	PTOX DPTOX	Source of explant material, Light, IAA, NAA, 2,4-D, Cytokinin/Kinetin ratios	Highest synthesis - Kinetin $10ml.L^{-1}$, IAA - $0.4ml.L^{-1}$, 2,4-D - $0.2ml.L^{-1}$; PTOX - increases in dark conditions (no difference between callus and suspension) DPTOX - increases when cultivated in the dark and has higher levels in callus than suspension.
Linum album (Suspension)	PTOX and related lignans	Illumination	0.2 % in dark 0.5 % in light
Linum album (Suspension, shake-flasks: 1000ml - 50mL medium, 300mL - 50 mL medium, establishment of bioreactor)	PTOX	Oxygen supply Increasing of shaker speed	Enhancement of PTOX accumulation.
L. altaicum (Callus, Suspension)	Justicidin B Isojusticidin B	Dark	Justicidin B - between 0.92 - 0.96%.
L. austriacum ssp. euxinum (Callus, suspension)	Justicidin B	Dark	Justicidin B - between 0.50 - 0.96%
Linum campanulatum (Callus, Suspension)	Justicidin B	Illumination, Kinetin, IAA, 2,4-D, Succrose	Lignan content: 1.41 % in the dark 0.40 % in light
L. leonii Hairy roots	Justicidin B	Agrobacterium strains	Justicidin B-10.8 $mg.g^{-1}$ dw 5 times higher than callus
L. lewisii (Callus, Suspension)	Justicidin B	Dark	Justicidin B - between 0.16 - 0.30%
L. linearifolium (Callus, Suspension)	PTOX 6MPTOX	Phytohormones, Medium composition	6.54 mg/g dw PTOX on day 30
Linum nodiflorum (Suspension)	6MPTOX	Illumination	Light - 0.6 % Dark - trace amounts

(Table 3). Contd…..

Species, (*In vitro* culture)	Lignans synthesized	Varying factors with impact on the culture	Results of optimization
Linum tauricum (Suspension, Shoots, Callus, Hairy roots)	4'-Demethyl-6-MPTOX 6MPTOX	Phytohormones Methyl Jasmonate Agrobacterium strains	Suspension - 4 mg.L^{-1} NAA, maximal production of 4'-demethyl-6-methoxy-podo-phyllotoxin (2.16 mg.g^{-1} dw) Callus - 0.1mg/L 2,4-D, 0.2mg/L IAA, 2.0mg/L Kinetin - maximal production of 6-methoxy-podophyllotoxin (3.99 mg/g dw)
Podophyllum hexandrum (Suspension)	PTOX	Illumination	Light - 0.03 % Dark - 0.09%
Podophyllum hexandrum (Callus)	PTOX	Phytohormones	2,4-D + Kinetin - 0.077%
Podophyllum hexandrum (Suspension, 3L-stirred-tank bioreactor)	PTOX	Mathematical model for developing nutritient-feeding strategies, low shear conditions in fed-batch modes of operation, prolonging the productive log-phase of cultivation.	Improvement to 48.8 mg.L^{-1} PTOX, corresponing volumetric productivity of 0.80 mg.L^{-1} per day
Podophyllum hexandrum (Suspension)	PTOX	pH, Phytohormones, Carbon source, Inoculum	pH 6.0 IAA - 1.25 mg.L^{-1} Glucose 72 g.L^{-1} Inoculum level - 8 g.L^{-1}
Podophyllum hexandrum (Suspension)	PTOX	Sugar, Nitrogen source, Phosphate	MS medium, NH_4+Salts: Nitrate = 1:2, 60 g.L^{-1} Glucose - highest growth and PTOX accumulation
Podophyllum peltatum (Callus)	PTOX	Red light (660 nm) Carbon source Phytohormones	Inhancement of production in red light Sucrose - 0.057 % Maltose - 0.023 % 2,4-D + Kinetin - 0.57 %
Rollinia mucosa (Jacq.) Bail. (Callus).	Furofuranic lignans: Epigambin, Magnolin, Epiyangambin	Origin of plant material, Explant type, Growth regulators (2,4-D, NAA, BA, PIC)	Foliar blade explants - biomass, synthesis nhancement; PIC - best biomass production; NAA, 2,4-D-Epiyangambin, Magnolin (dependent on explant source); PIC - Epiyangambin - Calli from foliar blade

The Data are citated in [4, 7-10, 23, 24, 41].

main human metabolizing enzyme cytochrome P450 3A4 (CYP3A4). Epipodophyllotoxin (Fig. **4**) has been detected as the only metabolite in yields up to 90%. Therefore, the heterologous expression of CYP3A4 in *E. coli* presents an interesting alternative for a large-scale production of epipodophyllotoxin, the direct precursor for the semi-synthesis of anti- cancer drugs etoposide.

8. SCALE-UP OF PLANT CELL CULTURES

Since plant cells produce unique pharmaceuticals, which can be harnessed, they need to be produced in large-scale bioreactors. Selection of the best performing cell line, its maintenance and stabilization are necessary prerequisites for its production in bioreactors and subsequent scale-up of the cultivation process to the industrial level. Scale-up of growth and product yield depends on a multitude of factors, such as growth medium, conditions of cultivation, inoculum, type of reactor and processing conditions. The composition of the growth medium, elicitors and precursors, etc. can markedly influence the production. Cell/tissue types such as cell suspension cultures, immobilized cells and hairy roots have been very ideal for scale-up. Configuration of bioreactors used for microbial cells cannot always be utilized directly for plant cells, owing to distinctive features, which are not favorable for plant cell cultivation. Plant cells are less stable in productivity, highly shear sensitive, exhibit low oxygen re-

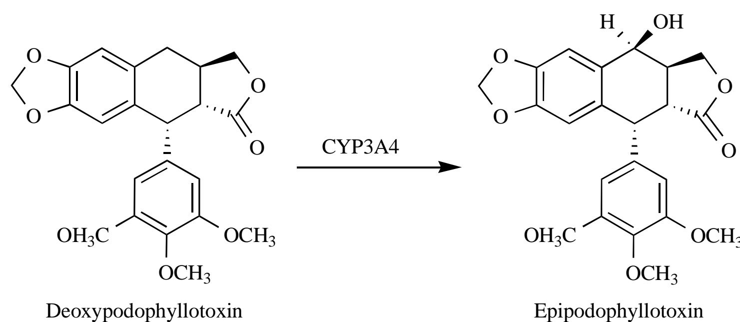


Fig. (4). Stereoselective hydroxylation of deoxypodophyllotoxin by recombinant CYP3A4 in *Escherichia coli* to epipodophyllotoxin.

quirements (ca. 1-mmol O2) slow growth (doubling time 25 - 100 h) and often occur as cell clumps of 2 - 4mm diameter. Bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology with several advantages for mass cultivation of plant cells:

- It gives better control for scale up of cell suspension cultures under defined parameters for the production of bioactive compounds;
- Constant regulation of conditions at various stages of bioreactor operation is possible;
- Handling of culture such as inoculation or harvest is easy and saves time;
- Nutrient uptake is enhanced by submerged culture conditions which stimulate multiplication rate and higher yield of bioactive compounds; and
- Since the biosynthetic efficiency of populations varies, for this purpose a high yielding variety should be selected as a starting material. The fundamental requirement in all this is a good yield of the compound, and reduced cost compared to the natural synthesis by the plants.

Bioreactor studies represent the final step leading to commercial production of economically important phytochemicals from plant cell cultures. Although a number of economically important compounds have been produced by plant cell and tissue culture techniques, production of compounds on an industrial scale is still restricted. Commercial production of podophyllotoxin using plant cell cultures is yet to be achieved.

Batch cultivation of *P. hexandrum* for production of podophyllotoxin was conducted in a 3 litre stirred tank bioreactor using optimized medium which resulted in biomass yield of 21.4g l^{-1} in 24 days and podophyllotoxin concentrations of 13.8mg l^{-1} in 26 days [47].

In another study, upto 0.2% podophyllotoxin (on dry weight basis) was obtained when *L. album* cell suspensions were cultured in a 20 litre bioreactor in a batch system, by optimizing aeration conditions. In the optimized conditions, cell cultures reached a maximum production of 130 mg/L PTOX [48].

In the last few years in Bulgaria, the fed-batch cultivation of *L. tauricum* suspension cultures in a 2 litre stirred tank bioreactor, using optimized medium leads to substantial increase (8-10 times higher than production in bach cultivation) of the levels of 6MPTOX and 4'DM-6MPTOX. These technologies are currently applying to improve the production in bioreactor culture of *L. linearifolium* (Linaceae) as a renewable source of podophyllotoxin [Ionkova, unpublished].

"Hairy root," is at the core of a promising new technique that could one day lead to "biofactories" that produce medicines derived from rare plants in huge quantities at a low cost. Recent advances in bioreactor design and construction allow hairy root-based technologies to be scaled up while maintaining their biosynthetic potential [49].

CONCLUSION

Due to the pharmaceutical importance and the low content in the plants the present review focuses on alternative production systems for podophyllotoxin and related lignans. Biotechnology is generally regarded as a key technology of the new century. Accumulation of lignans in plant tissue and organ cultures has been discussed based on the work of different authors. A survey of literature data has shown positive results in experiments with optimization of conditions of culturing, selection of high producing cell lines, influence of stress factors and feeding of precursors in plant *in vitro* cultures for the enhancement of production of lignans. Working with plant cells drastically reduces the preparation time, handling and storage costs associated with the traditional whole plant approaches. In our laboratory, we focus on the production of some important pharmaceuticals in plant cell cultures and have successfully established cell cultures for production of anticancer agents. The results were described in the present review. We hope that plant biotechnology will provide a more inexpensive and efficient method for producing anticancer drugs in the near future.

Exploration and development of biosynthetical pathways leading to 2,7′-cyclolignans may open paths for future metabolic engineering, such as the bioconversion of deoxypodophyllotoxin to epipodophyllotoxin employing a human hepatic enzyme heterologously expressed in Escherichia coli.

Recently, the interest of international pharmaceutical industries has been directed more and more to plant based anticancer compounds. In this case, it has been shown that production of podophyllotoxin and related lignans in cell cultures is possible. We believe that cell cultures of different

plants as source of biologically active lignans can play a role in this respect.

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ABBREVIATIONS

PTOX	=	podophyllotoxin
6MPTOX	=	6-methoxypodophyllotoxin
DPTOX	=	deoxypodophyllotoxin
4'DM-6MPTOX	=	4'-demethyl-6-MPTOX
HR	=	hairy root cultures

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