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Production of podophyllotoxin from *Podophyllum hexandrum*: a potential natural product for clinically useful anticancer drugs

Archana Giri & M. Lakshmi Narasu*
School of Biotechnology, Jawaharlal Nehru Technological University, Mahaveer marg, Hyderabad 500028, India
(Author for correspondence; E-mail: mln28@usa.net)*

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Abstract

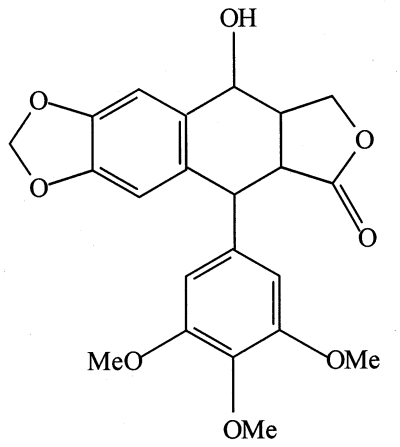
Podophyllum hexandrum Royle of family Berberidaceae is an endangered medicinal plant. Rhizome of *P.hexandrum* contains several lignans which posses antitumor activity. Podphyllotoxin is the most active cytotoxic natural product. It is used as starting compound for the synthesis of anticancer drug etoposide and teniposide. Podophyllotoxin acts as an inhibitor of microtubule assembly. These drugs are used for lung cancer, testicular cancer, neuroblastoma, hepatoma and other tumors. Besides this, it also shows antiviral activities by interfering with some critical viral processes. Availability of podophyllotoxin from plants has its limitations because of its intense collection from nature and lack of organized cultivation. The chemical synthesis of podophyllotoxin is considered to be very complicated as yet. The use of biotechnological approaches for the production of podophyllotoxin using cell cultures, organ cultures, and biotransformation route or by manipulating biosynthetic pathway proves to be an attractive alternative for production of podophyllotoxin. The present paper discusses the current status of research, limitations and future prospects for the production of podophyllotoxin *in vitro*.

Introduction

Plant kingdom has provided us with many important medicaments, anticancer agents being one of them. Higher plants have made important contributions in this regard (Cragg et al., 1997). Podophyllotoxin is a natural product isolated from *Podophyllum hexandrum* and has long been known to posses medicinal properties. Commercially exploitable sources of podophyllotoxin are few and currently it is obtained for drug use from dried rhizome and roots of *Podophyllum sps. viz Podophyllum hexandrum* (Indian Podophyllum) and *Podophyllum peltatum* (American Podophyllum) belonging to family Berberidaceae. These valuable plant species are distributed in Himalayas and North America, respectively. The resin of *Podophyllum* rhizome is the source of podophyllotoxin. Podophyllotoxin is used for the preparation of semisynthetic derivatives that are clinically applied as cytostatics in the treatment of several types of cancer. Successful development of anticancer drug etoposide and teniposide from natural podophyllotoxin has focussed attention on *Podophyllum* as an economic source of lignans. (Kamil and Dewick 1986, Holthuis 1988). American *Podophyllum* contains 4–5% podophyllum resin, whereas Indian sps. contains 7–16%. The variation in percentage of resin is attributed to seasonal differences, different sites of growth and age of the plant (Purohit et al., 1999). In certain areas as much as 20% resin has also been recorded. The highest percentage of resin is obtained in May-June during the flowering stage. Thus Indian podophyllum when collected at the proper season contains 2.5 times more resin compared to its American counterpart. Moreover, this resin has double the amount of podophyllotoxin (Fay and Ziegler, 1985; Drew et al., 1987; Thakur, 1993). Podophyllotoxin is commonly extracted from *P. hexandrum* that contains 6–12% resin of which the concentration of podophyllotoxin is around 40%. Availability of podophyllotoxin isolated from plant has its limitations, due to scarce occurrence of the plant because of intense collection from nature and lack of organized

cultivation (Gupta and Sethi, 1983). One of the major problems for the cultivation of this plant is its long juvenile phase and poor fruit setting ability. Moreover, its seeds take long period to germinate (Handa et al., 1989). As the yields of podophyllotoxin are low it is an expensive starting compound for the chemical synthesis of its derivatives. In addition the chemical synthesis of podophyllotoxin is very complicated and rather difficult because of the presence of four chiral centers, a rigid trans -lactone and an axially locked 1-aryl substituent (Rust and Roth, 1981; Forsey et al., 1989). It forms the starting material for the synthesis of anticancer drugs Etoposide and Teniposide. As chemical synthesis of podophyllotoxin is difficult and since it is the starting compound for synthesis of anticancer drugs the effective availability of these drugs will ultimately depend upon the supply of raw materials. Biotechnological means for production of podophyllotoxin using plant cell and organ cultures has been considered as an attractive alternative.

This review provides information about the plant *Podophyllum hexandrum*, a source of podophyllotoxin, describes the medical preparations of podophyllotoxin, their mode of action, uses and discusses the potential of biotechnology for production of podophyllotoxin.



PODOPHYLLOTOXIN

Product identification

The product podophyllotoxin is also available as synonym compounds such as podophyllin and podophyllum. The chemical formula of podophyllotoxin is $C_{22}H_{22}O_8$ with molecular weight of 414.41. It is marketed with product code J.T.Baker: 2898 Mallinckrodt: 7700.

Uses of podophyllotoxin

Podophyllotoxin is the best known lignan among hundreds of natural plant lignans. Podophyllin resin derivatives have been used as medicaments for over 250 years. Native Americans believed podophyllotoxin to be an effective antidote against snakebites. Since that time it has been used as a poison, an antihelminthic and a local antifungal (Kao et al., 1992; Chang et al., 1992). Catesby made the first report in the medical literature in 1731 in his natural history of Carolina, Florida and Bahama Islands (Schacter 1996). In western medicine *Podophyllum* was first used medicinally as laxative in the early 19th century. Since 1940 *Podophyllum* resin has been used topically for various skin lesions such as warts and condylomas. Kaplan 1942 first cited that an alcoholic extract of podophyllotoxin as topical treatment for veneral warts (condyloma acuminata) an ailment caused by a papilloma virus. Today podophyllotoxin continues to be used worldwide as first-line treatment for condyloma acuminata and also for some cathartic herbal preparations. It has also been used for the treatment of noncervical human papilloma virus for the treatment of genital infection (Mayeaux et al., 1995).

In the treatment of external condylomas, a standardized alcoholic solution of podophyllotoxin is used to obtain necrosis of external condylomas that are not very extensive. Penile warts can be safely treated with 0.5–2.0% podophyllin self applied by the patient at a fraction of the cost of commercially available podophyllotoxin. The shelf life of podophyllin extract is at least 3 months, this is relevant for countries where resources for health care are limited (White et al., 1997). A mixture of natural and semisynthetic glycosides from *Podophyllum hexandrum* has been used for many years for the treatment of rheumatoid arthritis but it shows gastrointestinal side effects. 25% solution of *Podophyllum* resin is efficacious and a cost effective treatment with minimal side effects for HIV-related oral hairy leukoplakia which is a symptom free lesion (Gowdey et al., 1995). Goel et al. (1998) reported a significant antitumour effect at subtoxic, well tolerated, sequential doses of aqueous extract of *Podophyllum hexandrum*. They also reported radioprotective properties of *P. hexandrum* that is

comparable to synthetic radioprotectors like diltizem etc. Podophyllotoxin is used as starting compound for the chemical synthesis of etoposide (VP-16–213) and teniposide (VM-26) (Baker et al., 1995; Clark and Slevin, 1987; Stahelin and Wortburg, 1989, 1991).

Pharmacology and mode of action

There are several modes of antiviral activities associated with lignans and podophyllotoxin is most notable among the tubulin binding lignans. Highly purified podophyllotoxin and preparation containing two semisynthetic podophyllotoxin glycosides inhibit mitogen induced lymphocyte proliferation and immunoglobulin synthesis (Truedsson et al., 1993). Apart from antitumor and antiviral activity insecticidal and phytotoxic activities of podphyllotoxin are also reported (Mac Rae and Towers, 1984; Inamori et al., 1986). Initially, Kaplan hypothesized that podophyllotoxin exerts its pharmacological effect by producing potent spasm in blood vessels with resultant ischemia, necrosis and sloughing. Controlled clinical trials demonstrated that podophyllotoxin exerts a colchicine like effect by arresting mitosis in metaphase resulting in epithelial cell death. The anti-mitotic effects of podophyllotoxin are the result of the drug's ability to act as an inhibitor of microtubule assembly. Podophyllotoxin also appears to attach to cell proteins and acts by increasing the incorporation of amino acids into proteins, inhibition of purine synthesis and inhibition of purine incorporation into RNA (Filley, 1982). It has also been found to have a direct effect on mitochondria by reducing the activity of cytochrome oxidase and succinoxidase (Ferguson, 1992). The mode of their antiviral activity is by tubulin binding, reverse transcriptase inhibition, integrase inhibition and topoisomerase inhibition. Podophyllotoxin is most notable among the tubulin binding lignans. By binding to tubulin these are able to disrupt the cellular cytoskeleton and interfere with some critical viral processes (Charlton, 1998). Their cytotoxic action is based on the inhibition of topoisomerase II, while podophyllotoxin acts as an inhibitor of microtubule assembly. Semisynthetic derivatives of podophyllotoxin show significant therapeutic activity against several human neoplasms.

Their action takes place at the level of microtubules. Podophyllotoxin a meiotic spindle poison inhibits the polymerization of tubulin and stops cell division at the beginning of the metaphase. Study of structure-activity relationships has made it possible to design semisynthetic derivatives with good activity and limited side effects. These products are demethylated at 4' belong to epi series and their hydroxyl in position 4 is part of a glycoside linkage with a glucose of which two of the hydroxyl groups (at 4" and 6") are blocked by acetalization as thianylidene (teniposide) or ethylidene (etoposide). However, these derivatives do not affect the microtubule assembly. Both block the cell cycle in two specific places: they block the phase between the last division and the start of DNA replication (the G1 phase) and they block the replication of DNA (the S phase). It is known to cause single-strand breaks in DNA. They also act via inhibition of topoisomerase II and activation of oxidation-reduction reactions to produce derivatives that bind directly to DNA. Topoisomerae II relaxes both negative and positive supercoils, their reaction is ATP dependent. The reaction is mediated by making a double-stranded break in one DNA duplex and passing another duplex region through it. The reaction probably represents a non specific recognition of duplex DNA in which enzyme binds any two double stranded segments that cross each other and forms a cleavable complex, which allows one double strand of DNA to pass through a temporary break in another double strand. Semisynthetic derivatives of podophyllotoxin bind and stabilize this complex that prevents the repair of double stranded break (Haskell, 1990; Chabner and Myers, 1989; Schacter 1996). Etoposide is cell cycle phase specific with predominant activity occurring in late S phase and G2 (Robles et al., 1999). Zhu et al 1999 reported that tris substituted aniline-4'-O-demethyl-podophyllotoxin derivatives have been evaluated as inhibitor of DNA topoisomerase II and tumour cell growth. They displayed significant growth inhibitory action against a panel of tumour cell lines including etoposide resistant subclones. It is ten folds more potent than etoposide in both cell killing and topoisomerase II inhibition. In addition to inhibition of tubulin polymerization and arresting cell growth by inhibiting DNA topoisomerase II, an unknown mechanism of action has been proposed for some of the recently modified podophyllotoxins (Owing to its severe toxic side effects, a number of modifications have been done on podophyllotoxin structure).

Recent developments on podophyllotoxin have led to structure-activity correlation, which have assisted in the design and synthesis of new derivatives of potential antitumour activity (Damayanthi and Lown, 1998; Robles et al., 1999). Greenwald et al., 1999

reported oncolytic activity of a series of water soluble acyl derivatives of PEG conjugated podophyllotoxin. Several 9-deoxy-9-substituted podophyllotoxin derivatives possess good anticancer activity against ovarian, renal and lung cancer cell lines (Subrahmanyam et al., 1999).

Preparations and dosing

Podophyllum resin is an amorphous powder, light brown to yellow in colour, when subjected to light it turns brown. It is a mixture of ligands with podophyllotoxin (podofilox) being the major constituent. α- and β-peltatins, demethylpodophyllotoxin and picropodophyllotoxin are also found in smaller concentrations. Crystalline podophyllotoxin is obtained by extracting crude podophyllotoxin with chloroform, evaporating the solution, dissolving the remaining compound in alcohol and diluting it with water and benzene.

General podophyllin resin formulations

- Podophyllin Resin Topical Solution USP 11.5% (in compound benzoin tincture 10% and ethanol 70.5%)
- Pod-Ben-25 25% (in compound benzoin tincture)
- Podben 25% (in compound benzoin tincture 10% and isopropyl alcohol 72%)

Podophyllin resin combinations

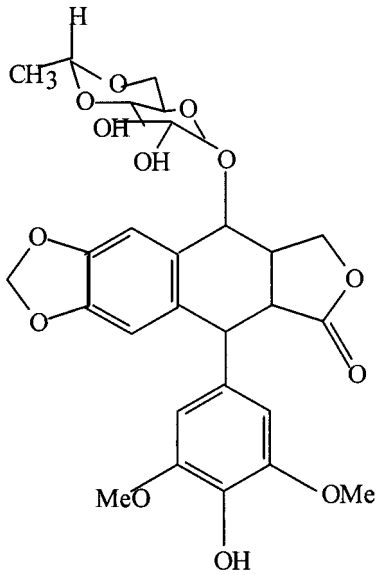
- Cantherone Plus 5% with Cantharidin 1% and salicylic acid 30%
- Verrusol-C + M 5% with Cantharidin 1% and salicylic acid 30%
- Verrex-C + M 10% with salicylic acid 30%

Pure podophyllotoxin formulation

- Condolyx 5%(in 95% ethanol)

Pure podophyllotoxin formulations are associated with less adverse effects and greater efficacy when compared with podophyllin resin formulations (Romanelli, 1996).

Etoposide



ETOPOSIDE

Etoposide (Demethylepipodophyllotoxin-ethylidene-glucopyranoside, EPE, epipodophyllotoxin) with synonyms: VP-16, VP–16–213, EPEG is sold by Bristol-Myers Squibb as Vepesid, aka VP-16. It is usually prescribed in multiple chemotherapy protocols (Stahelin and Von Wartburg, 1989, 1991). It is a highly active and widely used antineoplastic agent (Cai et al., 1999). It is active against many tumour types and used primarily as part of combination treatment for testicular tumours and leucopenia. This is most active single agent for small cell lung cancer (Chabner et al., 1996; Aapro, 1996). The product is available as an injectable solution to be administered by infusion or it is administered orally as liquid capsules (Montaldo et al, 1990).

Usual doses:

Intravenous- q1-4w: 100–250 mg/m^2, q2- 5w: 35–150 mg/m^2/day $\times$ 3–5 days. 100–150 mg/m^2/day $\times$ 3–7 days by continuous infusion.

Oral- IV, 50–150 mg/sq. m/day $\times$ 1–3 days per os, double doses.

Uses

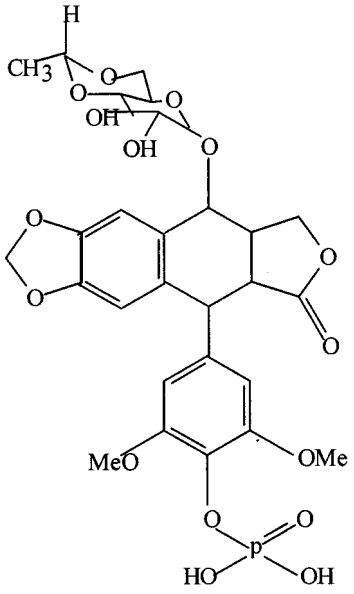
It is used mainly to treat a range of cancer *viz*. acute

lymphocytic leukemia, acute myelogenous leukemia, germ cell tumours, hodgkins disease, ovarian cancer, Rhabdomoysarcoma and newly diagnosed glioblastoma multiforma. It is used less frequently for brain tumours, wings sarcoma, histiocytosis, kaposis sarcoma and neuroblastoma (Viana et al., 1991; Santana et al., 1992; Montaldo et al., 1990; Krogh, 1993; Cai et al., 1999).

Combination of chemotherapy with etoposide and cisplatin and concomitant radiotherapy followed by removal of tumour is highly effective in the treatment of pediatric patients with primary intracranial yolk sac tumour and embryonal carcinoma (Ushio et al., 1999).

Etoposide in combimation with some other agents is an effective autologous transplantation preparative regimen for lymphoma with little toxicity (Przepiorka et al., 1999; Reiser et al., 1999).

Major side effects include hair loss, nausea, anorexia, diarrhoea and low leucocyte and platelet counts. Etoposide is known to cause fetal damage and birth defects, so pregnant or nursing women should not use it (Clark and Slevin, 1987; Rowinsky and Donehower, 1992; Winick et al., 1993; Dorr and Von Hoff, 1994).

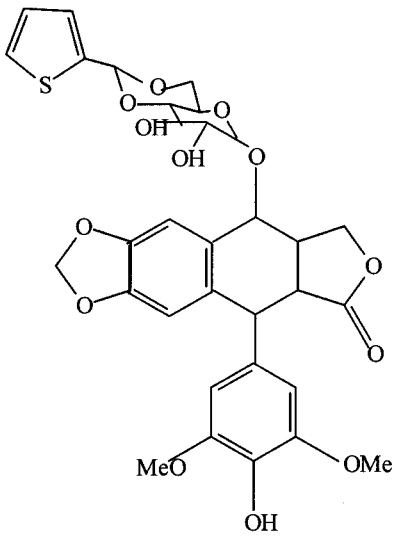


ETOPOSIDE PHOSPHATE

Etoposide phosphate

Etoposide phosphate (Etophos) is sold by Bristol-Myers Squibb Company (Princeton, NJ, USA). It is a water-soluble prodrug of etoposide that is rapidly and completely converted to the parent compound after intravenous dosing. While etoposide is widely used in the treatment of many cancers, it has a number of limitations due to its lack of water solubility. The pharmacokinetics profile of etoposide after treatment with etoposide or etoposide phosphate is same. It is easily soluble in water and can be made up to a concentration of 20 mg ml^{-1}, that can be given as a 5-minute bolus, in high doses in small volumes and as continuous infusion. Easier to use etoposide phosphate represents an improved formulation of etoposide. The main advantages of etoposide phosphate are its ability to be administered as a bolus without incidence of hypotension or other acute toxicities, increased convenience of high dose treatment and feasibility of chronic infusion (Schacter, 1996).

Teniposide



TENIPOSIDE

Teniposide with a synonym VM-26 is used for the treatment of lymphomas of acute refractory leukemia and that of brain and bladder tumors. It can be used in single drug therapy for induction of remission.

Usual doses: 30 mg/sq. m/day in 5 day cycles; 4–5 cycles 10–21 days apart or for maintenance (60 mg/sq. m/day) once weekly as well as in multiple drug chemotherapy (Montaldo et al., 1990; Chabner et al.,

1996). Toxicity is hematological, in addition, the excipient induces a risk of immediate anaphylactic type reaction with acute respiratory distress. It is used less often than etoposide (Richter et al., 1987).

NK 611

NK 611 is a novel podophyllotoxin derivative. Substitution of a glycosidic moiety with acrylamines produced enhanced activity modification in the sugar ring. Compared to etoposide NK 611 carries a dimethylamino group at the D-glucose moiety. The antitumour activity of NK 611 showed to be equal or superior to etoposide. (Damayanthi and Lown, 1998; Rassmann et al., 1999).

Biosynthetic pathway

Podophyllotoxin belongs to lignan group of compounds. Lignans are dimerization products of two phenylpropane units linked by β carbon atoms of their side chain (Dewick, 1989). Most of the pathways proposed involve phenolic oxidative coupling of C6-C3 monomers via shikimic acid pathway (Figure 1). Production of optically active lignan dimers is enzyme-controlled reaction. A series of compounds of considerable medicinal and commercial interest as clinically useful anticancer drugs are formed by reductive dimerization of cinnamic alcohols or cinnamic acid (Jackson and Dewick, 1984).

In vitro studies for production of podophyllotoxin

Since totally synthetic approaches to etoposide and teniposide are commercially unacceptable for the foreseeable future, the successful development of anticancer drugs from podophyllotoxin has focussed attention on podophyllotoxin as an economic source of lignans. Since podophyllotoxin is an expensive compound and its isolation in high quantities from plant is difficult, a continued supply of podophyllotoxin requires large scale cultivation of suitable species with selected lines being produced by micropropagation. Arumugam and Bhojwani (1990) reported *in vitro* multiplication of *P. hexandrum* via somatic embryogenesis. Biotechnological production of podophyllotoxin using *in vitro* techniques may prove to be a good alternative.

Use of callus and cell suspension cultures for the production of podophyllotoxin

Cell cultures have been used in the past for the production of natural products *in vitro* as an alternative. The production of podophyllotoxin from callus cultures has been reported for *Podophyllum peltatum* (Kadkade, 1981; Kadkade, 1982). It is very difficult to establish callus cultures in *Podophyllum hexandrum*. Van Uden et al., (1989) reported initiation of cell cultures from the roots of *P. hexandrum* and production of podophyllotoxin in undifferentiated callus and cell suspension cultures. It has also been reported that coniferin was a substrate for podophyllotoxin production (Van Uden et al., 1990). Callus cultures of *P. hexandrum* Royle were very difficult to initiate, Van Uden et al. (1989) tested hundreds of basal medium, hormone combinations and concentrations and found B_5 medium supplemented with 2% coconut milk, 4% sucrose, 4 mg l^{-1} Naphthalene acetic acid to be the best. Cell suspensions were initiated by transferring the callus to same liquid medium agitated on a shaker at 150 rpm. After ten passages homogeneous and undifferentiated suspensions were obtained. Good podophyllotoxin producing cultures were dark brown coloured when they changed to yellow green complete loss of podophyllotoxin production was observed. Therefore, only dark brown calli and cell suspensions were used in this study, the highest content of podophyllotoxin was 0.3% on dry wt. basis. Light grown callus cultures contained 3–4 times less podophyllotoxin in comparison to dark grown cultures. The highest cellular accumulation of podophyllotoxin in suspension cultures was measured at day 15 during stationary phase of growth cycle followed by decrease in content. The cell suspension cultures of *P. hexandrum* do not appear to secrete podophylloyoxin in the culture medium as the amount of podophyllotoxin in the medium was reported to be negligible. Podophyllotoxin production in callus cultures derived from rhizomes of *Podophyllum peltatum* was stimulated upon illumination. Culture conditions and other complex factors are involved in the biosynthesis of podophyllotoxin (Van Uden et al., 1989; Heyenga et al., 1990). Woerdenbag et al. (1990) reported increased podophyllotoxin content in *P. hexandrum* cell cultures after feeding coniferyl alcohol complexed with β-cyclodextrin. Cells cultured in media containing 4 mg L^{-1} NAA accumulated 0.001–0.002% podophyllotoxin on dry weight basis. Feeding of 3mM coniferyl alcohol, dissolved in culture medium as

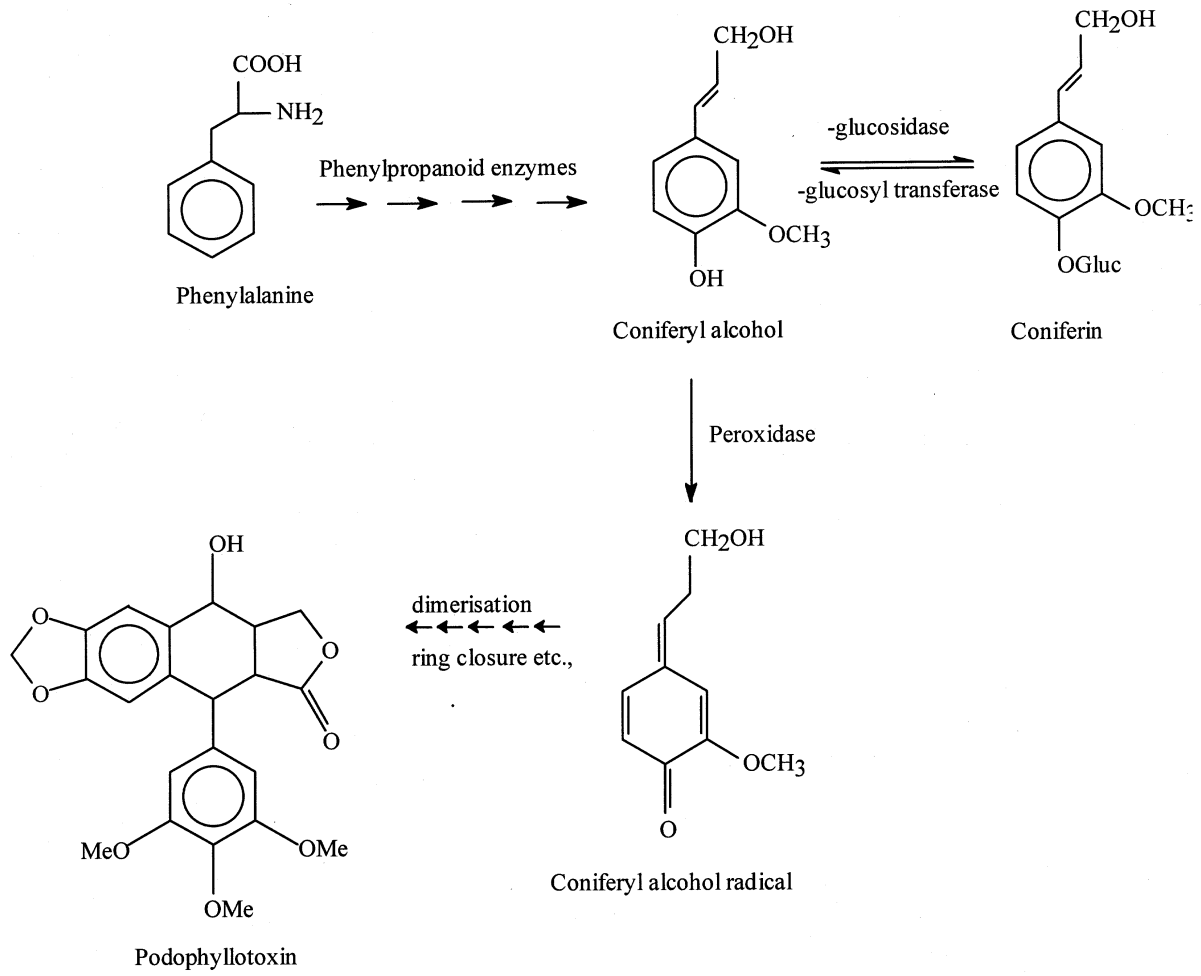


Figure 1. Biosythetic pathway for the production of podophyllotoxin (Van Uden et al., 1990).

β-cyclodextrin complex resulted in enhanced podophyllotoxin accumulation with a maximum of 0.012%. Noncomplexed medium enhanced it to a maximum of 0.006% (Van Uden et al., 1994).

Use of transgenic hairy roots for the production of podophyllotoxin

A new route for secondary metabolite production is the use of natural vector system. Genetically transformed hairy roots produced by infection of plants with *Agrobacterium rhizogenes*, a gram-negative soil bacterium, appear to be a promising tool for secondary metabolite production. These hairy roots are unique in their genetic and biosynthetic stability and their fast growth offers an additional advantage. These fast growing hairy roots can be used as a continuous source for the production of valuable secondary metabolites. Hairy roots are valuable source of root derived phytochemicals that are useful as pharmaceuticals cosmetics and ingredients in food. These hairy roots are best experimental system for production of secondary metabolites (Giri and Lakshmi Narasu, 1999). Podophyllotoxins are also produced from root cultures of *Linum flavum* (Berlin et al., 1988), cultures of *Linum flavum* produce 5 methoxy derivatives of podophyllotoxin (5 MPT). Higher production (5–10 fold) of 5 MPT is reported from hairy roots than untransformed cell suspension cultures (Oostdam et al., 1993).

As podophyllotoxin is extracted from the rhizome of the plant, it can prove to be an alternative source for production of podophyllotoxin by induction and culture of transgenic hairy roots. Our laboratory is

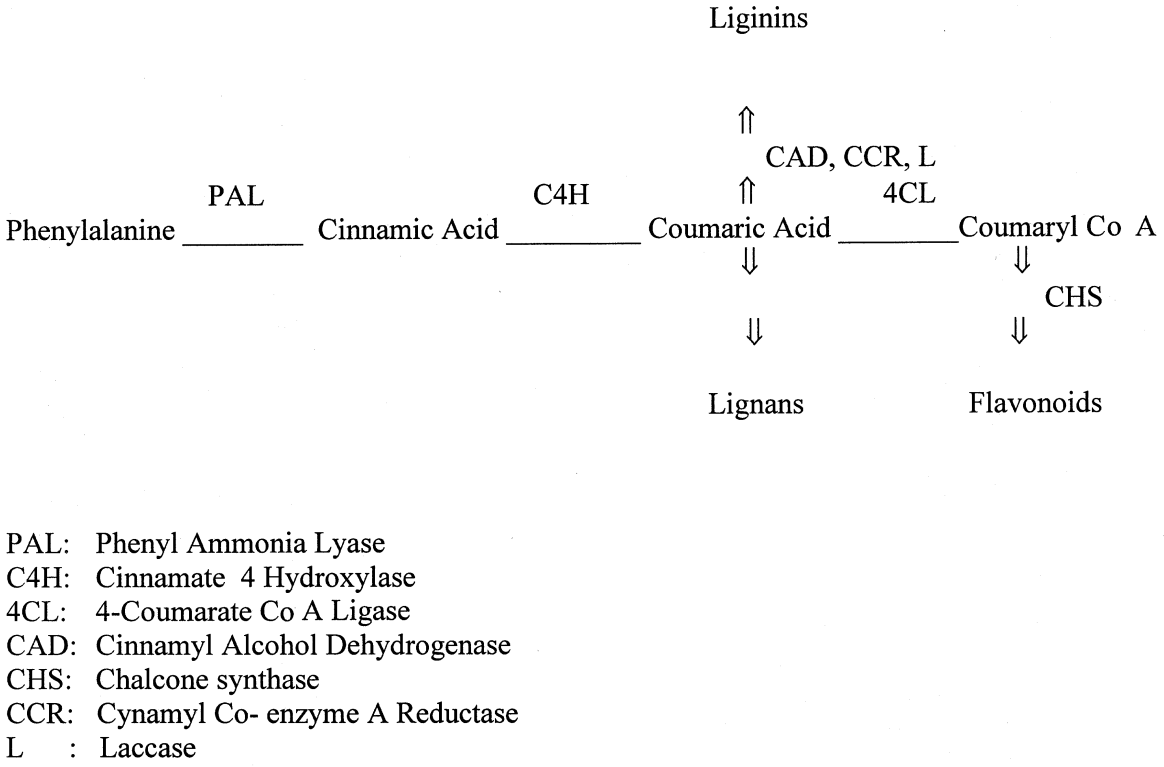


Figure 2. Phenyl propanoid pathway.

actively engaged to standardize the parameters for the production of transgenic hairy roots in *Podophyllum hexandrum*.

Podophyllotoxin production using biotransformation approach

Recently the potential of biotransformation using cell cultures for the production of etoposide has been emphasized. Etoposide is derived chemically from podophyllotoxin by two step conversion. It has been demonstrated that a cell line of *Podophyllum peltatum* active in the biosynthesis of podophyllotoxin was able to maintain repeated biotransformation by oxidative coupling of the butanolide to the podophyllotoxin analouge. It has been carried out in a bioreactor for a total of more than 15 cycles each of 24 h duration giving a yield of around 50%. This result indicates the feasibility of a biotransformation route to podophyllotoxin using a chemically synthesized precursor (Franssen and Walton 1999).

Metabolic engineering considerations for the production of podophyllotoxin

Genetic engineering of metabolic pathways has recently been proved to be highly beneficial for the manipulation of secondary metabolite production.

Podophyllotoxin, which belongs to lignan group of compounds, is the product of Phenyl Propanoid Pathway (PPP) (Figures 1 and 2). Isolation and cloning of gene for the key enzymes in secondary metabolite pathway branch chains for lignins are rather difficult. Alkaloid biosynthetic pathways that are too long can be modified using antisense or co-suppression technology so that desired alkaloid can be accumulated by blocking the side pathway or catabolic steps (Kutchan, 1995). The isolation and cloning of gene encoding Cinnamyl Alcohol Dehydrogenase (CAD) offers unique opportunity. Down regulation of CAD using antisense technology offers potential for the reduction of lignins which has major industrial and economic implications (Walter et al., 1988). Recently in addition to CAD enzymes like Cinnamyl Co-enzyme Reductase(CCR) and Laccase have been isolated which

has helped in the directed negative regulation of lignin biosynthesis. These developments for the reduction of lignin synthesis may offer possibility for channeling precursor for the increased synthesis of lignans in the branched chain in the PPP biosynthetic pathway. This diversion of biosynthetic pathway may offer the possibility to increase podophyllotoxin production.

Conclusion

In this paper an attempt has been made to bring about the importance and uses of podophyllotoxin for its pharmaceutical applications. The emphasis has been made how the cell and callus cultures, transgenic hairy root system and metabolic engineering considerations would be a possible alternative strategy for the production of podophyllotoxin.

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