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REVIEW ON PODOPHYLLOTOXIN: SOURCES, EXTRACTION, APPLICATIONS AND CURRENT PERSPECTIVES

Parul Sharma*[1], Abhay Kumar Verma[2], Sonia Chauhan[3], Somnath Kharwar[4] and Priyanka Shrivastava[5]

*[1,2,3,4,5]Spectrum Institute of Pharmaceutical Sciences and Research, Greater Noida, India.

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***Corresponding Author**
Prof. Parul Sharma
Spectrum Institute of Pharmaceutical Sciences and Research, Greater Noida, India.

ABSTRACT

Lignans are a class of secondary plant metabolites produced by oxidative *β-β* linked dimerization of two phenyl-propanoid units. Podophyllin resin obtained from ethanolic extract of *Podophyllum peltatum* and *Podophyllum emodi* belonging to family Berberidaceae, is a good source of the aryltetratin type lignin. Podophyllotoxin and its semi-synthetic derivatives exhibit several biological activities including antiviral and anti-neoplastic. Etoposide and Teniposide have emerged as successful antineoplastic agents after modifications of the basic skeleton in podophyllotoxin. The mechanistic studies and chemical manipulations to improve their bioactivity are still under investigation. The podophyllotoxin analogues have advanced in several therapeutic areas and developmental chemotherapy. Podophyllotoxin based multidrug regimens have been developed for better therapeutic efficacy.

KEYWORDS: Lignans, Podophyllum, Podophyllotoxin, HPTLC.

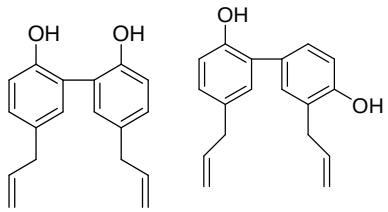
INTRODUCTION

India is one of the twelve-mega diversity centers with a varied variety of flaura and fauna marked by remarkable ecosystem and genetic diversity. Since a vast majority of medicinal plants have been recklessly exploited, it has become important to rationalize the use of some important medicinal plants.[1]

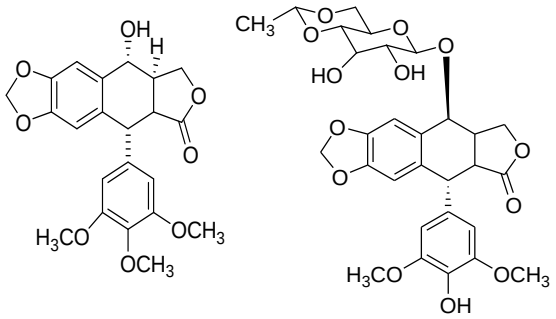
Plant synthetic derivatives have been employed in various therapies for malignant diseases, among these, majority are used against cancer. Lignans are a class of secondary plant metabolites produced by oxidative *β-β* dimerization of two phenyl-propanoid units. The term

lignin is assigned to the optically active dimers of phenyl-propanoids linked by the central carbon atoms of their side chains, while the term *neolignan* applies to dimers of two phenylpropanoids that are not connected by the bond between the central carbons of the side chains. Lignans are mostly present in nature in free form, while their glycoside derivatives are only minor components. They are widely distributed in the plant kingdom, found in more than seventy families representing pteridophytes, gymnosperms and angiosperms. Lignans are found in roots, rhizomes, stems, leaves, seeds and fruits. The wound resins of the trees and heartwood are good source of lignans and can be readily isolated in substantial quantities.[2]

In spite of their extensive distribution, their biological functions in plants are not clear. Since lignans are reported to have potent antimicrobial, antifungal, antiviral, antioxidant, insecticidal and anti-feeding properties, they play probably an important role in plant defense and may also participate in the plant growth and development. The o=n aryltetra-hydro-napthalene lignans from Podophyllum species, neolignan from *Magnolia officinalis* and aryl naphthalene, aryltetra-hydronapthalene and diarylbutanelignans from *Phyllanthus* species have received lot of attention for exhibiting varied therapeutic activities such as anti-inflammatory, anti-microbial etc, as depicted in figure 1.



Magnolol (1) Honokiol (2)



Podophyllotoxin (3) Etopside (4)

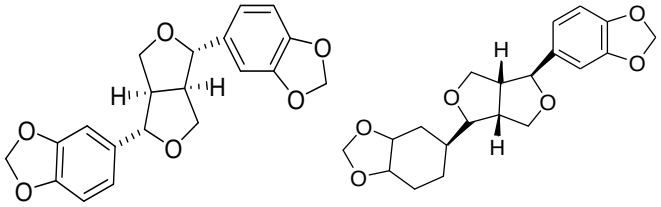


Fig: 1 Main lignans from *Magnolia*(1, 2), *Podophyllum*(3, 4) and *Sesamum*(5, 6) species

Podophyllum

Podophyllum is a genus of six species of herbaceous perennial plants belonging to family Berberidaceae, native of eastern Asia (five species) and eastern North America (one species, *P. peltatum*). They are woodland plants which typically grow in colonies derived from a single root. The stems grow 30–40 cm tall with palmately lobed umbrella-like leaves up to 20–40 cm in diameter surrounded by 3–9 deep cut lobes. The plants produce several stems from a creeping underground rhizome; some stems bear a single leaf and do not produce any flower or fruit. Flowering stems bear a pair or more of leaves with 1–8 flowers in the axil. The apical flowers with 6-9 petals are white, yellow or red, 2–6 cm in diameter. They mature into a green, yellow or red fleshy 2–5 cm long fruit. All the parts of the plant, except the fruit, are poisonous. Even the fruit can cause indigestion. They are also grown as ornamental plants for their attractive foliage and flowers. *Podophyllum peltatum* grows in moist, humus-rich soil and partially shaded spots.[20]

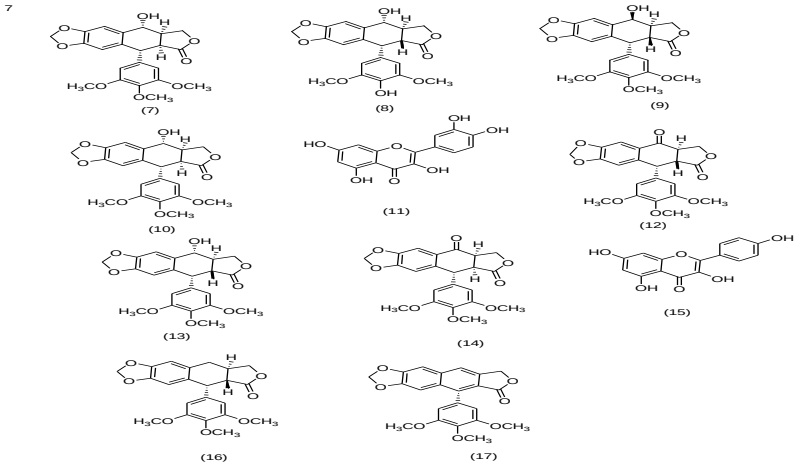


Fig:2 Structures of chemical constituents of Podophyllum (7) Picropodophyllinglucoside; (8) 4'-demethylpodophyllotoxin; (9) Epipodophyllotoxin; (10) Picropodophyllin; (11) Quercetin; (12) Podophyllotoxone; (13) Podophyllotoxin;

(14) Picropodophyllone; (15) Kaempferol; (16) Deoxypodophyllotoxin; (17) Dehydropodophyllotoxin[28][45][8]

DISCOVERY AND DEVELOPMENT

The first known identity and usage of Podophyllum was described by Linnaeus(Species Plantrum, 1753). Further as the active potential of Podophyllum went under expertise, its first chemical investigation was carried out by Podwyssotzki.[55][53], the correct empirical formula for podophyllotoxin was first assigned by Borsche and Niemann[3] which was later confirmed by Gensler.[22] The first total synthesis and the configuration of podophyllotoxin was established and confirmed by the analysis of crystal structure of easily obtained 2'-bromopodophyllotoxin.[51]

The alcohol-soluble portion of podophyllum was named podophyllum resin and podophyllin was included in United States Pharmacopoeia in 1820. Bently, in 1861, mentioned local anti-tumour effects of podophyllin. Later, Kaplan in 1942 reported that topical application of podophyllin in condyloma acuminatum (a type of venereal wart) produced very satisfactory clinical results, subsequently King and Sullivan in 1946 found that podophyllin caused pronounced cytological changes in normal human and rabbit skin and gave the mechanism of action of podophyllotoxin.[66] These reports revived medicinal interest in podophyllin. The gastrointestinal toxicity of Podophyllum indicated that the podophyllum lignans might occur naturally as glycosides. Various chemical modifications were carried out to inhibit enzymatic degradation. Research efforts were then focused on a program to chemically modify both the glucosides and aglucones of a wide range of podophyllotoxin derivatives eventually leading to discovery of the clinically active anticancer drugs etoposide and teniposide.[64][65] Interestingly, it was not until 20 years later that the interaction of this drug with DNA began to be understood and was recognised that the effects were mediated by topoisomerase II. In 1966 synthesis and biological evaluation of etoposide were done, further water-soluble phosphate ester prodrug of etoposide and etopophos was launched by Bristol-Myers Squib.[7] This prodrug was readily converted *in vivo* by endogenous phosphatase to the active drug.

Pomier and co-workers identified azatoxin using molecular modelling of the pharmacophore defined by topoisomerase II inhibitors in 1992. In 1993, scientists at Taiho Pharmaceutical Co. Ltd. synthesised a series of 4b-alkyl amino derivatives of 4'-O-demethyl-4-deoxypodophyllotoxin, from this series, TOP-53 was selected for further evaluation, TOP-53 displayed twice the inhibitory activity of etoposide against topoisomerase II and exhibits *in*

vivo superior antitumor activity than etoposide against several types of cancer and in view of high activity and good properties so it was progressed later to phase II clinical trials. In the year 1996 US launched the prodrug etopophos.[68][35][36] Series of continuous development and researches lead to the discovery of a novel compound Tafluposide in 2000 which was described as a novel catalytic inhibitor of topoisomerase I and II as illustrated below.

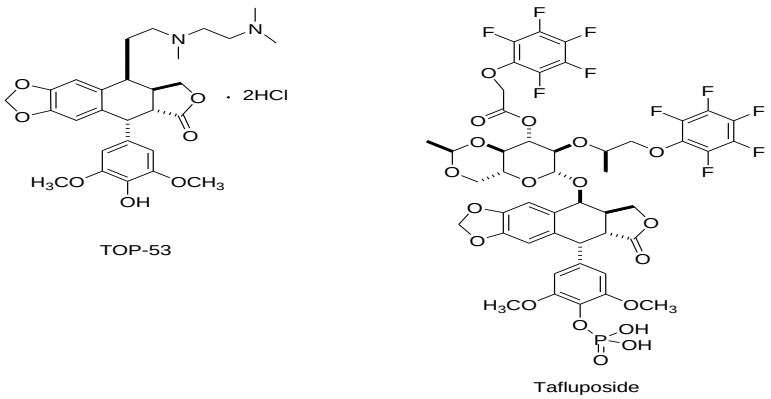


Fig: 3 Structures of TOP-53 and Tafluposide

EXTRACTION AND NEW APPROACHES

Podophyllotoxin has traditionally been isolated from podophyllin, resin of Podophyllum rhizome. *Podophyllum emodi*(Indian Podophyllum) is preferred to *Podophyllum peltatum* (American Podophyllum) because the first one gives more resin and is this is richer in podophyllotoxin.[56][24] The content in podophyllotoxin is about 4.3% of dry weight in *P.emodi* as compared to 0.25% in *P.peltatum.*[28] Recently, a new extraction process was described based in rehydration of powered tissues of *P.peltatum* prior to extraction with organic solvent. This allows endogenous β-glucosidases to hydrolyze lignans thus increasing the yield of podophyllotoxin of rhizomes and leaves. However, the collection of known plants that are natural sources of podophyllotoxin is limited and insufficient to supply the increasing demand of this compound as starting material for synthesis of etoposide and the semi-synthesis of new derivatives. The main causes were the availability of the endangered *P.emodi* and the difficulties in its cultivation which must be solved.[17] To overcome these new possibilities of in vitro propagation and optimisation of the cultivation of *Podophyllum* species have been studied.

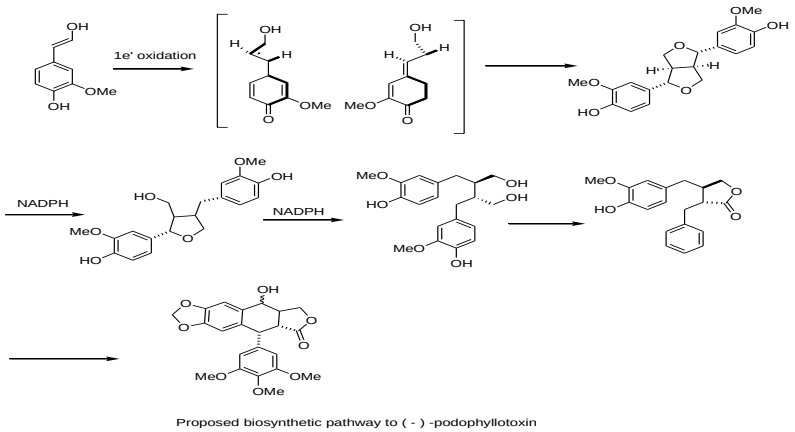


Fig: 4 Proposed biosynthetic pathway to (-)-podophyllotoxin (Canel et al., 2000)

Various new approaches in biotechnology have led to in vitro production of cyclolignans. These methods have lead to new method for sustainable development of endangered plants. Plant cell and organ culture are one of the strategies being developed[52] Since total chemical synthesis of podophyllotoxin is complicated and expensive, biotechnological approaches particularly plant cell and tissue cultures appear to be attractive alternatives for the production of this pharmaceutically important lignan. Induction of callus culture from *P. peltatum* and detection of podophyllotoxin from such cultures was first reported by Kadkade[31][70] initiated podophyllotoxin producing callus cultures from *in vitro* plantlets of the Indian Podophyllum; dark-grown cultures accumulated upto 0.3% podophyllotoxin (dry weight basis). After several tedious trials we initiated and established callus cultures of the Indian Podophyllum from *in vitro* grown acetic seedling explants and roots and rhizomes isolated from 1 year old mature plants on B5 and MS media supplemented with growth regulators. Podophyllotoxin could be detected from all the callus lines that survived after 1 year of initiation, induced from different juvenile and mature explants.[49] Callus cultures producing podophyllotoxin have also been initiated from needles of *Callitris drummondii*[69] leaves of *Juniperus chinensis*[61] Podophyllotoxin and its derivative 6-methoxypdophyllotxin have been obtained by in vitro production of differentiated organ cultures, mainly roots, undifferentiated calli and suspension cell cultures of different species of *Podophyllum, Linum*, *Juniperus* and *Callitris.*[20][9-1]

Transgenic hairy roots produced by infection of plants with *Agrobacterium rhizogenes*, have proved to be a valuable source of root derived phytochemicals and have been considered as the best experimental system for production of secondary metabolites. With this technique, Oostdam et al. (1993) reported a 5-10 fold higher production of 6-methoxypododphyllotxin

than in untransformed cell suspension cultures. Suspension cultures have been proposed to be a viable alternative for the production of economically important phytochemicals. Such cultures have a relatively fast growth rate and are easy to manipulate. Suspension cultures producing podophyllotoxin were initiated from different plant species which are tabulated below in table 1.

Table1. Podophyllotoxin production in cell suspension culture of different plant species

Species	References
Callitrisdrummondii	[38]
Linum album	[37]
L. nodiflorum	[69]
*L. mucronatum*spp. *Armenum*	48]
Podophyllumhexandrum	[10][1][42]
P. peltatum	[45][6]

BIOLOGICAL ACTIVITIES

Three semi synthetic derivates of podophyllotoxin, etopside, teniposide and etopophos are widely used as anticancer drugs and show good clinical effects against several types of neoplasms including testicular and small-cell lung cancers, lymphoma, leukemia, Kaposi's sarcoma etc.[2][59] Among the plethora of physiological activities and potential medicinal and agricultural application, the antineoplastic and antiviral properties of podophyllotoxin congeners and their derivatives are the most eminent from a pharmacological perspective. Semisynthetic derivatives of epipodophyllotoxin 3, e.g. Etoposide 11[1], etopophos 12 and teniposide 13 induce a premitotic block in late S or early G_2 stage (Hainsworth and Greco, 1995). This result due to binding of etoposide to topoisomerase II, an enzyme required for unwinding of DNA during replication. Topoisomerase II forms a transient, complex, which allows one double DNA strand of DNA to pass through a temporary break in another double strand. Etoposide binds and stabilizes the cleavable complex preventing repair of double-strand breaks.[59]

Compounds with C-4β configuration are less cytotoxic and inhibit DNA-topoisomerase II. All podophyllin based drugs possess modified 4β-D-glucoside moieties. However, highly active derivatives have been synthesized that have either amino or alkyl residues at C-4[26] with a few exceptions, the C-2α, C-3β trans configuration is of crucial importance for biological activity. On the basis of molecular modeling studies,[47] proposed a composite pharmacophore model with three structurally distinct domains: a DNA intercalating moiety,

the minor groove binding site and the molecular region that can accommodate a number of structurally diverse substituents, which might also bind to minor groove.

The similarity between the modes of action of aryl tetra lignans as anticancer and antiviral agents is noteworthy. Owing to their ability to bind tubulin, these lignans disrupt the cellular cytoskeleton and thus interfere with viral replication. In addition to tubulin binding, synthetic podophyllotoxin analogs show inhibition of reverse transcriptase which may be exploited to selectively combat RNA viruses such as the human immunodeficiency virus (HIV).

The challenge of stereo-selective synthesis is embedded in the formation of the four contiguous stereo centres and the presence of the base sensitive *trans*-lactone moiety.[63] Four general approaches to the synthesis of podophyllotoxin derivatives have been developed. Several variations and innovations have been introduced within each of the overall schemes. The four routes involve the elaboration of a $\varkappa$-oxo ester, the lactonization of dihydroxy acid, the cyclization of a conjugate addition route, or the utilization of a Diels-Alder reaction to construct the aryltetralin unit.

A number of synthesis, particularly some of those in the dihydroxy acid route, also put the Diels-Alder protocol to establish the aryltetralin molecular framework. The majority of useful stereo-selective routes to podophyllotoxin and its analogs/derivatives have also been designed according to the synthetic principles underpinning these approaches. Extensive modifications at various sites of the podophyllotoxin backbone have been introduced with a view to enhance its therapeutic activity or eliminate undesirable side effects.[33][32][72][47]

Yield of podophyllotoxin from *Podophyllum* species can be dramatically increased through use of selective processing techniques that allow the in-situ conversion of podophyllotoxin glucosides. The destructive effect of this resin on experimental cancer cells in animals is also described. The antiviral activity of aqueous extract of *Podophyllum peltatum* was investigated. Extensive research is being carried out all over the globe for obtaining podophyllotoxin and its analogues, these studies have also accounted for many patents as well.

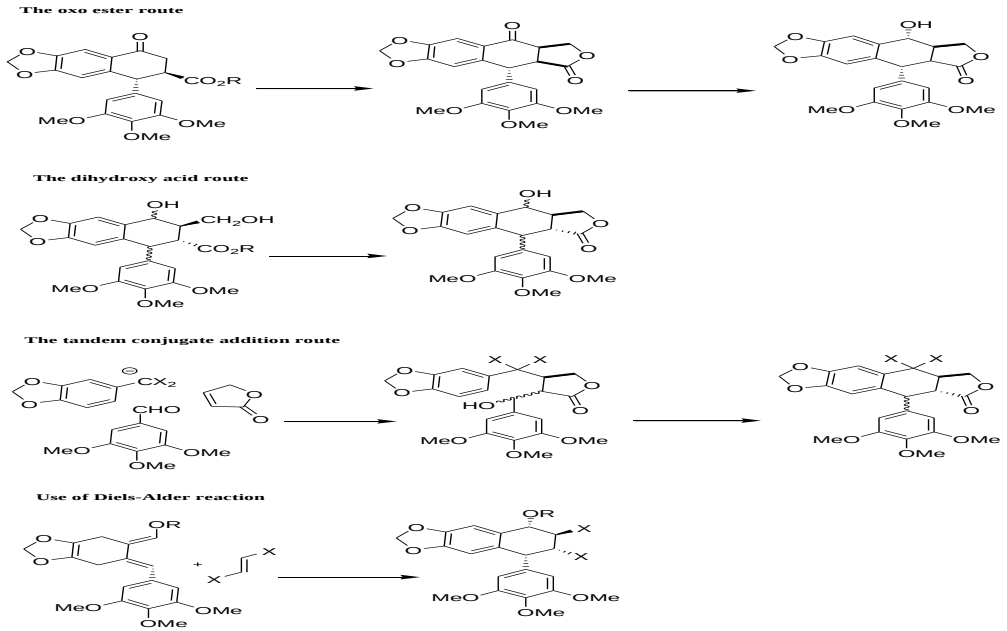


Fig: 5 General approaches to the synthesis of podophyllotoxin derivatives[6]

PREPARATION OF EXTRACTS AND QUANTITATIVE ANALYSIS

The rhizomes and leaf samples of *Podophyllum* species were collected from all the different locations. The rhizome samples were thoroughly washed under running tap water to free the plant material of soil, dust and extrinsic contamination. The samples after proper washing were air dried in hot oven at 30ºC overnight. The dried samples were powdered and were subjected to methanolic extraction in a Soxhlet by hot extraction (2 washes for 3 hours each). The solvent was removed and concentrated under vaccum using rotavapor at 50ºC. Removal of the solvent from the extract under reduced pressure gave a semi-solid gum which was re-suspended in HPLC grade methanol for analysis. The lignans were separated and quantified by HPLC as tabulate din the table below.[6]

Table 2. Compounds isolated under different HPLC conditions

Compounds	Sample	HPLC Conditions	Detection	References
Podophyllotoxin, α-peltatin, β-peltatin	*Podophyllum* resin	Column: Perkin-Elmer silica A (550×2.5mm). Mobile phase: $CHCL_3$ with 1.8%ethanol. Flow rate:0.8ml min^{-1}	UV 206 and 280 nm	Treppendahl et al., 1980
Seven diastereoisomers of podophyllotoxin	Mixtures of diastereoisomers	Column A: Hypersil silica gel column (250×5mm, 5µm). Mobile phase: *n*-heptane-CH_2CL_2-CH_3OH 90:10:4. Flow rate: 1.0ml min^{-1}. Column B: ODS-Hypersil (250×5mm). Mobile phase:	UV 280 nm	Lim et al., 1983)

		CH3CN-CH_3OH-H_2O 22.5:22.5:55 or CH_3CN-DMSO-CH_3OH-H_2O 20:20:10:50. Flow rate 2.0ml min^{-1}		
Podophyllotoxin	Podophyllum resins of different sources	Column: LiChrospher Si100. Mobile phase: CH_3OH-tetrahydrofuran-acetic acid-hexane 10:4:1:100. Flow rate 2.0 ml min^{-1}	UV 280 nm	Fay et al., 1985
Eight Podophyllumlignans and glucosides	Resins *P.peltatum* and *P.emodi*	Column: Taxsil (250×5 mm, 5µm). Mobile phase: complex gradient elution mode containing three solvents. Solvent A: reagent alcohol (Fisher Scientific Reagent Alcohol: 90.6% ethanol, 4.5%CH_3OH, 4.9%isoprppanol)-tetrahydrofuran-methyl-*t*-butyl ether 4:3:1. Solvent B CH_3OH-H_2O-acetic acid (15:84:1) containing0.1% ammonium acetate pH=3.46. Solvent C: CH_3CN. Flow rate:1.1ml min^{-1}	Photodiode array detection over the 210-200nm range, quantization at 240 nm	Lim et al., 1996
Podophyllotoxin	Fifty plants and callus cultures of *Podophyllumhexandrum*	Column: Nova Pak C18 (250×4.6mm). Mobile phase: CH_3CN-CH_3OH-H_2O 37:5:58. Flow rate: 1.5ml min^{-1}	UV 235nm	Sharma et al.,2000

Most methods for determining podophyllotoxin use High Performance Liquid Chromatography (HPLC). Other methods include enzymatic techniques. Methods that use a small volume of sample are desirable when estimation has to be done where collection of larger volumes is not feasible. At times techniques like RP-HPLC and RP-TLC techniques for the determination of podophyllotoxin in dry cell mass obtained by tissue culture or single plant basis. Since MS is now becoming a quite common detection approach in lignan analysis by means of HPLC or GC, the methods using the MS detection were mentioned in the parts dedicated to the given methodologies and not in the part devoted to hyphenated methods. Although HPLC-MS and GC-MS has simplified the lignan identification, on several occasions MS data alone is not sufficient for their structure elucidation. Further data especially NMR spectra are therefore demanded.

CURRENT PERSPECTIVES

Additive and synergistic laboratory interactions with other cytotoxic drugs are being continuously being exploited to allow development of podophyllotoxin-based multidrug regimens. These are showing activity in several malignancies and many of its related analogues will complement conventional pharmaceuticals in treatment, prevention and diagnosis of disease, while at the same time adding value to agriculture. Extensive structural modifications of podophyllotoxin have been performed in order to obtain more potent and less toxic antitumor agents, which have resulted in the widespread clinical introduction of two semisynthetic glucoconjugate analogues of etoposide and teniposide and newer agents with promising preclinical activity, are in various stages of clinical assessment. As knowledge of molecular and biochemical mechanisms of action and resistance continues to expand, newer and better podophyllotoxin-based strategies for treatment of malignant disease are likely to evolve.

While the traditional source of podophyllotoxin are being extensively utilised it is becoming scarcer whereas the demand for the compound continues to increase. In U.S. alone sales of etoposide tripled in 1995 and have since risen.[6] Such a growing demand for podophyllotoxin thus exerts severe pressure on the natural source, *P. Emodi* and has already drastically reduced the size of natural populations. We must search for alternative methods such as the development of shorter synthetic routes, the utilization of biotechnological and enzymatic approaches, manipulations of the biosynthetic pathway, alternative and renewable natural sources like the American P. peltatum in order to meet the increasing demand and utilisation patterns.

CONCLUSION

One of the major therapeutic areas where natural products have made a major impact on continuity and quality of life is in various malignant diseases. In fact, most of the important anti-neoplastic drugs are natural products, from plants or microorganisms. The introduction of etoposide 11, etopophos 12, teniposide 13 and various other analogs and derivatives of (-)-podophyllotoxin 1 as anti-tumor drugs, is an excellent example of the manner in which useful pharmaceuticals may be developed from folk medicines. Thus, more than fifty years after the first medicinal application of antimitotic activity of (-)-podophyllotoxin was proposed this aryltetra lignan continues to be the subject of extensive research.

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