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An overview of lignans with special reference to podophyllotoxin, a cytotoxic lignan

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ABSTRACT

Lignans are the natural products that are specifically made up of two hydroxycinmoyl alcohol units which are joined by C8 side chain. Lignans are originated from the same precursor as lignins but lignans are formed only by stereospecific coupling of monolignol unlike of lignins. It posses significant medicinal importance along with its age-old traditional uses, especially podophyllotoxin, for its cytotoxic and antiviral activity. Here we reviewed the basic structure of lignans and its variability along with history and the recent reconstruction of podophyllotoxin biosynthetic pathway from pluviatolide to (–)-4-desmethylepipodophyllotoxin. To elucidate the monolignol trafficking towards lignan and lignin, we discuss the regulatory mechanism of this trafficking by streospecific coupling by dirigent protein. Furthermore the medicinal importance of various lignans, especially of podophyllotoxin and its glycosidic derivatives, are conferred here with their medicinal efficacy for the benefit of human kind.

Keywords: Cytotoxic, (–)-4'-desmethylepipodophyllotoxin, Dirigent protein, Lignan, Podophyllotoxin

INTRODUCTION

Lignans are one of the most important group of plant secondary metabolites originated from the phenylpropanoid pathway. They have a significant role in plant defense and are most effective in human nutrition and medicine.[1] They are formed by the combination of two phenylpropane units and they can be classified into four groups, namely Lignans, Neolignans, Oxyneolignans and trimmers, higher analogues and mixed lignanoids.[2] In the case of cyclolignan, a carbocycle is formed by two carbone carbone bond through side chains and situated between two phenylpropane, one of them situated between β-β' position.[3] Plants containing lignans have been used since approximately 1000 years ago as folk remedies in traditional medicine of many diverse cultures. Plants with high lignan contents were commonly used in Chinese, Japanese and the Eastern world folk medicine, for example, *Kadsura coccinea* (Schizandraceae), *Fraxinus* sp. and *Olea europaea* (Oleaceae).[4] Lignans were isolated from more than 60 families of vascular plants and from their different parts namely roots, rhizomes, woody parts, stems, leaves, fruits, seeds and in other cases, from exudates and resins.[5,6,7] Lignans have also been detected in the urine of humans and other mammals, however, some of them are identical to possible components of the plant diet, others show distinct chemical functions, showing that the internal metabolic transformation may occur.[4] Several *Streptomyces* species were also found as a good source of lignans.[8] A cytotoxic lignan, namely podophyllotoxin and other 8-8′ linked lignan, are mainly recognized for their cytotoxicity and antitumor activity. Other lignans except podophyllotoxin, like (-) steganacin and (-) steganangin , isolated from the stem and bark of *Steganotaenia araliacea*, showed a good antileukemia activity as well. Lignans like podophyllotoxin and α-peltetin showed some antiviral activity beside of their cytotoxic activity[1]. Some other lignans are also known for anti hepatotoxic, anti HIV, anti inflammatory, antiasthmatic and antidepressant activity. Most effective cytotoxic lignan viz. podophyllotoxin obtained from *Podophyllum hexandrum* and *P. peltatum* were derivatized into their glycosidic form, namely etoposide and tenposide, and used as chemotherapeutic agents for small cell lung carcinoma, leukemia and cancer treatment.

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Chemical synthesis of cylolignan is not commercially feasible. To overcome this problem several biotechnological approaches were introduced, like biotransformations with whole cell fermentations,[9] especially transgenic hairy roots produced by infection of plants with *Agrobacterium rhizogenes*, is a valuable source of root derived phytochemicals[10] and have been considered as 'the best experimental system for the production of secondary metabolites'.[11] With this technique, Oostdam *et al*, (1993)[12] reported a 5–10 fold higher production of 6-methoxypodophyllotoxin than in untransformed cell suspension cultures. To meet this ever-increasing demand, cell culture-based production of cyclolignan namely podophyllotoxin has been investigated as well.[13,14,15,16,17] Not only podophyllotoxin, but also other related lignans were also identified from cell culture of the genus *Linum.*[18,19,20,21,22,23] In a recent study, hinokinin, the most potent anti-human hepatitis B virus agent, was isolated from *in vitro* cultures of *L. corymbulosum* focussing to resolve the molecular basis of lignan biosynthesis.[24] We also reported that the generation of *P. hexandrum* plantlets through direct organogenesis from rhizome explants.[25] Currently (–)-4′-desmethylepipodophyllotoxin (the etoposide aglycone), a naturally occurring lignan that is the immediate precursor of etoposide and unlike podophyllotoxin, a potent topoisomerase inhibitor was synthesized *in vivo* and isolated in a very small amount from the leaf of transgenic *Nicotiana tabacum* over expressing 6 novel enzymes.[26] Presently, our review is aimed to summarize the structural variability, occurrence, evolution of biosynthetic pathway, medicinal importance and uses of lignans from different plants. Here we also discuss about mode of action of cytotoxic cyclolignan, namely podophyllotoxin with their glycosidic derivatives. As lignin and lignan both are originated from the phenylpropanoid pathway so their regulation through regiochemical coupling of monolignol by dirigent protein oxidase is also discussed here.

CHEMICAL STRUCTURES AND OCCURRENCE OF LIGNAN

In the earlier of the 19th century, some plant secondary metabolites were isolated and they were named according to their tradition uses without knowing their chemical properties and sturucture. Guaiaretic acid (Figure 1A) is one of such lignan, the skeletal formula of which was proposed by Schroeter *et al*, (1918).[27] It was proposed that guaiaretic acid and its related compound belong from unique dimeric class phenylpropanoid substances and they are linked exclusively through 8-8′ bonds. This group of dimeric phenylpropanoid structure was named as lignane by Haworth (1936)[28] and considered pinoresinol, guaiaretic (Figure 1A,B) like substances as a lignan which contains two regiospecifically linked cinnamyl (C_6C_3) molecules to their C8 Carbon. According to Chunha *et al* [29] most of the known natural lignans are oxidized at C9 and C9´ and based upon the way in which oxygen is incorporated into the skeleton and on the cyclization patterns, a wide range of lignans of very different structural types can be formed.

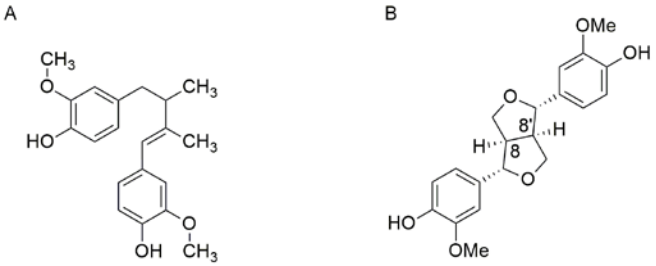

Figure 1. Dimeric phenylpropanoid substances ,that are lignans, connected through 8-8' bonds. (A) Guaiaretic acid, (B) Pinoresinol.

Due to this fact, lignans are classified into eight subgroups[30,31] and among these subgroups, the furan, dibenzylbutane and dibenzocyclooctadiene lignans can be further classified in "lignans with C9 (9´)-oxygen" and "lignans without C9 (9´)-oxygen". Later it was found that lignan also present as larger molecule (oligomeric lignan) in various plant species [1]. Some other substances like dehydrodiconiferyl alcohol (8-5′ linked), megaphone (8-1′ linked), coniferyl alcohol ether (8-O-4′ linked) (Figure 2) were named as neolignan.[32, 33] But these neolignan later grouped as allylphenol derived coupling products. Later neolignans were referred as lignan like substances which lack carbon at the C-9 or C-9′ position or a methyl group at aromatic mehoxyl group (Figure 2).[34,35,36]

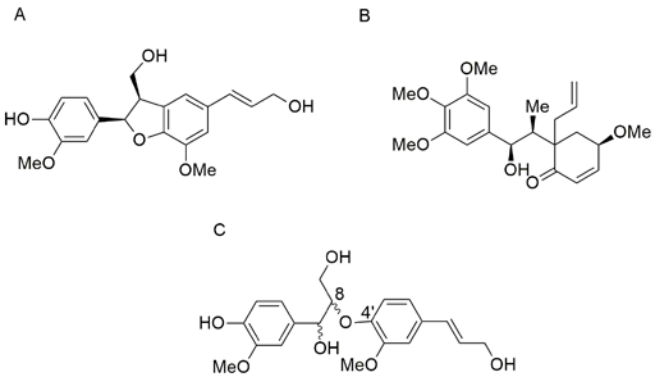

Figure 2. Different types of neolignan. (A) Dehydrodiconiferyl alcohol (8-5' linked), (B) Megaphone (8-1' linked), (C) Coniferyl alcohol ether (8-O-4' linked).

Among the all types of lignan, 8-8′ linked are the most abundant in nature and these are also classified furofurans, arylnaphthalenes, dibenzylbutanes etc.[1] Lignans can be found from a large range of vascular plant species, starting with very primitive like hornworts to woody angiosperm. Amount of lignan accumulation in plants varies with species to species. For example *Thuja plicata* heartwood contain dimeric lignan and oligolignan 20% of its dry weight.[37] Tissue specific localization of lignans also differs with different plant species. Most important cytotoxic podophyllotoxin mainly found from the rhizome and leaf of *Podophyllum sp.* Podophyllotoxin is also found from the different plant genera like *Jeffersonia* sp., *Diphylleia* sp.and *Dysosma* sp. (Berberidaceae), *Catharanthus* sp. (Apocynaceae), *Polygala* sp.(Polygalaceae), *Anthriscus* sp. (Apiaceae), *Linun* sp. (Linaceae), *Hyptis* sp. (Verbenaceae),

Teucrium sp., *Nepeta* sp. and *Thymus* sp. (Labiaceae), *Thuja* sp., *Juniperus* sp., *Callitris* sp. and *Thujopsis* sp. (Cupressaceae), *Cassia* Sp. (Fabaceae), *Haplophyllum* sp. (Rutaceae), *Commiphora* sp. (Burseraceae) and *Hernandia* sp. (Hernandiaceae).[38-49]

MAMMALIAN LIGNAN

According to Thompson *et al*, (1996)[50] Secoisolariciresinol diglucoside (SDG), a mammalian lignan precursor found in high-fiber foods, was isolated from flaxseed. SDG is metabolized by human colonic microflora by a series of hydrolysis, dehydroxylation and demethylation reactions to enterodiol [2,3- bis(3-OH phenyl) methylbutane-1,4-diol; ED; MW = 302] which can then be oxidized to enterolactone ([trans-2,3-bis(3-OH phenyl) CH3]-g-butyrolactone; EL; MW = 298) (Figure 3).[51] According to Setchell *et al*, (1981)[52] high concentration of these lignan in human urine and their cyclic pattern of extraction during menstrual cycle and their increased amount in early pregnancy clearly suggests its physiological activity in the case of pregnancy. These lignans also have cytotoxic activity *in vitro* at very high concentration. According to Kitts *et al*, (1999)[51] Enterodiol and enterolactone also have potent antioxidant activity.

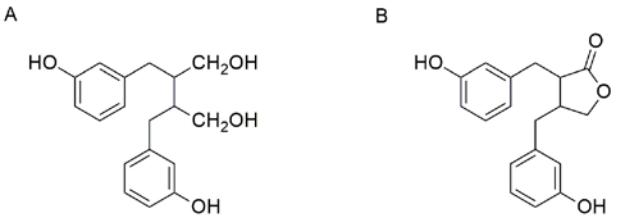

Figure 3. Mammalian lignan, metabolized from SDG. (A) Enterodiol, (B) Enterolactone.

LIGNAN BIOSYNTHETIC PATHWAY AND RECONSTRUCTION OF PODOPHYLLOTOXIN PATHWAY FROM PLUVIATOLIDE TO (–)-4′-DESMETHYLEPIPODOPHYLLOTOXIN

Formation of lignan by coupling of two phenylpropanoid units was first proposed by Erdtman (1933)[53] (from lignan biosynthetic pathway). Precursor of lignan is conferyl alcohol which is produced by cinnamyl alcohol dehydrogese (CAD) from the coniferaldehyde, a product phenylpropanoid pathway. Formation of optically pure (-)-secoisolariciresinol from an achiral phenylpropanoid monomer, coniferyl alcohol, was first reported *in vitro* using *Forsythia intermedia* as an enzyme source [54]. Selective oxidation of (-)-secoisolariciresinol to (-)-matairesinol by an enzyme preparation from *F. intermedia* was also discovered at the same time by Umezawa *et al*, (1990b,

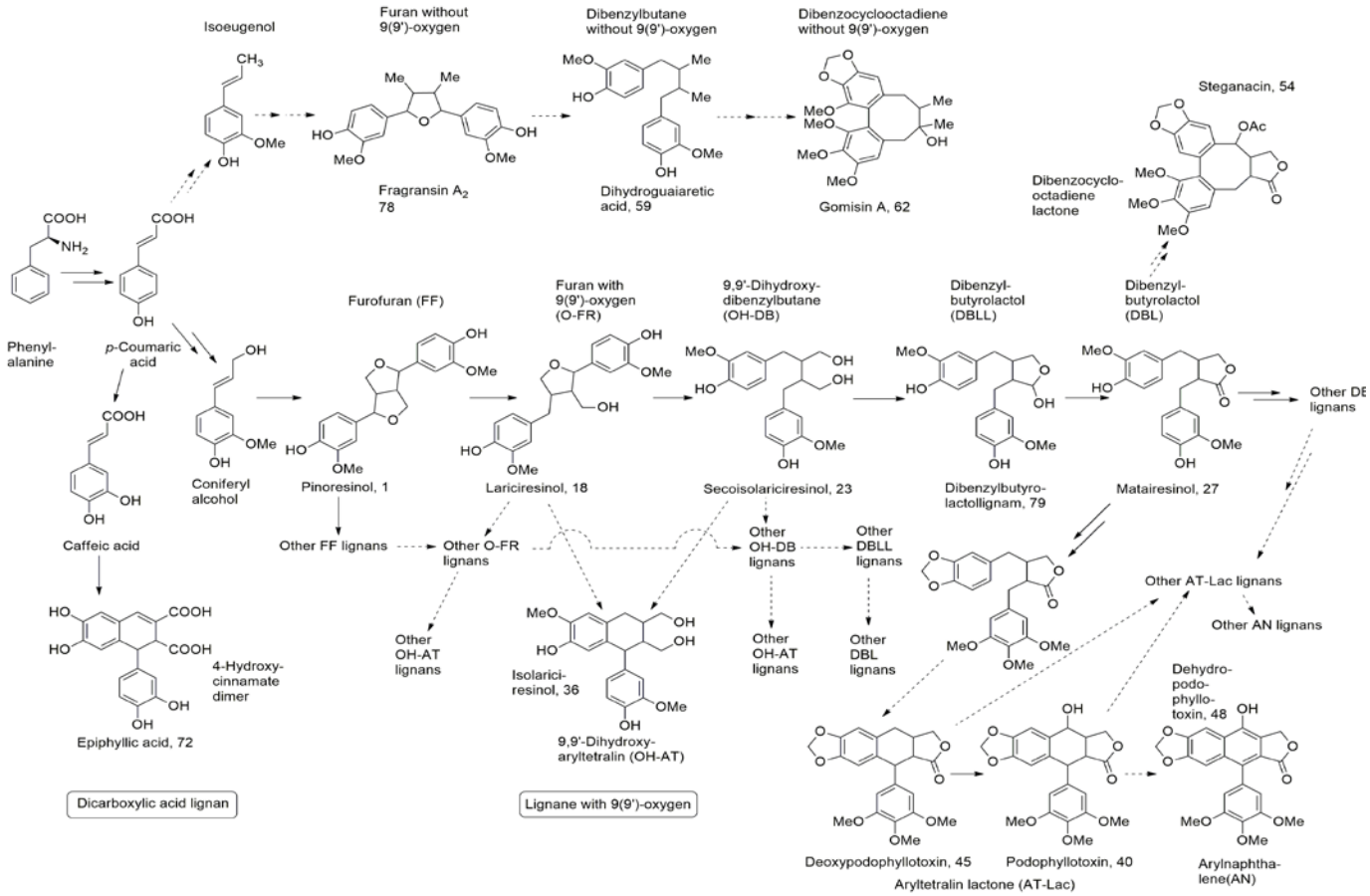

Figure 4. Possible biosynthetic pathways for various types of lignans. Solid and broken arrows represent pathways substantiated by experiments and assumed based on comparison of chemical structures, respectively. (Adapted from Umejawa et al, 2003[62])

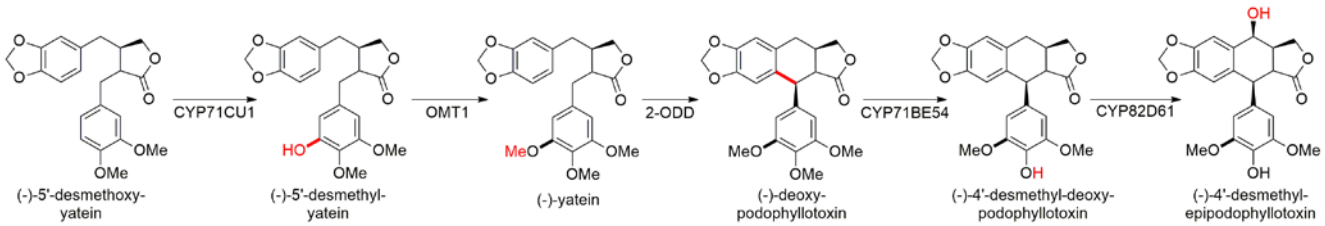

Figure 5. Reconstituted pathway from (–)-5′-desmethoxy-yatein to (–)-4′-desmethylepipodophyllotoxin. (Adapted from Lau et al, 2015[26])

1991).[55,56] Selective reduction of pinoresinol to secoisolariciresinol was also detected from *Forsythia* by Katayama *et al*, (1992), and Umezawa *et al*, (1994).[57,58] The enzyme, pinoresinol/lariciresinol reductase was cloned and purified by Chu *et al*, (1993); Dinkova-Kostova *et al*, (1996).[59,60] Later Davin *et al* discovered a unique protein, dirigent protein oxidase, that produces pinoresinol by enantioselective formation of (+)-pinoresinol by coupling of coniferyl alcohol, from *Forsythia*. Recently a cytochrome P450s, CYP719A23 from *P. hexandrum* and CYP719A24 from *P. peltatum* were isolated and both of these enzyme can covert the matairesinol into pluviatolide by catalyzing methylenedioxy bridge formation.[61] Many other lignans along with podophyllotoxin are formed from the same upstream basal lignans like pinoresinol, lariciresinol, secoisolariciresinol and matairesinol (Figure 4).[62]

Very recently Lau *et al*, (2015)[26] reconstitute the pathway from pluviatolide to (–)-4′-desmethylepipodophyllotoxin. They discovered the 6 novel enzyme among which O-methyltransferases3 (OMT3) catalyzes methylation of pluviatolide to generate (–)-5′-desmethoxy-yatein as the next step in the pathway. This (–)-5′-desmethoxy-yatein further coverted to (–)-4′-desmethylepipodophyllotoxin through 5 steps namely (–)-5'-desmethylyatein, (–)-yatein, (–)-deoxypodophyllotoxin, (–)-4'-desmethyl-deoxypodophyllotoxin and lastly (–)-4'-desmethylepipodophyllotoxin catalyzed by CYP71CU1, OMT1, 2-ODD, CYP71BE54, CYP82D61 respectively (Figure 5). Though (–)-4′-desmethylepipodophyllotoxin is a etoposide lignan and immediate precursor of etoposide lignan, but still pathway upto podophyllotoxin is unknown story.

REGIO- AND STEREOSPECIFIC COUPLING OF MONOLIGNOL BY A DIRIGENT PROTEIN OXIDASE (DPO) THAT CONTROLS THE LIGNIN AND LIGNAN BIOSYNTHESIS

According to Davin *et al*, (1997)[63] the regio- and stereospecificity of bimolecular phenoxy radical coupling reactions has especial importance in lignin and lignan biosynthesis and are clearly controlled in some manner *in vivo*; yet *in vitro* coupling by oxidases, such as laccases, only produce racemic products. One-electron oxidation of the monolignol, E-coniferyl alcohol, results in “random” bimolecular radical coupling to afford initially dimeric products, such as (±)-dehydrodiconiferyl alcohols, (±)-pinoresinols, and (±)-guaiacylglycerol (8-O-4-coniferyl alcohol ethers) (Figure 6A). Davin *et al*, (1997)[63] discovered a 78 kDa protein from *F. intermedia* which sterioselectively couple two E-coniferyl alcohol molecule by 8-8 linkage to produce (+)-pinoresinol(Figure 6B). According to Davin *et al*, (2000)[64] only in the presence of oxidases such as laccase/O_2 or peroxidase/ H_2O_2, the derigent protein was capable of engendering stereoselective coupling. Initial kinetic studies also suggested that the protein functioned in a very unique manner, whereby the oxidase first generates the free-radical intermediates, which are then presumed to be captured by the dirigent. These are bound and orientated in such a manner that coupling can only provide the product (+)-pinoresinol . Significantly, neither p-coumaryl nor sinapyl alcohols, which differ only in the degree of methoxylation of the aromatic ring, served as substrates for stereoselective coupling: This actually confirmed that dirigent selectively bound only coniferyl alcoholderived substrates; therefore, derigent proteins contain a distinct monolignol-derived binding sites. Later it was discovered that dirigent protein is a glycoprotein and the gene encodaed a protein is only about 18-19 kDa. The corresponding native subunit was found to be glycosylated with a subunit size of approximately 26 to 27 and 21 to 23 kDa.[65,66] According to Gang *et al*, (1999)[66] dirigent protein has role in both lignin and ligan formation. To prove the regio and steriospecific action of derigent protein in lignin and lignan formation dirigent protein was immune labeled and detected under transmission electron microscope. It was found that a strong signal was observed in the cambial region of the actively dividing cells relative to that of the preimmune serum, and a second was in the lignified tracheary elements. Closer examination of the lignified tracheary elements, revealed a huge amount of the label dirigent was in the S1 layer of the secondary wall, which region is associated with coniferyl alcohol targeting to the lignin initiation sites. A small amount of immunolabeling was also detected in the S3 layer which may have originally been associated with lignan biosynthesis nearing cell death [64]. So these can be concluded that regio and steriospecific coupling monolignol, namely conferyl alcohol, by derigent protein actually control the trafficking of monolignol towards lignin and lignan formation.

LIGNAN IN HEALTH PROTECTION AND DISEASE TREATMENT

From long back lignan has importance in medicine and nutrition. Dietary lignan like secoisolaricirecinol and matiresinol has significant effect on health protection specially in prostate cancer. As discussed previously these lignan metabolized into mammalian lignan enterodiol and enterolactone. This dietary lignan protecting against various sex hormone induced cancer. *Schizandra chinensis* fruit has been used as traditional medicine since long backe in Asia. Its extract kita-gomisi contain 8-8′, 2-2′ lignan such as gomisin and used

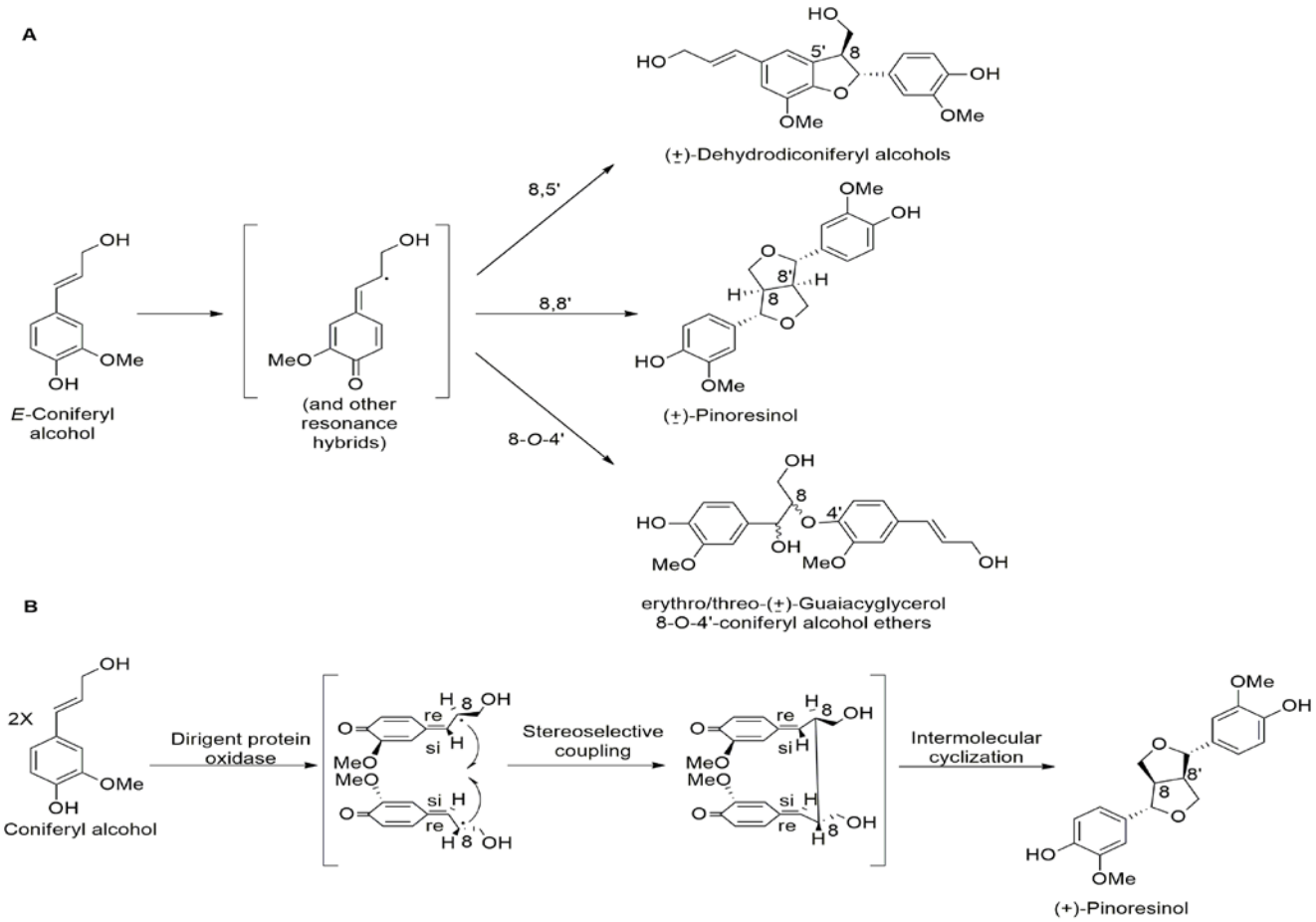

Figure 6. Bimolecular phenoxy radical coupling products from E-coniferyl alcohol. (A) Dimeric lignans formed via "random" coupling. (B) Dirigent-mediated formation of (+)-pinoresinol in *F. intermedia.* (Adapted from Davin et al, 1997[63] and Davin et al, 2000[64] respectively).

as antitussiv and tonic.[67,68] The fruits of that also used for acute hepatitis in Japan and East Asia. Another lignan (-) α –peltatin prevent development of murin of cytomegalovirus plaques in mouse 3T3-L1 cells.[69] Lignan like (-)arctigenin and tarchelogenin inhibits the replication of human immunodeficiency virus (HIV). (-)Arctigenin prevents the integration of proviral DNA into genomic DNA.[70] Kadsurenone is a lignan which act as platelet activating factor. Such effect is also found in faragesin from *Mangolia biondii.*[71,72] Magnoshinin and magnosalin isolated from *Magnolia salicifolia* buds also has anti-inflammatory effect. Lignan like prostalidins from *Justicia prostata* showed antidepressant activity. Lastly lignan of *Siberian ginseng* has cardiovascular effects. This lignan is widely used in Asia, helps in sustaining cardiovascular activity during prolonged exercise.[1]

CYTOTOXIC LIGNAN PODOPHYLLOTOXIN AND ITS MODE OF ACTION

From the long back *Podophyllum* species have been used by various cultures as antidotes against poisons, or as purgative, antihelminthic, vesicant, and suicidal agents.[4] According to Gordaliza *et al*, (2004)[3] Podophyllin was included in the US Pharmacopoeia in 1820 and the use of this resin was prescribed for the treatment of venereal warts, attributing this action to podophyllotoxin. Significant effect of this resin on cancer cell line is also reported. Antiviral activity of aqueous extract of *P. peltetum* was also studied.[73] Effect of podophyllotoxin in inhibiting the replication of measles and herpes simplex type I viruswas also reported.[74,75] In many pharmacopoeias podophyllotoxin had been described for the treatment of condyloma acuminatum caused by human papilloma virus (HPV) and other venereal and perianal warts.[76-79] Podophyllotoxin is also effective in the treatment of anogenital warts in children and against *molluscum contagiosum* that is generally a self-limiting benign skin disease that affects mostly children, young adults and HIV patients.[80] Another well known property of podophyllotoxin is its anti tumor activity. It is effective in the treatment of Wilms tumours, different types of genital tumors (carcinoma verrucosus, for example), non-Hodgkin and other lymphomas,[4] lung cancer.[81,82] Podophyllotoxin mainly acts through inhibiting the polymerization of β-tubulin and ultimately arrests the cell cycle.[4,42,83] According to Gordaliza *et al*, (1995)[84] podophyllotoxin group might work as alkylating agents through their C-9 methylene, rather than as acylating agents to prevent the polymerization of tubuline. Schonbrunn *et al*, (1999)[85] showed the crystallization of podophyllotoxin linked to a tubulin fragment.

GLYCOSIDIC DERIVATIVES OF PODOPHYLLOTOXIN AND THEIR MODE ACTION

Three very widely used glycosidic derivatives of podophyllotoxin are etoposide, teniposide and etopophos (Figure 7).

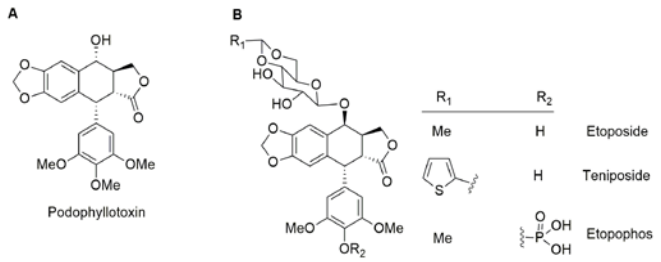

Figure 7. (A) Podophyllotoxin and (B) its Glycosidic derivatives (Adapted from Gordaliza et al, 2004[3])

These three derivatives showed significant effect against neoplasms including testicular and small-cell lung cancers, lymphoma, leukaemia, Kaposi's sarcoma, etc.[4,86] Modification of podophyllotoxin by demethylation at 4′ - position and the insertion of a b-glycosidic moeity in 7-position convert compounds into potent irreversible inhibitors of DNA topoisomerase II. These derivatives do not act through by inhibiting the polymerization but they form an irreversible drug –enzyme-nuclic acid complex, which induces breaks in single- and double-stranded DNA as the initial step in a series of biochemical transformations that eventually lead to cell death.[87,88] According to Eich *et al*, (1991)[89] inhibition of DNA topoisomerase II by etoposide, consisting of the binding of the OH in position 4′ to the phosphate unit of the nucleic acid and the formation of amides with topoisomerase II through the carbonyl group of the lignan, linking a covalent bond with the enzyme. Some other derivatives of cycloligna has also been introduced with lots of modification and better antitumor activity. Some examples of such derivatives are GP-11,[90] NK-611,[91,92] TOP-53,[81] NPF.[93]

CONCLUSION

In conclusion, it is worthwhile to mention that lignans, contain two hydroxyl cinamoyl alcohol moiety coupled through C8 side chain, has major medicinal importance, especially podophyllotoxin and its widely used glycosidic derivatives which have anticancer activity for small cell lung carcinoma and other lymphoma. Till date several leading groups across the world contributed significantly about the region and steriochemical coupling of monolignol which actually control the trafficking of monolignol towards lignin and lignan. Since long time a very limited knowledge is available about the podophyllotoxin biosynthetic pathway after the conversion of matirasinol to puviatilide, very recently six novel enzymes were discovered including some methyl transferase and cytochrome P450 which completes the pdophyllotoxin pathway up to a etoposide lignan, (–)-4′-desmethylepipodophyllotoxin, instead of podophyllotoxin. Still, some in-depth investigation is essentially required to reconstitute the pathway up to podophyllotoxin.[94]

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