Graphical Abstract

Abstract

Extensive phytochemical analysis of *Podophyllum hexandrum* revealed the presence of five compounds i.e., β-sitosterol glucoside (SR-101), kaempferol (SR-102), quercetin (SR-103), podophyllotoxin (SR-104) and podophyllotoxin glucoside (SR-105). β-sitosterol glucoside is reported for the first time from this plant species. The structures of these compounds were established on the basis of rigorous spectroscopic data analysis in the light of literature. Podophyllotoxin, the most abundant lignan isolated from *P. hexandrum*, was synthetically modified at C-4 position to four novel derivatives. All the synthesised analogs were subjected to MTT cytotoxicity screening against a panel of four different human cancer cell lines viz. prostate (PC-3), pancreatic (PANC-1), colon (COLO-205) and lung (A549). Among the synthesised analogs, SR-104b and SR-104c bearing a pinitol-diacetonide moiety at C-4 position, exhibited superior potency to etoposide against A549 cancer cell line with IC\textsubscript{50} of 2.48 and 2.50 µM respectively.

**Keywords:** *Podophyllum hexandrum*, podophyllotoxin, lignans, cytotoxic, A549 cells.
4.1. Introduction

*Podophyllum hexandrum* Royale (*P. emodi* Wall, ex Hook. f. & Thoms) is an endangered rhizomatous perennial herb distributed in lower elevations of Himalayan countries like Afghanistan, Pakistan, India, Nepal, Bhutan, and in S. W. China. In India *P. hexandrum* is mostly found in Alpine Himalayas of Jammu and Kashmir, Himachal Pradesh, Sikkim, Uttarakhand and Arunachal Pradesh at an altitude of about 1,800-4,000 m.1,2 The name *Podophyllum* is taken from *podos*, a foot, and *phyllon*, a leaf, and refers to the resemblance of the leaves to a duck's foot. It belongs to family Berberidaceae and is commonly known as Himalayan may apple or Indian may apple. It was known as Aindri (a divine drug) in ancient times and in Hindi and Ayurveda, its name is Bantrapushi or Giriparpat.3 In Kashmiri, it is known as Banwangun, since its red colour fruit (berry) is of the size of a small brinjal.3 *P. hexandrum* is tolerant to cold temperatures, as would be expected of a Himalayan plant, but sensitive to dry conditions. It can be propagated by seed or by dividing the perennial rhizome.3

4.2. Review of literature

4.2.1. Ethnopharmacology

Indian *Podophyllum* has a long history of usage amongst natives of the Himalayas. Many tribes consume brew from the powder as a laxative or to treat intestinal worms. The roots are used as a tonic for liver, lung, and stomach ailments. A decoction made by boiling the roots in water has been used to treat rheumatism. Many of these medicinal properties have been assigned to its antioxidant properties.4 It has been extensively used in Ayurvedic system of medicine for treatment of several ailments like constipation, cold, biliary fever, septic wounds, inflammation, burning sensation, mental disorder, monocytoid leukemia, Hodgkin’s and non-Hodgkin’s lymphoma.5 It has also been extensively exploited in traditional medicine for the treatment of lung, bladder and

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venereal warts. Utility of _P. hexandrum_ has also been reported against erysipelas insect bite, plague and to provide symptomatic relief in some of the allergic and inflammatory conditions of skin. It has also been employed in treatment of various types of cancers.

Podophyllin, a resin obtained by ethanolic extraction of the _Podophyllum_ roots and rhizomes has traditionally been used as a common cathartic, anthelmentic and as a lethal poison. The main constituents in podophyllin are the lignans podophyllotoxin, 4'-demethylpodophyllotoxin and α- and β-peltatin. It has also been used as a remedy in ophthalmia.

### 4.2.2. Phytochemistry and Pharmacology

_Podophyllum_ is a small genus represented by six species viz. _P. hexandrum_, _P. Peltatum_, _P. sikkimensis_, _P. delavayi_, _P. pleianthum_ and _P. versipelle_. This genus has been the focus of attraction for medicinal chemists primarily because of its bioactive compounds and has been the subject of numerous chemical and pharmaceutical studies. The main secondary metabolites of _Podophyllum_ species are lignans and flavonoids. Lignans form a group of important plant secondary metabolites. This group received much attention in the field of natural product chemistry ever since the discovery of podophyllotoxin. Lignans are defined as ââ-dimers of phenylpropanoid derivatives, and are widely spread throughout the plant kingdom. The present interest for this group of natural products is based on their fascinating pharmacological applications in the field cancer. _P. hexundrum_ is reported to contain a number of compounds with significant pharmacological properties, e.g. epipodophyllotoxin, podophyllotoxone, aryltetrahydronaphthalene lignans, flavonoids such as quercetin, quercetin-3-glycoside, podophyllotoxinglycoside, kaempferol and kaempferol-3-glucoside. The rhizomes and roots of the plant contain antitumor lignans such as podophyllotoxin, 4'-demethyl podophyllotoxin and podophyllotoxin-4-O-glucoside. Among these lignans, podophyllotoxin is most

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important for its use in the synthesis of antineoplastic drugs.\textsuperscript{10} Other than \textit{Podophyllum} species, podophyllotoxin has also been isolated from \textit{Anthriscus sylvestris} and \textit{Pulsatilla koreana}.\textsuperscript{11}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4_1.png}
\caption{Anticancer drugs derived from podophyllotoxin}
\end{figure}

Podophyllotoxin is one of the eye-catching results from medicinal plant research. However, the isolation of podophyllotoxin from the rhizomes of \textit{P. hexandrum} is not ideal as its rhizomes contain only 4\% of podophyllotoxin on a dry weight basis. Its supply has becomes increasingly limited due to both intensive collection and lack of cultivation.\textsuperscript{12} This species is listed in appendix II of CITES (convention for international trade in endangered species). This appendix lists species that are not necessarily threatened with extinction but that may become so unless trade is closely controlled.\textsuperscript{13} This supply problem forms the drive for a large number of scientists to search for alternative sources of podophyllotoxin. The chemical synthesis of podophyllotoxin is

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\begin{footnotesize}
\textsuperscript{10} Issell BF, Muggia FM, Carter SK. 1984. Etoposide (VP-16) - Current Status and New Developments. Academic Press; Orlando, USA.
\textsuperscript{13} World Conservation Monitoring Centre. 2001. Checklist of CITES species: a reference to the appendices to the Convention on international trade in endangered species of wild fauna and flora, CITES Secretariat/World Conservation Monitoring Centre, Chatelaine-Genève
\end{footnotesize}
\end{flushright}
possible,¹⁴ but largely hampered by the complicated stereochemical ring closure necessary to attain this compound. Synthetic production therefore only yields restricted quantities at high costs.

The attention of pharmacists for lignans in general and podophyllotoxin in particular is due to pronounced cytotoxic activity of these compounds and their derivatives. Podophyllotoxin shows strong cytotoxic activity against various cancer cell lines. It is effective in the treatment of Wilms tumors, various genital tumors and in non-Hodgkin’s and other lymphomas and lung cancer.¹⁵ The attempts to use podophyllotoxin in the treatment of human neoplasia were mostly unsuccessful due to complicated side effects such as nausea, vomiting, damage of normal tissues, etc.¹⁶ Because of this reason, podophyllotoxin as such is not used as a drug. Extensive structure modifications were performed to obtain more potent and less toxic anticancer agents, which resulted in production of three semi synthetic anticancer drugs. Most commonly used due to its anticancer properties is etoposide, which was developed in 1966 and received FDA approval in 1983. A phosphate analogue, etopophos, produced by Bristol-Myers Squibb Co. was FDA approved in 1996. The third derivative is teniposide, which is less frequently used for chemotherapy in comparison with etoposide.¹⁷ These are the most widely used derivatives for the treatment of lymphomas, acute leukemia, testicular cancer, small cell lung cancer, ovarian, bladder, brain cancers etc.¹⁸

The antineoplastic activity of Podophyllotoxin is achieved through the degradation of the microtubules, which results from the formation of podophyllotoxin-tubulin complexes. Cells treated with podophyllotoxin are arrested in the metaphase of the mitosis. Its mode of action is comparable to the alkaloid colchicin and for their mode of action such compounds are called spindle poisons. Other spindle poisons in clinical use are paclitaxel and vincristine like alkaloids.¹⁹,²⁰ The clinically applied podophyllotoxin-derivatives etoposide, teniposide and etopophos have a completely different mode of action. These

compounds are topoisomerase II inhibitors. Topoisomerase II is an enzyme that cleaves double-stranded DNA and seals it again after unwinding and is crucial in the processes of DNA replication and repair. For its function topoisomerase II binds covalently to the broken DNA. Etoposide and other derivatives stabilise the DNA-topoisomerase II complex in such a way that resealing of the DNA strands becomes impossible. Cells that are duplicating their DNA for the mitosis are very sensitive for this mechanism. The overall effect of these anticancer drugs is the arrest of the cells in late S or early G2 phase of the cell cycle.\textsuperscript{17,21} A major advantage of the etopophos (etoposide phosphate), a pro-drug of etoposide, is the improved solubility in water. After administration the phosphate group is hydrolysed in the human body to yield the bioactive etoposide. Because of its hydrophilic property etopophos can be administered much easier.\textsuperscript{22} In addition, podophyllotoxin is also the precursor for a new derivative CPH-82 (reumacon) that is being tested in Europe in phase III clinical trials for arthritis.\textsuperscript{23}  

\textsuperscript{22} Witterland AH, Koks CH, Beijnen JH. 1996. \textit{Pharm. World Sci.}, 18, 163.
4.3 Objectives of present work

- Collection and identification of plant material (*Podophyllum hexandrum*).
- Extraction and isolation of chemical constituents from plant extracts using column chromatography.
- Identification and characterization of natural isolates using spectral data analysis in light of literature.
- Synthesis of drug like molecules using podophyllotoxin as a key template.
- Bioevaluation of all the analogs for anticancer activity.

![Molecular structures of the isolated compounds.](image)

**Figure 4.2.** Molecular structures of the isolated compounds.

4.4. Results and discussion

4.4.1. Chemistry

*P. hexandrum* was collected from the upper reaches of Gulmarg, Kashmir, India in October 2011. The air dried powdered was extracted with dichloromethane:methanol (1:1) according to the NCI protocol to afford the respective extract. The concentrated dichloromethane: methanol extract was subjected to column chromatography over silica gel. Repeated column chromatography of hexane and acetone extracts using varied solvent polarity (hexane:ethyl acetate) and recrystallization techniques afforded five compounds (Figure 6.2).
4.4.1.1. Compound 1 (SR-101)

This compound was isolated as a white coloured amorphous solid. The HR-EIMS showed a molecular ion peak at \( m/z \) 576.4478. From elemental analysis, HR-EIMS and other spectral data (\(^1\)H NMR, \(^{13}\)C NMR), **SR-101** was assigned the molecular formula C\(_{35}\)H\(_{60}\)O\(_6\). The IR spectrum showed a hydroxyl band at 3550-3390 cm\(^{-1}\) and bands at 3050, 1660 and 817 cm\(^{-1}\) due to trisubstituted double bond.

![Figure 4.3. Structure of SR-101 (β-Sitosterol glucoside).](image)

**SR-101**, in its \(^1\)H NMR, displayed resonance signals due to two quaternary methyl groups at \( \delta \) 0.70 (3H, s, H-18) and 0.96 (3H, s, H-19) and three secondary methyl groups at \( \delta \) 0.91 (3H, d, \( J = 6.5 \) Hz, H-21) and 0.88 (6H, d, \( J = 5.2 \), H-26, H-27). The upfield chemical shift at \( \delta \) 0.82 as a triplet with the intensity of 3H and coupling constant of 6.9 Hz was assigned to the terminal methyl group at 29 position. The \(^1\)H NMR spectrum of this compound displayed one resonance signal at \( \delta \) 5.36 (1H, d, \( J = 3.1 \) Hz, H-6), due to olefinic proton. The \(^1\)H NMR spectrum of this compound also showed one resonance corresponding to the proton connected to the C-3 (-OH carbon) which appeared as a triplet of doublet of doublets at \( \delta \) 3.44. The chemical shifts in the region \( \delta \) 2.94-3.14 (5H) were assigned to five sugar protons and a doublet at \( \delta \) 4.45 (\( J_{H1'-H2'} \) 7.20 Hz) with anomeric carbon resonating at \( \delta \) 103.57 indicated that the sugar moiety is attached to the aglycone part via β-linkage. \(^{13}\)C NMR-DEPT spectrum exhibited the presence of thirty five carbon signals including six methyls, twelve methylene, fifteen methine and three quaternary carbons. This compound showed positive Liebermann-Burchard and Salkowski test specific for Δ\(^5\) sterols. 24 The EI-MS spectrum showed characteristic fragment ions at \( m/z \) 399, 396, 381, 329 and 303. Fragment ions 329 and 303 are

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considered to be diagnostic for sterols having $\Delta^5$-unsaturation.\textsuperscript{24} Comparison of physical characteristics and spectral data of \textit{SR-101}, with that reported in literature,\textsuperscript{25} confirmed it to be \textbf{\textit{\textbeta-Sitosterol-3-O-glucoside}}. This compound is reported for the first time from this plant species.

\textbeta-Sitosterol-3-O-glucoside is a common plant secondary metabolite and exhibits a variety of biological activities. It has recently been reported for its antimicrobial activity against \textit{N. gonorrhoea}, \textit{P. aeruginosa} and \textit{S. aecalis}.\textsuperscript{26} \textbeta-Sitosterol glucoside has also been reported to modulate the growth of estrogen-responsive breast cancer cells \textit{in vitro} and in ovariectomized athymic mice.\textsuperscript{27}

\textbf{4.4.1.2. Compound 2 (SR-102)}

This compound was obtained as yellow solid with melting point 274-277 °C. The HR-EIMS showed a molecular ion peak at $m/z$ 286.0563. From HR-EIMS and other spectral data ($^1$H NMR, $^{13}$C NMR), \textit{SR-102} was assigned the molecular formula C$_{10}$H$_{10}$O$_6$. The compound responded positively to Shinoda test (orange colour), sulphuric acid test (yellow colour) and gave light blue colour in Gibbs test indicating the presence of flavonoid skeleton. IR spectrum exhibited a hydroxyl band at 3317 cm$^{-1}$ and a carbonyl band at 1662 cm$^{-1}$. The UV spectrum showed absorption maxima at 329 and 269 nm characteristic of flavonoids.\textsuperscript{28}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure4_4}
\caption{Structure of SR-102 (Kaempferol).}
\end{figure}

The $^1$H NMR spectrum showed a pair of doublets ($J = 8.8$ Hz each) observed at $\delta_H$ 8.05 and 7.14 integrating for four protons were assigned to H-2', H-6' and H-3', H-5' respectively. The two other doublets ($J = 2.2$ Hz each) at $\delta_H$ 6.62 and 6.33 integrating for


one proton each, were attributed to H-8 and H-6 respectively. The $^{13}$C NMR-DEPT spectra showed the presence of fifteen carbons comprising of six methines and nine quaternary carbons. The signal at $\delta$C 177.7 was assigned to carbonyl carbon (C-4) and the signals at 165.7, 162.8, 160.9, 158.5 and 147.7 were assigned to oxygen deshielded carbons attributed to C-7, C-5, C-4', C-9 and C-2 respectively.

From the spectral evidence and comparing the physical data with the literature values, 29 SR-102 was identified as 3,4',5,7-tetrahydroxy flavone (Kaempferol) (Figure 6.4). This compound has already been reported from this plant species.

Kaempferol is a pharmacologically important bioactive molecule having a wide range of pharmacological activities. Numerous preclinical studies have demonstrated its antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, antiosteoporotic, anxiolytic, analgesic, and antiallergic activities.

4.4.1.3. Compound 3 (SR-103)

SR-103 was obtained as yellow solid with melting point 297-302 °C. The HR-EIMS showed a molecular ion peak at $m/z$ 302.0522. From HR-EIMS and other spectral data ($^1$H NMR, $^{13}$C NMR), it was assigned the molecular formula C$_{10}$H$_{10}$O$_{6}$. This compound also responded positively to Shinoda test (orange colour), sulphuric acid test (yellow colour) and gave light blue colour in Gibbs test confirming the presence of flavonoid skeleton. IR spectrum exhibited a hydroxyl band at 3550-3250 cm$^{-1}$ and a carbonyl band at 1665 cm$^{-1}$. The UV spectrum showed absorption maxima at 310 and 266 nm characteristic of flavonoids.

![Figure 4.5. Structure of SR-103 (Quercetin).](image-url)
The $^1$H NMR spectrum exhibited an ABX system at $\delta_H$ 7.73 (1H, d, $J = 2.0$ Hz, H-2'), 7.62 (1H, dd, $J = 2.0$, 8.0 Hz, H-6') and 6.87 (1H, d, $J = 8.0$ Hz, H-5') due to 3', 4' disubstitution of ring B and a typical meta-coupled pattern for H-6 and H-8 protons ($\delta_H$ 6.37 and 6.17, d, $J = 2.0$ Hz). The $^{13}$C NMR-DEPT spectra showed the presence of fifteen aromatic carbon signals comprising of five methines and ten quaternary carbons. The signal at $\delta_c$ 178.5 was assigned to carbonyl carbon (C-4) and the signals at 164.5, 161.5, 158.7, 147.2, 146.2 and 145.3 were assigned to oxygen deshielded carbons attributed to C-7, C-5, C-9, C-4', C-2 and C-3' respectively. Based on the spectral evidence and comparing the physical data with the literature values, SR-103 was identified as 3',4',5,7-pentahydroxy flavone (Quercetin) (Figure 6.5). This compound has already been reported from this plant species.

Quercetin is the most commonly occurring flavonoid and is an excellent antioxidant that is also suggested to possess other beneficial activities. Because of its potent antioxidant activity, it has been reported to be effective in inflammation, arteriosclerosis, bleeding, allergy and swellings. In addition, epidemiological data suggests that quercetin is associated with reduced risk of certain types of cancers. It is considered to be one of the most potent flavonoid which is capable of interacting with and modulating activity of a variety of enzyme systems including cyclooxygenase, lipoxygenase, phosphodiesterase and tyrosine kinase.

### 4.4.1.4. Compound 4 (SR-104)

This compound was obtained as white crystalline solid with melting point 182-186 °C. Its molecular formula was determined as C$_{22}$H$_{22}$O$_8$ by HR-EIMS (414.1397) and other spectral data ($^1$H NMR, $^{13}$C NMR). The absorption bands in the IR spectrum at 3350, 2900, 1745, 1610 and 1450 and 1030 cm$^{-1}$ indicated the presence of a hydroxyl, C-H, lactone carbonyl, aromatic C=C and C-O functionalities respectively. The UV spectrum showed absorption maxima at 280 and 210 nm suggesting the existence of the benzene rings.
The $^1$H-NMR spectrum of SR-104 revealed proton signals of three methoxyl groups and signals of four aromatic protons at $\delta_H$ 3.77 (6H, s, 3', 5'-OMe), 3.84 (3H, s, 4'-OMe), and 7.15 (1H, s, H-5), 6.55 (1H, s, H-8), 6.40 (2H, s), and a pair of proton signals of −CH$_2$− at $\delta_H$ 6.15 (1H, d, $J = 1.2$ Hz) and 6.10 (1H, d, $J = 1.2$ Hz). The doublet at $\delta_H$ 4.78 (1H, d, $J = 8.8$ Hz) could be safely assigned to 4β proton, besides the other aliphatic signals at $\delta_H$ 4.60 (2H, m, H-1), 4.10 (1H, t, $J = 9.5$ Hz) and 2.8 (2H, m) integrating for four protons (11α, H-11β, and H-2, 3). The $^{13}$C NMR-DEPT spectra revealed the existence of twenty two carbons comprising of nine quaternary, eight methines, two methylene and three methoxyls carbons. The signal at $\delta_C$ 174.7 was assigned to carbonyl carbon (C-12) of the lactone ring and the signals at 152.5, 147.5 and 136.9 were assigned to oxygen deshielded carbons attributed to C-3',5', C-6,7, and C-4' respectively. Based on the spectral evidence and comparing the physical data with the literature values, SR-104 was identified as Podophyllotoxin (Figure 6.6). This compound has already been reported from this plant species.

4.4.1.4.1. Synthesis of novel podophyllotoxin congeners

Podophyllotoxin is one of the representative antimitotic natural products. Currently, semisynthetic derivatives of podophyllotoxin i.e., etoposide, teniposide, and etopophos, are used in clinical practices for anticancer chemotherapy (Figure 6.1). These have shown a wide spectrum of clinical activity in the treatment of various tumour types as discussed earlier. However, there are several limitations in their clinical use. These drugs show

several side effects such as myleosupression, neutropenia, and nausea.\textsuperscript{38} Drug resistance is also an important barrier to effective treatment, which is a common problem in cancer chemotherapy. To get a more potent and effective drug, numerous derivatives have been prepared, even after the achievement of the synthesis of etoposide. Podophyllotoxin is a hot lead for medicinal chemists who are looking for a new anticancer agent, even in the 21\textsuperscript{st} century. To obtain better therapeutic agents, extensive synthetic efforts have been devoted to obtain a rationale for the improvement of topoisomerase II inhibition activity of podophyllotoxin derivatives. Podophyllotoxin contains a five-ring system (i.e., A, B, C, D and E rings). Molecular area-oriented chemical modification of podophyllotoxin has revealed structural features critical for the topoisomerase II inhibition:

- The $4\beta$-configuration is essential with various substitution accommodated at C-4.
- The free $4'$-hydroxy is crucial.
- The trans-lactone D ring with $2\alpha$, $3\beta$ configuration is very important.
- The dioxolane A ring is optimal.
- The free rotation of ring E is required.\textsuperscript{39}

Such structure-activity relationships (SARs) unambiguously demonstrate that C-4 is the only molecular area tolerable to significant structural diversification.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{structures.png}
\caption{Structures of GL-331 and TOP-53}
\end{figure}

Recently two $4\beta$-analogs i.e., GL-331\textsuperscript{40} and TOP-53\textsuperscript{41} (Figure 6.7), of podophyllotoxin have been found to have potential anticancer activity. GL-331, a $4\beta$-arylamino derivative,

was more active than etoposide both in vitro and in vivo\(^{42}\) and retained cytotoxicity against resistant cells.\(^{43}\) It is currently under phase II clinical evaluation against various cancer types, especially resistant malignancies.\(^{44}\) TOP-53, a 4β-alkylated derivative was a more potent topoisomerase II inhibitor than etoposide. It exhibited high activity to nonsmall cell lung cancer both in vitro and in vivo\(^{45}\) and showed much higher potency against a mutant yeast type II enzyme highly resistant to etoposide.\(^{46}\) TOP-53 is currently in phase I clinical trials.\(^{47}\) Both GL-331 and TOP-53 showed topoisomerase II inhibitory activity, antitumor spectra, and drug-resistance profiles significantly different from that of etoposide, which suggest an important role of various C-4 substitutions to the activity profiles of etoposide-related analogs and the feasibility of optimizing this class of compound through rational C-4 modifications. This postulation coincides with the composite pharmacophore model proposed by MacDonald et al.\(^{47}\) and the comparative molecular field analysis (CoMFA) models generated by Xiao et al.\(^{48}\) which suggest that the C-4 position could accommodate considerable structural diversity. The CoMFA model further demonstrated that bulky substituents at C-4 might be favorable for topoisomerase II inhibition. Accordingly a lot of derivatization has been done at C-4 position of epipodophyllotoxin and some of its derivatives were found to be very potent for their anticancer behaviour.\(^{49}\)

Based on the above cited findings and inspiration from the potential anticancer activity C-4 derived podophyllotoxin derivatives, a research programme was initiated towards the synthesis of drug like molecules of biological interest using podophyllotoxin as a key starting material. It was envisaged to introduce a pseudo-sugar D-pinitol at C-4 position of podophyllotoxin and 4′-O-demethyllepipodophyllotoxin to prepare derivatives analogous to etoposide (anticancer drug). The first step towards achieving the objectives was to convert podophyllotoxin (SR-104) into 4′-O-demethyllepipodophyllotoxin (SR-104a) by demethylation of 4′-OCH\(_3\) and simultaneous inversion of 4-hydroxyl to its β-

\(^{44}\) Choy W. Genelabs Technologies, personal communication.
configuration. SR-104a was synthesized as per the known procedure\textsuperscript{50} in which the podophyllotoxin is regiospecifically converted to corresponding demethyl iodo-derivative using CH\textsubscript{3}SO\textsubscript{3}H and NaI in CH\textsubscript{2}Cl\textsubscript{2} followed by weak base hydrolysis (water:acetone, BaCO\textsubscript{3}) to 4'-O-demethylepipodophyllotoxin (Scheme-1). In this methodology hydrogen iodide is formed \textit{in situ} from methanesulphonic acid/sodium iodide and this reagent system has been considered for its potential application for both the aspects of 4'-O-demethylation and C-4 epimerization in the podophyllotoxin system. Interestingly, when this reaction is carried out in MeCN as a solvent, 4'-O-demethylation product is not observed highlighting the role of chlorinated solvents like CH\textsubscript{2}Cl\textsubscript{2} or (CH\textsubscript{2})\textsubscript{2}Cl\textsubscript{2} in achieving selective 4'-O-demethylation.\textsuperscript{50} Formation of product (SR-104a) could easily be confirmed by the loss of singlet of C-4' methoxy protons in its \textit{1H} NMR spectrum. Additionally, the \textit{β}-configuration of 4-OH group could be confirmed by a doublet at $\delta_{\text{H}}$ 4.74 with coupling constant of 4.7 Hz accounting for H-4\textit{α}. Further characterization of SR-104a was done using $^{13}$C NMR-DEPT and HR-EIMS.

\begin{center}
\begin{tikzpicture}
\node at (0,0) {SR-104};
\node at (3.5,0) {SR-104a};
\node at (1.75,0) {MeSO\textsubscript{3}H/NaI \hfill CH\textsubscript{2}Cl\textsubscript{2}, rt, 5 h};
\node at (3,0) {H\textsubscript{2}O/Acetone \hfill BaCO\textsubscript{3}, rt, 30 min};
\end{tikzpicture}
\end{center}

\textbf{Scheme-4.1:} Synthesis of 4'-O-demethylepipodophyllotoxin (SR-104a) from podophyllotoxin (SR-104)

The second step towards accomplishing the objectives was the introduction of a D-pinitol moiety at C-4 position of SR-104 and SR-104a. This was achieved through BF\textsubscript{3}-O\textsubscript{Et}\textsubscript{2} promoted coupling of SR-104 and SR-104a with a 2,3,4,5-protected D-pinitol derivative in acetonitrile at -30 °C (Scheme-2, Scheme-3). The key step in the sequence is the attack of free hydroxyl of D-pinitol on the carbocation formed at C-4 of the lignan leading to the formation of a mixture of \textit{α} and \textit{β} substituted products. HPLC analysis of the mixture after 1 hour revealed that the reaction was essentially complete and showed the presence of coupled products 4\textit{α} and 4\textit{β} in a ratio of 1.5:8.5. The purification of the desired 4\textit{β}

products was achieved through recrystallization and chiral HPLC using MeOH:H₂O (3:7) as eluent at a flow rate of 1.0 ml/min. The formation of SR-104b and SR-104c was confirmed by the presence of C-4 β-proton doublets at δ_H 4.79 and 4.87 with coupling constant of 4.7 and 4.8 Hz respectively along with the signals of pinitol diacetonide. Further characterization of the products was done using HRMS data analysis.

Scheme-4.2: Synthesis of SR-104b from podophyllotoxin (SR-104)

Scheme-4.3: Synthesis of SR-104c from 4'-O-demethylepipodophyllotoxin (SR-104a)

Figure 4.8. HPLC chromatogram of the reaction mixture of SR-104a with pinitol-diacetonide (Scheme-4.3)
SR-104 (podophyllotoxin) was further derivatised to corresponding acids SR-104d and SR-104e using succinic and phthalic anhydride in presence of pyridine as base (Scheme-4). The formation of SR-104e could easily be confirmed by the appearance of additional aromatic signals for four protons at $\delta_H$ 7.60-7.90, while as the formation of SR-104d was confirmed by the appearance of extra signals in the aliphatic region at $\delta_H$ 2.6-2.7. Further characterization of was done using $^{13}$C NMR-DEPT and HR-EIMS.

Scheme-4.4: Synthesis of SR-104d and SR-104e from Podophyllotoxin (SR-104)

4.4.1.5. Compound 5 (SR-105)

SR-105 was obtained as white crystalline solid with melting point 219-225 °C. Its molecular formula was determined as C$_{28}$H$_{32}$O$_{13}$ by HR-EIMS (576.1928) and other spectral data ($^1$H NMR, $^{13}$C NMR). The absorption bands in the IR spectrum at 3450-3330, 2890, 1745, 1615 and 1456 and 1028 cm$^{-1}$ indicated the presence of a hydroxyl, C-H, lactone carbonyl, aromatic C=C and C-O functionalities respectively. The UV
spectrum showed absorption maxima at 279 and 209 nm suggesting the existence of the benzene rings.

The $^1$H-NMR spectrum of SR-105 displayed proton signals for three methoxyl groups and signals of four aromatic protons at $\delta_H$ 3.71 (6H, s, 3′, 5′′-OMe), 3.73 (3H, s, 4′-OMe), and 7.39 (1H, s, H-5), 6.44 (1H, s, H-8), 6.40 (2H, s, H-2′, 6′) respectively. A pair of signals at $\delta_H$ 5.95 (1H, d, $J = 1.2$ Hz) and 5.93 (1H, d, $J = 1.2$ Hz) was assigned to –CH$_2$– protons. The doublet at $\delta_H$ 5.07 (1H, d, $J = 8.8$ Hz) could be safely assigned to a 4β proton. The chemical shifts in the region $\delta_H$ 3.3-3.4 (4H) and 3.76 (1H) were assigned to five sugar protons and a doublet at $\delta_H$ 4.40 ($J_{H1'-H2'} = 7.6$ Hz) with anomeric carbon resonating at $\delta$ 103.5 indicated that the sugar moiety is attached to the aglycone part via β-linkage. The $^{13}$C NMR-DEPT spectra revealed the existence of twenty eight carbons comprising of nine quaternary, thirteen methines, three methylene and three methoxyls carbons. The signal at $\delta_c$ 177.1 was assigned to carbonyl carbon (C-12) of the lactone ring and the signals at 150.1, 148.8 and 133.9 were assigned to oxygen deshielded carbons attributed to C-3′,5′, C-6,7, and C-4′ respectively. The chemical shifts of the aglycone of SR-105 were very similar to those of podophyllotoxin, except for the signals due to the sugar moiety. Based on the spectral evidence and comparing the physical data with the literature values,$^{51,52}$ SR-105 was identified as Podophyllotoxin-4-O-β-D-glucopyranoside (Figure 6.7). This compound has already been reported from this plant species.

4.4.2. Biology

Podophyllotoxin (SR-104), a naturally occurring lignin, exerts cytotoxic activity by inhibition of microtubule assembly.$^{53}$ Its two semisynthetic glucoconjugates, etoposide and teniposide are novel DNA topoisomerase II inhibitors marketed in several countries. C-4 modified podophyllotoxins have elicited widespread interest due to their far superior antitumor activity compared to podophyllotoxin. Some non-sugar substituted analogs, particularly N-linked congeners, exhibit superior pharmacological properties to etoposide, and consequently, several newer-generation clinical candidates, including

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NPF, GL-331, and TOP-53 have emerged through C-4 modification as alternatives to overcome the drawbacks of etoposide. Based on these facts, the C-4 modified podophyllotoxin analogs were screened for cytotoxic activity against a panel of four different human cancer cell lines viz. prostate (PC-3), pancreatic (PANC-1), colon (COLO-205) and lung (A549) using MTT cytotoxicity screening assay. Preliminary cytotoxicity screening (% Growth inhibition) of the analogs was carried out at 10.0 µM concentration and cell death was determined (Table 4.1). Etoposide was used as positive control in this assay. All the synthesized analogs displayed broad spectrum cytotoxic effect in a dose dependent manner. The analogs which exhibited significant cytotoxic effect, greater than 50% growth inhibition at the preliminary screening concentration were further assayed at different concentrations (0.01-10 μM) to generate the IC$_{50}$ values (Table 4.2). The values are the average of triplicate analysis.

Table 4.1: % Growth inhibition (GI) data of podophyllotoxin analogs against prostate (PC-3), pancreatic (PANC-1), colon (COLO-205) and lung (A549) cancer cell lines at 10 µM using MTT assay.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Entry</th>
<th>PC-3 (%)</th>
<th>PANC-1 (%)</th>
<th>COLO-205 (%)</th>
<th>A549 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SR-104</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>SR-104a</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>SR-104b</td>
<td>98.8</td>
<td>99.6</td>
<td>99.7</td>
<td>99.2</td>
</tr>
<tr>
<td>4</td>
<td>SR-104c</td>
<td>99.0</td>
<td>99.3</td>
<td>99.3</td>
<td>99.5</td>
</tr>
<tr>
<td>5</td>
<td>SR-104d</td>
<td>98.2</td>
<td>98.4</td>
<td>98.3</td>
<td>97.8</td>
</tr>
<tr>
<td>6</td>
<td>SR-104e</td>
<td>97.4</td>
<td>96.7</td>
<td>97.2</td>
<td>96.0</td>
</tr>
<tr>
<td>7</td>
<td>SR-105</td>
<td>97.8</td>
<td>96.8</td>
<td>97.6</td>
<td>97.0</td>
</tr>
<tr>
<td>8</td>
<td>Etoposide</td>
<td>99.6</td>
<td>99.9</td>
<td>99.9</td>
<td>99.4</td>
</tr>
</tbody>
</table>

Etoposide was used as positive control

Table 4.2: IC$_{50}$ (µM) values of podophyllotoxin analogs against prostate (PC-3), pancreatic (PANC-1), colon (COLO-205) and lung (A549) cancer cell lines using MTT assay.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Entry</th>
<th>PC-3</th>
<th>PANC-1</th>
<th>COLO-205</th>
<th>A549</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC$_{50}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>SR-104</td>
<td>0.020 ± 0.0089</td>
<td>0.023 ± 0.0076</td>
<td>0.021 ± 0.0091</td>
<td>0.031 ± 0.0078</td>
</tr>
<tr>
<td>2</td>
<td>SR-104a</td>
<td>0.025 ± 0.0121</td>
<td>0.018 ± 0.0038</td>
<td>0.016 ± 0.0034</td>
<td>0.023 ± 0.0091</td>
</tr>
<tr>
<td>3</td>
<td>SR-104b</td>
<td>1.92 ± 0.153</td>
<td>1.11 ± 0.124</td>
<td>1.54 ± 0.145</td>
<td>2.48 ± 0.143</td>
</tr>
<tr>
<td>4</td>
<td>SR-104c</td>
<td>1.89 ± 0.268</td>
<td>1.06 ± 0.096</td>
<td>1.12 ± 0.265</td>
<td>2.50 ± 0.133</td>
</tr>
<tr>
<td>5</td>
<td>SR-104d</td>
<td>3.90 ± 0.223</td>
<td>3.30 ± 0.133</td>
<td>2.70 ± 0.298</td>
<td>4.88 ± 0.231</td>
</tr>
<tr>
<td>6</td>
<td>SR-104e</td>
<td>5.40 ± 0.154</td>
<td>7.34 ± 0.234</td>
<td>4.57 ± 0.283</td>
<td>7.22 ± 0.224</td>
</tr>
<tr>
<td>7</td>
<td>SR-105</td>
<td>4.50 ± 0.133</td>
<td>5.60 ± 0.076</td>
<td>4.80 ± 0.033</td>
<td>6.70 ± 0.241</td>
</tr>
<tr>
<td>8</td>
<td>Etoposide</td>
<td>1.56 ± 0.283</td>
<td>0.82 ± 0.0062</td>
<td>0.88 ± 0.005</td>
<td>2.55 ± 0.189</td>
</tr>
</tbody>
</table>

IC$_{50}$ values are indicated as the mean ± SD of three independent experiments. Etoposide was used as positive control.

All the podophyllotoxin analogs displayed potent cytotoxic effects against all the tested human cancer cell lines. Among these analogs, \textit{SR-104b} and \textit{SR-104c} exhibited superior potency to etoposide against A549 cancer cell line with IC$_{50}$ of 2.48 and 2.50 µM respectively, besides showing interesting cytotoxic activity profile against PC-3, PANC-1 and COLO-205. Additionally, \textit{SR-104d} and \textit{SR-104e} displayed reduced cytotoxic activity as that of etoposide. These observations revealed the beneficial influence of the pinitol-diacetonide moiety on the cytotoxic activity of podophyllotoxins. With the potent cytotoxic profiles, compounds \textit{SR-104b} and \textit{SR-104c} merit further development as the new generation of podophyllotoxin and epipodophyllotoxin-derived antitumor agents.

4.5. Conclusion

Five natural products were isolated and characterized from the rhizomes of \textit{P. hexandrum}. dichloromethane:methanol extract yielded five compounds i.e., β-Sitosterol-3-O-glucoside (\textit{SR-101}), Kaempferol (\textit{SR-102}), Quercetin (\textit{SR-103}), Podophyllotoxin (\textit{SR-104}) and Podophyllotoxin-4-O-β-D-glucopyranoside (\textit{SR-105}). Compounds \textit{SR-102, SR-103, SR-104} and \textit{SR-105}, were earlier reported from this plant species while as \textit{SR-101} is reported for the first time from this plant species. In addition to this, four new podophyllotoxin derivatives were prepared and evaluated for cytotoxicity against four different human cancer cell lines. From the derived cytotoxicity data it was found that \textit{SR-104b} and \textit{SR-104c} with a pinitol-diacetonide moiety on C-4 position are having activity comparable with that of the standard drug etoposide. It can be concluded that these lignans are compounds of interest for the pharmaceutical industry. Podophyllotoxin is an important starting material to prepare semi-synthetic cytoptatics. An investigation of the novel synthesized derivatives may lead to new cytoptatic compounds, which can form the basis for new anticancer drug discovery.

4.6. Experimental

4.6.1. General experimental procedures

\textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectra (with chemical shifts expressed in $\delta$ and coupling constants in Hertz) were recorded on Bruker DPX 500 and 400 instruments using CDCl$_3$ and DMSO-d$_6$ as the solvents with TMS as internal standard. Infrared spectra were
recorded as KBr pellets in cm$^{-1}$ on a Hitachi 270-30 spectrophotometer. Melting points were determined on a Buchi melting point apparatus. UV spectra were scanned in methanol on specord S100. HR-EIMS were recorded on an Agilent Technologies G6540-UHD LC/MS Q-TOF. Column was run using silica gel (60-120 mesh). TLC plates were visualized under UV light and after exposure to iodine vapour in iodine chamber.

### 4.6.2. Plant Material

The rhizomes of *P. hexandrum* were collected from the upper reaches of Gulmarg (J&K), India. After proper identification, a voucher specimen (No. 2109/11) was deposited in the Herbarium of the Indian Institute of Integrative Medicine, Srinagar, India.

### 4.6.3. Extraction and Isolation

Air-dried and coarsely powdered plant material (aerial part, 5.0 Kg) was extracted with hexane for 48 hours. The marc was dried and extracted with dichloromethane:methanol (1:1) according to the NCI protocol to afford the respective extract for 48 hours. The extract thus obtained was concentrated under reduced pressure to give 250.0 g of dichloromethane:methanol extract. The extract thus obtained was dissolved in minimum amount of methanol and adsorbed on silica gel to form slurry. The dried slurry was subjected to silica gel column chromatography leading to Fr-I (20% ethyl acetate in petroleum ether), Fr-II (40% ethyl acetate in petroleum ether) and Fr-III (80% ethyl acetate in petroleum ether). Repeated column chromatography of these fractions and acetone extract using different percentages of petroleum ether-ethyl acetate afforded five compounds viz. β-Sitosterol-3-O-glucoside (*SR-101*), Kaempferol (*SR-102*), Quercetin (*SR-103*), Podophyllotoxin (*SR-104*), Podophyllotoxin-4-O-β-D-glucopyranoside (*SR-105*).
Figure 4.10. Flow chart depicting preparation of extracts and isolation of compounds from the rhizomes of *Podophyllum hexandrum*.

4.6.3.1. SR-101 (**β**-Sitosterol-3-O-glucoside)

White amorphous solid; yield: 55 mg, mp. 285-289 °C; IR (KBr) \( \nu_{max} \) cm\(^{-1}\): 3500-3390, 3050, 2850, 1660, 1445, 1360, 1257, 1160, 1105 & 1020, 817; EIMS \( m/z \): 576, 414, 399, 396, 381, 276, 273, 285, 213, 198, 174, 160, 149, 146, 138, 135, 106, 83, 81, 71, 69, 55; \(^1\)H NMR (CDCl\(_3\), 500 MHz) \( \delta \): 5.36 (1H, d, \( J = 3.1 \) Hz, H-6), 4.45 (1H, d, \( J_{H1'-H2'} = 7.20 \) Hz, H-1’), 3.21 (1H, m, H-3), 2.94-3.14 (m, 5H, glc-H), 2.14 (3H, m, H-4, 25), 1.72 (12H, m, H-7, 15, 16, 17, 22, 23), 1.33, (8H, m, H-2, 11, 12, 28), 1.31 (1H, m, H-20), 1.24 (2H, m, H-1), 1.22 (4H, m, H-8, 9, 14), 1.13 (1H, m, H-24), 0.96 (3H, s, H-19), 0.91
(3H, d, J = 6.5 Hz, H-21), 0.84 (6H, d, J = 5.2, H-26, 27), 0.82 (3H, t, J = 6.9 Hz, H-29), 0.70 (3H, s, H-18); $^{13}$C NMR (CDCl$_3$, 125 MHz) δ: 140.52 (C-5), 122.15 (C-6), 103.57 (C-1'), 81.12 (C-3), 77.02 (C-3'), 76.63 (C-5'), 74.77 (C-2'), 70.93 (C-4'), 61.58 (C-6'), 57.31 (C-14), 56.26 (C-17), 50.41 (C-24), 50.21 (C-9), 44.01 (C-4), 42.97 (C-13), 40.33 (C-12), 39.73 (C-22), 38.82 (C-1), 37.38 (C-20), 37.15 (C-10), 33.19 (C-7), 33.07 (C-8), 29.93 (C-2), 29.74 (C-16), 29.48 (C-23), 25.94 (C-25), 25.72 (C-15), 23.70 (C-28), 21.82 (C-11), 20.51 (C-27), 19.83 (C-21), 19.37 (C-19), 18.35 (C-26), 12.46 (C-18), 11.62 (C-29); HR-EIMS: 576.4478 (calculated for C$_{29}$H$_{50}$O, 576.4390).

4.6.3.2. SR-102 (Kaempferol)

Yellow amorphous solid; yield: 160 mg, mp. 274-277 °C; UV $\lambda_{\text{max}}$ nm MeOH: 269, 329, 370; IR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3317, 1662, 1612, 1568, 1506, 1440, 1383, 1302, 1251, 1224, 1176, 1087, 1008, 974, 883, 846, 817, 794, 723, 680, 621; $^1$H NMR (DMSO-d$_6$, 500 MHz) δ: 8.05 (2H, d, J = 8.8 Hz, H-2',6'), 7.14 (2H, d, J = 8.8 Hz, H-3',5'), 6.62 (1H, d, J = 2.2 Hz, H-8), 6.33 (1H, d, J = 2.2 Hz, H-6); $^{13}$C NMR (DMSO-d$_6$, 125 MHz) δ: 177.7 (C-4), 165.7 (C-7), 162.8 (C-5), 160.9 (C-4'), 158.5 (C-9), 147.7 (C-2), 137.5 (C-3), 130.9 (C-2',6'), 123.7 (C-1'), 116.4 (C-3',5'), 104.8 (C-10), 99.7 (C-6), 94.9 (C-8); HR-EIMS: 286.0563 (calculated for C$_{15}$H$_{10}$O$_6$, 286.0477).

4.6.3.3. SR-103 (Quercetin)

Yellow amorphous solid; yield: 145 mg, mp. 297-302 °C; UV $\lambda_{\text{max}}$ nm MeOH: 266, 310, 375; IR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3550-3250, 1665, 1640, 1510, 1452, 1388, 1020; $^1$H NMR (DMSO-d$_6$, 500 MHz) δ: 7.73 (1H, d, J = 2.0 Hz, H-2'), 7.62 (1H, dd, J = 2.0, 8.0 Hz, H-6'), 6.87 (1H, d, J = 8.0 Hz, H-5'), 6.37 (1H, d, J = 2.0 Hz, H-8), 6.17 (1H, d, J = 2.0 Hz, H-6); $^{13}$C NMR (DMSO-d$_6$, 125 MHz) δ: 178.5 (C-4), 164.67 (C-7), 161.52 (C-5), 158.74 (C-9), 147.27 (C-4'), 146.22 (C-2), 145.33 (C-3'), 134.7 (C-3), 122.49 (C-1'), 121.04 (C-6'), 115.97 (C-5'), 115.21 (C-2'), 104.81 (C-10), 98.47 (C-6), 93.74 (C-8); HR-EIMS: 302.0522 (calculated for C$_{15}$H$_{10}$O$_7$, 302.0427).

4.6.3.4. SR-104 (Podophyllotoxin)
White crystalline solid; yield: 6.2 g, mp. 182-186 °C; UV $\lambda_{\text{max}}$ nm MeOH: 280, 210; IR (KBr) $v_{\text{max}}$ cm$^{-1}$: 3350, 2900, 1745, 1610, 1450, 1030; $^1$H NMR (DMSO-d$_6$, 500 MHz) $\delta$: 7.15 (1H, s, H-5), 6.55 (1H, s, H-8), 6.40 (2H, s, H-2', 6'), 6.15 (1H, d, $J = 1.2$, OCH$_2$O), 6.10 (1H, d, $J = 1.2$ Hz, OCH$_2$O), 4.78 (1H, d, $J = 8.8$ Hz, H-4$\beta$), 4.60 (2H, m, H-1, 11$\alpha$), 4.10 (1H, t, $J = 9.5$ Hz, H-11$\beta$), 3.84 (3H, s, 4'-OMe), 3.77 (6H, s, 3', 5'-OMe), 2.8 (2H, m, H-2, 3); $^{13}$C NMR (DMSO-d$_6$, 125 MHz) $\delta$: 174.7 (C-12), 152.5 (C-3', 5'), 147.5 (C-6, 7), 136.9 (C-4'), 135.7 (C-1'), 133.3 (C-10), 130.9 (C-9), 110.1 (C-8), 108.3 (C-2', 6'), 106.5 (C-5), 101.3 (-OCH$_2$O-), 72.2 (C-4), 71.5 (C-11), 60.7 (4'-OMe), 56.1 (3', 5'-OMe), 45.4 (C-2), 44.2 (C-1), 40.1 (C-3); HR-EIMS: 414.1397 (calculated for C$_{22}$H$_{22}$O$_8$).

**4.6.3.4.1 Synthesis of 4'-O-demethylepipodophyllotoxin (SR-104a)**

To a solution of podophyllotoxin (414 mg, 1 mmol) in dry CH$_2$Cl$_2$ (10 ml), NaI (447 mg, 3 mmol) was added and stirred for 5 min. To this stirred suspension, MeSO$_3$H (288 mg, 3 mmol) was added dropwise with syringe at 0 °C and the stirring was continued for 5 h at room temperature. Nitrogen was bubbled through the solution to drive of the excess hydrogen iodide. This solution was then evaporated in vacuo and used for the next reaction without further purification. To the above crude product BaCO$_3$ (395 mg, 2 mmol), 0.5ml of water was added in 10 ml of acetone and stirred for 30 minutes at room temperature. The reaction mixture was then filtered, diluted with ethyl acetate and washed with water and 10% Na$_2$S$_2$O$_3$ solution, dried and purified via column chromatography on silica gel with ethyl acetate/hexane as eluent to afford the title compound (66.0 %). White crystalline solid; IR (KBr) $v_{\text{max}}$ cm$^{-1}$:3550, 1770, 1603, 1383, 1338; $^1$H NMR (DMSO-d$_6$, 500 MHz) $\delta$: 6.94 (1H, s, H-5), 6.51 (1H, s, H-8), 6.21 (2H, s, H-2', 6'), 6.00 (2H, d, $J = 4.6$ Hz, H-13), 4.74 (1H, d, $J = 4.7$ Hz, H-4$\alpha$), 4.50 (1H, d, $J = 1.2$ Hz, H-1), 4.34 (1H, m, H-11$\alpha$), 4.14 (1H, m, H-11$\beta$), 3.62 (6H, s, H-3'- OMe, 5'-OMe), 3.26 (1H, m, H-2), 2.75 (1H, m, H-3); $^{13}$C NMR: 175.5 (C-12), 148.6 (C-7), 147.1 (C-6), 146.9 (C-3', 5'), 134.6 (C-4'), 133.1 (C-9), 131.7 (C-10), 130.6 (C-1'), 110.0 (C-8), 109.9 (C-5), 108.4 (C-2', 6'), 101.2 (C-13), 71.5 (C-4), 67.9 (C-11), 56.5 (3', 5'-OMe), 43.7 (C-1), 40.7 (C-2), 38.6 (C-3); HR-EIMS: 400.1231 (calculated for C$_{28}$H$_{32}$O$_{13}$, 400.1158).
4.6.3.4.2 General procedure for the synthesis of SR-104b and SR-104c

25 ml two neck round-bottom flask with a stir bar and a septum was charged with lignan (SR-104/SR-104a, 0.5 mmol), dry 4 Å molecular sieves (1.0 g), D-pinitol diacetonide (0.6 mmol), anhydrous acetonitrile (5.0 ml). The solution was stirred until homogeneous and then cooled to -30 °C. Boron trifluoride etherate (0.20 ml, 1.6 mmol) was added dropwise over 2 min. The reaction was held at -30 °C for 60 min. Pyridine (2.0 ml) was added to quench the Lewis acid. The solution was filtered to remove remaining solids. Extractive workup with ethyl acetate (3x20 ml) afforded the coupled product in organic layer. The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo to an off-white solid. The desired β product was purified by recrystallization and chiral HPLC to afford the title compound.

4.6.3.4.2.1 SR-104b

White amorphous solid; ¹H NMR (CDCl₃, 400 MHz) δ: 7.18 (1H, s), 6.55 (1H, s), 6.42 (2H, s), 6.14 (1H, d, J = 1.2), 6.10 (1H, d, J = 1.2 Hz), 4.79 (1H, d, J = 4.7 Hz), 4.60 (2H, m), 4.15 (5H, m), 3.84 (3H, s), 3.77 (6H, s), 3.51 (3H, s), 3.26 (1H, m), 3.20 (1H, m), 2.8 (2H, m), 1.53 (6H, s), 1.49 (6H, s); HR-EIMS: 671.2688 (calculated for C₃₅H₄₂O₁₃, 670.2625).

4.6.3.4.2.2 SR-104c

Cream coloured amorphous solid; ¹H NMR (CDCl₃, 400 MHz) δ: 7.28 (1H, s), 6.49 (1H, s), 6.49 (2H, s), 5.96 (2H, d, J = 4.5), 4.87 (1H, d, J = 4.8), 4.54 (1H, d, J = 1.2 Hz), 4.44 (1H, m), 4.32 (2H, m), 4.22 (m, 4H), 3.77 (6H, s), 3.50 (3H, s), 3.27 (1H, m, H-2), 3.21 (1H, M), 2.80 (1H, m, H-3), 1.58 (6H, s), 1.52 (6H, s); HR-EIMS: 657.2532 (calculated for C₃₄H₄₀O₁₃, 656.2469).

4.6.3.4.3 General procedure for the synthesis of SR-104d and SR-104e

To a solution of podophyllotoxin (104 mg, 0.25 mmol) in dry CH₂Cl₂ (5 ml), the corresponding anhydride (succinic anhydride/phthalic anhydride, 0.5 mmol) was added followed by the addition of pyridine and refluxed for 6 hours. The reaction progress was
monitored with TLC. After completion of reaction, the contents were concentrated and extracted with ethyl acetate (3x20 ml). The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo to an off-white solid (98.0%). The compound was purified via column chromatography on silica gel with ethyl acetate/hexane as eluent.

4.6.3.4.3.1 SR-104d

White amorphous solid; ¹H NMR (CDCl₃, 500 MHz) δ: 7.90 (1H, m), 7.60 (3H, m), 6.87 (1H, s), 6.53 (1H, s), 6.41 (2H, s), 6.11 (1H, d, J = 1.2), 5.93 (1H, d, J = 1.2 Hz), 4.75 (1H, d, J = 8.8 Hz), 4.60 (1H, t, J = 8.8 Hz), 3.78 (3H, s), 3.73 (6H, s), 3.04 (1H, m), 2.97 (1H, m); ¹³C NMR (CDCl₃, 125 MHz) δ: 175.7, 170.1, 169.9, 152.1 (2C), 147.1 (2C), 135.5, 135.1, 133.3, 132.8, 132.2, 131.7, 131.1, 130.5, 127.8, 127.4, 109.9, 107.8 (2C), 106.1, 100.9, 73.9, 71.5, 60.7, 56.1 (2C), 45.5, 44.0, 39.9; HR-EIMS: 562.1538 (calculated for C₃₀H₂₆O₁₁, 562.1475).

4.6.3.4.3.2 SR-104e

White amorphous solid; ¹H NMR (CDCl₃, 500 MHz) δ: 6.83 (1H, s), 6.54 (1H, s), 6.40 (2H, s), 6.00 (1H, d, J = 1.2), 5.95 (1H, d, J = 1.2 Hz), 4.72 (1H, d, J = 8.8 Hz), 4.61 (1H, m), 4.42 (1H, t, J = 8.8 Hz), 4.21 (1H, m), 3.80 (3H, s), 3.77 (6H, s), 2.99 (1H, m), 2.72 (1H, m), 2.70 (2H, m), 2.61 (2H, m); ¹³C NMR (CDCl₃, 125 MHz) δ: 174.9, 174.0, 173.5, 152.7 (2C), 148.4, 147.9, 137.0, 135.4, 132.4, 128.4, 109.8, 108.2 (2C), 107.2, 101.8, 74.0, 71.8, 60.8, 56.2 (2C), 45.6, 44.4, 38.9, 29.8, 29.1; HR-EIMS: 514.1542 (calculated for C₂₆H₂₆O₁₁, 514.1475).

4.6.3.5. SR-105 (Podophyllotoxin-4-O-β-D-glucopyranoside)

White crystalline solid; yield: 135 mg, mp. 219-225 °C; UV λmax nm MeOH: 279, 209; IR (KBr) νmax cm⁻¹: 3450-3330, 2890, 1745, 1615, 1456, 1028; ¹H NMR (DMSO-d₆, 500 MHz) δ: 7.39 (1H, s, H-5), 6.44 (1H, s, H-8), 6.40 (2H, s, H-2', 6'), 5.95 (1H, d, J = 1.2 Hz, OCH₂O), 5.93 (1H, d, J = 1.2, OCH₂O), 5.07 (1H, d, J = 8.8 Hz, H-4β), 4.70 (1H, dd, J = 8.4, 6.7 Hz, H-11α), 4.55 (1H, d, J = 1.2, H-1), 4.40 (1H, d, J = 7.6 Hz, H-1’), 4.23 (1H, t, J = 9.6 Hz, H-11β), 3.91 (1H, dd, J = 11.7, 2.0 Hz, H-6”α), 3.76 (1H, dd, J = 11.7, 5.5 Hz, H-6”β), 3.73 (3H, s, 4’-OME), 3.71 (6H, s, 3’, 5’-OME), 3.3-3.4 (4H, m, H-2’, 3”, 4’, 5’), 2.99 (1H, m, H-2), 2.92 (1H, m, H-3); ¹³C NMR (DMSO-d₆, 125 MHz) δ:
177.1 (C-12), 150.1 (C-3', 5'), 148.8 (C-6, 7), 133.9 (C-4'), 135.9 (C-1'), 132.8 (C-9), 132.5 (C-10), 110.2 (C-8), 109.5 (C-2', 6'), 108.9 (C-5), 103.5 (C-1''), 102.5 (-OCH2O-), 78.4 (C-3''), 78.1 (C-5''), 77.9 (C-4), 75.3 (C-2''), 72.7 (C-11), 71.5(C-4''), 62.9 (C-6''), 60.5 (4'-OMe), 56.8 (3', 5'-OMe), 46.5 (C-2), 44.9 (C-1), 40.3 (C-3); HR-EIMS: 576.1928 (calculated for C_{28}H_{32}O_{13}, 576.1843).

4.6.4. HPLC analysis

Reverse phase chromatography with a chiral column was used to separate the reaction mixture. The most suitable mobile phase system was established after several tests and it consisted of MeOH:H_{2}O (3:7, v/v). The mobile phase flow rate was 1.0 ml/min for the analysis. The column temperature was maintained at 32 °C and the elution was monitored at 220 nm for the products. UV spectra were recorded with a DAD detector from 200 to 700 nm.

4.6.5. Cell culture and reagents

Human cancer cell lines, viz: prostate cancer (PC-3), pancreatic cancer (PANC-1), colon cancer (COLO-205) and lung cancer (A-549) were purchased from European Collection of Cell Cultures (ECACC). Cells were cultured in Minimum Essential Medium (MEM), RPMI-1640 and DMEM (Invitrogen) with Penicillin G, Streptomycin from Sigma and 10% foetal bovine serum (FBS) from GIBCO. Trypsin-EDTA was obtained from Sigma and cells were incubated at 37 °C with 5% CO_{2} incubator (Eppendorf). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Etoposide, Paraformaldehyde, Triton X-100, UltraCruz DAPI mounting media and Dimethylsulfoxide (DMSO) were obtained from Sigma Chemicals.

4.6.5.1 Cell viability assay

The cell viability was determined by standard MTT dye uptake method. Briefly PC-3, PANC-1, COLO-205 and A-549 cells from moderately confluent flasks were trypsinized properly and plated into 96 well tissue culture plates at a density of 2.5 x10^{3} cells/well. Treatment to the cells was given with different concentrations of test compounds along with etoposide (+ve control) in triplicates so that the final concentration of DMSO
solvent was 0.2%. After 44 h incubation, MTT solution (2.5 mg/ml) was added and further incubated at 37 °C and 5% CO2 for 4 h. The amount of coloured formazan derivatives was determined by measuring optical density (OD) using TECAN microplate reader (Infinite M200 PRO) at 570 nm. The percentage of cell viability was determined and the IC50 values were calculated using GraphPad Prism software.

Representative Graphs

$\text{H} \text{ & } \text{C NMR spectra of SR-104 (Podophyllotoxin)}$
1H & 13C NMR spectra of SR-104a (4'-Demethyl epipodophyllotoxin)
$^{1}$H & $^{13}$C NMR spectra of SR-104c (αβ-mixture)
$^1$H spectra of SR-104c and SR-104e
Chapter 4

Podophyllum hexandrum

1H & 13C NMR spectra of SR-104d