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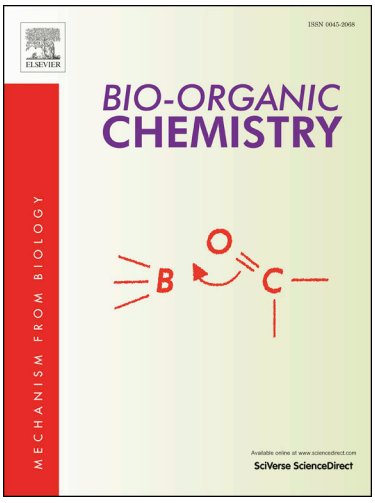

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Synthesis of D-ring modified Acid hydrazide derivatives of Podophyllotoxin and their anticancer studies as Tubulin inhibiting agents

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ABSTRACT

A new series (except compound 3a) of D-ring modified acid hydrazides of podophyllotoxin were synthesized by cleaving of its D-ring with various hydrazines. Furthermore, the synthesized compounds were screened for their anticancer activity against human tumor cell lines i.e., MCF-7, HeLa and A-549 and among the synthesized compounds **3c** and **3f** have shown significant anticancer activity almost similar to that of standard drug etoposide. Molecular modelling studies were also conducted for active compounds and found that the free energies obtained were in good agreement with the observed IC_{50} values.

The crude extract isolated from the roots and rhizomes of the plants of podophyllum species is called Podophyllum resin and it has been used as a cathartic, antihelminthic agent and a remedy for condyloma acuminatum for several years.[1,2] The active component of this resin is an aryltetralin lignan named podophyllotoxin **(1)**, a promising anticancer molecule. Its analogues Etoposide **(2)** and Teneposide **(3)** were proved to be potential antitumor drugs for various types of cancer ailments.[3] Currently some of its structural analogues such as GP-11 **(4)**, GL-331 **(5)** and TOP-53 **(6)** are drug leads for cancer treatment (Fig. 1).[4] GL-331 is currently in phase-II clinical trials against gastric carcinoma, colon cancer, non-small cell carcinoma and etoposide resistant malignancies.[5] However, their clinical use has encountered certain problems, such as poor water solubility, drug resistance, metabolic inactivation and certain toxic effects.[6,7]

Antitumor property of podophyllotoxin and its analogues was mainly due to its mechanism of action which involves inhibition of Tubulin polymerization.[8] Tubulin inhibiting agents interfere with microtubule dynamics and hinder the assembly of Tubulin into microtubules. Microtubules are vital cytoskeletal filaments that found in eukaryotes and play key role in numerous cellular functions, such as cell motility, vesicle transport and cell division. Disruption of this equilibrium will lead to cell cycle arrest or cell apoptosis. Given their significant role in the growth and function of cells, microtubules are among the most important molecular targets for cancer chemotherapeutic agents. Colchicine is the first drug that is well known to bind Tubulin, and its binding site has been characterized recently. In the past number of small molecules were discovered which bind to Tubulin, interfering with the polymerization or depolymerization of microtubules and then inducing cell cycle arrest, resulting in cell death.[9,10]

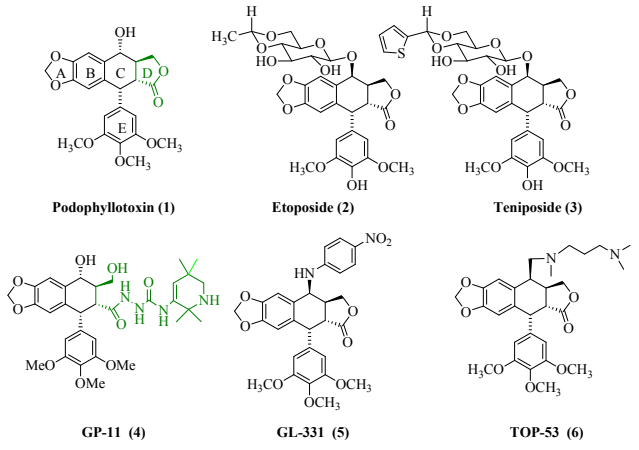


Figure 1. Podophyllotoxin, its anticancer drugs and drug lead compounds.

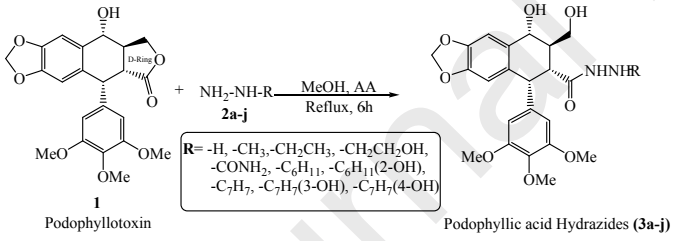
On the other hand, acid hydrazides have wide applications as drugs, chemical preservers for plants, for manufacturing

polymers, glues, etc., in industry and for many other purposes.[11] A wide variety of heterocyclic compounds like pyrroles, pyrazoles, pyrazolines, oxadiazolethiones, thiazoles, oxadiazoles, triazoles, thiadiazoles and tetrazines can be synthesized starting from acid hydrazides using Gewald reaction, Curtius rearrangement, Dimroth rearrangement, Horner-Emmons reaction and Reid-Heindel reaction.

Based on the SAR studies, initially it was presumed that intact trans-lactone ring of Podophyllotoxin i.e., D-ring was responsible for its anticancer property. But reports thereafter revealed that it may not be the case. For example **GP-11(4)** and other D-ring modified derivatives have disproved it and moreover the former is equipotent to a well-known cancer drug, etoposide.[12] In addition, the clinical success of GP-11, a derivative of Podophyllic acidhydrazide, encouraged us to take up synthesis and anticancer screening of similar derivatives of podophyllotoxin.

To overcome the toxic effects, poor water solubility and other issues associated with the earlier reported podophyllotoxin derivatives, Herein we have shown some necessary modifications to Lactone ring (D-ring) of podophyllotoxin to synthesize podophyllic acid hydrazides. The synthesized compounds were also tested for their efficacy in inhibiting tumor growth of three human tumor cell lines (MCF-7, HeLa and A-549).

Initially Podophyllotoxin (**1**) was dissolved in methanol, and then, acetic acid (AA) and corresponding anhydrous hydrazines (**2a-j**) were added with stirring.[13] The reaction mixture was refluxed for about 6-10 h to afford podophyllic acid hydrazides (**3a-j**) in good to excellent yields (Scheme 1).



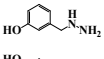
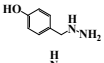
Scheme 1. Synthesis of podophyllic acid hydrazides.

Podophyllotoxin was made to react with structurally different hydrazine derivatives and the results obtained were summarized in Table 1. It was clear from the table that among various hydrazines, aliphatic hydrazines (Table 1, Entries 1-4) performed well by giving good yields in short reaction times. Semicarbazide and alicyclic hydrazines (Entries 5-7) were took longer times to cleave the ring and gave lower yields than that of aliphatic hydrazines. Even benzylic hydrazines (Entries 8-10) were also equally good at cleaving D-ring of podophyllotoxin to give corresponding acid hydrazides. However, aromatic hydrazines (Entries 11 and 12) were unable to cleave the D-ring of podophyllotoxin and there were no products formed even after refluxing for 24 h. This inability of aromatic hydrazines to cleave D-ring may be attributed their lower basicity than the corresponding aliphatic and other hydrazines of present study.

The NMR and HRMS techniques were used to elucidate the structures of the synthesized acid hydrazides. The formation of acid hydrazides from podophyllotoxin can be realised by comparing the ^{1}H-NMR & ^{13}C-NMR spectra of parent compound and formed acid hydrazides. The appearance of new peak in ^{1}H-NMR spectrum of acid hydrazides at around 9.16δ (corresponds to CO-NH proton) clearly indicates the formation of acid hydrazides from the parent compound. This peak was not there in the ^{1}H-NMR spectrum of parent compound. In ^{13}C-NMR spectrum, the peak corresponding to carbonyl carbon of lactone ring was experienced a downfield shift due to deshielding effect with D-ring opening and thus moved from~175 to ~172 δ. The same downfield shift was also seen in the case of methylene carbon of lactone ring i.e., from ~72 to 68.7 δ. Further confirmation of acid hydrazide formation was also done by HRMS spectra.

Table 1.
Showing the results for the synthesis of podophyllic acid hydrazides from structurally diverse hydrazines

.

Entry	Hydrazine	Product [a]	Reaction time (hour), (yield, %)[b]	Entry	Hydrazine	Product [a]	Reaction time (hour), (yield, %)[b]
1		**3a**	6(94)	7		**3g**	10(75)
2		**3b**	6(92)	8		**3h**	7(90)
3		**3c**	6(94)	9		**3i**	8(88)
4		**3d**	6.5(90)	10		**3j**	8(85)
5		**3e**	8(65)	11[c]		**3k**	No Reaction
6		**3f**	10(72)	12[c]		**3l**	No Reaction

[a]All products were characterized by ^{1}H/^{13}C NMR and mass spectral analysis.[b] Isolated yields after column chromatography. [c] 24 h, reflux

In vitro cytotoxicity assay: The synthesized podophyllic acid hydrazides (**3a-j**) were evaluated for *in vitro* cytotoxic ability against a panel of human cancer cell lines, including MCF-7, HeLa and A549 using a MTT assay.[14] The results were summarized in Table 2 and well-known standard drug etoposide was used as a reference. The newly synthesized podophyllic acid hydrazides have shown moderate to good anticancer activity against most of the cell lines in this investigation. Among them, compounds **3c** and **3f** were exhibited significant anticancer activity with IC_{50} values ranging from 12-20 μM and the observed activity of these compounds was almost similar to that of standard drug etoposide. As far as structure activity relationship is concerned, the compounds having ethyl and cyclohexyl substituents on hydrazine i.e., the compounds **3c** and **3f** have shown the most promising apoptotic activity than the other substituents (for 3c, 15±0.04 μM against MCF-7, 18±0.18 μM against HeLa and 16±0.02 μM against A-549; for 3f, 12±0.06 μM against MCF-7, 20±0.07 μM against HeLa and 15±0.12 μM against A-549). In the case of compound **3g,** introduction of hydroxyl group next to the hydrazine of cyclohyxyl ring significantly reduced the activity (38±0.01 μM to 45±0.25 μM). Particularly, the compounds having benzyl moiety on hydrazine i.e., **3h, 3i** and **3j** have shown very moderate activity ranging from 30±0.02 μM to 45±0.05 μM.

Table 2
in vitro cytotoxic activity of podophyllic acidhydrazides (**3a-j**) on human cancer cell lines[a] (IC_{50} μM) [b].

Entry	Compound	MCF-7(Breast)	HeLa (Cervical)	A-549 (Lung)
1	3a	30±0.03	35±0.01	30±0.02
2	3b	35±0.05	40±0.13	32±0.14
3	**3c**	**15±0.04**	**18±0.18**	**16±0.02**
4	3d	37±0.01	45±0.24	40±0.01
5	3e	45±0.01	43±0.04	38±0.45
6	**3f**	**12±0.06**	**20±0.07**	**15±0.12**
7	3g	42±0.05	45±0.25	38±0.01
8	3h	40±0.02	35±0.06	42±0.01
9	3i	30±0.02	35±0.11	45±0.05
10	3j	34±0.02	35±0.01	38±0.10
11	Etoposide	10±0.32	15±0.37	12±0.12

[a] Data represent as mean ± SEM values. Cytotoxicity as IC_{50} for each cell line, is the concentration of compound which reduced by 50% the optical density of treated cell with respect to untreated cells using the MTT assay.
[b] Data represent as mean ± SEM values of these independent determinations.

Molecular docking studies: Docking studies of the active compounds **3c** and **3f** were performed with Auto Dock software to reveal the interactions of these compounds with the active sites of Human γ-Tubulin with PBD ID 1Z5V. The results were impressive showing high binding affinity of 3c and 3f with the enzyme and free energies of -7.8 & -7.6 kcal/mole were observed for the compounds respectively (Table 3).

Table 3.
Binding energies for active molecules **3c** and **3f**

Sl. No.	Compound	Binding Energy (PBD ID 1ZXN)	Residues
1	3c	-7.8	ASP A60, GLY A129, GLY A130, THR A131, ASN A88, VAL A164
2	3f	-7.6	GLN A11, CYS A12, GLN A15, PHE A208 SER A126, VAL A164

Docking studies of **3c** revealed that the compound docked well with enzyme through various types of interactions such as Hydrogen bonding, pi-alkyl and carbon hydrogen interactions (Figure 2). The cleaved D-ring was actively involved in bonding with GLY A130, THR A131 and ASN A88 through H-bonding. Other notable interactions of 3c include H-bonding between carbonyl oxygen of Asparagine and Hydrogen of C-ring OH.

On the other hand, compound **3f** also interacted well with amino acid residues of the enzyme through various connections which include pi-pi stacked, pi-alkyl and hydrogen bonding. In the case of **3f**, the E-ring efficiently binds with the amino acid residues like GLN A11 and CYS A12 through hydrogen bonding involving oxygens of methoxy groups and hydrogens of amino groups of respective amino acids. The other prominent interactions include hydrogen bonds between etheric oxygen of A-ring and GLN A 15 and between SER A 126 and carbonyl oxygen of cleaved D- ring.

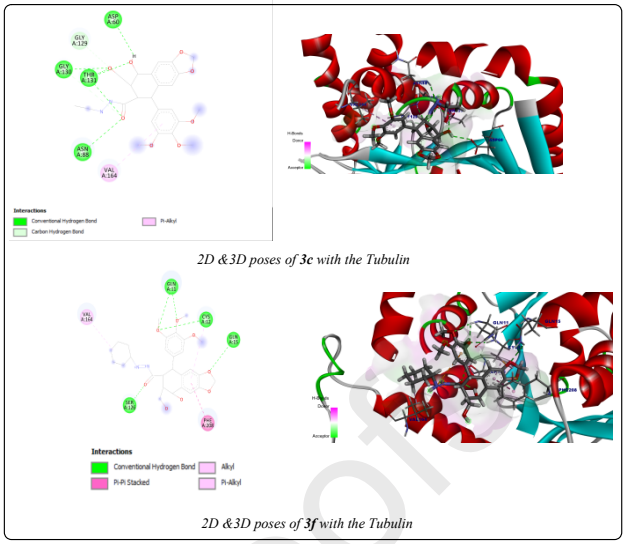


Figure 2. The docking poses of **3c** & **3f** with Human γ-Tubulin

In conclusion, we synthesized Podophyllic acid hydrazides in good yields and estimated the relative basicities of diverse hydrazines in cleaving lactone ring of podophyllotoxin. In future, this chemistry would eventually lead to synthesis of pharmacologically novel heterocyclic analogues of podophyllotoxin as acid hydrazides are important synthetic precursors. Among the synthesized compounds **3c** and **3f** have shown impressive *in vitro* anticancer activity and thus they are identified as promising lead compounds. Molecular docking studies of synthesized compounds as Tubulin inhibiting agents have also supported the observed cytotoxicity.

Supplementary data

Experimental procedures, Spectral data and antitumor evaluation methods for compounds **3a-j** can be found, in the online version.

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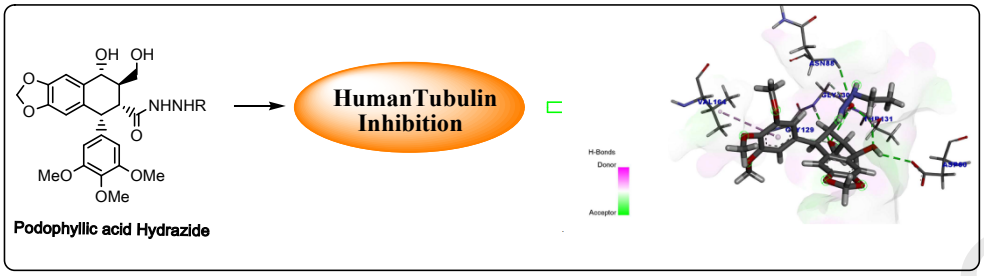
Highlights

- D-ring modified Podophyllic Acid hydrazides were successfully synthesized
- Among synthesized compounds, **3c** and **3f** have shown potential anticancer activity
- Tubulin inhibiting efficacy of active compounds were proved by Docking studies

Graphical Abstract



'Declarations of interest: none.'