Synthesis, anticancer and antimitotic activity of analogues of podophyllotoxin on B16F10 melanoma cell lines and *Allium cepa L.*

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ABSTRACT

Introduction and Aim: As a timely need for potent anticancer drugs, we attempted to synthesize analogues of podophyllotoxin which are related to etoposide and tenoposide presently in the market.

Materials and Methods: Compounds 8-13 were synthesized at standard condition by modifying the ring C structure of the parent podophyllotoxin and characterized by IR, NMR, Mass spectra and elemental analysis. Anticancer (MTT assay) activity for synthetic compounds was carried out on B16F10 mouse melanoma cell lines and antimitotic activity (cytotoxic) assay was on mitotic cells of root tips of *Allium cepa L*.

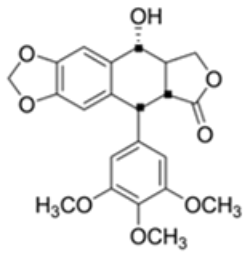
Results: Analogues 8 and 9 exhibited greater anticancer activity with the IC_{50} values of 1.6 and 1.75mM respectively, and strong inhibition of mitosis with the ID_{50} values of 1.85mM and 2.10mM respectively, whereas the analogues 10, 11, 12 and 13 showed moderate anticancer activity.

Conclusion: Analogues 8 and 9 would become the novel anticancer drugs in future for cancer chemotherapy after further investigations.

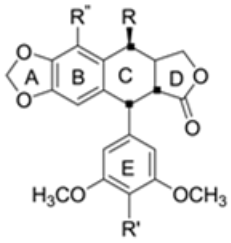
Keywords: Synthesis; podophyllotoxin; anticancer; antimitotic.

INTRODUCTION

At present, the deadly disease cancer remains one of the major causes for the increasing death toll over the globe. Currently, the discovery of anticancer drugs became an extraordinary challenge to the current researchers over the course of years. The number of plant origin and synthesized products having potent antimitotic and anticancer, cytotoxic (1, 2), cathartic, antiviral, antibacterial, antiangiogenic activity have been reported worldwide. Camptothecin, an alkaloid from *Camptothera accuminata* as an anticancer drug having a unique mode of action that is inhibition of DNA topoisomerase I (3, 4). Podophyllotoxin 1 (5), otherwise known as Podofilox (Fig. 1, *Podophyllum* species), is a well-known naturally occurring aryltetralin lignin, and it shows strong cytotoxic activity against various cell lines (6). It is effective in the treatment of Wilm's tumors, various genital tumors and in non-Hodgkin and other lymphomas and lung cancer (7, 8). Recently, several synthetic analogues of podophyllotoxin and its related compounds reported antiviral and cytotoxic activity (9, 10). The attempt to use podophyllotoxin in the treatment of human neoplasia is restricted due to its complicated side effects (11, 12) such as nausea, vomiting, damage of normal tissues etc., Because of this reason, podophyllotoxin as such is not used as a drug. Extensive structural modifications were done to obtain more potent and less toxic anticancer agents, which resulted in two semisynthetic glycosidic cyclic acetals of epipodophyllotoxin, etoposide and tenoposide (6). These are the most widely used derivatives for the treatment of lymphoma, leukemia, testicular cancer, small cell lung cancer, ovarian, bladder, brain cancer, etc., (13). Analogues of podophyllotoxin etoposide and tenoposide (Fig. 2) are also potent antitumor agents which are clinically in use still retaining side effects (14). This observation has prompted the authors to prepare new analogues and examine them for their antimitotic (cytotoxicity) and anticancer activity.

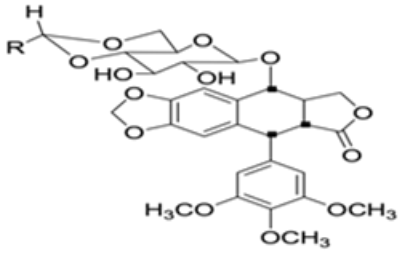


1



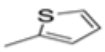
2 & 3

Fig: 1



SP-G, R=C_6H_5;

R = VM-26

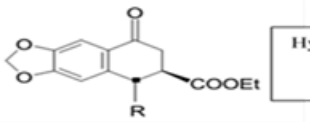


tenoposide

VP-16-213, R =CH_3 etoposide

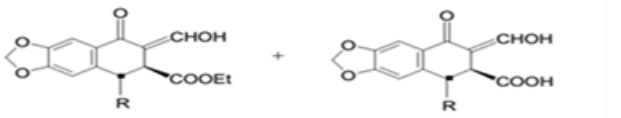
Fig: 2

2) R = H, R' = R" = OH
3) R = H, R' = OCH_3, R" = OH
4) R = O-Glucosyl, R' = OCH_3, R"= OH
5) R = R' = H, R" = O-Glucosyl
6) R = H, R' = OCH_3, R" = O-Glucosyl
7) R = O-Glucosyl, R' = R" = H



14a-c

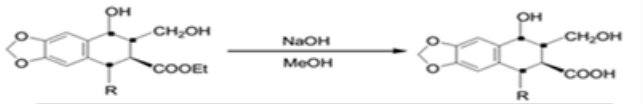
Hydroxyl methylation



15a-c 16a-c

$NaBH_4$ MeOH

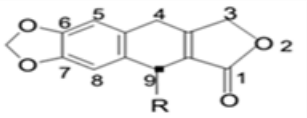
$NaBH_4$ MeOH



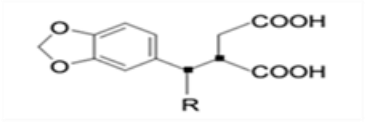
17a-c 18a-c

p-TsCl Dry pyridine

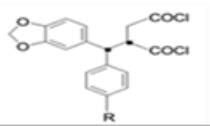
a : R= p- Cl-C_6H_4
b : R= p- F-C_6H_4
c : R= p- NO_2-C_6H_4



Scheme -1 8, 9 and 10



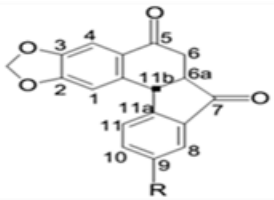
$SOCl_2$ Dry C_6H_6



19a-c 20a-c

Anhyd. $AlCl_3$ Dry CH_2Cl_2

a : R= p- Cl-C_6H_4
b : R= p- F-C_6H_4
c : R= p- NO_2-C_6H_4



Scheme-2 11, 12 and 13

MATERIALS AND METHODS

General: All the chemicals and reagents required were purchased from Sigma Aldrich and Merck Company. Melting points were determined on a SONAR melting point apparatus and are uncorrected. Reactions were monitored by TLC on 0.2mm precoated silica gel 60 F254 plates (E. Merck). Infrared spectra were recorded on Perkins Elmer spectrum-1000 (450-4000cm-1) spectrometer on KBr disc or Nujol mull. The ^{1}HNMR spectra were recorded with a Varian T-60 and Brucker DRX-300 (300MHz FT NMR) spectrometer using tetra methyl silane (Me^4Si, δ=0) as an internal standard in $CDCl_3$, J values are given in Hz. The mass spectra were recorded on a JEOL SX102A spectrometer. Elemental analysis data were recorded on Elemental Analyzer Vario EL III. The preparation of hydroxy methylene tetralone esters 15a-c and hydroxy methylene tetralone acids 16a-c; Formylation of the previously synthesized tetralone esters 14a-c with ethyl formate using sodium hydride as the base at room temperature gave two products (15, 16).

A typical procedure is described for the preparation of 4-(p-chloro phenyl)-1-oxo-2-hydroxymethylene-3-ethylcarboxy-6,7-dioxy methylene- 1,2,3,4 tetrahydro naphthalene 15a (6).

Sodium hydride (0.3216g, 0.0134 moles) was added to a mixture of absolute ethanol (10 ml) and dry benzene (150 ml) and stirred for 1h. Ethyl formate (10 ml) was added dropwise to the above mixture and stirred for another1 hour followed by dropwise addition of 14a (5g, 0.0134 moles) in dry benzene (100 ml) over a period of 1h. After stirring the red coloured mixture at room temperature for 12hrs, it was poured into 2N H_2SO_4 (100 ml) in ice (100 g). The separated organic layer was washed with water (3x50 ml) and extracted into a saturated sodium bicarbonate solution (3x50 ml), followed by a 1% sodium hydroxide solution (3x50 ml).

The sodium hydroxide extract was acidified with 2N H_2SO_4 to give a yellow solid. It was recrystallized from ethanol to give a yellow crystalline solid in 80.73 % yield (4.34g). M.p.102-104ºC. IR (KBr): 3500-3200 (OH), 1740 (ester C=O),1695 (tetralone C=O),1630 (conjugated C=C), 1595 (aromatic C = C)cm-1; PMR (CDCl3): δ 4.2 (q, J = 4Hz, 2H, ester CH2), 5.9 (s, 2H, OCH2O), 6.8 (m, 6H, Ar-H), 5.6 (s, 1H, vinyl OH), 8.3 (s, 1H, vinylic), 3.9 (d, J=4Hz, 1H, C4- H), 3.6 (d, J = 4 Hz, 1H, C3-H), 1.1 (t, 3Hz, 3H, ester CH3). Anal. calcd. for $C_{21}H_{17}O_6Cl$: C, 62.93; H, 4.27%. Found: C, 62.90; H, 4.25%. The bicarbonate extract was acidified with 2N H_2SO_4 to give a yellow solid. It was recrystallized from ethanol to give a yellow crystalline solid 51a in 3.20 % yield (0.16g). M.p.99-110º C.

4-(p-Fluorophenyl)-1-oxo-2-hydroxymethylene-3-ethylcarboxy-6,7-dioxymethylene-1,2,3,4-tetrahydro naphthalene 15b.

Prepared from 14b (5g, 0.0140 mole), sodium hydride (0.03360g, 0.0140 mole) and ethyl formate (10 ml) as a yellow crystalline solid in 83.40% yield (4.53g). M.p. 102ºC. IR (KBr): 3500 – 3200 (OH), 1738 (ester C=O), 1690 (tetralone C=O), 1635 (conjugated C=C), 1600 (aromatic C=C) cm-1; PMR (CDCl3): δ 4.1 (q, J=4Hz, 2H, ester CH2), 0.9 (t, J =4Hz, ester CH3), 3.5 (d, J = 4Hz, 1H, C3-H), 3.9 (d, J = 4Hz, 1H, C4-H), 8.3 (s, 1H, vinyl), 5.6 (s,1H, Vinyl OH), 6.7 (m, 6H, Ar-H), 6.0 (s, 2H, OCH2O); Anal. calcd. for $C_{21}H_{17}O_6F$: C, 65.62; H, 4.46%. Found: C, 65.59; H, 4.44%. The bicarbonate extract was acidified with 2N H2SO4 to give a yellow crystalline solid of 15b in 3.6% yield (0.18g). M.p. 98-100ºC. PMR (CDCl3): δ 9.9 (bs, 1H, COOH) and no ester group signal. Anal calcd. for $C_{19}H_{13}O_6F$: C, 64.05; H, 3.68%. Found: C, 64.03; H, 3.67%.

4-(p-Nitrophenyl)-1-oxo-2-hydroxymethylene-3-ethylcarboxy-6,7-dioxymethylene-1,2,3,4-tetra hydronaphthalene 15c.

Prepared from 14c (5g, 0.0130 mole), sodium hydride (0.3120g, 0.0130 mole) and ethyl formate (10 ml) as a pale yellow solid in 78.48 % yield (4.21g). M.p. 88-90ºC. IR (KBr): 3550–3200 (OH), 1740 (ester C=O), 1695 (C=O), 1630 (conjugated C=C), 1600 (aromatic C=C) cm-1; PMR (CDCl3); δ 3.8 (q, J = 4Hz, 2H, ester CH2), 1.0 (t, J = 4 Hz, 3H, ester CH3), 3.6 (d, J = 4Hz, 1H, C3-H), 4.1(d, J = 4Hz, 1H, C4-H), 8.2 (s, 1H, Vinylic), 5.7 (s, 1H, vinyl OH) , 6.7 (m, 6H, Ar-H), 5.9 (s, 2H, OCH2O). Anal. calcd. for $C_{21}H_{17}O_8N$: C, 61.32; H, 4.17; N, 3.41%. Found: C, 61.29; H, 4.15; N, 3.37%. The bicarbonate extract was acidified with 2N H2SO4 to give a yellow crystalline solid 16c in 3.0% yield (0.15g). M.p. 96-98oC. PMR (CDCl3): δ 9.8 (bs, 1H, COOH) and no ester group signal. Anal. calcd. for $C_{19}H_{13}O_8N$: C, 59.54; H, 3.42; N, 3.65%. Found: C, 59.52; H, 3.44; N, 3.64%.

A typical procedure is described for the preparation of 1-hydroxy-2- hydroxymethyl-3-ethylcarboxy-4-(p-chlorophenyl)-6,7-dioxy methylene- 1,2,3,4, tetrahydronaphthalene 17a (6).

To a solution of 15a (4g, 0.01 mole) in absolute methanol (60 ml), sodium borohydride (0.38g, 0.01mole) in absolute methanol (60 ml) was added during 1h at room temperature. At an hourly interval, a solution of sodium borohydride (1.0g) in methanol (10 ml) was added three times. The reaction mixture, after stirring at room temperature for 5h, was concentrated to 40 ml, acidified with 2N HCl and then the pH of the solution was adjusted to 8 by adding 1% aqueous ammonium hydroxide solution. The product was extracted into ether (3x50 ml), the ether layer was washed with cold 1% sodium

hydroxide solution (2x40 ml), water (2x40 ml) and then dried over anhydrous $Na_2 SO_4$. A pink colored semi solid was obtained in 72.77 % yield (2.94g), after evaporating the solvent. IR (Nujol): 3550-3200 (OH), 1735 (ester C=O), 1600 (aromatic C=C) cm-1; PMR (CDCl3): δ 4.2 (q, J = 3Hz, 2H, ester CH2), 1.0 (t, J=3Hz, 3H, ester CH3), 3.3 (m, 2H, C1-H, C3-H), 6.8 (m, 6H, Ar-H) , 2.4 (m, 3H, C2-H, & CH2OH), 4.1 (d, J = 3Hz, 1H, C4-H), 5.3(s, 1H, CH2OH), 5.9 (s, 1H, OCH2O); Anal. calcd. for $C_{21}H_{21}O_6Cl$: C, 62.30; H, 5.23 %. Found: C, 62.28; H, 5.22%.

1-Hydroxy- 2- hydroxymethyl- 3-ethylcarboxy- 4-(p-fluorophenyl)- 6, 7- dioxymethylene-1,2, 3, 4, tetrahydronaphthalene 17b.

Prepared from 15b (4g, 0.0104mole) and sodium borohydride (0.3934g, 0.0104 mole) in methanol (80 ml) as a reddish yellow colored semi solid in 76.69 % yield (3.10g). IR (Nujol): 3560 – 3200 (OH), 1742 (ester C=O), 1590 (aromatic C=C) cm-1; PMR ($CDCl_3$): δ 4.2 (J = 4Hz, 2H, ester CH_2), 0.9 (t, J=4Hz, ester CH_3), 6.8 (m, 6H, Ar-H), 2.4 (m, 3H, C2-H, & CH_2OH), 4.0 (d, J=4Hz, 1H, C4-H), 3.6 (m, 2H, C1-H & C3-H), 5.9 (s, 2H, OCH_2O), 5.6 (s, 1H, CH_2OH); Anal. calcd. for $C_{21}H_{21}O_6F$: C, 64.94; H, 5.45%. Found: C, 64.91; H, 5.43%.

1-Hydroxy-2-hydroxymethyl-3-ethylcarboxy-4-(p-nitrophenyl)-6,7-dioxymethylene-1,2,3,4-tetra hydronaphthalene 17c.

Prepared from 15c (4g, 0.01 mole) and sodium borohydride (0.3783g, 0.01mole) in methanol (80 ml) as a yellow colored semi solid in 71.30 % yield (2.88g). IR (Nujol): 3500 – 3200 (OH), 1735 (ester C=O), 1595 (aromatic C=C) cm-1; PMR ($CDCl_3$): δ 4.3 (q, J = 4Hz, 2H, ester CH2), 1.0 (t, J=4Hz, ester CH_3), 6.7(m, 6H, Ar-H), 2.5 (m, 4H, C2-H & CH_2OH), 4.0 (d, J = 4Hz 1H, C4-H), 3.5 (m, 1H, C3-H) , 6.0 (s, 2H, OCH_2O), 5.7(s, 1H, CH_2OH); Anal. calcd. for $C_{21}H_{21}O_8N$: C, 60.72; H, 5.10; N, 3.37%. Found: C, 60.71; H, 5.08; N, 3.34%.

1-Hydroxy-2-hydroxymethyl-3-carboxy-4-(p-chlorophenyl)-6,7-dioxymethylene-1,2,3,4-tetra hydro naphthalene 18a.

A solution of 17a (3g, 0.0074 moles) in methanol (30 ml) and 5% sodium hydroxide (40 ml) was refluxed for 3h. After removing the methanol under reduced pressure, the alkaline solution was acidified with 2N HCl to give a yellow colored solid which on recrystallization from methanol gave a pale yellow colored crystalline product in 73.06 % yield (2.04g). M.p. 87-89ºC. IR (KBr): 3520-3200 (OH), 1715 (carbonyl C=O), 1600 (aromatic C=C) cm-1; PMR ($CDCl_3$): δ 9.9 (s, 1H, COOH); Anal. calcd. for $C_{19}H_{17}O_6Cl$: C, 60.57; H, 4.55%. Found: C, 60.53; H, 4.53%.

1-Hydroxy-2-hydroxymethyl-3-carboxy-4-(p-fluorophenyl)-6,7-dioxymethylene-1,2,3,4-tetra hydronaphthalene 18b.

Prepared from 17b (3g, 0.0077 mole) in methanol (30 ml) and 5% sodium hydroxide (40 ml) as a pale yellow crystalline solid in 76.80% yield (2.85g). M.p. 91-92ºC. IR (KBr): 3500-3200 (OH), 1710 (carbonyl C=O), 1600 (aromatic C=C) cm-1; PMR (CDCl3): δ 9.8 (s, 1H, COOH); Anal. calcd. for $C_{19}H_{17}O_6F$: C, 63.33; H, 4.76%. Found: C, 63.32; H, 4.72%.

1-Hydroxy-2-hydroxymethyl-3-carboxy-4-(p-nitrophenyl)-6,7-dioxymethylene-1,2,3,4-tetra hydronaphthalene 18c.

Prepared from 17c (3g, 0.0072mole) in methanol (30 ml) and 5% sodium hydroxide (40 ml) as a yellow crystalline solid in 75.07 % yield (2.80g). M.p. 92-94ºC. IR (KBr): 3500-3200 (OH), 1708 (carboxyl C=O), 1595 (aromatic, C=C) cm-1; PMR (CDCl3): δ 9.9 (s, 1H, COOH); Anal. calcd. for $C_{19}H_{17}O_8N$: C, 58.92; H, 4.42; N, 3.62%. Found: C, 58.90; H, 4.41; N, 3.59%.

A typical procedure is described for the preparation of 6,7-dioxymethylene-9-*p*-chloro phenyl naphtho [2, 3-C] furan-1-(3H, 4H, 9H) one 8a.

A mixture of 18a (2g, 0.0053 mole), p-toluene sulfonyl chloride (1.01 g, 0.0053 mole) and dry pyridine (30 ml) in dry benzene (60 ml) was refluxed for 3h. The reaction mixture was cooled to room temperature, washed with 2N HCl (3x50 ml) and then with water (2x40 ml). The solvent was removed by distillation under reduced pressure to give a thick brown residue. The crude product was column chromatographed over silica gel in 10x30 cm column using benzene as an eluent. The solvent was removed and evacuated at 50º C on a rotary evaporator to give an orange red crystalline solid in 71.32 % yield (1.29g). M.p. 76-78ºC. IR (KBr): 1775 (lactone C=O), 1670(shoulder tetra substituted C=C) 1605 (Ar C=C,)cm-1; PMR (CDCl3): δ 4.0 (s, 1H, C3-H,), 6.8 (m, 6H, Ar-H), 3.8 (s, 2H, C4-H), 4.4 (s, 2H, C9-H), 5.9 (s, 2H, OCH_2O); Mass (m/z, % of abundance): 341(M^+, 83), 339 (28), 112 (11), 245 (18), 228 (36); Anal. calcd. for $C_{19}H_{13}O_4Cl$: C, 66.97; H, 3.85%. Found: C, 66.95; H, 3.82%.

6,7-Dioxymethylene–9-*p*-fluorophenyl naphtho[2, 3-C] furan-1-(3H, 4H, 9H) one 9 was prepared from 18b (2g, 0.0056 mole), *p*-toluene sulfonyl chloride (1.07g, 0.0056 mole) and dry pyridine (25 ml) in dry benzene (60 ml) as orange red colored crystalline colored compound in 73.33 % yield (1.32g). M.p. 74- 76ºC. IR (KBr): 1756 (lactone C=O), 1666 (shoulder tetra substituted C=C), 1595 (aromatic C=C) cm-1. PMR (CDCl3): δ 4.3 (d, J=3Hz, 1H, C9-H), 6.8 (m, 6H, Ar-H), 3.7 (s, 2H, C4- H), 4.1 (s, 2H, C3-H), 6.0 (s, 2H, OCH2O); Mass (m/z, % abundance): 324 (M+, 93), 322 (19), 229

(23), 95 (15), 228 (41); Anal. calcd. for $C_{19}H_{13}O_4F$: C, 70.37; H, 4.04%. Found: C, 70.33; H, 4.02%.

6,7-Dioxymethylene–9-p-nitrophenyl naphtho[2, 3-C] furan-1-(3H, 4H, 9H) one 10 was prepared from 18c (1.5 g, 0.0039 mole), p-toluene sulfonyl chloride (0.74g, 0.0039 mole) and dry pyridine (25 ml) in dry benzene (60 ml) as light yellow crystalline solid in 70.56 % yield (1.28g). M.p. 72-73ºC. IR (KBr):1761 (lactone C=O), 1661 (shoulder tetra substituted C=C), 1590 (aromatic C=C) cm^{-1}; PMR (CDCl3): δ 4.3 (s, 1 H, C9-H), 6.7 (m, 6H, Ar-H), 3.8 (s, 2H, C4-H), 4.1 (s, 2H, C3-H), 5.9 (s, 2H, OCH2O); Mass (m/z, % abundance): 351 (M^+, 97), 349 (24), 202 (29), 201 (44), 122 (12); Anal. calcd. for $C_{19}H_{13}O_6N$: C, 64.96; H, 3.73; N, 4.0%. Found: C, 64.95; H, 3.71; N, 3.97%.

A typical procedure is described for the preparation of 6, 6a-dihydro- 2,3-dioxymethylene-9-chloro-11bH benzo [c] fluoren-5,7-dione 11.

A mixture of benzhydryl succinic acid 19a (2g, 0.0076mole) and thionyl chloride 40 ml) was refluxed for 2h. The excess thionyl chloride was distilled off. A pale yellow residue was obtained as a gummy product 20a in 85.32 % yield (1.88g). IR (KBr): 1777 (C=0 of acyl chloride) cm^{-1} and no -OH group peaks. A solution of 20a (1.5g, 0.0038 mole) in dry dichloromethane 50 ml was added into a stirred solution of anhydrous aluminium chloride (0.51g, 0.0038 mole) in dry dichloromethane at 0ºC for 8h. The reaction mixture was treated with cold 5N HCl (15 ml). The organic layer was washed with 10% NaOH solution (2x50 ml) and finally with water. The solvent was removed by distillation to give a light yellow residue. The crude product was column chromatographed over silica gel (1 cm x 30) using chloroform as the eluent. The solvent was removed and evacuated at 50ºC on the rotary evaporator to give a dark yellow solid. It was recrystallized from ethanol in 75.02% yield (0.92g). M.p.172-174ºC. IR (KBr): 1716 (Indanone carbonyl), 1673 (tetralone carbonyl), 1600 (aromatic C=C) cm-1; PMR (CDCl3): δ 2.8 (d, J=4Hz, 2H, C6-H), 3.7 (q, J=4Hz, 1H, C6a-H), 4.4 (d, J=4Hz, 1H, C11b-H), 6.9 (m, 5H, Ar-H) , 5.9 (s, 2H, OCH_2O); Mass (m/z), % abundance): 327 (M^+, 26), 299 (42), 271 (18), 243 (33), 122 (64); Anal. calcd. for $C_{18}H_{11}O_4Cl$: C, 66.17; H, 3.39%. Found: C, 66.14; H, 3.37%.

6, 6a-Dihydro-2, 3-dioxymethylene-9-fluoro-11bH benzo [c] fluoren-5, 7- dione 12 Prepared from benzhydryl succinic acid 19b [6] (2g, 0.0058 mole) and thionyl chloride (40 ml) gave 20b in 84.05 % yield (1.86g). 20b (1.5 g, 0.0039 mole) was cyclized to 12 using anhyd. aluminium chloride (0.52g, 0.0039mole) in dry dichloromethane (50 ml) to give yellow crystalline solid. It was recrystallized from ethanol in 77.39 % yield (0.94g). M.p. 188-190ºC. IR (KBr): 1711 (Indanone carbonyl), 1667 (tetralone carbonyl), 1595 (aromatic, C=C) cm^{-1}; PMR (CDCl3): δ 2.7(d, J=4Hz, 2H, C6-H), 3.5 (q, J=4Hz, 1H, C6a-H), 4.3 (d, J=6Hz, 1H, C11b-H), 6.7 (m, 5H, Ar-H), 5.9 (s, 2H, OCH2O); Mass (m/z, % abundance): 310 (M+, 21), 282 (41), 254 (28), 226 (32), 105 (64); Anal. calcd. for $C_{18}H_{11}O_4F$: C, 69.68; H, 3.57%. Found: C, 69.66; H, 3.54%.

6, 6a-dihydro-2, 3-dioxymethylene-9-nitro-11bH benzo[c] fluoren-5,7- dione 13 Prepared from 19c (2g, 0.0054 mole) and thionyl chloride (40 ml) gave 20c in 86.46 % yield (1.5g). The compound 20c (1.5g, 0.0037mole) was cyclized to give 13 using anhyd. aluminium chloride (0.50g, 0.0037 mole) in dry dichloromethane (50 ml) gave pale yellow crystalline solid. After recrystallization, the yellow solid is obtained in 73.79 % yield (0.91g). M.p.166-168ºC. IR (KBr): 1713 (Indanone C=O), 1670 (tetalone C=O), 1590 (aromatic C=C) cm -1; PMR (CDCl3): δ 2.7 (d, J=4Hz, 2H, C6-H), 3.6 (q, J=4Hz, 1H, C6a-H), 4.5 (d, J = 4Hz, 1H, C11b-H), 6.8 (m, 5H, Ar-H) , 5.9 (s, 2H, OCH2O); Mass (m/z, % abundance): 337 (M^+, 22), 309 (38), 281(23), 253 (28), 132 (62); Anal. calcd. for $C_{18}H_{11}O_6N$: C, 64.10; H, 3.29; N, 4.15% Found: C, 64.00; H, 3.26; N, 4.13%.

Anticancer assay

The newly synthesized analogues were screened for anticancer activity by determining the ability of compounds to inhibit the proliferation of B16F10 mouse melanoma cells following the in-vitro antiproliferative method (17) The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0x105 cells/ml using medium containing 10% fetal bovine serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once using PBS pH 7, 4.2mM and 100: l of different drug concentrations were added to the cells in microtitre plates. The plates were then incubated at 37ºC for 3 days in 5% CO_2 atmosphere, and microscopic examination was done. After 72 h, the drug solutions in the wells were discarded and 50: l of MTT in HBSS was added to each well. The plates were gently shaken and incubated for 3 hours at 37ºC in 5% CO_2 atmosphere. The supernatant was removed and 50: l of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540nm. The percentage growth inhibition was calculated using the following formula:

$$\% \text{ of inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

Antimitotic assay

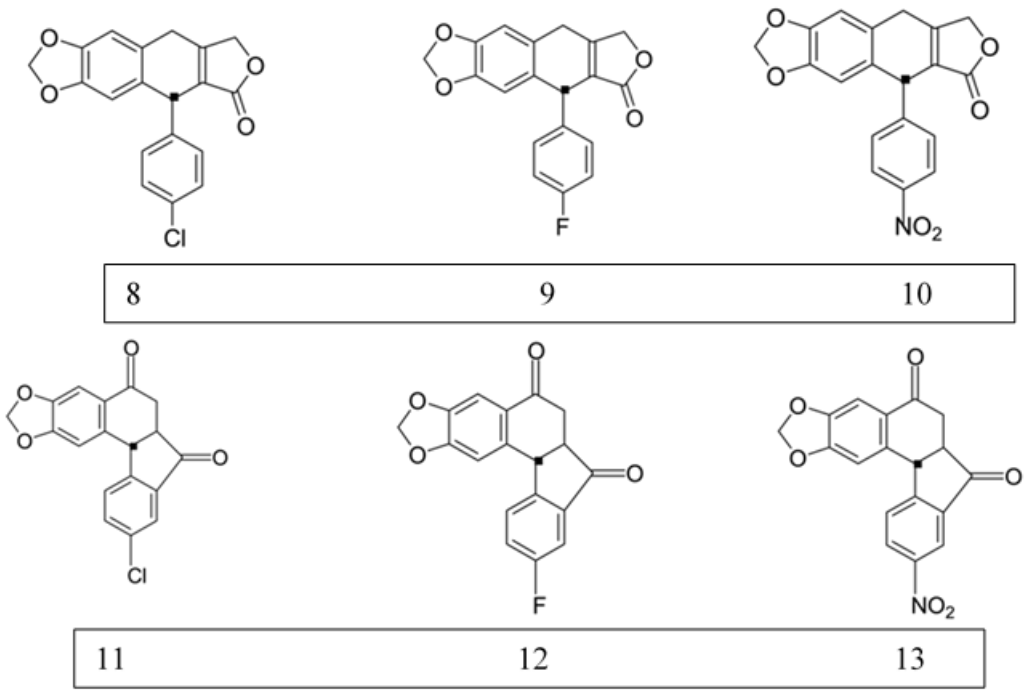
The antimitotic activities of the synthesized analogues of podophyllotoxin were examined using onion root tip method (18) The ID_{50} (concentration for 50% inhibition of mitosis) was determined. Test solution prepared by dissolving exactly known weight (0.001 to 0.003g) of synthetic analogue in 3ml of absolute ethanol and diluted with distilled water to 250 ml in a standard flask. All the tested synthesized products gave a clear solution in the above process. Onion base was immersed to an extent of about half a centimeter in a sample tube (7x3 cm) after removing the old roots from it and immersion was continued for two days for germination. After two days, germinated root tips were removed and placed on the sample tube containing fixing solvent (absolute ethanol-glacial acetic acid, 3:1v/v). After 24hrs, fixing solvent was decanted carefully and the root tips were washed with preserving solvent (70% ethanol) and kept immersed in the same solvent. An onion root tips were also allowed to germinate in a control solution (3 ml of absolute ethanol diluted with distilled water to 250 ml) without the synthetic analog in exactly the same way as done in preparing solution of synthetic analogues. Root tips were placed on a clean watch glass containing stain solution (orcein solution-HCl solution 7:1 v/v) and heated on the flame until fumes come out. It was then cooled to room temperature. Root tips were then placed on the micro slide, a drop of stain solution was added and the root tips were squashed by a razor blade and slides were prepared. The prepared slide was mounted for observation under a compound microscope. The total number of cells and the number of dividing cells were counted. The percent of the number of dividing cells compared to the control and the percent inhibition of mitosis by the test antimitotic agent at a given concentration against a control were calculated. The inhibition studies for each synthetic product were done for three different concentrations. The statistical data are presented in the table. A graph of concentration, verses percent inhibition for each test compound was drawn. The concentration needed for 50% inhibition (ID_{50}) was extrapolated from the graph as per the method of Thomas *et al.*, (19). ID_{50} values for the synthetic derivatives for antimitotic activity were calculated individually.

RESULTS AND DISCUSSION

Chemistry of synthetic compounds, 8-13

The Podophyllotoxin analogues 8-13 were synthesized by formylation of the tetralone esters 14a-c previously (15) with ethyl formate using sodium hydride as the base, afforded the hydroxy methylene tetralone esters 15a-c and hydroxy methylene tetralone acids 16a-c by following walker's method (16). Reduction of 15a-c and 16a-c with sodium borohydride in methanol gave the dihydroxy esters 17a-c and dihydroxy acids 18a-c respectively in excellent yield (6). Saponification of the dihydroxy esters 17a-c with 5% aqueous sodium hydroxide and methanol gave the dihydroxy acids 18a-c which is refluxed with *p*-toluene sulfonyl chloride in pyridine gave the podophyllotoxin analogues 8, 9 & 10 in excellent yield (Scheme 1).

The diketones 11, 12 & 13 were also synthesized by Friedel-Crafts intramolecular acylation reaction of 19a-c (6). The benzhydryl succinic acids 19a-c were converted into the benzhydryl succinyl chlorides 20a-c which were then cyclized by using anhydrous aluminium chloride in dry dichloromethane to yield the diketones 11, 12 & 13 - Scheme 2.



The IR spectra of 15a-c showed characteristic absorptions in the region of 3500–3200 cm^{-1} and 1635-1625 cm^{-1} assigned to vinylic hydroxyl and conjugated double bond groups respectively. The IR absorptions for the tetralone carbonyl group and the ester carbonyl were not much displaced when compared to that of the tetralone esters 14a-c. The compounds 16a-c showed broad peaks at 3600 – 3200 cm^{-1} and a sharp peak in the range 1630-1620 cm^{-1} due to the vinylic hydroxyl as well as carboxylic hydroxyl groups and conjugated double bonds respectively. The carbonyl group of the carboxylic acid absorbed in the range of 1720-1710 cm^{-1} and the tetralone carbonyl at 1695-1710 cm^{-1}.

The PMR spectra of 15a-c resembled each other except the differences due to the substituents. A broad singlet centered in the range δ 5.6-5.8 due to vinylic hydroxyl proton, which was exchangeable with D_2O and a broad singlet in the range of δ 8.2-8.4 due to the vinylic proton. The compounds 16a-c showed a similar type of PMR spectra due to the absence of ethyl group, but a broad singlet in the range of 9.9 assigned to carboxylic OH proton(6).

Sodium borohydride has been extensively used to reduce ketones as well as α, β unsaturated ketones to 1, 3-diols without affecting the ester functional group. The hydroxy methylene tetralone esters 15a-c were reduced to corresponding dihydroxy esters 16a-c by sodium borohydride. Compounds 15a-c were dissolved in absolute methanol and then excess sodium borohydride in absolute methanol was added during 1h at room temperature. The reaction mixture was stirred for 5h at room temperature, which on usual work up gave a brown pasty mass in good yields (6). Based on Walker's work (16) in a similar reduction, it was assumed that a mechanism involving 1,4 attack on the keto enol system which involved in the sodium borohydride reduction of 15a-c to 17a-c. The substituent groups at positions 1 and 2 in 17a-c are assumed to be cis to each other, similar to the views of walker. The IR spectra of 17a-c showed a broad absorption in the region 3600 – 3200 cm-1, assigned to the OH groups, and a sharp absorption at 1737 cm-1 assigned to the ester carbonyl group (6).

Hydrolysis of the esters 17a-c with 5% aqueous sodium hydroxide in methanol was affected smoothly at reflux temperature to give the dihydroxy carboxylic acids 18a-c in 73-78% yield. During alkaline hydrolysis of 17a-c inversion of the carboxyl group did not occur under these conditions since the compounds 16a-c on reduction with sodium borohydride in absolute methanol gave the identical products 18a-c. The hydroxy methylene tetralone acids 16a-c was dissolved in methanol and then sodium borohydride in absolute methanol was added during 1h at room temperature. The excess of sodium borohydride was decomposed by dilute HCl and the separated solid on recrystallization from methanol gave white feathery crystals. The IR spectra of 18a-c from both routes were identical. An absorption in the region of 3550 – 3200 cm-1 was assigned to carboxylic hydroxyl groups and other primary and secondary hydroxyl groups (6).

The *p*-toluene sulfonyl chloride in pyridine has been used as a dehydrating agent in many organic syntheses. The same reagent was used to convert podophyllotoxin (1) to β-apopicropodophyllin in a single step by Murthy and Rai (20). Following the same procedure, the dihydroxy acids 18a-c were successfully dehydrated with concomitant isomerization to the lower corresponding podophyllotoxin analogues 8, 9 and 10 respectively (6).

Compounds 18a-c in dry benzene were mixed with *p*-toluene sulfonyl chloride in pyridine and refluxed for 3 hr. After the usual workup, the crude products were column chromatographed over silica gel using chloroform as the eluent. The products 8 & 9 are orange red solids and 10 is yellow solid from the eluents. They showed the absence of OH group in their respective IR spectra, but a strong absorption in the region of 1750 – 1740 cm-1 due to the presence of an α,β, unsaturated-γ-lactone carbonyl group and a shoulder at the range 1665 – 1635 cm-1 due to the tetra-substituted C=C bond. These observations corresponded very well to those of β-apopicropodophyllin as observed by Gensler (21) and Murthy (20). The PMR spectra of the analogues 8, 9 & 10 showed singlets at 3.3 and 4.5 for C4 & C3 protons, a singlet at δ 5.9 due to the1, 3-methylenedioxy protons and a singlets at δ 7.2 and 7.4 for C5 & C8 protons respectively (6). The mass spectra of 8, 9 and 10 showed molecular ion peaks respectively at 341, 324 and 351.

The diketones 11, 12 & 13 were synthesized by Friedel-Crafts intramolecular acylation reaction of 20a-c previously reported by Sathisha *et al*., (6). This method has two steps, first, the benzhydryl succinic acids 19a-c were converted into the benzhydryl succinyl chlorides 20a-c, which were then cyclized by using anhydrous aluminium chloride in dry dichloromethane. The products formed were found to be the diketones 11, 12 & 13, which were characterized by IR, PMR, Mass spectra and elemental analysis data (Scheme 2). The IR absorption in the region of 1710-1700 cm-1 is assigned to the six membered carbonyl group and 1745-1730 cm^{-1} assigned to the five membered carbonyl group (6). PMR of 11, 12 & 13 showed a doublet of a double in the region of δ 2.6 assigned to C_6-H, a doublet at 4.1and a quartet at δ 3.7 to 6a-H. The mass spectra of 11, 12 & 13 showed molecular ion peaks (M^+) at their respective mass numbers m/z 327, 310 and 337 respectively.

Anticancer activity: As any new molecules that are developed for the treatment of cancer, should be subjected to the preliminary investigations. In line to this, there are several methods known to carry out the anticancer activity. Among these, in-vitro MTT assay (17) to check the cellular viability was used in our study and was the most convenient as per the facility available in our laboratory. We have used the mouse melanoma B16F10 cell line for conducting anticancer activity experiments.

The antiproliferative (anticancer) assay was carried out for the synthesized podophyllotoxin analogues 8-13. Fig. 3 by modifying the ring C of parent compound, using the in-vitro MTT assay method. The analogues 8 and 9 have chloro and fluoro groups respectively showed a greater antiproliferative activity compared to control at low concentration whereas the remaining analogues showed moderate inhibition of cancer cell growth Fig. 3.

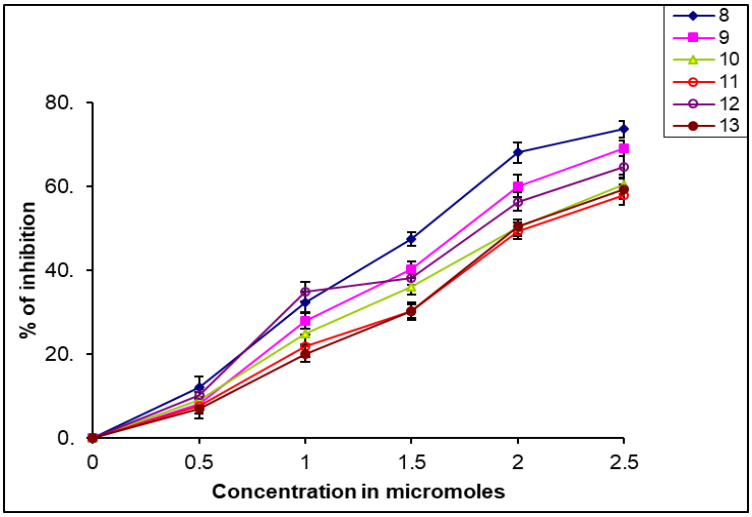


Fig. 3: Dose dependent antiproliferative assay (MTT assay)

The compounds were screened individually in the different dose range from 0-2.5µM with mouse melanoma B16F10 cell line, incubated for 3 days, absorbance was read at 570nm, against the control and percentage inhibition and IC50 values were calculated. The standard mean ±SEM, for n= 4.

The dose dependent assay was carried out for all the analogues to monitor the IC_{50} values. The analogues 8, 9, 10, 11, 12 and 13 have given the IC_{50} values 1.6, 1.75, 2.0, 2.15, 1.82 and 2.0µM respectively (Table 1).

Antimitotic activity (cytotoxicity): The cytotoxic effect of the new analogues 8-13 were studied in terms of inhibiting the cell cycle (mitosis) using the onion root tip method (18). The analogues 8 and 9 showed the maximum antimitotic activity, 10 and 11 moderately, whereas the rest of the analogues 12 and 13 showed comparatively very less activity (6). The podophyllotoxin analogues earlier reported were known to potent antimitotic activity (22) in which the ring C was modified. The above analogues 8- 13 were synthesized by modifying the ring C of parent compound using chloro, fluoro and nitro groups and these analogues showed maximum antimitotic activity by arresting the cell cycle Fig. 4. The analogues 8, 9, 10, 11 and 12 gave the ID_{50} values in 1.85, 2.1, 2.25, 2.5 and 2.42mM respectively when dose dependent assay was carried out (Table-1).

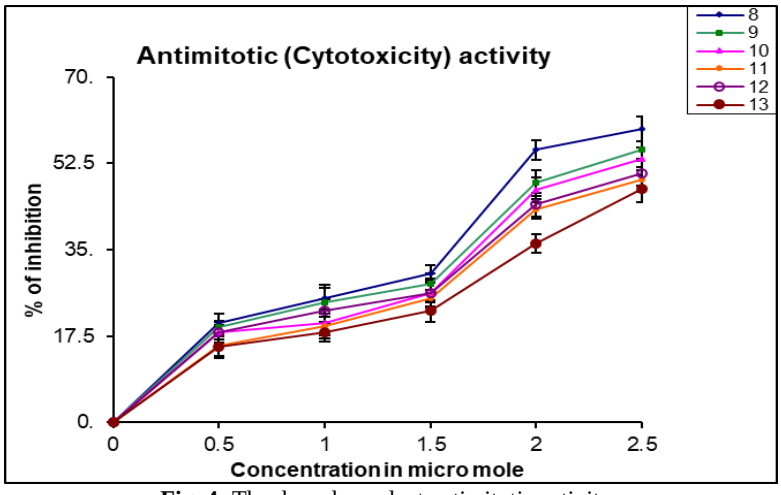


Fig. 4: The dose dependent antimitotic activity

The compounds were taken in micromolar concentration, the above said method is followed. The standard mean ±SEM, for n=4. Control is treated as same without the compound treatment and taken as 100%.

Table 1: Anticancer and antimitotic activity with IC_{50} and ID_{50} values

Compound	Anticancer activity IC_{50} µM/L	Antimitotic activity ID_{50} µM/L
1	3.4	3.0
8	1.6	1.85
9	1.75	2.10
10	2.0	2.25
11	2.15	2.5
12	1.82	2.42
13	2.0	-

CONCLUSION

Currently, there is an immediate need of potent anticancer drugs. Keeping that view, after an extensive literature survey, the podophyllotoxin analogues 8-13 were synthesized and characterized. These analogs were subjected to a preliminary screening where they rendered significant anticancer and antimitotic activity. Among these, Compounds 8 and 9 were having strong growth inhibitory effects on mouse melanoma cancer cell lines as well as mitotic cells of onion root tips. These experimental observations conclude that compound 8 and 9 would become novel drugs in future cancer chemotherapy after further investigations.

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