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Effect of etoposide on grass pea DNA topoisomerase II: an in silico, in vivo, and in vitro assessments

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Abstract

Background: Etoposide is one of the most potential anti-cancerous drugs that targets topoisomerase II (topoII) and inhibits its activity by ligation with the DNA molecule.

Results: In silico study confirmed that the etoposide-binding sites of topoII are conserved among the plants and human. The efficacy of the drug on plant system was initially assessed using germinated grass pea (*Lathyrus sativus* L.) seedlings (in vivo) in relation to radicle length and mitotic index. The callus system (in vitro) was also used to elucidate the effect of etoposide on callus growth kinetics. Furthermore, it was observed that etoposide able to inhibit the division of polyploid cells induced by colchicine treatment (0.5%, 8 h). To determine the molecular interaction, topoII was isolated from young grass pea leaves using polyethylene glycol fractionation and ammonium sulphate precipitation followed by column chromatography on CM-Sephadex (C-25). The plasmid linearization assays by isolated plant topoII in the presence of etoposide significantly revealed the functional similarity of plants and human topoII. Results indicated that the effect of etoposide on plant topoII is significant.

Conclusions: This study may pave the way to develop a plant-based assay system for screening the topoisomerase targeted anti-cancerous drugs, as it is convenient and cost-effective.

Keywords: Callus growth kinetics, Etoposide, *Lathyrus sativus* L., Mitotic index, Topoisomerase II, Topoisomerase assay

Background

New drug discovery for cancer treatment is facing serious challenges due to limited number of approvals because of complex screening processes (Batra and Sharma 2013; Denayer et al. 2014), high cost, and stringency in the use of animal model/cell culture (Astashkina et al. 2012). It widens the necessity to explore a simple and cost-effective novel approach for drug discovery based on plant system. Plants and animals originated from a common ancestor (Meyerowitz et al. 2002) though they are structurally and functionally different but their basic cellular activities namely, cell cycle, DNA replication, and its regulations are conserved in nature (Elledge 1996; Shirahige 1998; Jensen 2006; Sclafani and Holzen 2007). Like in animal system, it may be assumed that in

plant system as well, cell cycle regulatory proteins or check points can be the potential target for anti-cancer compound(s) to block the different steps of cell cycle and cell division.

Etoposide is an important and widely used anti-cancerous drug mostly effective against lung cancer, lymphoma, and leukemia (Issell 1984; Hande 1998; Baldwin and Osheroff 2005). The functional activity of etoposide is to inhibit the topoisomerase II (topoII), which is an essential enzyme for DNA replication. TopoII is responsible for relaxing, unknotting, and untangling the DNA by generating the transient double strand breaks in a separate segment of DNA (Wang 1995; Nitiss 1998; Champoux 2001; Wang 2002). Etoposide, topoII, and DNA form a drug-enzyme-DNA ternary complex, which is a critical stoichiometry for the drug activity (Nitiss 1994; Fortune and Osheroff 2000). Etoposide stabilizes the cleavage complex created by topoII with DNA and thereby inhibits the functional activity of the enzyme. Etoposide contains five rings namely A, B, C, D, and E

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(McClendon and Osheroff 2007). The E ring is mainly responsible for interaction with G-478, D-479, L-502, and R-503 amino acids of human topoI (Wu 2011) which is an isoform of topoII. The drug prevents cell cycle progression in late S and G₂ phase, and when exists in sufficient concentration trigger apoptosis (Loike and Horwitz 1976; Felix 1998; Kaufmann 1998; Kaufmann et al. 1998; Rowley 1998; Wilstermann and Osheroff 2003).

Isolation and characterization of topoII in higher plants are rather meager maize embryos (Carballo et al. 1991), cauliflower (Fukata and Fukasawa 1982, 1983), pea leaves (Rudenko 1992), and *Arabidopsis* inflorescence (Xie and Lam 1994). Furthermore, from available literature, it seems that there is no report about the effect of etoposide on plant topoII. The present article describes the effect of etoposide on plant system using *Lathyrus sativus* L., Family: Fabaceae in vivo and in vitro. In silico study from available gene database is performed to assess the structural and functional similarities between human and plant topoII. The objective of the work is to foresee the effectivity of etoposide on plant system which can open up the possibility of using plant system for screening of topoisomerase-targeted anti-cancerous drugs. The plant system as a model is convenient to use and is cost-effective.

Materials and methods

Plant material

Grass pea (*Lathyrus sativus* L., Family: Fabaceae) seeds obtained from plants grown in the experimental garden of the Haldia Institute of Technology (22.03° N and 88.06° E) during mid-April.

Treatments

Seeds collected from mature plants were air-dried surface sterilized (0.1% HgCl₂) for 5 min, washed by distilled water (3 times, 10 min each), and finally soaked in triple distilled water (12 h). The seeds were then kept on Petri plates lined with cotton-soaked etoposide (GLS Pharma Ltd., India; MW 588.557) solution (25, 50, 75, 100, 125, and 150 μM) for 72 h and for time-dependent assay solution with drug (50, 100, and 150 μM) treated for 24, 48, and 72 h and then on distilled water (25 ± 2 °C). Colchicine (aqueous solution – 0.5 %, 8 h; prior treatment)-treated seeds were also kept in different concentrations of etoposide for 72 h.

The seeds soaked in distilled water (12 h) have been marked as control A while colchicine (0.5 %, 8 h)-treated seeds considered as control B.

In silico study

Amino acid sequence of human and plant topoIIβ was retrieved from NCBI database (<http://www.ncbi.nlm.nih.gov/>). The sequences that share 95% or higher identity were considered as likely alleles (Zang et al. 2001; Wang

et al. 2004) and aligned in CLUSTALW multiple sequence alignment tool (Thompson et al. 1997). From this alignment, the cleavage core of drug interacting residues (40 aa) was analyzed using WebLogo (<http://weblogo.berkeley.edu/logo.cgi>) software for visualizing the similarities of drug interacting amino acid residues among plant species and human beings.

Assessment of radicle length and determination of mitotic index (MI)

Radicle length (mm) was measured from germinating seedlings (randomly 25 seedlings from each set) on the 3rd day of treatment (72 h). Seedling morphology was also studied. Experiment was performed with 3 replicas including control A and control B.

Mitotic study was performed from root tips (2 mm) collected from the control and treated material, fixed in acetic alcohol (1:3) for 3 h, preserved in 70% alcohol, and kept under refrigeration. The tips were stained in 2% orcein-HCl (1 N) mixture and squashed in 45% acetic acid. Mitotic index (MI) was determined using the formula: MI = (dividing cells/total number of cells) × 100. Frequency of polyploid cells was estimated from dividing cells.

Callus growth kinetics in response to etoposide treatments

Grass pea callus line was initiated in accordance with protocol reported earlier (Samanta et al. 2014). Callus growth was initiated using hypocotyls as explant on MS media (Murashige and Skoog 1962) supplemented with 16.11 μM NAA (α-naphthalene acetic acid) and 2.32 μM KIN (Kinetin). Different concentrations of etoposide (25, 50, 75, 100, 125, and 150 μM) solution were sterilized (using 0.2 μm membrane filter) and added to the different sets of medium. For each experiment (10 sets), 0.1 g of callus was transferred to fresh medium (control and treatments). The fresh weight of the callus was measured, and the morphology of the calli was also observed at an interval of 5 days up to 35 days to calculate its growth kinetics followed by the equation of Hunt and Loomis 1976.

Isolation of DNA topoII from young grass pea leaf

Freshly collected grass pea leaves (100 g) were homogenized with 250 ml TEM buffer (50 mM Tris-HCl at pH 8, 1 mM EDTA, and 5 mM β-mercaptoethanol) and centrifuged at 12,000 g for 45 min at 10 °C. PEG-6000 (10%) and 2 M NaCl were added in the supernatant to remove the nucleic acids. Supernatant was collected and precipitation was done by solid ammonium sulphate (55% saturation). Following centrifugation at 12,000 g for 30 min at 10 °C, a protein cake was accumulated at the middle phase between PEG and ammonium sulphate. The protein cake was collected and dissolved in 250 ml TGD buffer (50 ml Tris-HCl at pH 8, 20% glycerol, 0.05

mM dithiothreitol) and again centrifuged to get the clear supernatant. The supernatant was loaded on CM-Sephadex column (C-25) pre-equilibrated with TGD buffer. The column was extensively washed with the TGD buffer. The bound topoII enzyme was eluted with KCl with five-step gradients (100 mM, 120 mM, 140 mM, 160 mM, and 180 mM) in TGD buffer. The elution was collected by fraction collector and protein-containing fractions were stored by measuring their optical density (OD) at 280 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (1970) to identify the topoII in different samples using a mini gel apparatus (Bio-Rad): crude (before ion-exchange) and different fraction from CM-Sephadex column (C-25). Samples (0.1 µg of protein) were diluted in SDS-containing sample buffer with β-mercaptoethanol (under reducing condition) prior to loading. Electrophoresis was performed at room temperature at 16 mA during stacking and 18 mA per plate during resolving. Protein-containing bands were visualized by silver staining method (Oakley et al. 1980). The protein bands were analyzed by comparing with standard MW marker.

Time-dependent plasmid linearization assay by isolated plant topoll to confirm its activity

The DNA topoisomerase activity assay was performed on relaxation of negatively supercoiled duplex plasmid

DNA (pBluecript SK+; pBS) as was described earlier by Nitiss et al. (2012) and visualized by agarose gel electrophoresis. The standard reaction mixture (25 µl) composed of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM ATP, 1 mM dithiothreitol, 160 µg/ml bovine serum albumin (BSA), 0.5 µl plasmid DNA, and 5 µl enzyme solution. The reaction mixtures were incubated at 35 °C and reactions were stopped after different time interval (0, 10, 20, 30, and 60 min) by adding either of 3 µl of 5% sodium dodecyl sulphate (SDS) or 3 µl of etoposide (5 µM). A second reaction mixture comprising of 10 X EcoR1 buffer, 0.5 mm³ plasmid DNA and 2 µl EcoR1 to prepare fully relaxed DNA. Agarose gel electrophoresis, ethidium bromide staining, and gel documentation were done as described previously (Datta et al. 2008).

Plasmid linearization assay by isolated plant topoll at different concentration of etoposide

The plasmid linearization assay in the presence of increased etoposide concentration was performed using the same reaction mixture (25 µl) as described previously, 0.5 µl plasmid DNA, and 5 µl enzyme solution with etoposide (10, 20, 30, and 40 µM). The final reaction mixtures were incubated at 35 °C for 60 min. A second reaction mixture of 10X EcoR1 buffer, 0.5 µl plasmid DNA, and 2 µl EcoR1 was also incubated for 60 min at 35 °C to prepare fully relaxed DNA.

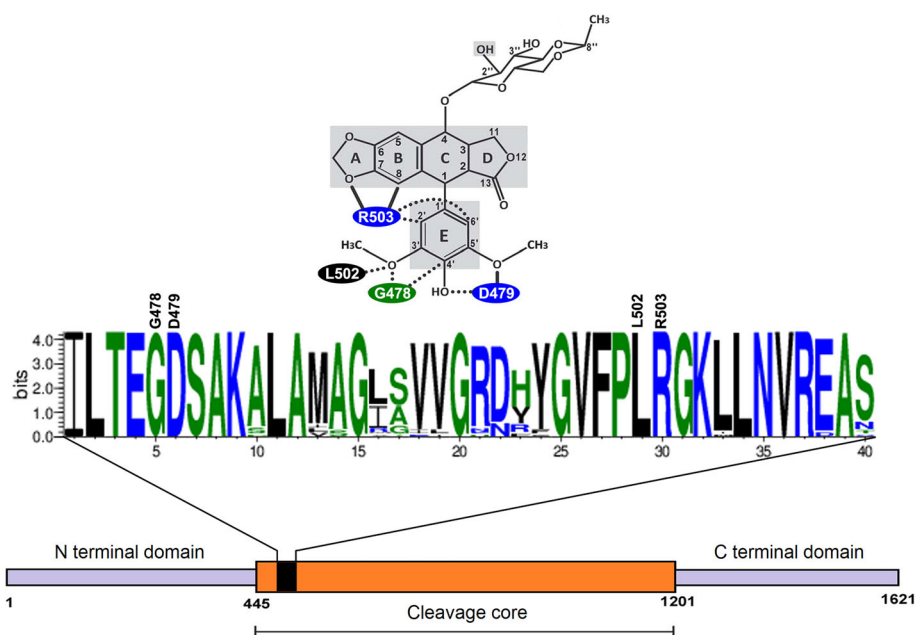


Fig. 1 The etoposide-binding region inside the cleavage core of topoisomerase II is shown with WebLogo result. Conserved amino acid sequences of 38 different plant species and human are represented in this cleavage core. The data for this logo consists of 40 amino acids. The chemical structure of the drug and its interacting sites (G-478, D-479, L-502, R-503) (above) and topoisomerase II domain structure (below) are shown in the figure

Results

Structural similarities of DNA-binding cleavage core domain of plants topoII and human topoII β

The length of human topoII β polypeptide is about 1621 amino acids long (Wu et al. 2011), and it contains N-terminal domain, cleavage core, and C-terminal domain (Mirski and Cole 1995; Adachi et al. 1997; McClendon and Osheroff 2007). The phylogenetic tree (Additional file 1: Figure S1) and multiple alignment of the amino acid sequence among plant and human (Additional file 2: Figure S2) indicate that the cleavage core domain of topoII is conserved. The WebLogo represents that the cleavage core domain of topoII and the specific etoposide interacting residues with topoII G-478, D-479, L-502, and R-503 are well conserved among plants and humans (Fig. 1).

Assessment of radicle length and mitotic index

Radicle length (control A, 19.7 mm \pm 1.2; treatments, 11.8 mm \pm 1.1 to 3.9 mm \pm 0.7) and mitotic index (control A, 12.11%; treatments, 5.48 to 0.05%) are found to decrease with an increase concentration of etoposide (Fig. 2a and Fig. 3a). Radicle length and mitotic index

also decrease with time (24, 48, and 72 h) when treated with etoposide (Table 1). All the attributes significantly correlate ($p \leq 0.05$) between themselves suggesting that etoposide possesses the potential effect on cell division.

Effect of colchicine and its reversal

Colchicine treatments (control B) induce bulging of radicle tips (Fig. 3b) of grass pea seedlings. The frequency of dividing polyploid cells is found to be 5.60% along with an enhancement in cell size and nuclear volume. Bulging of radicle tips shows diminishing tendency with the increase concentration of etoposide, and it is observed phenotypically normal from 75 μ M concentration onwards. Frequency of polyploid cells is found to reduce from 2.98% (25 μ M) to 0.66% (75 μ M) and finally to 0.0% from 100 μ M etoposide concentration onwards (Fig. 2b) indicating that etoposide could inhibit polyploid cell formation in grass pea. In relation to control B, mitotic index (control, 8.90%; treatments, 3.25 to 0.00 %) and radicle length (control, 8.01 mm \pm 1.57; treatments, 6.59 mm \pm 1.11 to 4.16 mm \pm 0.89) also reduce with the increased concentration of etoposide.

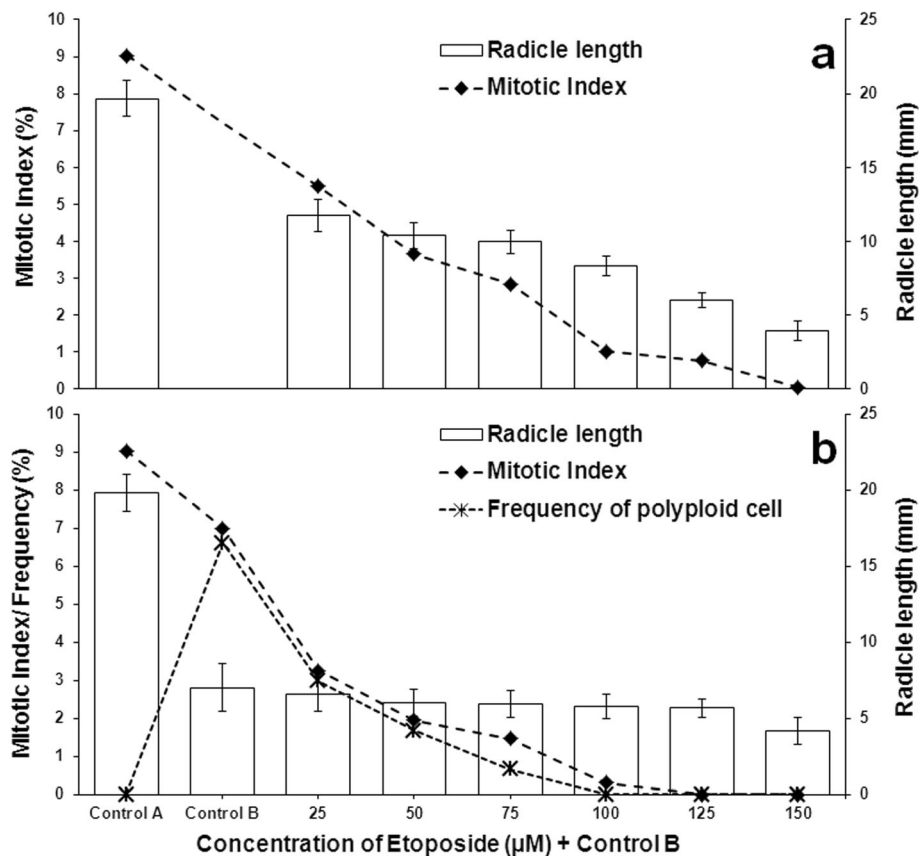


Fig. 2 Effect of different concentrations of etoposide in grass pea seedlings for 72 h. **a** Radicle length and mitotic index in the presence of etoposide (72 h). **b** Assessment of radicle length, mitotic index, and frequency of polyploid cells with the treatment of colchicine (8 h) followed by different concentrations of etoposide (72 h)

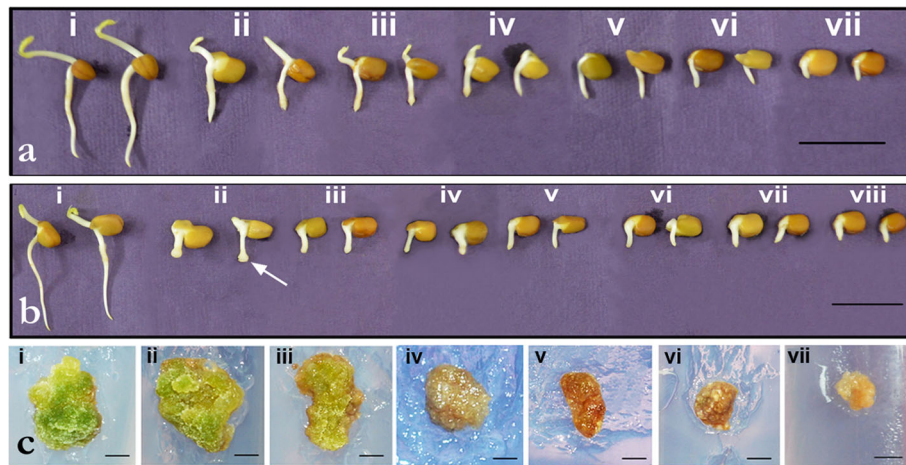


Fig. 3 Effect of different concentrations of etoposide in grass pea for 72 h. **a** Treatments showing reduced radicle length with increasing concentration of the etoposide (i to vii: 0, 25, 50, 75, 100, 125, and μM) treated for 72 h. **b** Assessment of radicle length, with the treatment of colchicine (8 h) followed by different concentrations of etoposide for 72 h. i, control A; ii, control B showing distinct bulging of root tips; iii to viii, significant decrease in bulging tendency of root tips with lower to higher concentrations of drugs added. **c** Callus growth inhibition with increased concentration of etoposide (i to vii: 0, 25, 50, 75, 100, 125, and 150 μM). The callus picture representing the 30th day's morphology. i, control; ii to vii, showing a decrease in callus size with lower to higher concentrations of etoposide added in the medium. Scale bar = 10 mm (Fig. 3a,b); 5 mm (Fig. 3c)

Effect of etoposide on callus growth kinetics

Etoposide shows dose-dependent inhibition in callus growth (Fig. 4a). The effect of etoposide on callus growth rate has been presented in Fig. 4b, a good estimation of maximum specific growth rate of callus. Browning and necrosis of callus tissues are also observed from 75 μM concentration onwards (Fig. 3c).

Isolation and purification profile of DNA topoII

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been performed for identification of the topoII in different samples. SDS gel electrophoresis of the active fractions of isolated topoII after ion-exchange chromatography on CM-Sephadex column (C-25) shows protein bands near 100 kDa (Fig. 5; lanes 1, 2, and 3).

Time-dependent plasmid linearization assay by isolated grass pea topoII to confirm its activity

DNA relaxing activity of enzyme converts supercoiled pBS DNA into nicked and linear forms (Fig. 6a, b) exhibiting

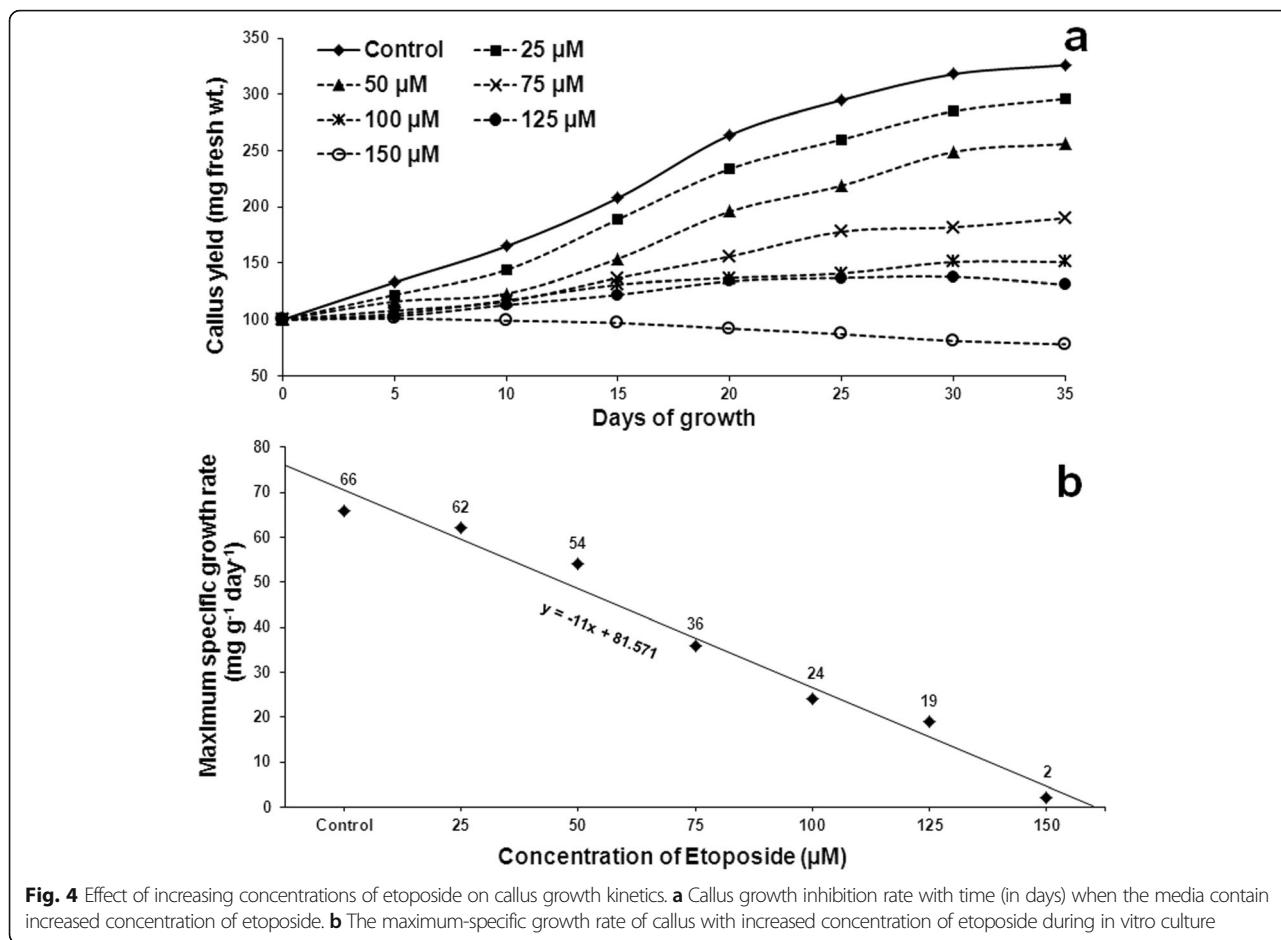
relatively low electrophoretic mobility. When the reactions were stopped at different time intervals (T_0 – T_{60}) by adding the SDS or etoposide, there has been a decrease in amount of supercoiled plasmid with concomitant increase of nicked and linear DNA (Fig. 6a, b).

Degradation of supercoiled plasmid DNA in the presence of topoII with increased concentration of etoposide

The purified plant topoII converts supercoiled pBS DNA into nicked and linear forms. Treatment of supercoiled plasmid by the topoII shows supercoiled, nicked, and some linear DNA (Fig. 7). When the same experiment is done in the presence of increased concentrations of etoposide (10 to 40 μM), initially there has been a decrease in the amount of nicked DNA with an increase in amount of linear DNA. On the other hand, increasing concentration of etoposide ($\geq 20 \mu\text{M}$) has been a concomitant decrease in band intensity and smearing of DNA, which signifies the cleaving of DNA at multiple sites. It seems that like human topoII, etoposide acts in a similar fashion on plant topoII as well.

Table 1 Time-dependent effect of etoposide on radicle length and mitotic cell division

Etoposide concentration (μM)	24 h		48 h		72 h	
	Radicle length (mm)	Mitotic index %	Radicle length (mm)	Mitotic index %	Radicle length (mm)	Mitotic index %
Control	19.70 \pm 1.2	9.02	19.70 \pm 1.2	9.02	19.7 \pm 1.2	9.02
50	16.21 \pm 1.3	5.40	12.44 \pm 1.6	4.90	10.4 \pm 0.9	3.66
100	12.84 \pm 1.2	5.00	10.60 \pm 0.8	3.00	8.3 \pm 0.7	1.01
150	10.36 \pm 0.9	3.40	6.32 \pm 0.9	1.30	3.9 \pm 0.7	0.05
LSD ($P \leq 0.05$)	1.22	0.67	0.52	0.39	0.75	0.35



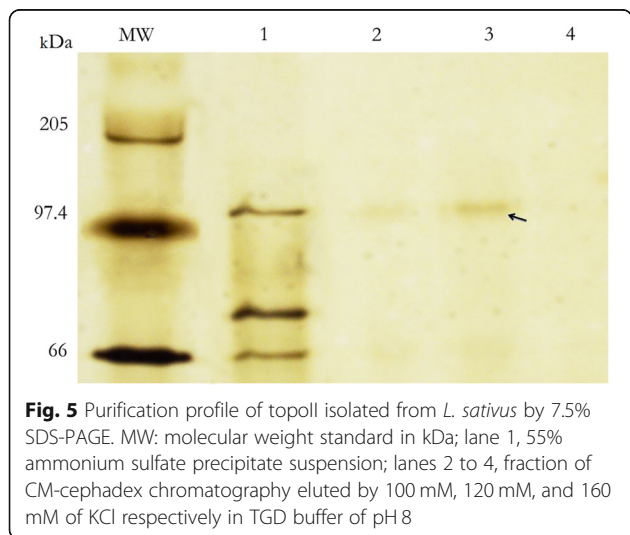
Discussion

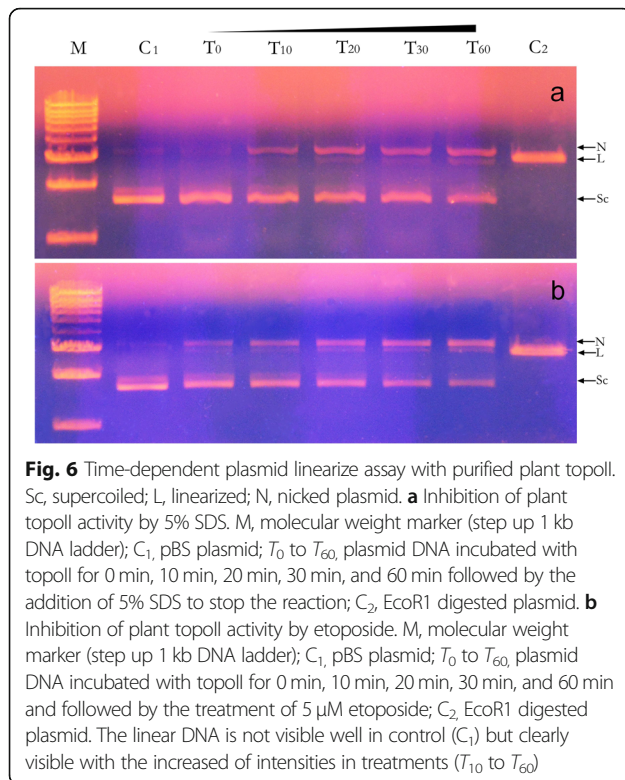
The structural relationship between plants and human topoIIβ

The present investigation reveals that the basic structure of etoposide-binding domain is similar in both plants and

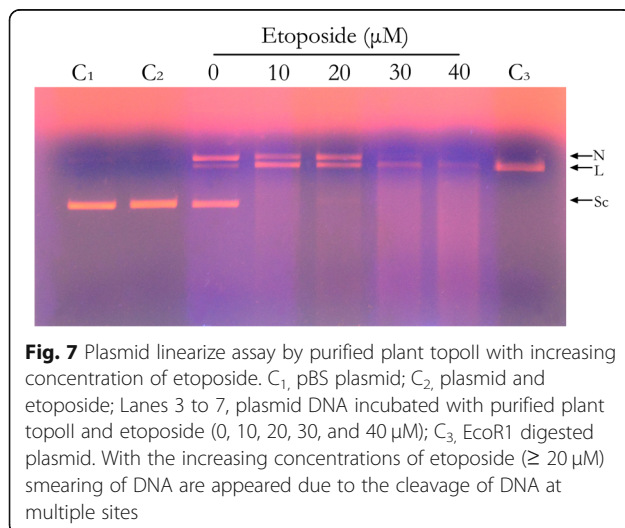
humans. Therefore, it is expected that etoposide can bind with plant topoII and inhibits its function. The etoposide-binding domain is present inside the cleavage core. The specific etoposide interacting residues (G-478, D-479, L-502, and R-503) of topoII are conserved between plants and human (Wu et al. 2011). The overall structural conformation of topoIIs are similar in bacteria and yeast as well (Dong and Berger 2007; Bax et al. 2010; Laponogov et al. 2010). Etoposide inhibits the topoII activity by stabilizing cleavage complex created by enzyme during DNA replication (McClendon and Osheroff 2007). Plant cell also possesses topoII with similar function as that of animals (Fukata and Fukasawa 1982; Xie and Lam 1994).

The drug etoposide contains five rings namely A, B, C, D, and E (McClendon and Osheroff 2007). The E ring interacts with glycine (G) 478, aspartate (D) 479, and leucine (L) 502 of topoIIβ protein. A Van der Waals interaction is also observed with the glycosidic group of etoposide and glutamine (Q) 778 and methionine (M) 782 of topoI (Wu et al. 2011). Wu et al. (2011) also determined the high-resolution crystal structure of the DNA-binding and cleavage core domain of the human topoIIβ isoform (residues 445 to 1201; here-in-after





abbreviated human topoll β core) in complex with DNA and the anti-cancer drug etoposide. The overall structure of the human topoll β core dimer adopts a more open quaternary conformation than in other DNA-bound structures as previously reported for bacterial and yeast topolls (Dong and Berger 2007; Bax et al. 2010; Laponogov et al. 2010) with significant changes in the relative orientations between the main DNA-contacting domains of the two etoposide monomers. The topoll activity has been partially purified and biochemically



characterized from cauliflower inflorescence (Fukata et al. 1986) and pea (*Pisum sativum*) leaves (Rudenko 1992). Their biochemical properties of topoll are similar to those of the other eukaryotic topolls. The enzyme topoll has also been found in higher plant chloroplasts (Lam and Chua 1987) and also evident by immunological analyses (Pyke et al. 1989). The chloroplast enzyme may be similar to bacterial gyrase, since both can relax supercoiled DNA substrates in the presence of ATP (Lam and Chua 1987) and has an apparent molecular weight of about 100 kDa (estimated by Western blot with a heterologous anti-topoll antiserum) (Pyke et al. 1989). Thus, it apparently appears that etoposide which is a potent inhibitor of human topoll also binds with plant topoll and possibly inhibits the enzyme action in a similar way.

The functional relationship between plants and human topoll β

Time-dependent activity study of isolated plant topoll using supercoiled pBS shows that concentration of relaxed and nicked DNA increases with concomitant decrease of supercoiled DNA (Fig. 6a, b) with respect to the time of incubation. Results of plasmid linearization assay support that etoposide can also inhibits the functional activity of plant topoll, thereby suggesting that etoposide acts in the similar way in plant and human topoll. In the present study, grass pea topoll shows a protein band near 100 kDa as identical to that reported (Pyke et al. 1989) in *Arabidopsis thaliana*.

The plasmid linearization assay with increasing concentration of etoposide indicates that pBS is present in supercoiled form in the first two lanes where no topoll is present (Fig. 7). In 3rd lane, topoll causes nicking of plasmid and create relaxed and nicked form of plasmid. With the increasing concentrations of etoposide (≥ 20 μ M), smearing of DNA appears suggesting cleavage of DNA at multiple sites in lanes (4th to 7th). Higher concentrations of etoposide prevent the relegation activity of topoll (Pommier et al. 2010).

In vivo and in vitro studies

The reduction of radicle length, mitotic index, and inhibition of polyploid cells are observed from 25 μ M concentration of etoposide onwards in a dose-dependent manner signifying the effect of etoposide on cell division. The result of drug treatment with time-dependent manner shows that the removal of drug also has the similar effect like dose-dependent manner. The attributes are significantly correlated ($p \leq 0.05$) between themselves. All the result signifies the effectivity of the drug on plant system as an animal model. Higher concentration of etoposide has been found to reduce the callus growth. Such findings highlight the potentiality of the drug etoposide in the inhibition of cell division and growth in plant system.

Conclusion

Present investigation evidently elucidates that etoposide, which is a potent anti-cancerous drug, having discernible anti-topoisomerase activity is also effective towards the inhibition of plant topoisomerase. The results establish a new possibility to screen out the anti-topoisomerase drugs using grass pea in vitro and in vivo system for preliminary screening of anti-cancerous lead molecule(s). Therefore, it may give an excellent opportunity to develop the anti-cancerous drugs using an easy and economical approach. This integrated approach would lead to save cost and time.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s42269-019-0217-4>.

Additional file 1: Figure S1. Dendrogram representing the cleavage core of topoisomerase II domains of plants (38 plant species) and human are well conserved.

Additional file 2: Figure S2. Comparison of etoposide-binding region of topol cleavage core domain sequence of plants (38 plant species) and human.

Abbreviations

EDTA: Ethylenediaminetetra acetic acid; KIN: Kinetin; MS: Murashige and Skoog; NAA: α -Naphthalene acetic acid; PEG: Polyethylene glycol

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Authors' contributions

TRM and AS performed the experiments, analyzed the data, and wrote the manuscript. SD, AKD, and SD designed the work and corrected the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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