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Evaluation of the effects of 5-fluorouracil and cyclophosphamide on *Lathyrus sativus* L.

Priyadharshini Sarkar¹, Aveen Samanta^{1,2}, Tilak Raj Maity¹ and Siraj Datta^{1*}

Abstract

Background: The effect of 5-fluorouracil (5FU) and cyclophosphamide (CP) on grass pea (*Lathyrus sativus* L.) has been studied.

Results: The effect of the selected drugs on radicle length, colchicine induces polyploidy and in vitro callus growth has been studied (concentration used: 5-Fluorouracil—0.01, 0.1, 1 and 10 mM; Cyclophosphamide—1, 10, 20 and 30 mM). The biochemical studies on superoxide dismutase, catalase and lipid peroxidase activity also studied. Radicle length, polyploidy percentage and callus growth decrease in both the drugs in a dose-dependent manner. The SOD, catalase and LP activity decrease with the increase in drug concentration except for low dose (for 5FU—0.01 mM and 1 mM for CP). Induced polyploidy (Control B) than water germinated seedling (Control A) shows higher enzyme activity but a decrease in the increased dose of drugs.

Conclusions: The present work has been done to assess the effective potentiality of two anticancerous drugs 5FU and CP with an objective to establish plant system as a model for preliminary screening of anticancerous lead compounds. The result of the present work would pave the way for the screening of unknown lead compounds with the potentiality to act as base analogue and DNA cross-linking drugs. This system is faster, cost-effective and convenient than animal model.

Keywords: Grass pea, Anticancerous drug, Mitotic index, Callus growth, Polyploid cell, Catalase, Lipid peroxidation, Superoxide dismutase

Background

5-Fluorouracil (5FU) and cyclophosphamide (CP) are the two anticancerous drug used as chemotherapeutic agent (Machover et al. 1982; Fleming 1997). 5FU binds with thymidylate synthase (TS) enzyme and blocks DNA replication and RNA synthesis (Hardy et al. 1987; Lazar et al. 1993; Cox et al. 1999; Calvert 2002). The drug 5FU primarily catabolizes in liver and then enters inside cell to transform into Fd UMP that forms a stable ternary complex with enzyme (Longley et al. 2003). 5FU is most effective against stage III colorectal cancer (Douillard et al. 2000; Giacchetti et al. 2000). The drug is also

effective against solid tumors (Johnston and Kaye 2001). Cyclophosphamide causes inter-strand and intra-strand cross-linking of DNA at N-7 position of guanine, ultimately causing DNA lesions and blocking DNA replication and cessation of cell division (Attardi et al. 2004). Cyclophosphamide is rapidly absorbed by cell and in the liver the mixed function oxidase enzymes (cytochrome P450 system) convert it to active metabolites, phosphoramide mustard and acrolein (Boddy and Yule 2000). Inside cell cyclophosphamide can produce other cytotoxic products (Zhang et al. 2005; Wang and Wang 2012). The drug is recognized by pregnane X receptor (PXR, NR1I2) of mammalian liver (Xie et al. 2000; Wang and Le Cluyse 2003).

The present study aims to find out the affectivity of the two known anticancerous drugs on germinating grass pea seedling on the basis of radicle length, mitotic

*Correspondence: dattasiraj@gmail.com

¹ Department of Biotechnology, Haldia Institute of Technology, Haldia, Purba Medinipur, West Bengal 721657, India

Full list of author information is available at the end of the article

index, in vitro callus growth and biochemical activity of antioxidant enzymes. Callus which is the uncontrolled cell mass (Ikeuchi et al. 2013) is chosen as model for tumorous cells. The selected drugs are also assessed on radicle growth and induced polyploidy cells by colchicine treatment (Ravelli et al. 2004; Caperta et al. 2006).

The present work has been done with the objective of assessing the affectivity of the two known anticancerous drugs on plant species.

Methods

Plant material and treatments

Seeds of grass pea (*L. sativus*; Family: Fabaceae) were collected from experimental field of Haldia Institute of Technology (originally collected from Bidhan Chandra Krishi Vishwavidyalaya, Kalyani, West Bengal, India extension farm). After collection seeds were dried and sterilized with a fungicide (1% bavistin) for 15 min, then rinse two times (5 min each) with distilled water and treated for 5 min with 0.1% HgCl₂ and washed in sterile distilled H₂O (3 times; 5 min each). Triple distilled water soaked seeds (12 h) were kept in different concentrations (5-Fluorouracil—0.01, 0.1, 1 and 10 mM; cyclophosphamide—1, 10, 20 and 30 mM) of Fluracil (fluorouracil injection, 250 mg/ 5 mL vial; Biochem pharmaceutical industries limited; MW 130.08) and Endoxan (50 mg cyclophosphamide tablets, Zydu-sonco sciences Baxter, MW 261.083) in Petri plates (25 ± 2 °C) lined with cotton soaked in respective solution for 3 days. In another set of experiments, seeds were treated with aqueous solution of colchicine (0.5%, 8 h) prior to treatments in the test samples with different concentrations for 72 h. Seeds soaked for 12 h in distilled water were served as control A, whereas seeds soaked in colchicine solution (0.5%) for 8 h were considered as control B. All necessary dilutions for preparing different concentrations were made in triple distilled water. Doses administered were based on pilot trial experiments.

Assessment of radicle length and mitotic index (MI)

On the 3rd day from treatments germinating seedlings were measured for radicle length. Germinating root tips (2 mm) were fixed in 1:3 aceto-alcohol for overnight and preserved in 70% alcohol for calculating mitotic index (MI). The root tips were stained in 1% acetoorcein and HCl (1 N) mixture.

Frequency of polyploid cells was estimated from colchicine-induced dividing cells.

Callus growth in response to treatments

Callus was initiated using hypocotyls on MS medium (Murashige and Skoog 1962) containing 16.11 μM NAA (α-naphthalene acetic acid) and 2.32 μM KIN (Kinetin). Different concentrations of the test materials (5-Fluorouracil—0.01, 0.1, 1.0 and 10.0 mM; cyclophosphamide—1, 10, 20 and 30 mM) were added to different sets of medium. 0.1 g of callus was inoculated to medium and the fresh weight of the callus was noted at an interval of 5 days for 30 days.

Antioxidant enzymes activity

The enzyme extract for superoxide dismutase and catalase was prepared by grinding 0.5 g fresh leaves in ice cold extraction buffer (0.1 M potassium phosphate buffer of pH 7.6 containing 0.5 M EDTA). The homogenate was centrifuged (Remi C-24 plus) at 4 °C for 15 min at 15,000 rpm and the supernatant was used to determine both SOD & CAT enzyme activity.

Superoxide dismutase activity was estimated by the decrease in absorbance of nitro-blue tetrazolium dye (Dhindsa et al. 1981; Datta Gupta and Datta 2003). 3 ml of mixture was prepared containing 100 mM potassium phosphate buffer (pH—7.6), 100 mM methionine, 1 mM nitro-blue tetrazolium (NBT), 0.5 M EDTA and 100 μL supernatant as enzyme extract. The reaction started once 0.1 mM riboflavin was added and placed under fluorescent lamps for 15 min. On withdrawal of the light source the reaction stopped. The mixture without enzymes was used as a standard. A non-irradiated enzymatic mixture served as a baseline. Finally, absorption beams were recorded at 560 nm, and a unit of enzymatic activity was taken as the amount of enzyme that reduced the absorption reading of the samples by 50% compared to the standard reaction mixture.

CAT activity was estimated by the decrease in absorbance of reaction mixture at 240 nm (Aebi 1984; Datta Gupta and Datta 2003). The reaction began by the addition of H₂O₂ into the reaction mixture (50 mM potassium phosphate buffer pH -7, 50 μL enzyme extract), and the decrease in the absorbance value was recorded for 30-s interval. Hence, the amount of H₂O₂ decomposed was calculated and thus the amount of enzyme activity was estimated.

The amount of thiobarbituric acid reactive substances (TBARS) was estimated to find out the lipid peroxidation

$$\text{Mitotic Index (MI)} = \left(\frac{\text{dividing cells}}{\text{total number of cell}} \right) \times 100$$

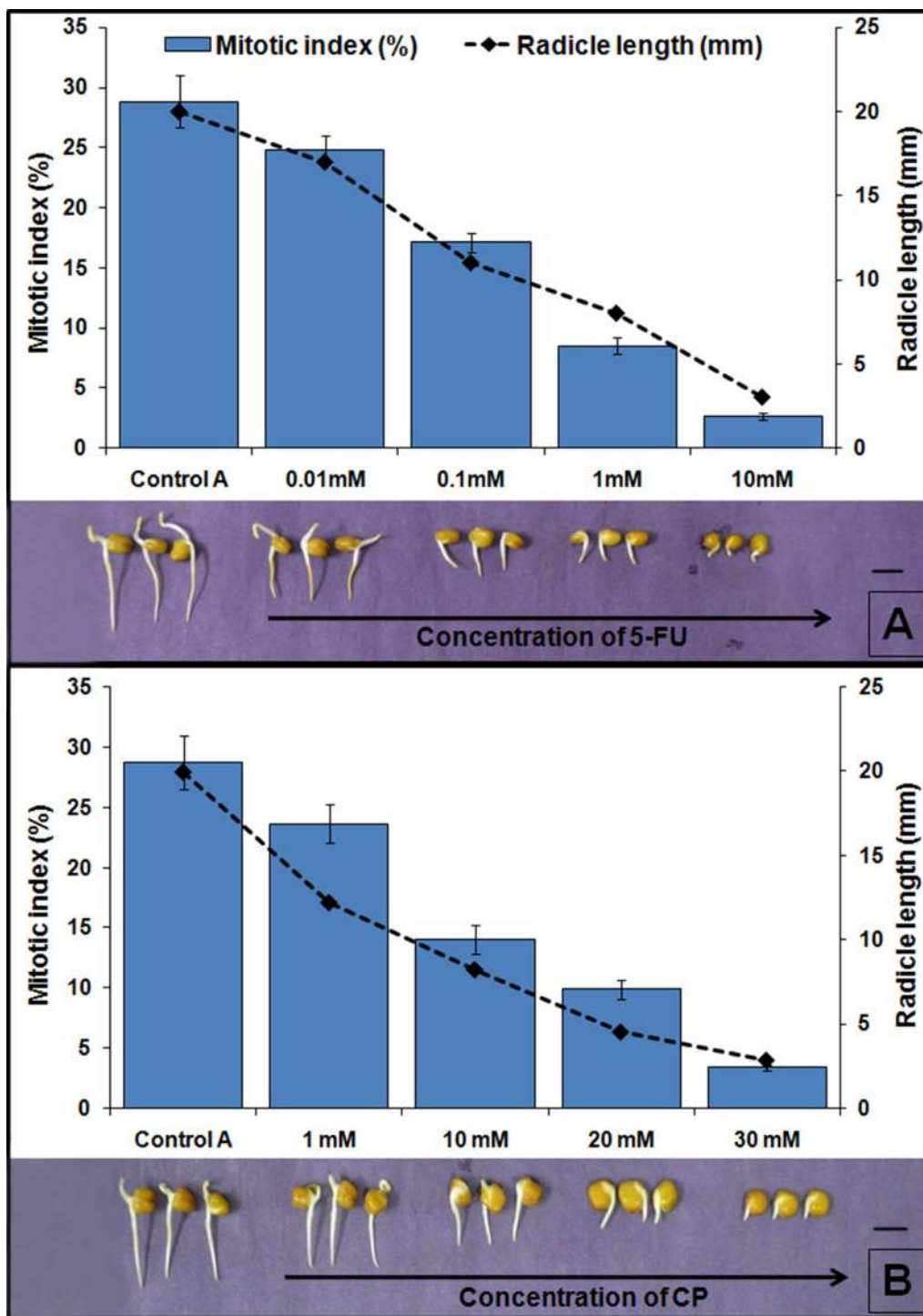


Fig. 1 Effect of different concentrations of (A) 5-Fluorouracil and (B) Cyclophosphamide on radicle length and mitotic index of *Lathyrus sativus*. Scale bar = 10 mm

level (Health and Packer 1968). The procedure for measurement of lipid peroxidase activity involved extraction of the fresh leaves extract (0.5 g) using 0.1% trichloroacetic acid (TCA). The extract was then centrifuged at

10,000 rpm for 15 min. The mixture (supernatant aliquot added with 0.5% thiobarbituric acid in 20% TCA) was then heated at 95 °C for 30 min followed by rapid cooling in an ice bath. After centrifugation at 10,000 rpm

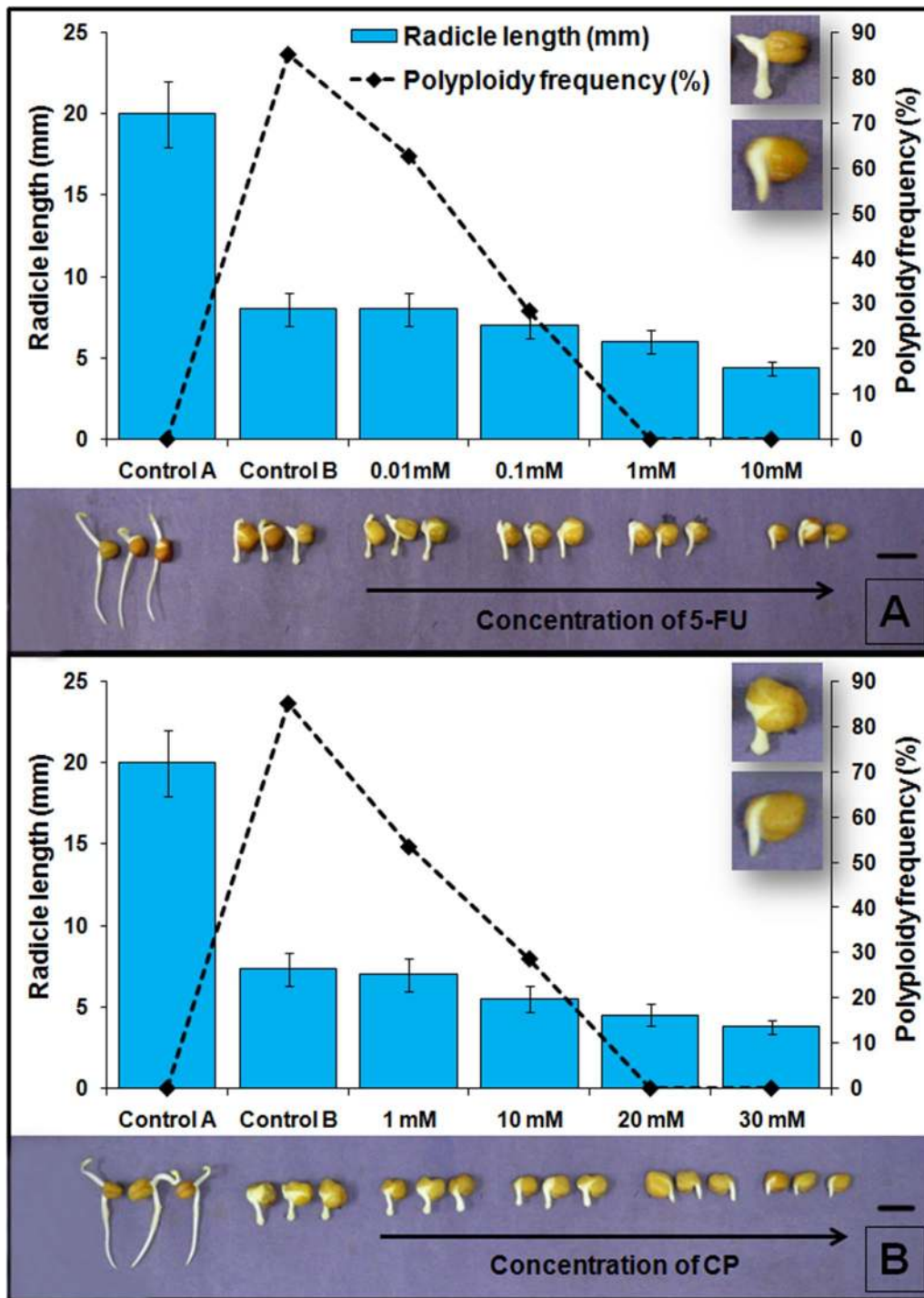
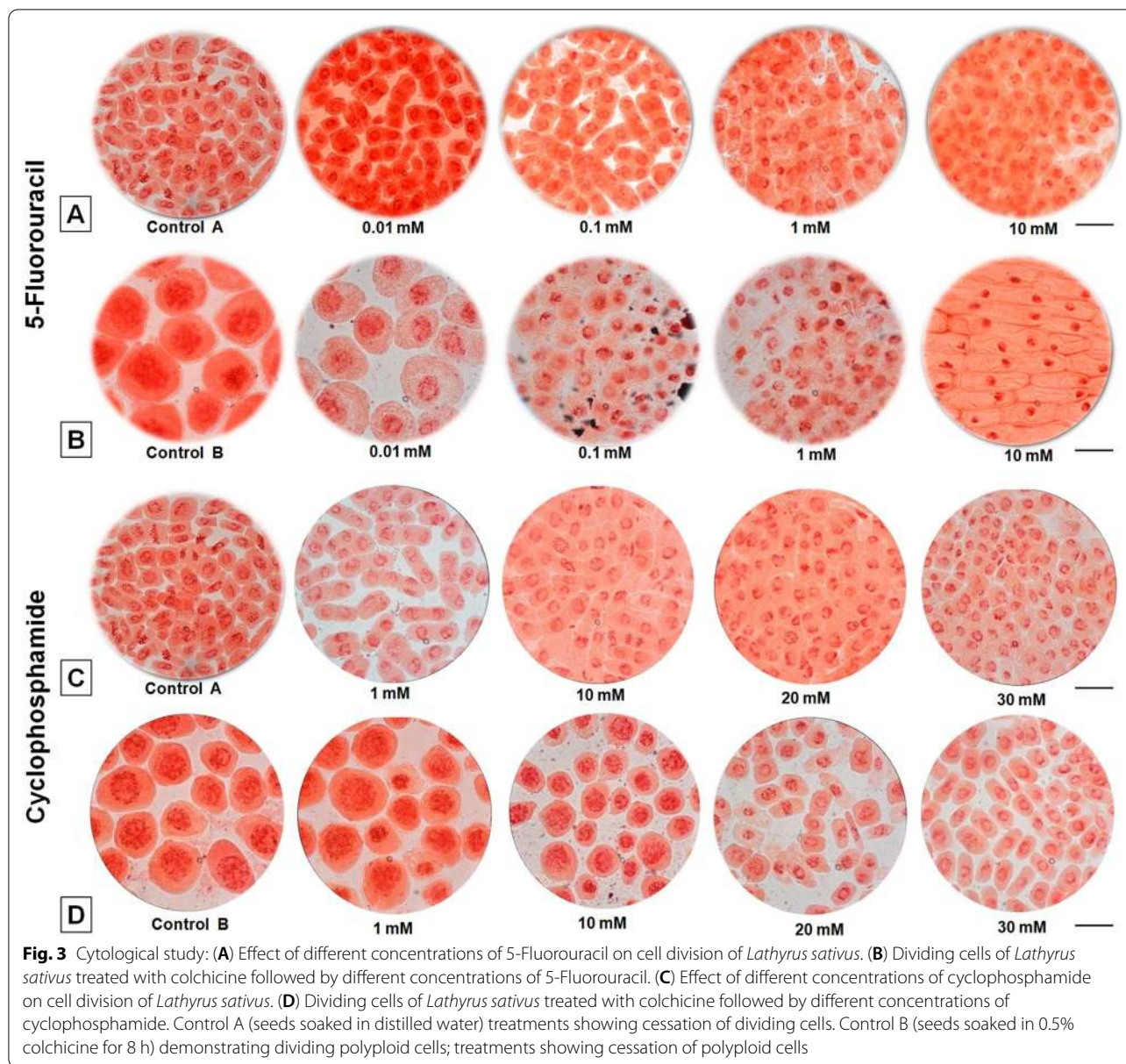


Fig. 2 Assessment of radicle length and frequency of polyploid cells treated with colchicine followed by different concentrations of (A) 5-fluorouracil and (B) cyclophosphamide. [Control B showing distinct bulging of root tips, other treatments decrease in bulging tendency of root tips from lower to higher concentrations of 5-FU and CP]. Scale bar = 10 mm

for 10 min the absorbance of supernatant was recorded at both 532 nm and 600 nm. The TBARS content

was calculated according to its extinction coefficient [$155 \text{ mM}^{-1} \text{ cm}^{-1}$].



Statistical analysis

Least significance difference ($P \leq 0.05$) was performed with the help of ANOVA test using Microsoft Excel data analysis tools.

Results

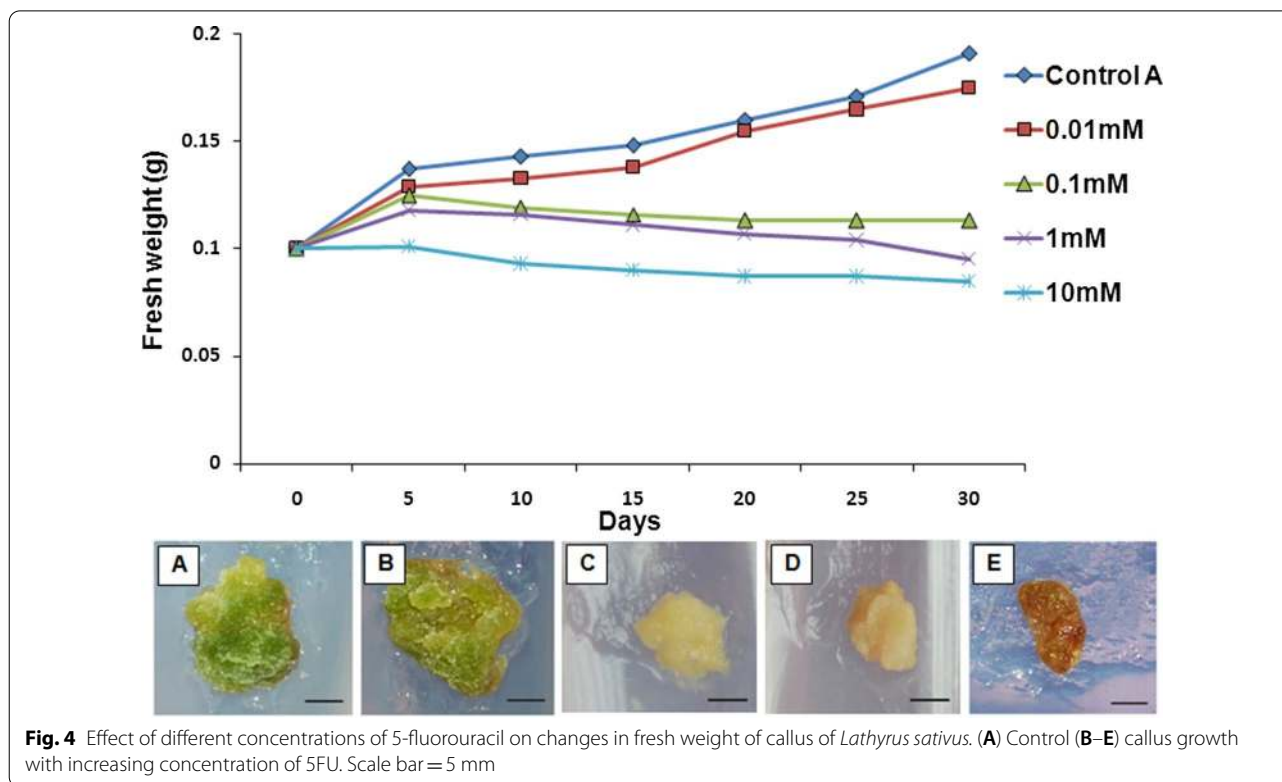
Assessment of radicle length and mitotic index

The radicle length gradually decreases with increasing the different concentrations of 5-fluorouracil (Fig. 1A) and cyclophosphamide (Fig. 1B) in relation to control A (20 mm). Mitotic index also decreases in comparison with control A with higher doses of both the

drug concentrations (Fig. 3A, C). Reduction of radicle length and mitotic index is significant ($P \leq 0.05$) in dose-dependent manner with 5-fluorouracil and cyclophosphamide treatment.

Effect of colchicine on cytology and its reversal anticancerous drugs

Treatments of colchicine (control B) to the radicle induce bulging in the tips. Root tips are phenotypically normal in control A. Diminishing tendency of bulging of radicle tips is observed with an increase in concentration of the test materials and becomes phenotypically normal in



higher concentrations (0.1 mM and 10 mM onward for 5FU and CP, respectively) (Fig. 2). The cytological study of the root tips shows reduced polyploidy cell (Fig. 3B, D) division in higher doses supporting the phenotype result.

Callus growth inhibition

The test materials are found to inhibit callus growth showing significant ($P \leq 0.05$) negative correlation with doses (Fig. 4). The in vitro study showed that with the increasing days of treatment the callus became brownish and in higher concentration, it became necrotic. The fresh weight of callus decreased with the increasing concentration of drugs. The drug concentrations from 0.1 mM for 5-Fluorouracil and 10 mM for cyclophosphamide on treated callus show browning and necrosis of tissues from 35 d onward.

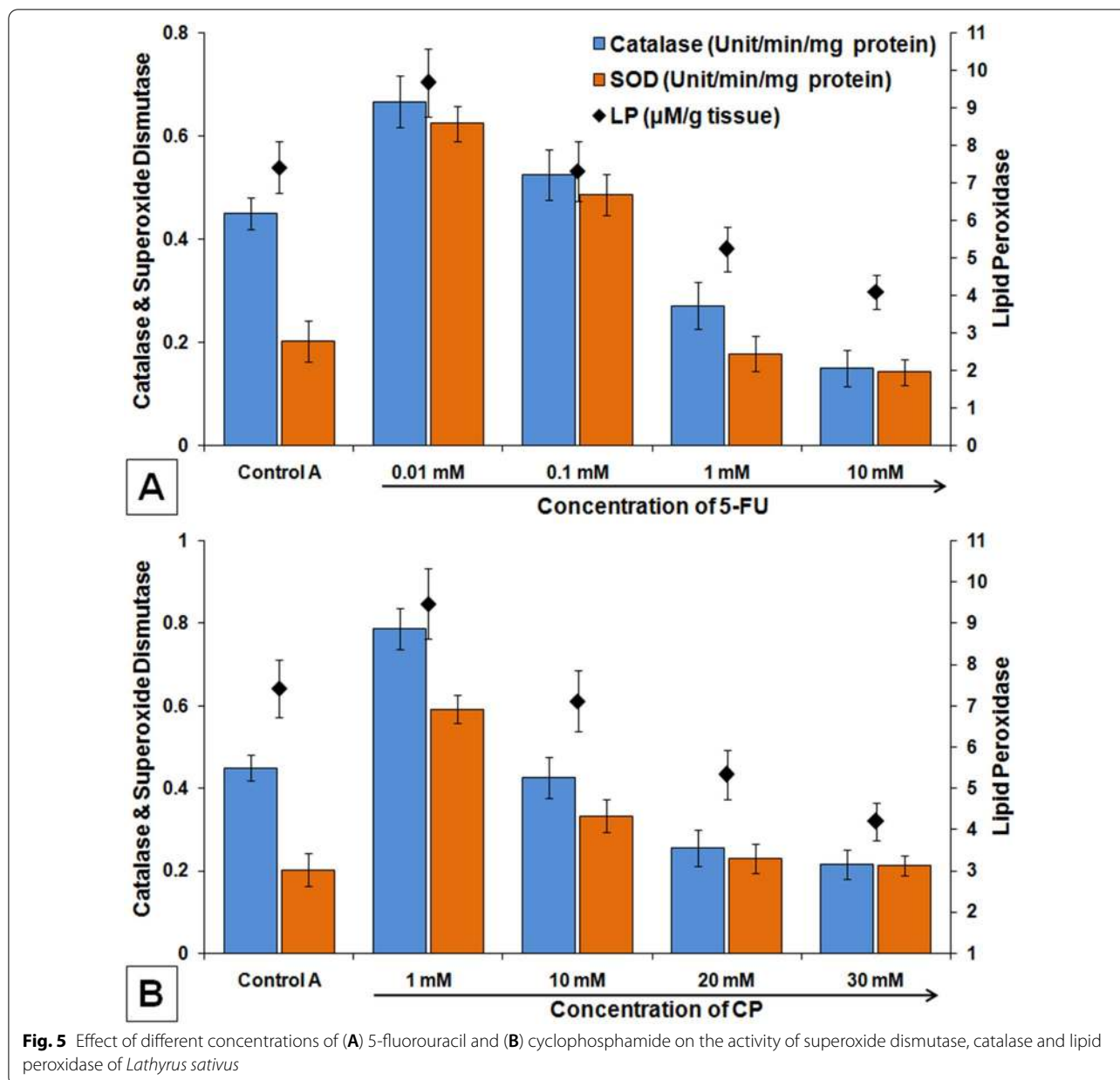
Enzymatic assay of germinated seedlings treated with different concentrations of Fluorouracil and Cyclophosphamide

The germinating seeds treated with different concentrations of 5FU and cyclophosphamide is an antimetabolite which interferes with cell division and stops the cell cycle at S phase, thus reducing cell division and production of reactive oxygen species (ROS) and simultaneously

reduction in antioxidant enzymes. But it was observed at initial concentration of 5FU and CP the SOD, catalase and LP level increase as the cell faces an oxidative stress, thus to combat this situation the level of antioxidant enzyme increases initially. At the lower concentration, i.e., 0.01 mM and 1 mM of 5FU and CP, respectively, the SOD level is $0.625 \text{ Units min}^{-1} \text{ mg}^{-1} \text{ protein}$ and $0.592 \text{ Units min}^{-1} \text{ mg}^{-1} \text{ protein}$, respectively (Fig. 5), it is raised as compared to control A ($0.201 \text{ Units min}^{-1} \text{ mg}^{-1} \text{ protein}$) as the concentration of the drug increases the level of SOD also decreases from 0.1 mM to 10 mM of 5FU and 10 mM to 30 mM of CP, respectively, it will lead to inhibition of cell division according to the increase of doses.

Similarly, in catalase enzyme assay it was observed that compared to control A, $0.45 \text{ Units min}^{-1} \text{ mg}^{-1} \text{ protein}$, the level of catalase has increased at the concentration of 0.01 mM and 1 mM of 5FU and CP, respectively ($0.667 \text{ Units min}^{-1} \text{ mg}^{-1} \text{ protein}$ and $0.786 \text{ Units min}^{-1} \text{ mg}^{-1} \text{ protein}$, respectively). The level of catalase gradual decreases in its level from in concentrations 0.1 mM to 10 mM of 5FU and 10 mM to 30 mM of CP (Fig. 5).

Lipid peroxidase at concentration of 0.01 mM and 1 mM of 5FU and CP is $9.67 \mu\text{M TBARS content g}^{-1}$ and $9.48 \mu\text{M TBARS content g}^{-1}$, respectively, as compared with control A ($7.42 \mu\text{M TBARS content g}^{-1}$). It



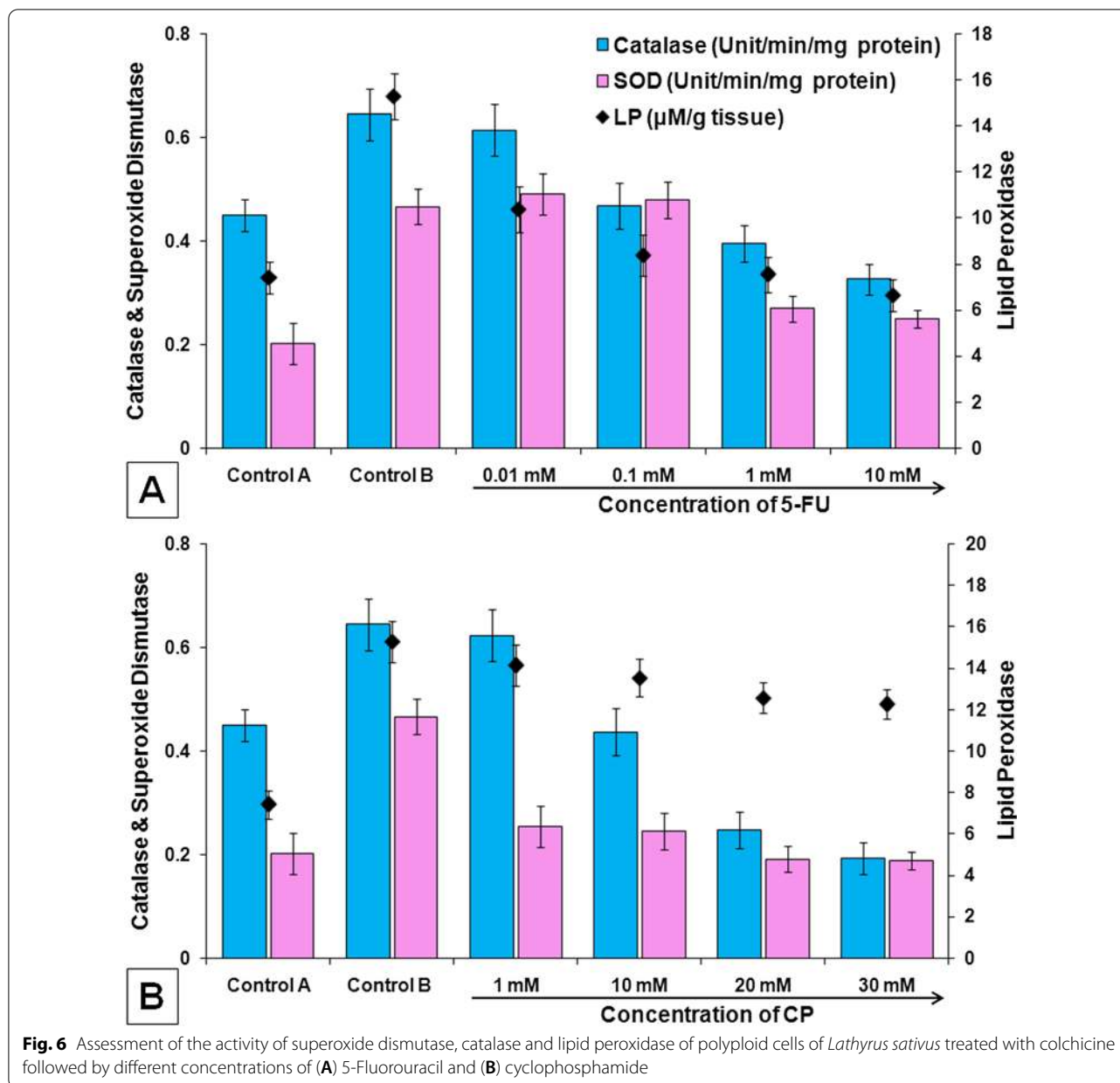
gradually decreases at higher dose as the cell divisional phase decreases of 5FU and CP (Fig. 5).

Enzymatic assay of polyploidy cells treated with different concentrations of 5FU and CP

SOD activity had been measured in control A and control B along with the sets where preincubation of seed had been performed with various concentrations of 5FU and CP. Compared to control A of 5FU and CP (0.201 Units $\text{min}^{-1} \text{mg}^{-1}$ protein) higher SOD activity had been noticed in control B of 5FU and CP (0.47

Units $\text{min}^{-1} \text{mg}^{-1}$ protein). In the presence of increasing concentration of 5FU and CP, SOD activity decreased sharply for both the test drugs. All the results signified ($P \leq 0.05$) that increase in the concentration of 5FU and CP diminishes the content of polyploidy cells simultaneously decreasing SOD activity. However, in higher concentrations of 5FU and CP transformed the polyploidy cells to normal state with decreased SOD activity (Fig. 6).

Lipid peroxidation level, compared to control A of 5FU and CP (7.42 μM TBARS content g^{-1}), increased in control B of 5FU and CP (15.29 μM TBARS content g^{-1}) as polyploid cell synthesized more lipid. Lipid peroxidation



was considered to be a marker of cellular stress and had been estimated from the malonaldehyde generated from the cellular lipid by the free radical oxidation. Increasing the concentration of 5FU and CP, the LP activity decreased gradually and at 1 mM of 5FU ($7.54 \mu\text{M TBARS content g}^{-1}$) it was almost normalized (Fig. 6).

In the presence of H_2O_2 the catalase activity increased in control B of 5FU and CP ($0.645 \text{ Units min}^{-1} \text{ mg}^{-1} \text{ protein}$) due to the rise in the total H_2O_2 content, as compared to control A of 5FU and CP ($0.45 \text{ Units min}^{-1} \text{ mg}^{-1} \text{ protein}$). However, at higher concentrations this activity reduced (Fig. 6).

Discussion

These results show the negative effect of 5FU and CP on polyploid cell division as well as uncontrolled chromosome numbers of grass pea. From the doses administered and results obtained, it seems that the efficiency of 5FU is more than CP. This result signifies the proposal of the inhibitory effect of other anticancerous drugs, namely Methotrexate, Cisplatin, Etoposide and Vinblastine on polyploidy and uncontrolled cell division of callus (Samanta et al. 2014, 2015, 2019). The activity of SOD was estimated by recording the decrease in absorbance of nitro-blue tetrazolium dye (Dhindsa et al. 1981; Datta Gupta and Datta 2003). CAT activity was obtained

by monitoring the decrease in absorbance due to hydrogen peroxide (H_2O_2) at 240 nm (Aebi 1984; Datta Gupta and Datta 2003). The level of lipid peroxidation depends on the terms of thiobarbituric acid reactive substances (TBARS). Thus, the amount of TBARS was used to determine the lipid peroxidation level (Health and Packer 1968). An enhancement in the H_2O_2 concentration, a commonly occurring ROS has been reported from past studies in *L. stocksii* seedlings as well as in other halophiles under conditions of increasing salinity (Jithesh et al. 2006; Hameed et al. 2015; Ozgur et al. 2013). Past reports conducted on effect of salt stress/salinity on SOD activity demonstrate a rise in SOD function with increased salinity (Rahnama and Ebrahimzadeh 2005; Gao et al. 2008). Report is also available on the effect of different anticancerous drugs on cell division and induced polyploidy of grass pea (Samanta et al. 2015). In contrast to plant system, animals/human cancer cells exhibit low level of antioxidant enzyme activity profile (Oberley and Oberley 1997; Carmeliet and Jain 2000; Chang et al. 2002; Chabner and Roberts 2005). However exception is evident in certain cancers like adenocarcinoma, renal cell carcinoma and mesothelioma where elevated level of manganese superoxide dismutase and catalase activity has been reported (Oberley and Oberley 1997). Gene expression of manganese superoxide dismutase alleviates fivefold over malignant phenotype in human prostate carcinoma cells arresting cell growth (de Haan et al. 1996). Plants are unique from animal because of the presence of plant-specific ROS absorbing enzymes like monodehydroascorbate reductase (MDAR), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHR). Being placed in a superior position regarding detoxification of ROS due to the presence of multiple oxidases and peroxidases, it is noteworthy that plants are better scavenger of ROS compared to animals. This implies probable beneficial roles of ROS in plants. In current thoughts it has been considered that ROS in a concentration below the cytotoxic level and above cytostatic range conducts biological redox reactions, supports cellular proliferation, stress management, promotes differentiation and development, defense against pathogen, transduces signal and triggers programmed cell death (apoptosis or regulated necrosis) in plants. With the increase of cell division, the level of ROS increases to control the oxidative stress, the level of antioxidant enzymes like SOD, catalase and lipid peroxidase also increases. The decrease in level of all the antioxidant enzymes with the increase of the drug concentration signifies the affectivity on plant system in reduced cell division (Butler 2004; Balunas and Kinghorn 2005; Koehn and Carter 2005).

Conclusions

The present investigation reveals that 5FU and CP can inhibit cell division, callus growth and frequency of polyploid cell formation which are significant parameters associated with cancer. It can be predicted that the plant system can also respond similarly to the different anticancerous drugs as human beings. The results suggest that the modes of action of the test drugs are more or less similar to that of plants. Present findings can lead to the possibility of using the plant system as a model for preliminary screening of novel unknown compounds. Use and maintenance of plant system is rather convenient for their simplicity and cost-effective for the purpose.

Abbreviations

5FU: 5-Fluorouracil; CP: Cyclophosphamide; ROS: Reactive oxygen species; SOD: Superoxide dismutase; LP: Lipid peroxidase; CAT: Catalase; TBARS: Thiobarbituric acid reactive substance; LSD: Least significant difference.

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Authors' contributions

PS, TRM and AS performed the whole experiment, data analysis, and composed the manuscript. AS and SD designed the work and corrected the primary 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.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interest.

Author details

¹Department of Biotechnology, Haldia Institute of Technology, Haldia, Purba Medinipur, West Bengal 721657, India. ²Department of Botany, Prabhat Kumar College, Contai, Purba Medinipur, West Bengal 721401, India.

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