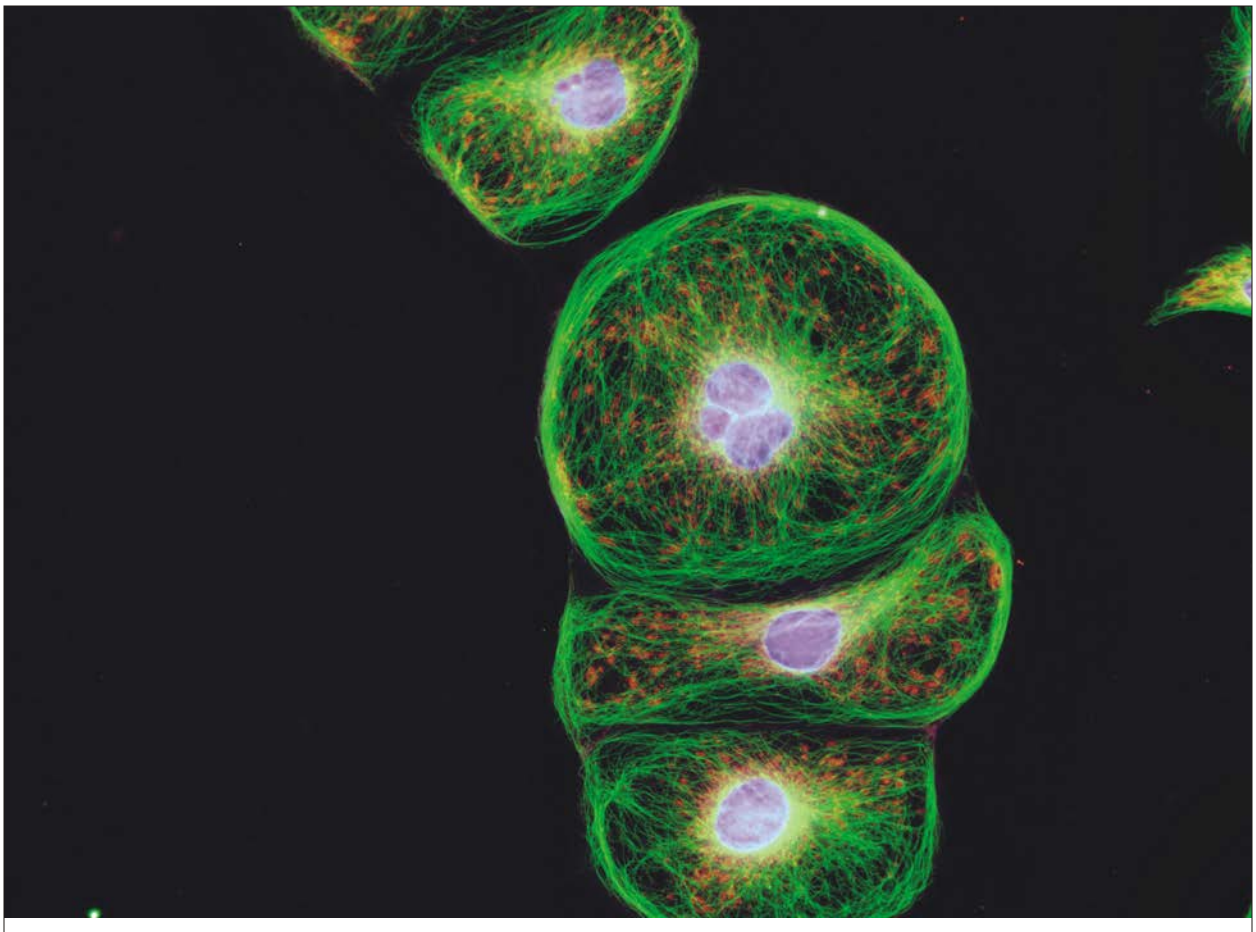

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Cell Proliferation and DNA Damage Study by SCGE in Fission Yeast Exposed to Curcumin and 5-fluorouracil

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ABSTRACT

Curcumin has a concentration based effect on normal cells and its mechanism of action is very intriguing. Effect of various concentrations of curcumin on wild type *Schizosaccharomyces pombe* cells was studied with an aim to evaluate possibility of combining curcumin with chemotherapeutic drugs and to gain an insight into its mechanism of action in normal cells. Effect of 5-fluorouracil or curcumin independently and in combination on cell growth, reactive oxygen species generation, antioxidant enzyme activity, DNA integrity and cell cycle arrest were studied. Curcumin at 5 μM and above concentrations reduced the cell growth, increased reactive oxygen species and arrested the cell cycle in G1/S phase in untransformed cells. Treatment of 5-fluorouracil also reduced cell growth and led to increased reactive oxygen species. However, combination of lower concentration of curcumin with 5-fluorouracil could control reactive oxygen species levels and prevent DNA damage. The cells never entered into apoptosis due to curcumin treatment up to 10 μM concentration, as observed by DNA pattern in gel. Curcumin prevented the DNA damage by activation of natural antioxidation mechanism in cells as observed by increase in total glutathione level and catalase activity. In conclusion, curcumin showed concentration based effect on growth of *S. pombe* cells and its combination with 5-flurouracil was found to be beneficial in controlling the cell growth and toxic reactive oxygen species levels.

Key words: Cell cycle arrest, curcumin, reactive oxygen species, *Schizosaccharomyces pombe*

INTRODUCTION

The toxicity of chemotherapeutic drugs to normal tissues has been one of the major obstacles to successful cancer chemotherapy. Therefore, several chemotherapy regimens and chemopreventive medicine are often combined with herbal drugs for enhancement of the treatment effect and to reduce the toxicity of these medicines. The endorsement of curcumin as a chemopreventive agent has encouraged its use along with traditional chemotherapeutic drugs to minimize the drug toxicity. In contemporary medicine, curcumin (1, 7-bis (4-hydroxy 3-methoxy phenyl)-1, 6-heptadiene-3, 5-Dione) has emerged as an important tool for chemoprevention of cancer (Rahman *et al*., 2006). Curcumin possesses a variety of pharmacological qualities including anti- inflammatory, antioxidant, antiangiogenic and apoptogenic activities which are well suited for efficacy in relation to cancer chemoprevention (Banjerdpongchai, 2006; Hail, 2008). Curcumin has been found to inhibit cell growth, cell invasion, metastasis and angiogenesis by affecting multiple signaling pathways (Ravindran *et al*., 2009). It has been demonstrated that curcumin at

low concentrations exerts its anticancer activity in HL-60 cells, while at high concentrations promotes Reactive Oxygen Species (ROS) generation (Chen *et al.*, 2005). Although toxicological studies indicated that curcumin is non-toxic even at high dosages (Du *et al.*, 2006), its time and concentration dependent cellular and genetic toxicity was also reported (Balaji and Chempakam, 2010). In spite of the extensive studies in last half a century to investigate curcumin's mechanism of action on cancer cells, its role in normal cell cycle remains enigmatic. The present study was designed to investigate (i) the possibility of combining curcumin with chemotherapeutic drugs (ii) to study the effect of various concentrations of curcumin alone as well as in combination with FDA approved chemotherapeutic drug 5-fluorouracil (5-FU) on normal yeast cells.

Schizosaccharomyces pombe (*S. pombe*) is considered a good model for study in cellular proliferation, apoptosis and in medicine (Matsuyama *et al.*, 1999; Fleury *et al.*, 2002; Mager and Winderickx, 2005). In the present study, wild type *S. pombe* was used to study the effect of curcumin on normal cells along with widely used chemotherapeutic drug 5-FU. As 5-FU happens to be analogue of Thymine, it gets incorporated in DNA and inhibits cellular proliferation.

MATERIALS AND METHODS

Chemicals: Curcumin (C7727, = 94% curcuminoid content, = 80% curcumin) and Glutathione Reductase (GR) (G3664-100UN) were purchased from Sigma-Aldrich and 5-Flurouracil (5- Fu) was purchased from Celon lab, India; 2, 4, 6 -Tripyridyl-2-triazine, GSSG (oxidized glutathione), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), 4',6-diamidino-2-phenylindole (DAPI), ethidium bromide and components of culture media were purchased from Hi-media.

Yeast strain and Growth conditions: A wild type strain of *S. pombe* was procured from Microbial Type Culture Collection, Chandigarh, India and was grown in liquid Yeast Extract Supplement (YES) medium containing 0.5% yeast extract, 3% glucose and 22.5 mg % of each of adenine, histidine, leucine, lysine hydrochloride and uracil as supplements. Cells were grown on a rotary shaker at 150 rpm or on solid YES medium containing 2% agar, at 30°C temperature.

Curcumin and 5-flurouracil effect on cell growth and proliferation: Curcumin concentration ranging from 2.5, 5.0, 10.0, 20 μM and 5-FU at a concentration of 50 μg mL^{-1} were used to treat the *S. pombe* cells. The selection of drug 5-FU and its effective concentration was based on earlier studies (Seiple *et al.*, 2006). Equal numbers of *S. pombe* cells were inoculated in liquid YES medium and allowed to grow up to exponential phase. Various concentrations of curcumin and 50 μg mL^{-1} 5-FU were added to exponentially growing cells independently and in combination. The cells were further allowed to grow for 24 h and growth rate was determined by cell counts using haemocytometer. Cell viability was assessed by trypan blue viability assay.

The effect of curcumin at higher concentrations on *S. pombe* cells was also studied by growing *S. pombe* cells on solid medium. *S. pombe* cells were treated with various concentrations of curcumin alone and in combination with 50 μg mL^{-1} of 5-FU for 24 h, washed with liquid YES medium and plated on solid YES medium. The cell growth was determined by colony counts after 24 h treatment.

FRAP Assay (Ferric reducing antioxidant power assay): FRAP assay was performed as described by Benzie and Strain (1999). In brief, for FRAP assay cell lysate free suspension was prepared by lysing the cells with 1X Tris-EDTA (TE) buffer and glass beads. To 0.1 mL sample,

3.0 mL of FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM 2,4,6-Tripyridyl-2-triazine in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ in ratio of 10:1:1) was added and absorbance was measured at 593 nm using 1 mM ascorbic acid as standard.

FRAP value of sample was calculated in μM as change in absorbance of sample from 0 to 4 min/change in absorbance of standard from 0 to 4 min×FRAP value of standard (1000 μM).

Total glutathione (GSH) content: Total GSH content was determined by measuring absorbance of standard GSSG at 412 nm (Sies and Akerboom, 1984). In a final volume of 0.5 mL, the reaction mixture contained; 100 mM phosphate buffer (pH 7.0), 1 mM EDTA, 0.24 mM NADPH, 0.0756 mM DTNB and 0.06 units glutathione reductase. Then, 100 μL of the appropriate GSSG standard or 100 μL of the crude extract was added to each reaction mixture. The absorbance of known concentrations of GSSG was used to construct a standard curve.

Catalase activity: The catalase activity in both treated and untreated S. *pombe* cells was measured by continuous spectrophotometric rate determination method (Beers and Sizer, 1952).

DAPI staining: The *S. pombe* cells treated with curcumin and 5-fluorouracil independently and in combination as well as untreated cells were fixed with glutaraldehyde and washed three times in ice-cold 1×Phosphate Buffered Saline (PBS). The cells were then suspended in 100 μL of 1×PBS. From the bottom, 10 μL of cell suspension and 10 μL of DAPI (50 μg mL^{-1}) were mixed on a glass slide and covered with cover slip and observed under fluorescence microscope (excitation at 360-370 nm and emission at 420 nm) (Eishi Noguchi Lab Protocol).

DNA fragmentation study: DNA isolation of treated and untreated cells was carried out and the isolated DNA was electrophoresed in 0.8% agarose gel. Briefly, treated and untreated *S. pombe* cells were harvested by centrifugation at 2000 rpm for 10 min at 4°C. The supernatant was removed and cells were resuspended in 0.5 mL of distilled water. The cells were transferred to screw-cap microfuge tube and 0.2 mL of lysis buffer (2% Triton X-100, 1% Sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris HCl, 1 mM EDTA) was added. To this 0.2 mL of phenol: chloroform: isoamyl alcohol (25:24:1) and acid-washed glass beads to the level of meniscus of the solution was added. It was microfuged for 5 min and upper aqueous layer was transferred to fresh tube, followed by addition of 1/10 volume 3 M sodium acetate and 2-5 volume of 100% ethanol. This was microfuged for 5 min, pellette was washed once with 70% ethanol, air dried and resuspended in 20-30 μL tris-EDTA buffer. Agarose Gel electrophoresis was carried out using 0.8% agarose and TAE buffer. Images of pre stained electophoresed agarose gel was captured by Olympus camera and analyzed by image analyzer (Alpha Digi Doc).

DNA damage study by single cell gel electrophoresis (SCGE): SCGE was carried out according to the method of Banerjee *et al.* (2008) with some modifications. The treated and untreated *S. pombe* cells were washed in PBS and kept in ice-cold PBS for preparation of single cell suspension and were used in minigel. The minigel was made on frosted microscopic slides using 1% normal melting point agarose (110 μL) as first layer and 0.5% low melting point agarose (LMP) (containing the mixture of 10 μL yeast cell suspension and 65 μL LMP as second layer. The slides were then submerged in chilled lysing solution 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris HCl, 1% Triton X-100, 10% DMSO, pH 10) overnight at 4°C in dark. Post lyses, the slides were

transferred to a jar containing alkaline electrophoresis buffer (1 mM di sodium EDTA, 300 mM NaOH, pH >13) for 20 min at 4°C and slides were transferred to an electrophoresis tank containing the same alkaline buffer and subjected to an electric field of 0.86 V/cm for 20 min at 4°C. These slides were neutralized in neutralization buffer (0.4 M Tris HCl, pH 7.5) and finally rinsed in double distilled water. Staining of DNA was carried out by ethidium bromide (25 μL per slide) for 20 min and the DNA damage was visualized using fluorescence microscope (excitation at 510-550 nm and emission at 590 nm) and recorded.

Statistical analysis: Results are presented as Mean±SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's Least Significant Difference (LSD) test. SPSS software 17 was used for the statistical analysis.

RESULTS

Effect of curcumin and 5-FU on growth and proliferation of *S. pombe* cells: Curcumin was found to inhibit the growth of wild type *S. pombe* cells in a dose dependent manner (Fig. 1). While at concentrations of 5 μM and above, cell numbers reduced significantly ($p<0.0001$), at 2.5 μM of curcumin treatment, the cell numbers decreased marginally ($p>0.5$) (Fig. 1). Therefore 2.5 μM and 5 μM curcumin were found to be non-cytotoxic and selected to study the effect of curcumin in combination with chemotherapeutic drugs. Treatment with 50 μg mL^{-1} of 5-FU also resulted in significant decrease in growth rate ($p<0.0001$). Combined treatment of 5-FU with 2.5 μM of curcumin showed almost same growth as only 5-FU treatment ($p>0.5$), however increased concentration of curcumin treatment (5 μM) resulted in further decrease in growth rate ($p = 0.5$) (Fig. 1).

Effect of curcumin and 5-FU on FRAP value in *S. pombe* cells: To determine the reason for cytotoxicity of curcumin at above 5 μM concentration and lack of cytotoxicity at below 5 μM

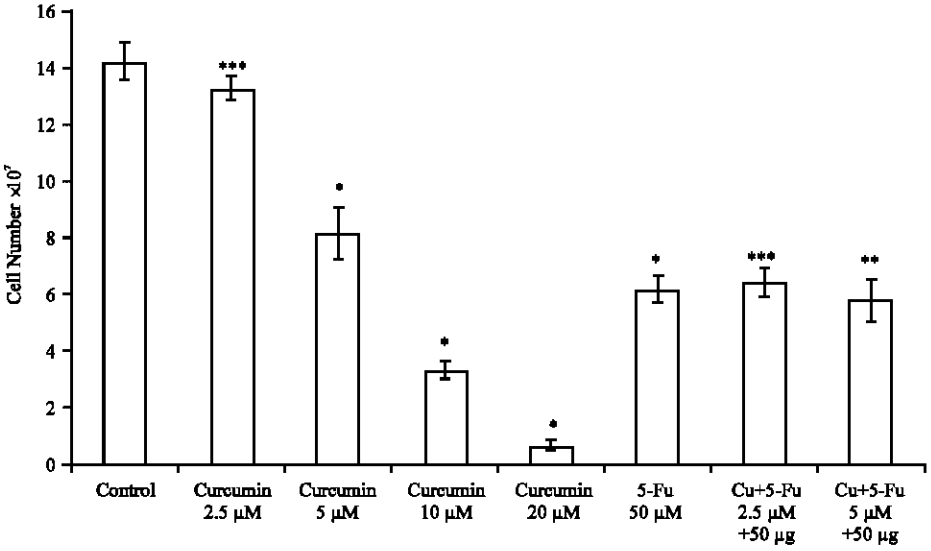

Fig. 1: Effect of curcumin, 5-fluorouracil and curcumin+5-fluorouracil after 24 h on growth of *S. pombe* cells after 24 h, Data are presented as Mean±SD,*$p<0.0001$, **$p = 0.5$, ***$p>0.5$; 5-FU = 5-fluorouracil

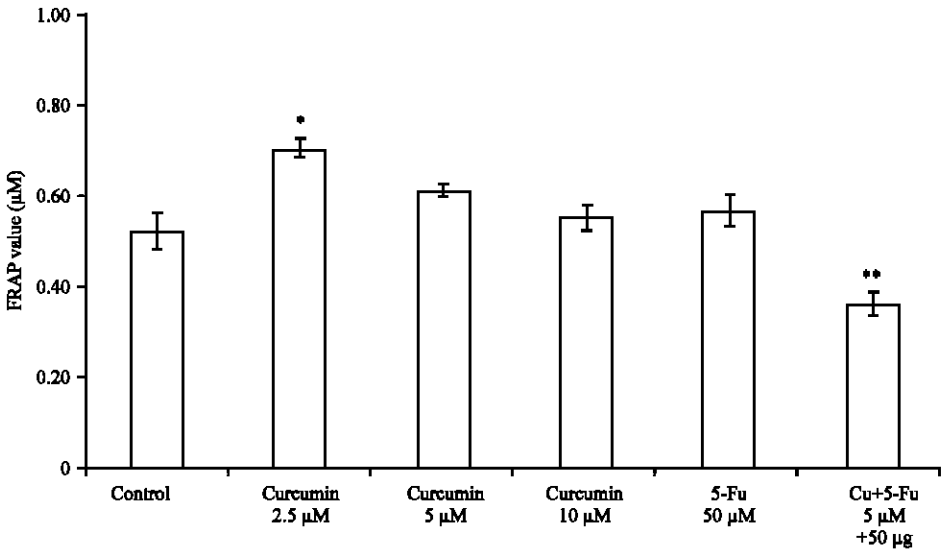

Fig. 2: Effect of curcumin, 5-fluorouracil and curcumin+5-FU on antioxidation capacity of *S. pombe* cells after 24 h, Data are presented as Mean±SD, *p = 0.001, **p<0.05, 5-FU = 5-fluorouracil

concentration on *S. pombe* cells, Reactive Oxygen Species (ROS) in cells were estimated by FRAP analysis. Treatment with 2.5 µM of curcumin resulted in higher FRAP values in *S. pombe* cells compared to control (p = 0.001), indicating lower ROS level in cells. However, with increased curcumin concentration, the FRAP values were found to be reduced. It has clearly showed that addition of higher concentration of curcumin increases the ROS levels in cells (Fig. 2). Independent treatment of 5-FU to S. pombe cells had higher FRAP value, indicating low ROS (p>0.5). Combined treatment with curcumin and 5-FU resulted in lower FRAP value (p<0.005).

Effect of curcumin and 5-FU on GSH levels in *S. pombe* cells: The cytotoxicity caused by higher concentration of curcumin was also confirmed by estimating GSH levels which is a useful measure of cytotoxicity. Treatment with 2.5 µM of curcumin increased the GSH levels compared to control (p = 0.001). However, GSH levels were found to be reducing significantly with increasing the concentrations of curcumin treatment (p<0.05). Five-fluorouracil at concentration of 50 µg mL^{-1} was also found to increase GSH levels (p<0.001). Increased in GSH levels upon treating S. pombe cells with curcumin (5 µM) along with 5-FU was highly significant (p<0.0001) (Fig. 3).

Effect of curcumin and 5-FU on catalase activity in *S. pombe* cells: As 2.5 µM of curcumin was not found to increase ROS levels, catalase activity was found higher in these cells, whereas the catalase activity declined significantly with increasing concentration of curcumin (p<0.005). The activity of catalase was also significantly low with 5-FU (when compared with that in control, p = 0.001) and activity was further declined when *S. pombe* cells were treated with curcumin and 5-FU together (p<0.0001) (Fig. 4).

Effect of curcumin and ascorbic acid on growth of *S. pombe* cells: As increase in curcumin concentration led to decrease in cell viability and increase in ROS either in independent or

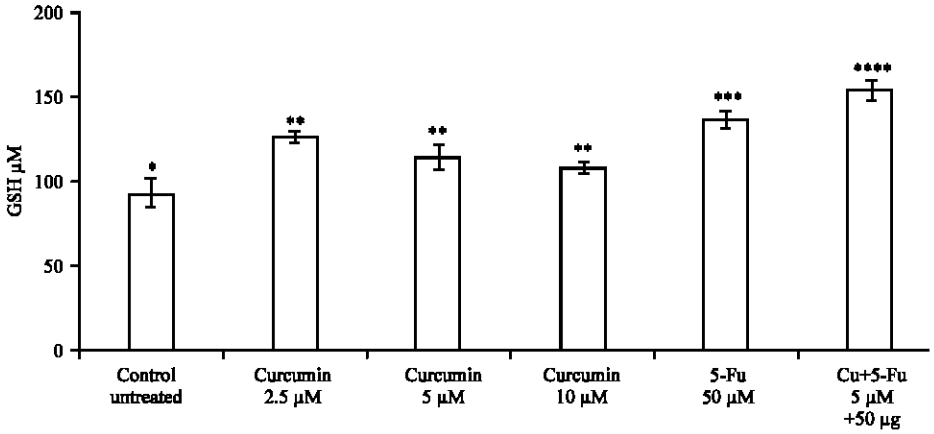

Fig. 3: Effect of curcumin, 5-fluorouracil and curcumin+5-fluorouracil on GSH content of *S. pombe* cells after 24 h, Data are presented as Mean±SD, * p=0.001, **p<0.05, ***p<0.001, ****p<0.0001; 5-FU=5-fluorouracil

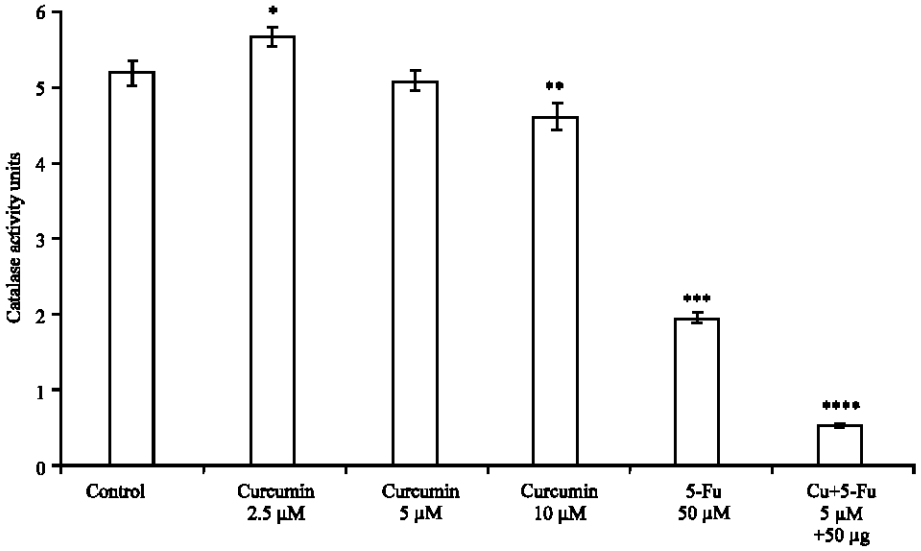

Fig. 4: Effect of curcumin, 5-fluorouracil and curcumin+5-fluorouracil on Catalase activity in *S. pombe* cells after 24 h. Data are presented as Mean±SD, *p<0.05, ** p<0.005, ***p = 0.001, ****p<0.0001; 5-FU = 5-fluorouracil

combined treatment, whether ROS is responsible for reduction in cell viability or not was determined by giving simultaneous treatment of ascorbic acid, as an antioxidant. Treatment with 10 µM of Ascorbic acid independently as well as in combination with curcumin resulted in decrease in growth rate of *S. pombe* cells compared to control (p<0.005, p<0.001)) (Fig. 5).

Effect of curcumin and 5-FU on DNA integrity: The DNA was found to be intact with 2.5, 5 and 10 µM of curcumin treatment. While 5-FU treatment caused DNA fragmentation, combination of 5 µM curcumin and 50 µg mL^{-1} of 5-FU prevented the DNA fragmentation (Fig. 6).

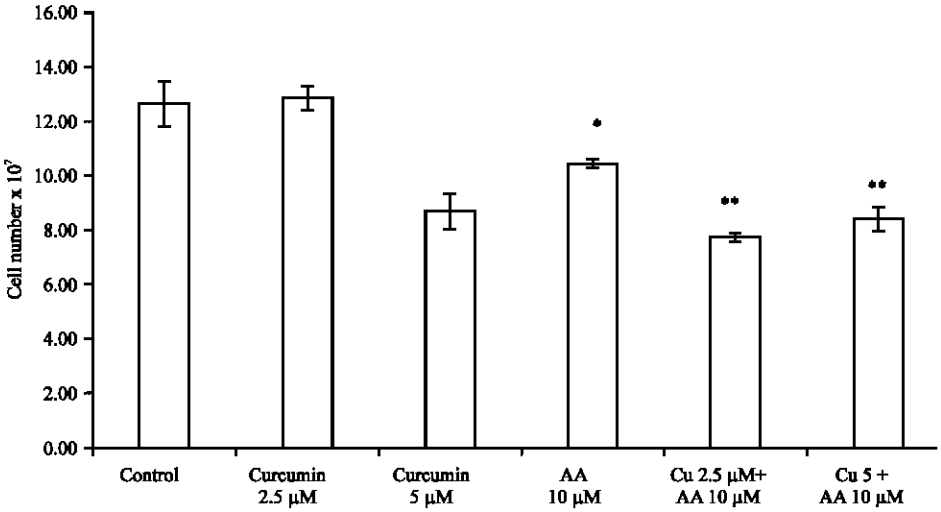

Fig. 5: Effect of curcumin and Ascorbic acid on growth of *S. pombe* cells after 24 h, Data are presented as Mean±SD. * p<0.005, **p<0.0001; Cu = Curcumin, AA = Ascorbic acid

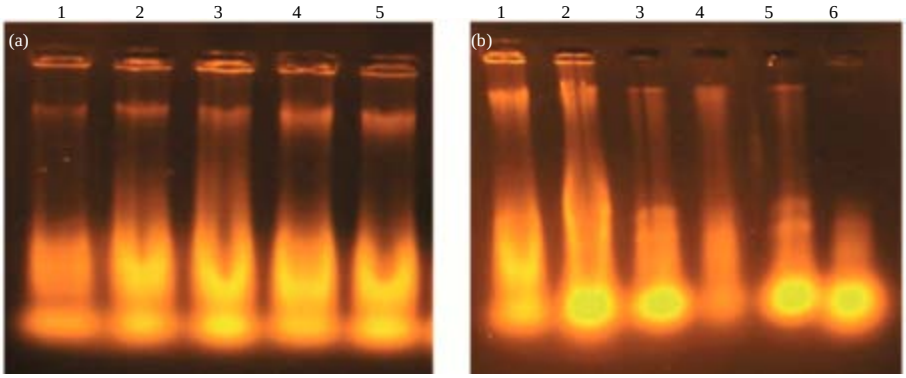

Fig. 6(a-b): (a) Effect of various concentrations of curcumin on integrity of DNA of S. *pombe* cells. Intact DNA bands without any damage are clearly seen. Lane 1: DNA extracted from untreated cells (control), Lane 2: DNA extracted from cells treated with 1.0 µM of curcumin, Lane 3: DNA extracted from cells treated with 2.5 µM of curcumin, Lane 4: DNA extracted from cells treated with 5.0 µM of curcumin, Lane 5: DNA extracted from cells treated with 10.0 µM of curcumin and (b) Effect of curcumin, 5-fluorouracil and curcumin+5-fluorouracil on integrity of DNA of *S. pombe* cells after 24 h. DNA damage is clearly seen in 5-fluorouracil treated sample (Lane 4) and combined treatment of curcumin+5-fluorouracil prevents DNA damage (Lane 5). Lane 1: DNA extracted from untreated cells (control), Lane 2: DNA extracted from cells treated with 2.5 µM of curcumin, Lane 3: DNA extracted from cells treated with 20 µM of curcumin, Lane 4: DNA extracted from cells treated with 50 µg of 5-fluorouracil, Lane 5: DNA extracted from cells treated with 5.0 µM of curcumin and 50 µg of 5-fluorouracil, Lane 6: DNA extracted from cells treated with acetic acid (positive control)

Curcumin arrests the *S. pombe* cells: Nuclear staining of *S. pombe* cells with DAPI showed that after treatment of curcumin at various concentrations, cells were arrested in G1/S phase of cell cycle. While 5-FU caused nuclei fragmentation, combined treatment (curcumin+5-FU) prevented nuclei fragmentation (Fig. 7), visualized as bright nuclei stained with DAPI.

Curcumin damages DNA of *S. pombe* cells at very high concentration: SCGE revealed that high concentration of curcumin and 5-FU caused DNA damage. Compared to untreated *S. pombe* cells, the cells treated with 20 μM of curcumin and 50 μg mL^{-1} 5- FU were having dispersed DNA ran out of cells. Combined treatment of 2.5 μM of curcumin and 50 μg mL^{-1} 5-FU prevented the DNA damage (Fig. 8).

DISCUSSION

Many phytochemicals preferentially inhibit the growth of tumor cells by arresting cell cycle or inducing apoptosis (Sa and Das, 2008). Curcumin has been shown to selectively inhibit the proliferation of almost all types of tumor cells either by activation of cell death pathways or by the inhibition of growth/proliferation pathways, with reportedly no effect on normal cells (Ravindran *et al.*, 2009; Syng-Ai *et al.*, 2004). Therefore, the present study was undertaken to investigate the effect of curcumin on normal cells.

When *S. pombe* cells were exposed to various concentrations of curcumin (2.5 to 10 μM), the growth rate declined in a dose dependent manner (Fig. 1). Treatment with 5-FU also resulted in reduction of growth rate of *S. pombe* cells. Synergistic effect of decrease in growth rate was observed when combined exposure of 2.5 μM of curcumin and 50 μg mL^{-1} of 5-FU were given to

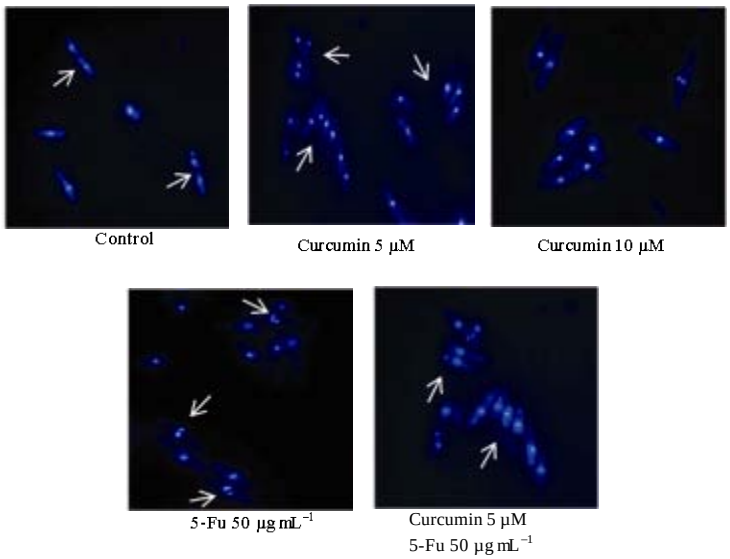

Fig. 7: Effect of lower and higher concentrations of curcumin on nuclei of *S. pombe* cells. Intact nucleus in the centre of cells showing cell cycle arrest is clearly seen in 5 and 10 μM curcumin treated cells in comparison to control where cells are seen in various phases of cell cycle whereas nuclei degradation is observed in 5-fluorouracil treated cells. Prevention of nuclei degradation is seen with combined treatment of curcumin and 5-FU; 5-FU = 5-fluorouracil

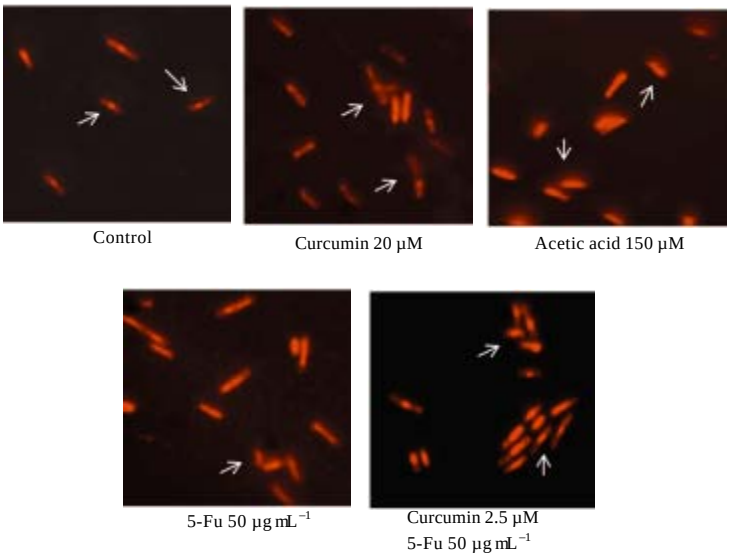

Fig. 8: Effect of high concentration (20 µM) of curcumin on DNA of normal *S. pombe* cells. DNA damage is clearly seen in 20 µM curcumin treated and in positive control cells in comparison to untreated (control) cells. Treatment with 5-fluorouracil also shows DNA damage. Combined treatment with 2.5 µM curcumin and 5-fluorouracil prevented the DNA damage.; 5-FU = 5-fluorouracil

S. pombe cells. Synergistic effect of growth inhibition due to combination of curcumin with other such chemotherapeutic drugs had also been observed by other workers (Chirnomas *et al*., 2006; Du *et al*., 2006; Lev-Ari *et al*., 2005).

Curcumin and 5-FU were found to increase the antioxidation capacity of cells as observed by FRAP assay and GSH measurement (Fig. 2, 3). Antioxidant and redox enzymes are required for defense against reactive oxygen species in cells (Penninckx, 2000). Treatment of *S. pombe* cultures with hydrogen peroxide have been shown to strongly induce GSH levels in different types of cells (Lee *et al*., 2003). Curcumin has been shown to increase the levels of GSH in several other systems like rat hepatocytes (White *et al*., 1998), kidney cells (Venkatesan *et al*., 2000) and human leukemia cells (Piwocka *et al*., 2001). However with increased concentration of curcumin, the antioxidant capacity of the cells was found to reduce. While a low concentration of curcumin (2.5 µM) elevated catalase activity significantly, higher concentration of curcumin (5 and 10 µM) lowered it. Decrease in catalase activity at higher concentration of curcumin reflects absence of activation of cells' natural antioxidation system which could be one of the reasons for its cytotoxicity at higher concentrations. Treatment with 5-FU also significantly reduced the catalase activity in *S. pombe* cells (Fig. 4). These observations indicate that lower concentration of curcumin (2.5 µM) could effectively control the cell growth and maintained antioxidant capability of cells whereas higher concentration of curcumin disturbed the normal cell metabolism and caused oxidative stress.

The oxidative stress generated due to curcumin treatment may not be the reason for decline in growth rate of *S. pombe* cells as addition of antioxidant (ascorbic acid) along with curcumin also resulted in decline of growth rate (Fig. 5). The reduction in growth rate of *S. pombe* cells exposed to curcumin was due to arrest of cell cycle as observed by DAPI staining (Fig. 7). Exposure of up

to 10 μM of curcumin did not affect the integrity of the DNA (Fig. 6), because the oxidative stress generated was controlled by antioxidants of the cells. However treatment with very high concentration of curcumin (20 μM) disturbed the antioxidant capacity of cells and DNA damage could be seen (Fig. 8). Treatment with 5-FU did cause the DNA damage (Fig. 6). In study by Lu *et al.* (2009) has showed dose and time-dependent increase in DNA damage with curcumin treatment which was confirmed by comet assay as well as agarose gel electrophoresis in mouse-rat hybrid retina ganglion cell line N18 cells.

CONCLUSION

Curcumin exhibited concentration based effect on growth of *S. pombe* cells. At 2.5 μM, it does not affect growth rate much but at 5 μM and above concentration it severely reduces growth rate of *S. pombe* cells. It restricts the cell growth by arresting the cell cycle in G1 phase. Treatment of curcumin induced reduction in GSH levels and catalase activity which could be responsible for prevention of DNA damage in spite of generation of high ROS levels. Treatment of a very high concentration of curcumin (20 μM) did result in DNA damage, as confirmed by comet assay.

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