

Anti cancer potential of 4*H*- chromene spirooxindole hybrid molecules against Dalton's Lymphoma (DL) cells

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ABSTRACT

The anticancer efficacy of 4*H*- Chromene Spirooxindole hybrid molecules **4i-m¹ (Samples 1- 5)** was investigated on Dalton's lymphoma (non-Hodgkin lymphoma) cells. Dalton's lymphoma (DL) is a spontaneous and highly invasive T-cell lymphoma that develops as an ascitic tumor in murines. It was observed that treatment of DL cells with compounds showed significant decrease in cell viability, altered morphology, nuclear disintegration and DNA fragmentation into low molecular weight DNA fragments and thus characteristic of apoptosis. In this view, these compounds may be used as potential therapeutic regimen to treat this type of cancer in experimental mice.

Figures : 07

References : 05

Table : 00

KEY WORDS : Anti-cancer potential, Apoptosis, Dalton's lymphoma cells, Viability

Introduction

Cancer is a life-threatening disease worldwide⁵, and still remains one of the leading cause of death in the world, in both high-income as well as in lower-income countries, which already account for 70% of world cancer deaths.^{4, 5}

Tumors are generally treated with platinum-based drugs (carboplatin oxaliplatin, cisplatin,) alone or co-administered with other chemotherapeutic agents.² Nevertheless, new anti-cancer drugs are still being actively in demand due to the emergence of platinum resistance and also severe side effects associated with these chemotherapeutic treatments.³

In the present study, we investigated here anti-tumor/anti-cancer activity of some 4*H*- Chromene Spirooxindole hybrid molecules 4i-m¹ [samples 1-5 (S1-S5)] against Dalton's Lymphoma cells.

Experimental

A. General

All chemicals were procured from Aldrich, USA and

E. Merck, Germany and used without further purification. RPMI-1640 culture medium was purchased from HiMedia, Mumbai, India and Fetal Calf Serum (FCS) was obtained from Invitrogen, Grand Island, NY, USA. MTT [3-(4, 5-dimethylthiazol 2-yl) -2, 5-diphenyltetrazolium bromide], Con-A (concanavalin-A), proteinase-K and chelerythrine were purchased from Sigma chemical company, Bangalore, India. DNA ladder were purchased from Promega, Masison WI, USA.

B. Cell Culture and Drug treatments

Dalton's lymphoma cells (DL cells) (Transplantable lymphoma of spontaneous origin in thymus of mice) were maintained in humidified atmosphere of 5% CO₂ at 37°C in RPMI-1640 supplemented with 10% FBS. Growth of microbes was prevented using penicillin (50-100 µg/ml), streptomycin (50-100 U/ml), L-glutamine (4mM), 1% nonessential amino acids, and 1% of sodium pyruvate. Purified non-adherent cell population (1×10⁶ cells) was incubated with samples 1-5 (S1-S5) to studied dose kinetics followed by time kinetics. Cells were cryopreserved for future references.

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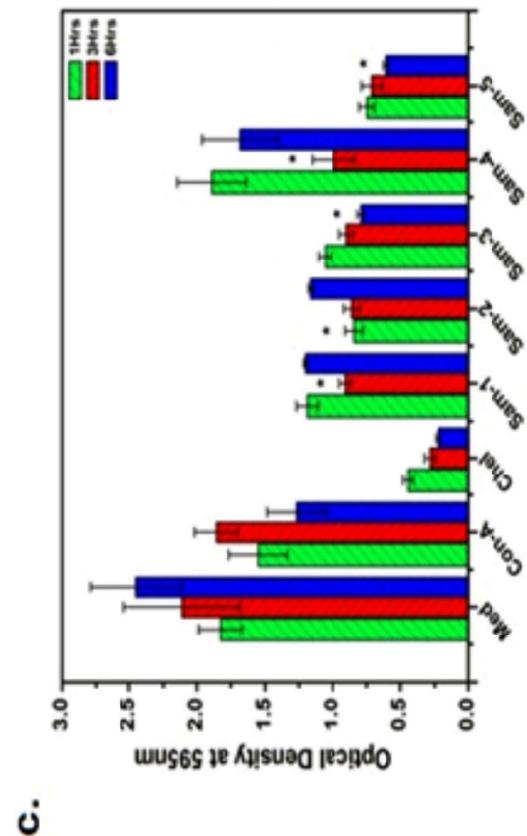
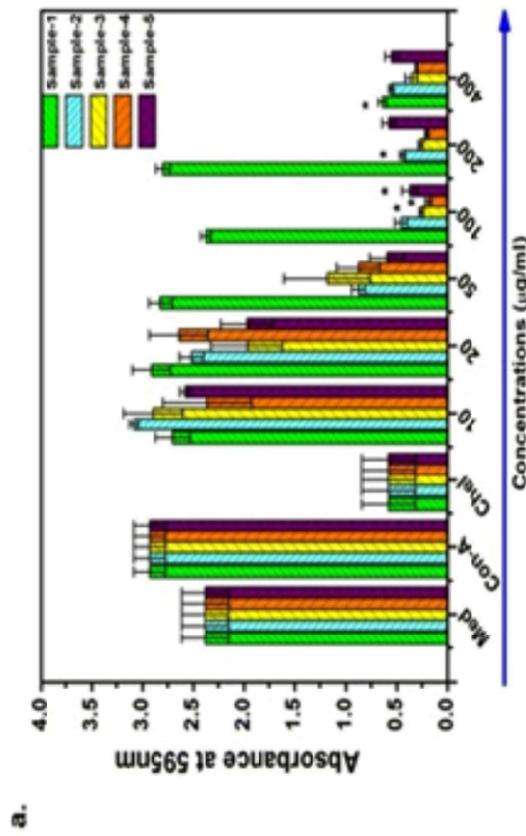
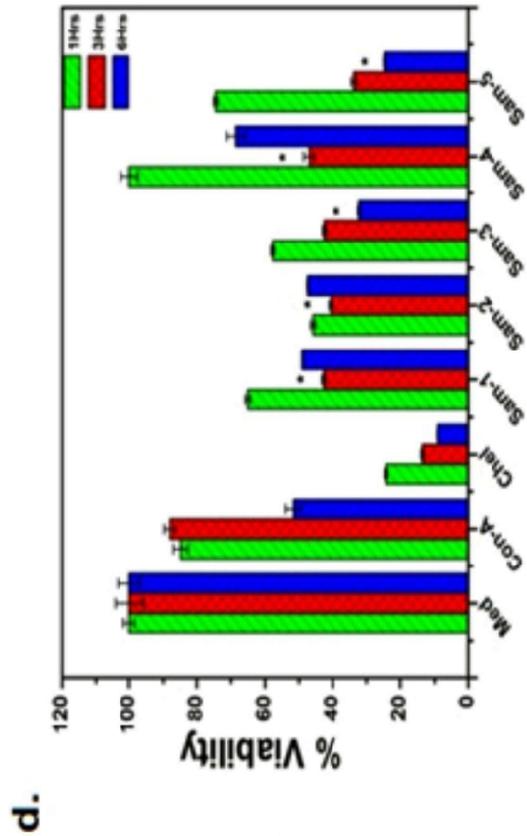
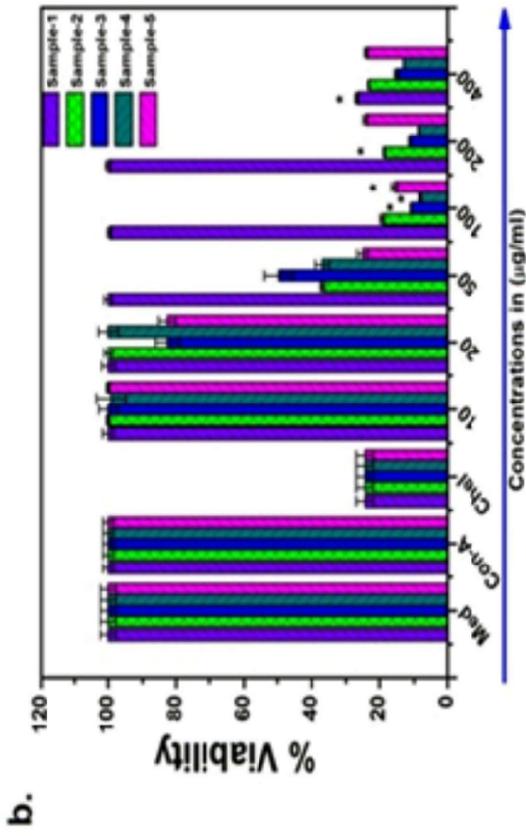


Fig.1 : (a & b) show dose kinetics and (c & d) show time kinetics of samples 1-5 (S1-S5).

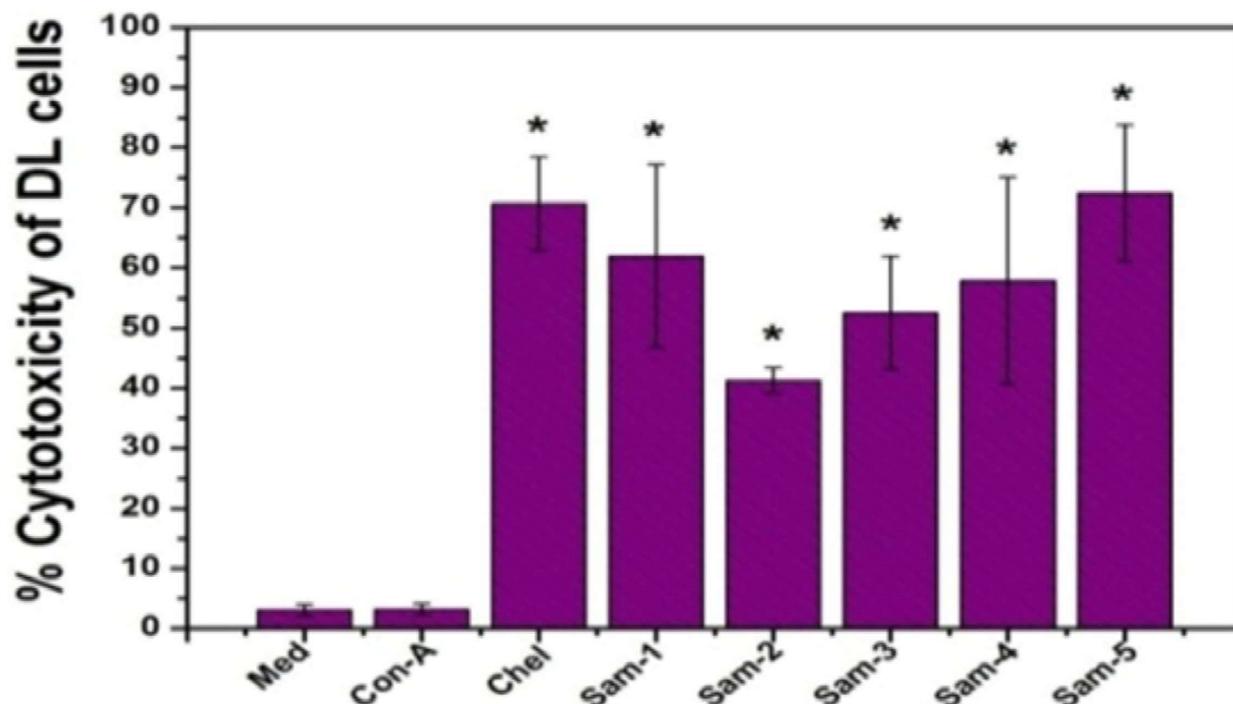


Fig. 2 : Samples 1-5 (S1-S5) induced cytotoxicity in DL cells

C. Analysis of cell viability

In order to know what impact was made by samples 1-5 (S1-S5) on DL cells viability, cells were seeded on 96 wells plate at a density 4.0×10^4 cells/well using RPMI-1640 enriched with fetal calf serum (10% v/v). Cells were incubated for 48hrs with increasing concentration (10-400 μ g/ml) of samples 1-5 (S1-S5) and chelerythrine in concentration of 10 μ g/ml was used as a standard. PBS (0.01M) was used as vehicle for control cells. Cell viability was manifested by the conversion of tetrazolium salt MTT to a coloured formazan by the mitochondrial dehydrogenases. Optical density was measured using a Bio-Rad spectrophotometer at 595nm after cells lyses in DMSO. Optical density of untreated cells was used as a control reference. The viability was determined using the following formula.

$$\% \text{Viability} = \frac{\text{Mean OD (Sample)}}{\text{Mean OD (Control)}} \times 100$$

D. Determination of toxicity

In order to investigate the level of cytotoxic of samples 1-5 (S1-S5) on DL cells, cells (1×10^6) were incubated with 50-200 μ g/ml of samples 1-5 (S1-S5) for 1hr, 3hrs and 6hrs respectively. Thereafter the number of dead cells was determined by trypan blue exclusion methods. To know this cells and dye (1:1 ratio) were mixed and observed under inverted light microscope. The percent of dead cells were determined using following

formula.

$$\left[\% \text{ Dead Cells} = \frac{\text{Number of Dead Cells}}{\text{Total Number of cells}} \times 100 \right]$$

E. Morphological Examinations

Giemsa-Eosin staining

To examine morphological changes, DL cells were incubated for 1hr, 3hrs and 6hrs in the presence of samples 1-5 (S1-S5) in a humidified atmosphere in 5% CO₂. Treated cells were washed and uniformly smeared on neat and clean slides, thereafter cells were permealized and fixed in 4% formaldehyde solution. Cells were stained with Geimsa (nucleic acid binding dye) and a counter stain eosin (stained to cytoplasm) and cells were observed under simple light microscope to study membrane blebbing and nuclear changes.

EtBr-Acridine staining

For morphological examination, cells were incubated with 400 μ g/ml of sample 1 (S1), 200 μ g/ml of sample 2 (S2) and 100 μ g/ml of samples 3-5 (S3-S5) for 1hrs, 3hrs and 6hrs in 5% CO₂ at 37°C in RPMI-1640 enriched by 10% v/v FCS. Thereafter cells were washed, smeared uniformly on clean slides, permealized with 4% formaldehyde solution freshly prepared in PBS (0.01M) and stained with EtBr (DNA binding dye) and a counter stain acridine orange (cytoplasm staining dye). Live cells photographs were captured under Fluorescence

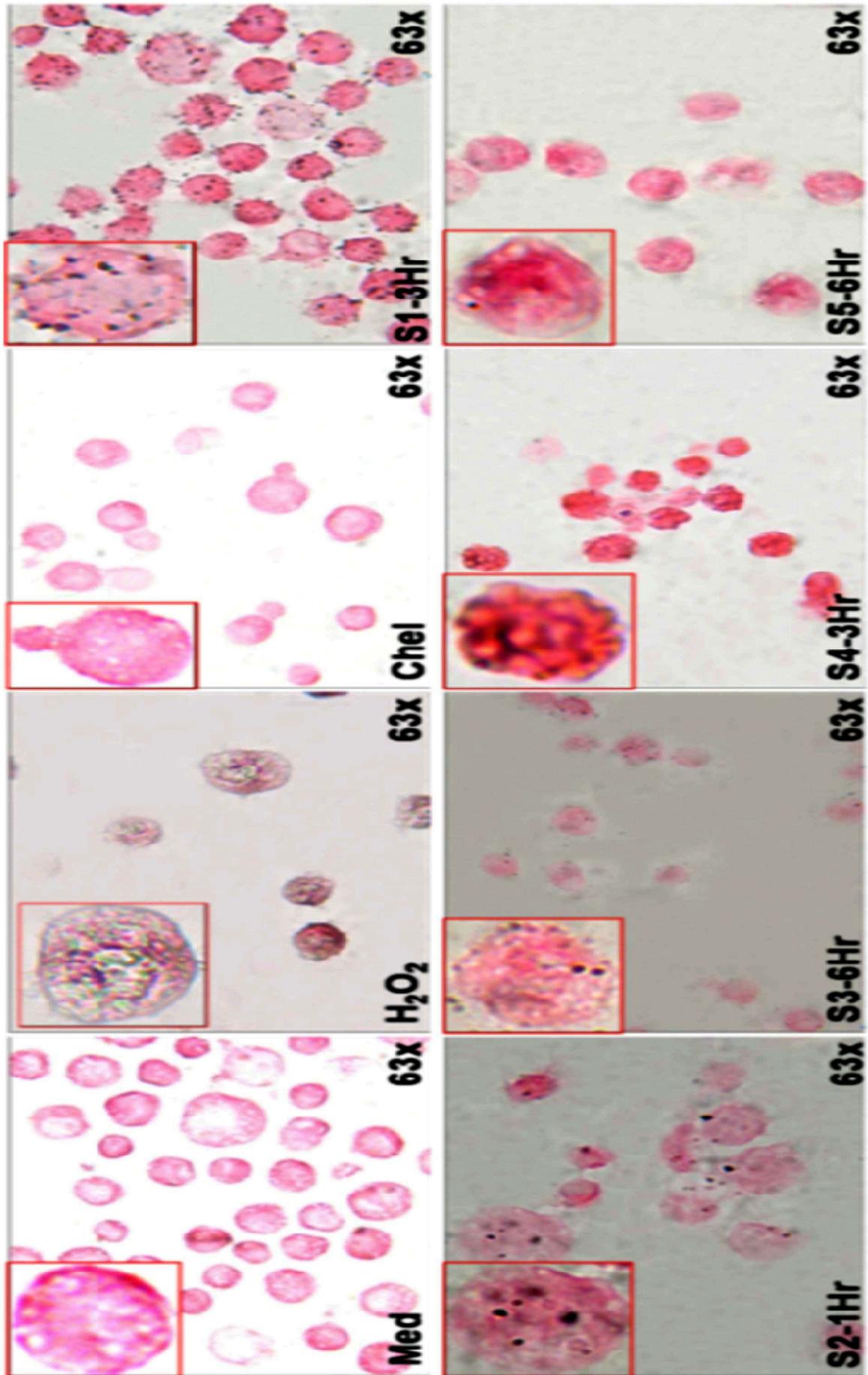


Fig. 3: Samples 1-5 (S1-S5) induced morphological changes in DL cells

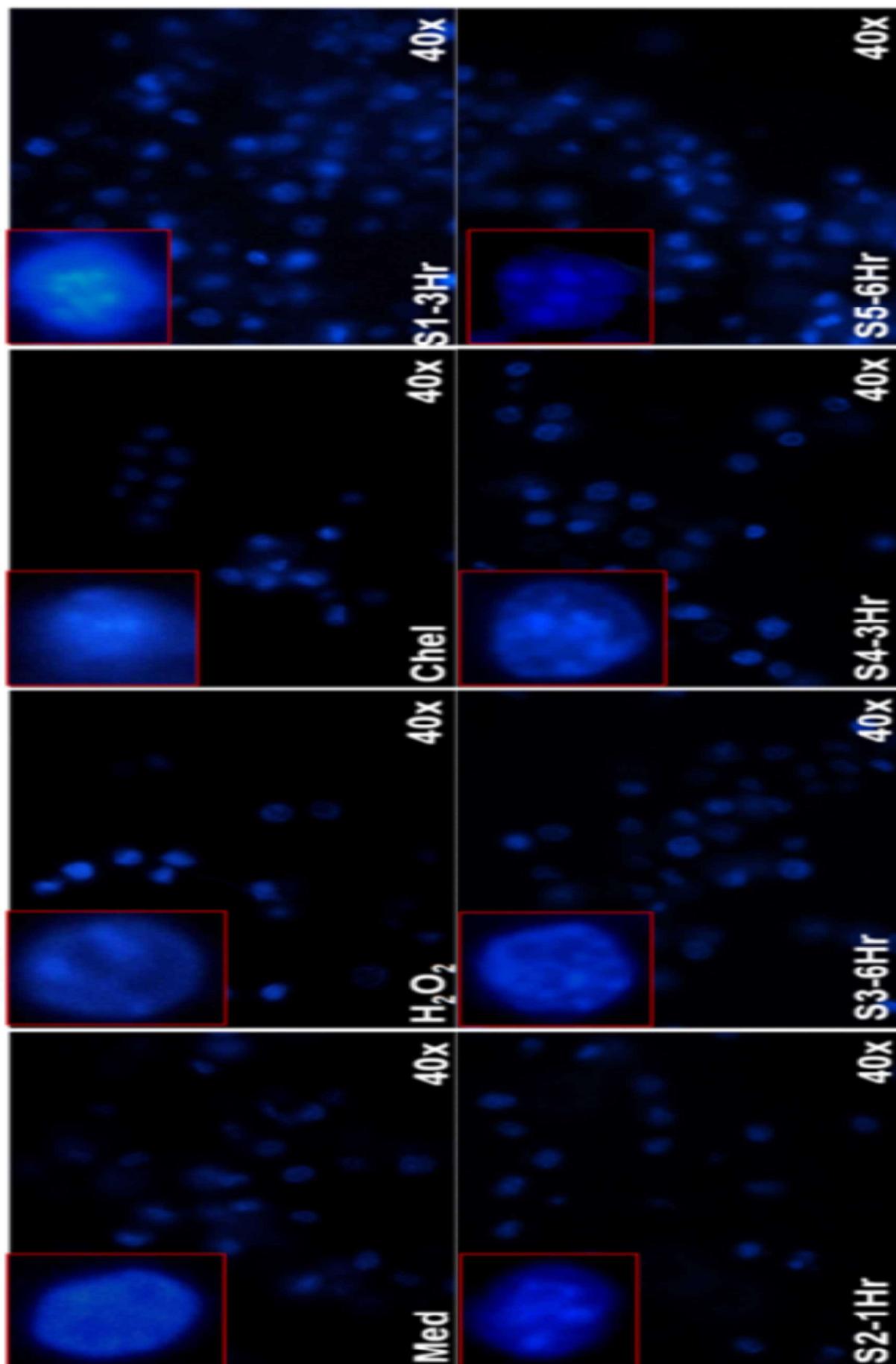


Fig. 4: Samples 1-5 (S1-S5) induced morphological changes in DL cells

microscope (Zeiss, India, Pvt. Ltd., Bangalore, India).

Hoechst staining

To study nuclear morphology, DL cells were treated with 400µg/ml of sample 1 (S1), 200µg/ml of sample 2 (S2) and 100µg/ml of samples 3-5 (S3-S5) in afore mentioned experimental conditions. Cells were washed, smeared uniformly on clean slides, permeabilized and stained with Hoechst-33342 (10µl) in ambient environment. Nuclear changes were observed under fluorescent microscope at 446nm band pass filter (Zeiss, India, Pvt. Ltd., Bangalore, India).

$$\left[\% \text{ Apoptotic Cells} = \frac{\text{Number of Apoptotic Cells}}{\text{Total Number of Cells}} \times 100 \right]$$

F. Analysis of DNA fragmentation

To analyze DNA fragmentation, DL cells were incubated with 400µg/ml sample 1 (S1), 200µg/ml of sample 2 (S2) and 100µg/ml of samples 1-5 (S1-S5) for 1hr, 3hrs and 6hrs, washed and lysed in 500µl of lysis buffer [10mM Tris-HCl, pH 8.0, 10mM EDTA, 1% Nonidet P-40 (NP-40), and 100µg/ml proteinase-K and 50µg/ml RNase] for overnight at 55°C in water bath. NaCl (50µl of 6M) and sodium acetate (50µl of 3M) were added, centrifuged at 4°C until clear supernatant was obtained. Apoptotic DNA was precipitated with phenol and chloroform (1:1)/isopropanol which was resuspended in Tris-EDTA buffer. Estimated concentration of DNA (2-5µg/well) was separated electrophoretically on 2% agarose gel containing EtBr (0.5µg/ml). The gels were being visualized and photographed under gel documentation system (Bio-Rad, India, Pvt. Ltd.).

Results

A. Samples 1-5 (S1-S5) delayed growth and survival of DL cells

It was found that cells treated with 400µg/ml of sample 1 (S1), 200µg/ml of sample 2 (S2) and 100µg/ml of samples 3-5 (S3-S5) showed significant decrease in cell viability in a dose-dependent manner when compared to control. The studied compounds were tested at concentration ranging from (10-400µg/ml) for 48 hrs (Fig. 1a and 1b). More than 50% inhibition of growth (IC_{50}) after 48hrs was observed when cells incubated with samples 1-5 (S1-S5) at 400µg/ml of sample 1 (S1), 200µg/ml of sample 2 (S2) and 100µg/ml of samples 3-5 (S3-S5) (Fig. 1a and 1b). Further, the number of viable cells after incubation with 400µg/ml of sample 1 (S1), 200µg/ml of sample 2 (S2) and 100µg/ml of samples 3-5 (S3-S5) showed decrease DL cells viability as compared to control when cells incubated for 1hr, 3hrs, and 6hrs respectively (Fig. 1c and 1d). These findings indicate that cells were more sensitive to samples 1-5 (S1-S5) at 400µg/ml

ml of sample 1 (S1), 200µg/ml of sample 2 (S2) and 100µg/ml of samples 3-5 (S3-S5) concentrations of studied compounds. These results were found corresponded to chelerythrine which was used as standard.

B. Samples 1-5 (S1-S5) induced cytotoxicity in DL cells

Level of toxicity was found significant after treatment with samples 1-5 (S1-S5) but highly significant level was obtained after treatment with samples 1, 4, 5 (S1, S4, S5) (Fig. 2). When data were compared to viability, it was found that as the percent toxicity increased, viability decreased corresponding in all the experimental conditions (Fig. 2).

C. Samples 1-5 (S1-S5) induced morphological changes in DL cells

It was found that cells incubated with samples 1-5 (S1-S5) produced morphological changes at the levels of membrane and nucleus included membrane blebbing and bulging (Fig. 3). But, control cells failed to produce these changes without any treatment (Fig. 3). Therefore, these results collectively indicate that DL cells exhibited more sensitivity towards these studied samples 1-5 (S1-S5) after 3 and 6hrs as compared to 1hr and control (Fig. 3). Here, we only studied the effect of samples 1-5 (S1-S5) on DL cell morphology after 3 and 6hrs.

Furthermore, It was observed that 3 and 6hrs incubation of DL with samples 1-5 (S1-S5) results in chromatin condensation and nuclear disintegration as compared to control cells (Fig. 4). Hoechst-33342 binds in minor groove of DNA and exhibits distinct fluorescence emissions at 460-490nm in dye: base ratio dependent manner (Fig. 4).

Moreover, our results suggest that viable cells excluded EtBr but permeable to acridine orange, which intercalated into DNA to produced green fluorescent nuclei (Fig. 5). However, cells that had yellow chromatin in condensed nuclei and often had membrane blebbing were considered as apoptotic (Fig. 5).

Further the percent of apoptotic DL cells was determined after manual calculations with the help of alphascope which showed the percent of apoptotic cells was observed significantly higher after treatment with samples 1-5 (S1-S5) as compared to control cells (Fig. 6). In contrast to apoptotic percentage, the percent of necrotic cells was found insignificant in all treated as well as untreated groups however necrotic cells showed orange nuclei but there was no marked increase in the number of necrotic cells (Figure 6).

D. Sample 1-5 (S1-S5) induced DNA fragmentation and apoptosis in DL cells

It was found that cell incubated with samples 1-5

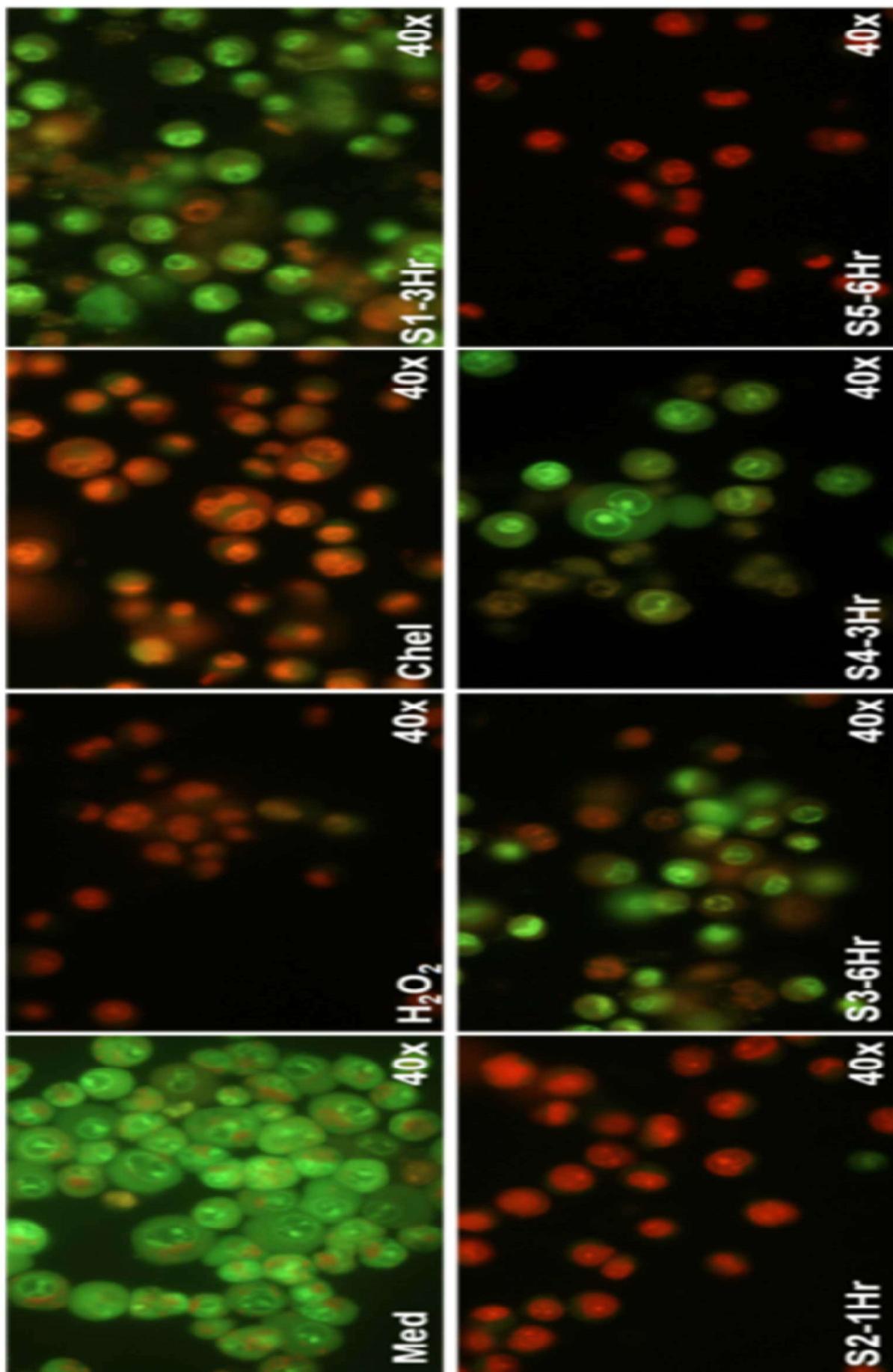


Fig. 5: Samples 1-5 (S1-S5) induced morphological changes in DL cells

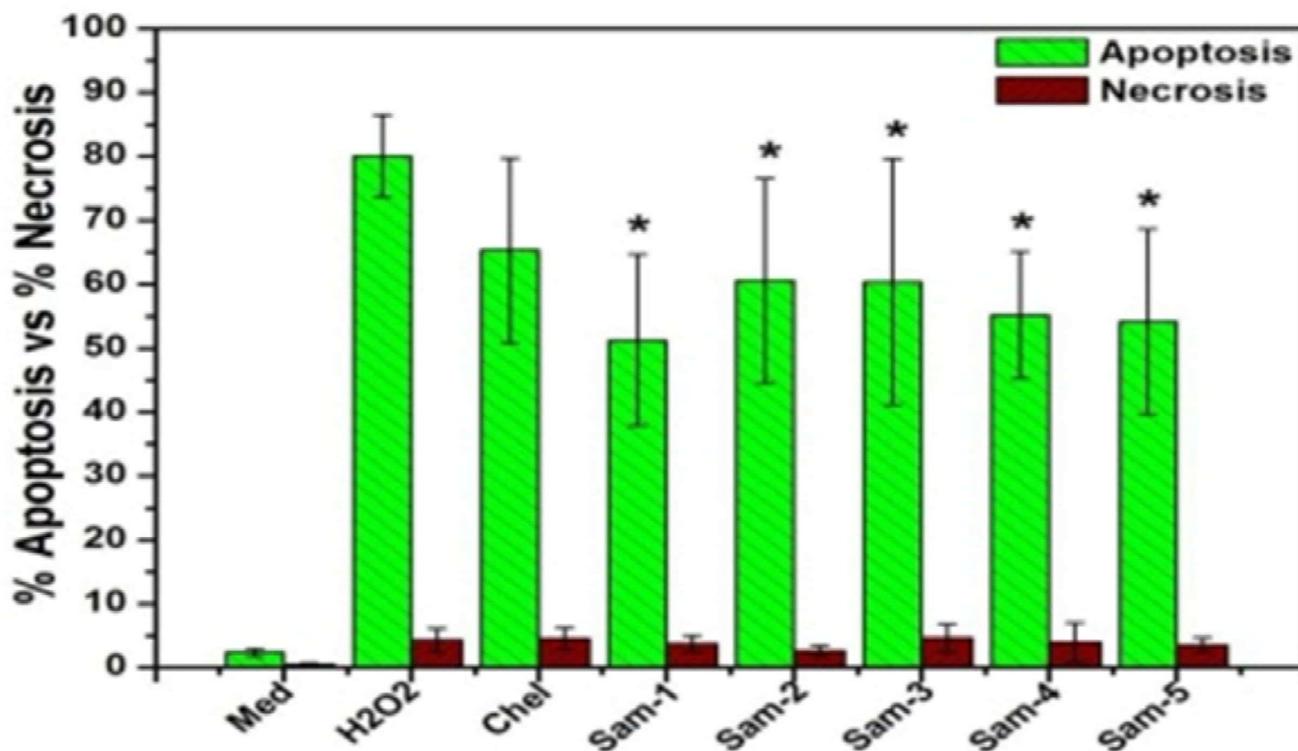


Fig. 6 : Effect of samples 1-5 (S1-S5) on percent apoptosis vs percent necrosis in DL cells

(S1-S5) for 1hr, 3hrs and 6hrs showed appearance of low molecular weight DNA (below 300bp) as compared to control (Fig. 7). Although, we observed similar pattern of DNA fragmentation in treated cells with samples 1-5 (S1-S5) but samples 1, 2, 3, 5 (S1, S2, S3, S5) showed greater intensity of DNA fragmentation as compared to sample 4 (S4) (Fig. 7). These results suggest that samples 1-5 (S1-S5) induced morphological changes (includes membrane blebbing, bulging and nuclear condensation), DNA fragmentation and apoptosis in DL cells.

Discussion

In the present investigation, we anticipated that synthesized 4*H*- Chromene Spirooxindole hybrid molecules {samples 1-5 (S1-S5)} delayed tumor growth and progression by induction of apoptosis in Dalton's lymphoma cells and showed anti-cancer potential against Dalton's lymphoma. Further, emphasis of our study concentrated on samples 1-5 (S1-S5) mediated morphological changes (plasma membrane blebbing, nuclear disintegration) and degradation of DNA into low molecular weight fragments and thus apoptosis of DL cells.

It was found that samples 1-5 (S1-S5) delayed tumor growth and proliferation of DL cells in concentration dependent manner. These results suggest that DL cells showed more sensitivity towards sample 1-5 (S1-S5) at 400µg/ml of sample 1 (S1), 200µg/ml of sample 2 (S2) and 100µg/ml of samples 3-5 (S3-S5) as compared to

control. Presented results were supported by morphological examinations after stained with Geimsa and eosin which showed acentric nuclei and membrane blebbing and bulging as compared to control cells. Treatment with samples 1-5 (S1-S5) for 1hr, 3hrs and 6hrs led to a marked increase in the number of cells possesses apoptotic like characteristic but this increase was observed maximum after 3 and 6hrs respectively.

The difference in the susceptibility of cells to samples 1-5 (S1-S5) may be due to their DNA binding ability in DL cells. The studied samples 1-5 (S1-S5) have different substituents at *N*-atom of isatin moiety. This may be a cause to show different effect on DL cells. Samples 1-5 (S1-S5) showed nuclear alterations (Hoechst-33342 stained cells) and increased number of apoptotic cells however no significant increase was observed in normal spleenocyte cells. In contrast to apoptosis, the number of necrotic cells was observed insignificant in all treated groups. Moreover, we found similar pattern of morphological as well as nuclear changes when treated cells were stained with EtBr/Acridine orange. These findings were further confirmed by gel based assay which suggest that treatment with samples 1-5 (S1-S5) for 1-6hrs showed degradation of DNA into low molecular weight/short fragments below 300bp in DL cells.

Conclusions

In conclusion, it was observed that DL cells transplantable lymphoma of spontaneous origin in thymus

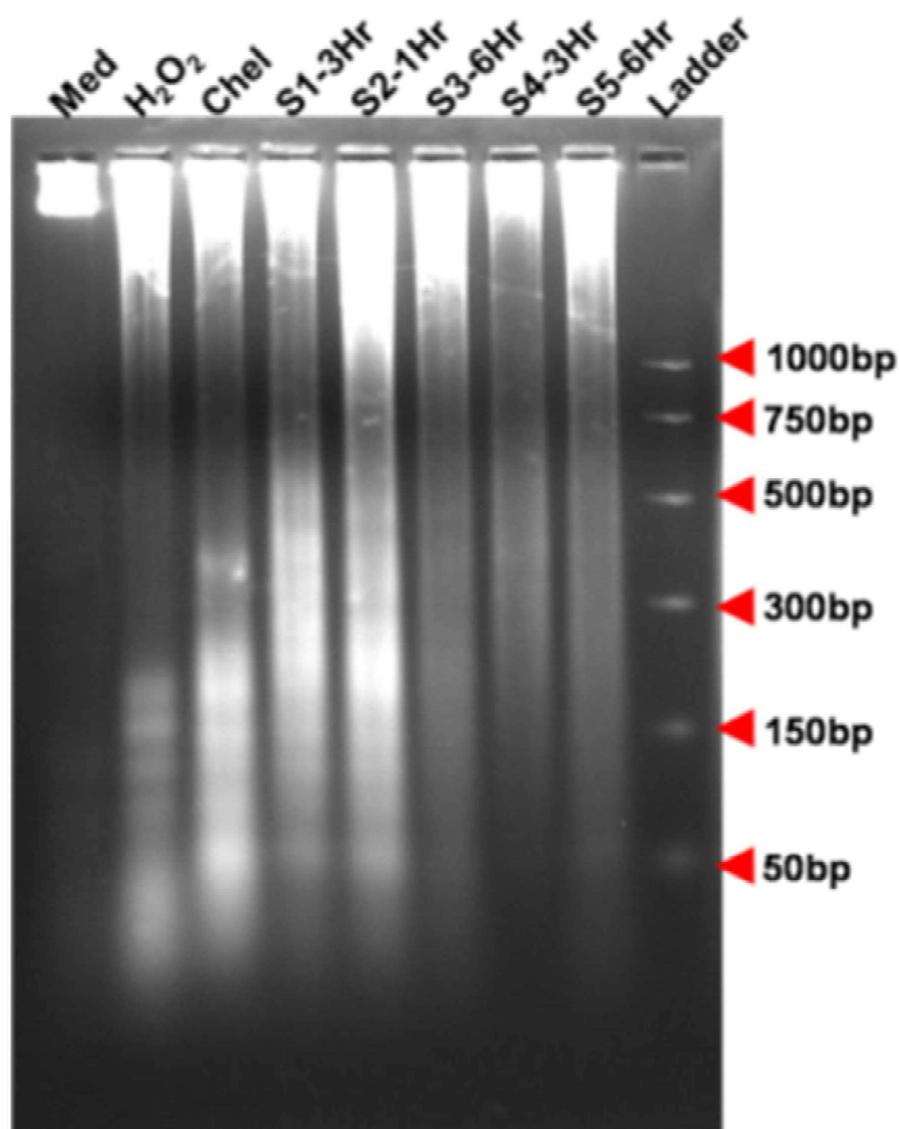


Fig. 7: Samples 1-5 (S1-S5) induced DNA fragmentation and apoptosis in DL cells

of mice in experimental mice were more sensitive to compounds [samples 1-5 (S1-S5) excluding S4]. These facts confirm that samples 1-5 (S1-S5) are capable to modulate cell growth and proliferation which further induced morphological changes corresponded to apoptosis,

intense DNA fragmentation and apoptosis in DL cells. These results were collectively suggested that samples 1-5 (S1-S5) may be better therapeutic. However, additional studies are needed to uncover the different facts of samples 1-5 (S1-S5). The challenges for future studies will improve the understanding of researchers.

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