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A report on spiro-oxindoles and quinoxaline derivatives induce apoptosis in dalton's lymphoma cells through inhibition of *in vitro* growth and proliferation

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Abstract: Cancer is a major human health issue that remains the second highest cause of deaths, in high-income countries as well as in lower-income countries also, which already account for 70% of world cancer mortality. Quinoxaline and its derivatives are versatile nitrogen containing heterocyclic molecules that possess a variety of biological activities and also presence of spirocyclic structure in various known natural products also adds interest in the investigation of spirocyclic- molecules. In the view of the above, we have synthesized spirooxindoles and quinoxaline derivatives (2a-f) by the reaction of *N*-substituted isatins with 1, 2- diaminobenzene under microwave irradiation. After that, anticancer efficacy of synthesized spirooxindoles and quinoxaline derivatives (2a-f) was investigated on Dalton's lymphoma (non-Hodgkin lymphoma) cells. DL cells were incubated with these synthesized compounds (2a-f) for 12 h, 18 h and 24 h and viability was evaluated. Further, toxicity, morphology and degradation of DNA were investigated after treatment of DL-cells with studied molecules (2a-f). It was observed that DL cells are more sensitive to molecules (2a, 2c-f) than those of compound (2b). These facts confirm that compounds (2a-f) are capable to modulate cell growth and proliferation, morphology and DNA fragmentation in DL cells. These results were collectively suggested that compounds (2a-f) showed significant anti-cancer potential by decrease cell viability, induction of morphological changes and degradation of nuclear DNA into fragments which resulted in apoptosis. Therefore, the studied compounds (2a-f) may be better therapeutic regimens to eradicate cancer problem from this world without affecting the normal cells.

Keywords: *N*-Substituted isatin derivatives, Spirooxindoles, Quinoxaline derivatives, Bentonite Clay, Apoptosis, DL Cells, Viability, Anti-cancer Potential.

1. Introduction

Cancer is a major human health issue that remains the second highest cause of

deaths, in high-income countries as well as in lower-income countries also, which already account for 70% of world cancer mortality. [1-3].

Quinoxaline derivatives are considered versatile nitrogen containing heterocyclic compound and a wonder nucleus that posses various biological activities, such as AMPA/GlyN receptor antagonist [4-5], angiotensin-II receptor antagonists [6,7], anti-cancer [8, 9], anti-infection [10-11] and immunomodulating activities. Spirocyclic system containing tetrahedral carbon atom common to two rings are structurally interesting [12, 13].

The asymmetric structure arises due to the chiral-spiro carbon atom of the molecule which is one of the most important criteria of biological activities [14-15]. The presence of the sterically constrained spiro structure in various natural products also adds interest in the investigation of spiro-compounds [16].

They also act as potent inhibitors of monoamine oxidase in human urine and rat tissues [17] and acetylcholinesterase [18], antagonist of in vitro receptor binding by atrial natriuretic peptide [19] and have a wide range of CNS activities [20].

Spirooxindole strucrure is also a part of many alkaloids with miscellaneous biological activities such as paraherquamide-A which possess anti-parasitic and anti-nematodal activities.

Keeping these points in view, the aim of the present investigation was to determine the anti-tumor/anti-cancer potential of spirooxindoles and quinoxaline derivatives (**2a-f)** on Dalton's lymphoma cell. Dalton's lymphoma, a cancer of T cell was used in the present investigation which owes the properties of rapid growth, highly deleterious and immunosuppressive. It was found that the cells treated with compounds **(2a-f)** results in reduce cell viability, changed morphology and degradation of DNA into fragments.

DNA fragmentation is one of the hallmarks of apoptosis. Therefore, the studied compounds may be used as novel anti-cancer agents to treat several types of tumor but detailed investigations are needed to explore the exact mechanism.

2. Materials and methods

2.1 Chemicals [9]

All chemicals were procured from Aldrich, USA and E. Merck, Germany and used without further purification. *N*-substituted isatins were prepared by earlier reported procedures. TLC was carried out on SiO_2 gel (HF_{254}, 200 mesh). The solvent system employed was ethyl acetate: n-hexane (11: 9) and the spots were identified by placing the plate in Iodine chamber. IR spectra were recorded on a PerkinElmer FT/IR version10.03.05 spectrometer.

NMR spectra were run on a JEOL AL300 FTNMR spectrometer; chemical shifts are given in δ ppm, relative to TMS as internal standard. Elemental microanalysis was performed on Exeter Analytical Inc Model CE-440 CHN Analyzer. Melting points were measured in open capillaries and are uncorrected. The microwave assisted reactions were carried out in a "MAS-II, Microwave Synthesis System" manufactured by Sineo microwave chemistry technology co. Ltd, having an output energy range

of 0 to 1000 watts, based on temperature of reaction and individual sensor for temperature control with attachment of reflux condenser with both magnetic and mechanical stirring device (thus avoiding the risk of high pressure development) and synthesis on preparative scales. RPMI 1640 culture medium was purchased from HiMedia, Mumbai, India and Fetal Bovine Serum (FBS) 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. All other chemicals stated otherwise were obtained either from HiMedia, Mumbai, India or Super Religare Laboratory (SRL), Mumbai, India.

2.2 General procedure for synthesis of compounds 2a-f [19]

1, 2-Diaminobenzene (0.001 mol) was added to a mixture of isatin or *N*-substituted isatin derivatives (**1a–f**, 0.001 mol), bentonite clay (20 mol%) and EtOH/H_2O (v/v, 3:1, 20 ml). The reaction mixture was stirred at 70 ^{0}C under microwave irradiation of 500 W. After completion of reaction, the catalyst was filtered, filtrate was concentrated under reduced pressure, and the residue was crystallized from ethanol.

2.3 Cell cultures

Dalton's lymphoma cells (DL cells) were cultured in RPMI-1640 supplemented with 10% FBS at 37^{0}C in 5% CO_2 humidified incubator. 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. Cells were also cryopreserved for future references.

2.4 Cell Growth and Cell Viability

To determine the effect of compounds (**2a-f)** on the viability of DL cells, 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 48 h with increasing concentration of 200 µg/ml of compounds (**2a-c, 2e & 2f),** 100 µg/ml of compound **(2d)** and chelerythrine in concentration of 10 µg/ml used as a standard. PBS (0.01M) was used as vehicle for control cells. Cell viability was marked 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 595 nm after cells lyses in DMSO. Optical density of untreated cells was used as a control reference. The viability was determined using the following formula.

$$\text{Percent Cell Viability} = \frac{\text{Mean OD}}{\text{Control OD}} \times 100$$

2.5 Cytotoxicity Assay

The toxicity of compounds **(2a-f)** on DL cells was carried by trypan blue exclusion methods. Cells at the density of 1×10^6 cell/ml were incubated with compounds in concentration of 200 µg/ml of compounds (**2a-c, 2e & 2f),** 100 µg/ml of compounds **(2d)** for 12 h, 18 h and 24 h respectively. Thereafter cells and trypan blue dye (1:1 ratio) was mixed and observed under inverted light microscope. The percent of dead cells were determined using following

formula.

$$\text{Dead Cells}\ (\%) = \frac{\text{Number of Dead Cells}}{\text{Total Number of cells}} \times 100$$

2.6 Cell Morphology and Nuclear Visualization

For morphological examination cells were incubated with 200 µg/ml of compound (**2a-c, 2e & 2f),** 100 µg/ml of compound **(2d)** for 12 h, 18 h and 24 h in 5% CO_2 at 37 ^{0}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 Haematoxylin and a counter stain Eosin. DPX was used to mount slides. Photographs were captured under light microscope (Leica, Alwarpet, Chennai, India).

To study nuclear morphology, DL cells were treated with 200 µg/ml of compounds (**2a-c, 2e & 2f),** 100 µg/ml of compound **(2d)** in aforementioned experimental conditions. Cells were washed, smeared uniformly on clean slides, permealized and stained with Hoechst-33342 (10µl) in ambient environment. Nuclear changes were observed under fluorescent microscope at ~ 460nm band pass filter (Zeiss, India, Pvt. Ltd., Bangalore, India).

2.7 Determination of DNA fragmentation in apoptotic DL cells

To analyze DNA fragmentation, DL cells were incubated with 200µg/ml of compounds (**2a-c, 2e & 2f),** 100 µg/ml of compound **(2d)** for 12 h, 18 h and 24 h, washed and lysed in 500 µl of lysis buffer [10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% Nonidet P-40 (NP-40), and 100 µg/ml proteinase-K and 50 µg/ml R Nase] for overnight at 55 ^{0}C in water bath. NaCl (50 µl of 6 M) and sodium acetate (50 µl of 3 M) were added, centrifuged at 4 ^{0}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 (2-5 µg/well) was separated electrophoretically on 2% agarose gel containing EtBr (0.5 µg/ml). The gels were being visualized and photographed under gel documentation system (Bio-Rad).

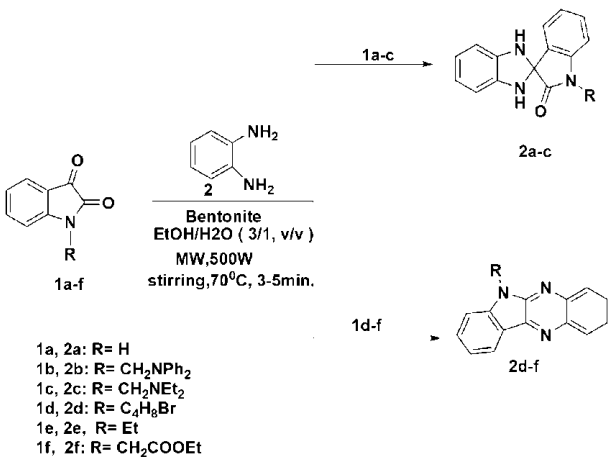
2.8 Statistical Observations

Data are represented as mean ±SE. One-way ANOVA followed by Bonferroni as post-hoc test was used for statistical calculations. Data were considered to be significant at $p<0.05$ as applicable.

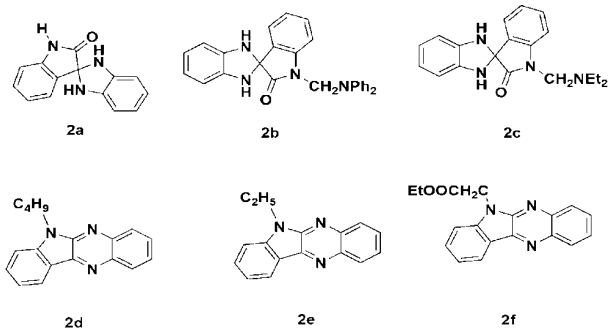
3. Results and discussion

3.1 Chemistry

The condensation of isatin derivatives with 1, 2-diamninobenzene was carried out by using catalytic amount of bentonite clay under microwave irradiation. The condensation reaction of compounds **1a**, **1b** and **1c** with 1, 2-diaminobenzene results selectively spirooxindoles **2a**, **2b** and **2c** while in the case of compounds **1d**, **1e** and **1f** the sole product quinoxaline derivatives **2d**, **2e**, **2f** was obtained after reaction with 1, 2- diaminobenzene (**Scheme 1**). The chemical structure of all synthesized products was confirmed by chemical and spectral data [21].



Scheme 1



Scheme 2

Library of synthesized compounds which were investigated (Compounds **2a-c**, spirooxindoles, compounds **2d-f**, quinoxaline derivatives)

3.2 Effect of compounds (2a-f) on the viability of DL cells

To examine the effect of compounds **(2a-f)** on the viability of DL cells, MTT-assay was carried out. Results show that cells treated with 200 µg/ml of compounds (**2a-c, 2e & 2f)** and 100 µg/ml of compound **(2d).** Compounds **(2a-f)** showed a significant decrease in cell viability in a dose-dependent manner when compared to control **(Figure 1a).** The studied compounds were tested at concentration ranging from (10-400 µg) for 48 h. More than 50% inhibition of growth (IC_{50}) after 48 h incubation with compounds (**2a-f)** was observed at 200 µg of compounds (**2a-c, 2e & 2f)** and 100 µg/ml of compounds **(2d) (Figure 1a**). Further, percent viability of cells was calculated with help of formula described in materials and methods **(Figure 1b)**. In addition, cells showed sensitivity to compounds (**2a-f)** at 200 µg/ml of compounds (**2a-c, 2e & 2f)** and 100 µg/ml of compounds **(2d)** in a time dependent fashion **(Figure 1c)**. This was confirmed at quantitative scale by percent apoptotic cells **(Figure 1d)**. These results correlated with chelerythrine, a standard drug using as anti-cancer agents in clinical practice.

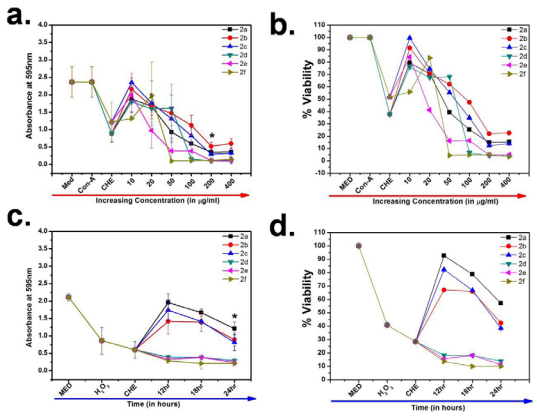


Figure 1. Effect of compounds (**2a-f**) on the viability of DL cells. Cells were incubated with increasing concentration of compounds (**2a-f**) for 48hr in RPMI-1640 supplemented with 10% FCS in 5% CO2. Optical density of signals was detected at 595nm. Figure (a) shows absorbance of dose kinetics, (b) shows percent viability of dose kinetics, (c) shows absorbance of time kinetics and (d) shows percent viability of time kinetics

Next, to determine the toxicity of compounds (**2a-f**) in an *in vitro* system, toxicity was assayed as described in materials and methods. The number of dead cells was manually calculated under light microscope in naubar's chamber using equal ratio of trypan blue dye and cells.

It was observed that treatment with 200 µg/ml of compounds (**2a-c, 2e & 2f)** and 100 µg/ml of compounds **(2d)** showed significant increase toxicity as compared to control cells **(Figure 2).**

A time-dependent increase in toxicity level of DL cells was observed but up to certain extent (**Figure 2**).

Further, a statistically significant toxic level was observed after 24 h but it was found weaker after 24 h and before 12 h (Data not shown). As the toxicity increases, viability decreases correspondingly **(Figure 2)**.

These observations were further confirmed at the levels of morphological ground and gel based assay such as DNA fragmentation.

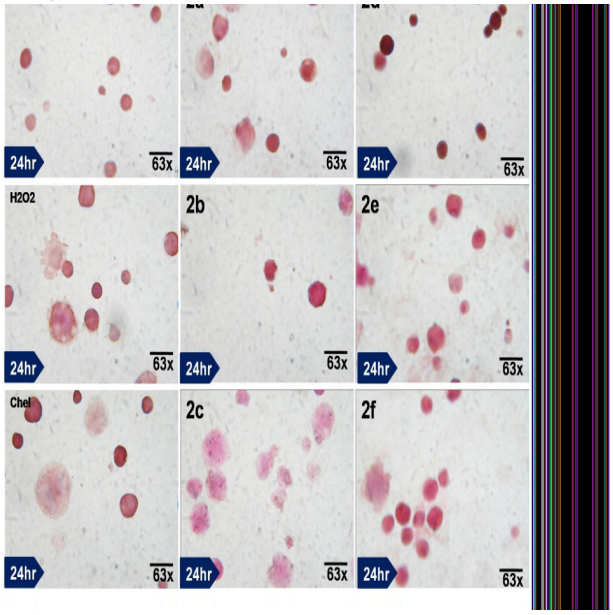


Figure 2. Effect of compounds (**2a-f**) on cytotoxicity of DL cells. Cells were treated with 200µg/ml of (**2a-2c, 2e** and **2f)** and 100µg/ml of **(2d)** for 12hr, 18hr and 24hr in RPMI-1640 supplemented with 10% FCS in 5% CO2. The number of dead cells were determined by Trypan Blue exclusion method and expressed in percent of dead cells.

3.3 Effect of compounds (2a-f) on morphology of DL cells

In order to investigate morphological changes, cells were treated with 200 µg/ml of compounds (**2a-c, 2e & 2f)** and 100 µg/ml of compounds **(2d)** for 12 h, 18 h and 24 h. Haematoxilin/Eosin, Etbr/Acridine and Hoechst-33342 staining was carried out.

It was observed that cells incubated with 200µg/ml of compounds (**2a-c, 2e & 2f)** and 100 µg/ml of compounds **(2d)** showed morphological alteration at the levels of membrane and nucleus such as membrane blebbing and bulging than that of control cells (**Figure 3 and 4**).

Two different concentrations of studied compounds were used for different time interval but remarkable changes were observed after 24 h (**Figure 3 and 4**).

It was found that the number of apoptotic cells was decreased after 24 h and necrosis become predominant (data not shown). The number of apoptotic cells was also observed after 12 h and 18 h but this was quite weaker than 24 h. Therefore, the effect of studied compounds **(2a-f)** on DL cell morphology after 24 h was shown here.

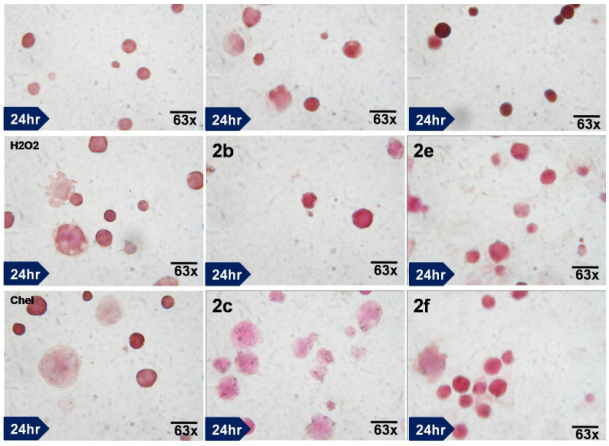


Figure 3. Effect of compounds (**2a-f**) on morphology of DL cells. Cells were treated with 200µg/ml of (**2a-2c, 2e** and **2f)** and 100µg/ml of **(2d)** for 12hr, 18hr and 24hr and stained with Giemsa and a counter stain eosin. To study morphological changes, cells observed under light microscope.

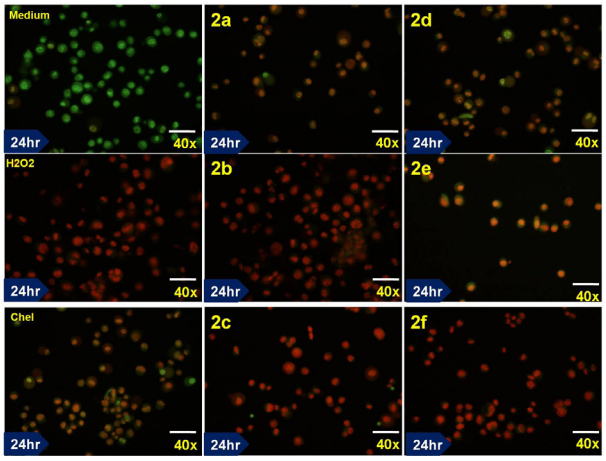


Figure 4. Effect of compounds (**2a-f**) on nuclear morphology of DL cells. Cells were incubated in the presence of 200µg/ml of (**2a-2c, 2e** and **2f)** and 100µg/ml of **(2d)** for 12hr, 18hr and 24hr and stained with a DNA binding dye Hoechst-33342. Nuclear morphology was examined under fluorescent microscope.

To visualize nuclear disintegration, treated cells were stained with Hoechst-33342 (2'-[4-ethoxyphenyl-1-1piperazinyl]-2, 5'-bi-1benzimidazole trihydrochloride trihydrate), binds preferentially to A-T rich regions. It was observed that that treatment with 200 µg/ml of compounds (**2a-c, 2e & 2f)** and 100 µg/ml of compounds **(2d)** resulted in chromatin condensation and nuclear disintegration in DL cells as compared to control cells (**Figure 5**). Hoechst-33342 binds in minor groove of DNA and exhibits distinct fluorescence emissions at 460-490 nm in dye: base ratio dependent manner in the present report (**Figure 5**).

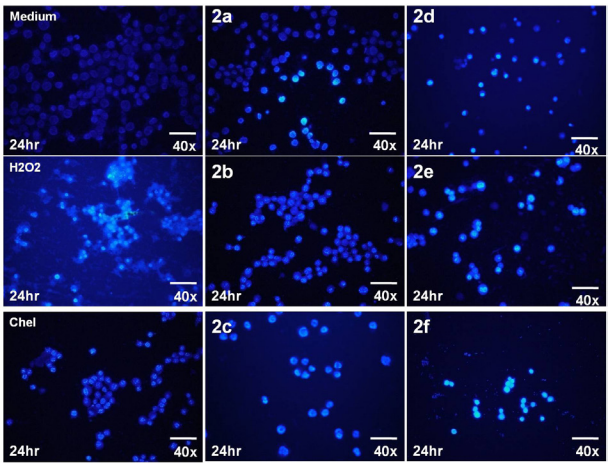


Figure 5. Effect of compounds (**2a-f**) on apoptotic changes in DL cells. Cells were incubated with 200µg/ml of (**2a-2c, 2e** and **2f)** and 100µg/ml of **(2d)** for 12hr, 18hr and 24hr and stained with a DNA binding dye EtBr and a counter stain acridine orange. To study apoptosis, cells were treated with studied compounds **(2a-2f)** and observed under fluorescence microscope.

Further, to confirm this, EtBr/Acridine staining was carried out. It was found that viable cells excluded EtBr but permeable to acridine orange, which intercalated

into DNA to produced green fluorescent nuclei (**Figure 4**). However, cells that had yellow chromatin in condensed nuclei and often had membrane blebbling were considered as apoptotic (**Figure 4**). There was no marked increase was observed in the number of necrotic cells.

3.4 Effect of compounds (2a-f) on DNA fragmentation and apoptosis of DL cells

In order to determine, degradation of DNA at quantitative scale, estimated concentration of isolated DNA was run on 2% agarose gel. Results showed that treatment with 200 µg/ml of compounds (**2a-c, 2e & 2f)** and 100 µg/ml of compounds **(2d)** for 24 h showed appearance of low molecular weight DNA fragments (below 200bp) than those of control (**Figure 6**). The anti-cancer effect of studied compounds was observed almost similar but it was more pronounce **(2b, 2c and 2f)** that those of **2a, 2d** and **2e** in DL cells (**Figure 6**). These results are in corroboration with viability, toxicity and morphological observations.

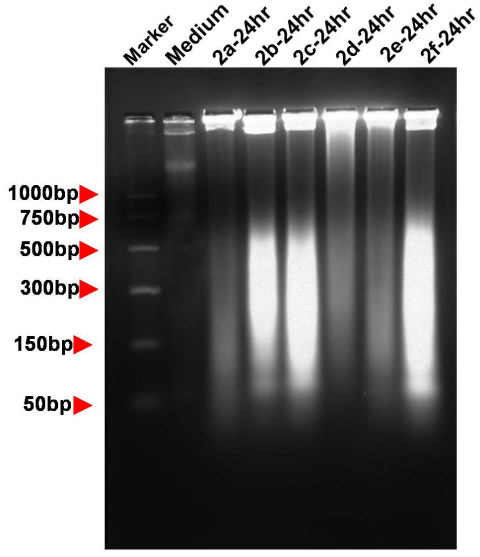


Figure 6. Effect of compounds (**2a-f**) on degradation of DNA in DL cells. Cells were treated with 200µg/ml of (**2a-2c, 2e and 2f)** and 100µg/ml of **(2d)** for 12hr, 18hr and 24hr, lysed and DNA was isolated. Estimated concentration of DNA was run on 2% agarose gel to study DNA fragmentation at quantitative scale in DL cells.

3.5 Discussion

Spirooxindoles and quinoxaline derivatives have been reported to possess variety of biological properties on various types of cancer cells. However, the mechanism of their action has not been fully elucidated yet and the synthesized spirooxindoles and quinoxaline derivatives (**2a-f)** have not been studied in Dalton's lymphoma cell. In the present report, it was anticipated that synthesized compounds **(2a-f)** showed strong anti-cancer potential against Dalton's lymphoma cells. The present investigation was concentrated on compounds **(2a-f)** mediated morphological changes, degradation of DNA into low molecular weight fragments and apoptosis of DL cells.

It was observed that compounds **(2a-f)** inhibit cell growth and proliferation of DL cells in concentration dependent manner. These results were corroborated with the previous findings. Further, it was observed that DL cells were more sensitive to compounds **(2a, 2c-f)** than those of compound **2b**. In evident to this, Geimsa and eosin stained cells showed acentric nuclei and membrane blebbing and bulging as compared to control cells. These results again corroborated with the previous findings. In addition, a marked increase in the number of cells possesses apoptotic like characteristic was observed

after treatment with compounds **(2a-f)** for 24 h.

Further, difference in the susceptibility of cells to compounds **(2a-f)** may be due to their DNA binding ability in DL cells. In addition, compounds **(2a-f)** showed nuclear disintegration (Hoechst-3342 stained cells) which is in agreement with previous findings. On the basis of molecular ground, gel based assay suggested that treatment with compounds **(2a-f)** for 24 h results in degradation of DNA into low molecular weight/short fragments below 200bp in DL cells. Strands breakage of super coiled duplex DNA led to reduction of the size of large molecule which can be visualized on 2% gel electrophoresis. Although, the treatment of compounds **(2a-f)** showed considerable amount of low molecular weight DNA (below 200bp) which was similar to our previous findings as discussed above that DL cells were highly sensitive to compounds **(2a-f)**.

4. Conclusion

In conclusion, it was observed that DL cells are more sensitive to compounds **(2a, 2c-f)** than those of compound (**2b**). These facts confirm that compounds (**2a-f)** are capable to modulate cell growth and proliferation, morphology and DNA fragmentation in DL cells. These results were collectively suggested that compounds (**2a-f)** may be better therapeutic regimens to eradicate cancer problem from this world without affecting the normal cells.

However, additional studies are needed to uncover the different facts of compounds (**2a-f**). The challenges for future studies will be improve the understanding of readers and researchers.

Declaration of Interest:

There is no conflict of interest among the authors. The authors alone are responsible for the content and writing of the paper.

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