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THE ANTINEOPLASTIC ACTIVITY OF ECHITAMIDINE-N-OXIDE-GLUCOPYRANOSIDE ISOLATED FROM STEM BARK EXTRACT OF ALSTONIA SCHOLARIS IN KB CELLS

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ABSTRACT

The antineoplastic activity of an isolated bioactive constituent, EOG (Echitamidine-N-Oxide-Glucopyranoside) from stem bark of Alstonia scholaris was studied in KB cells by clonogenic, micronucleus, comet and apoptosis assays. Exposure of KB cell to 0, 1, 5, 10, 20, 30, 40, 50 or 100 µg/ml EOG for varying times caused a concentration-dependent reduction in the cell survival up to 6 h post-treatment followed by a marginal but non-significant decline thereafter up to 24 h. Therefore, 6 h treatment time was considered as an optimum time for EOG exposure and further studies were conducted using this treatment time. The inhibitory concentration (IC_{50}) of EOG was found to be 25 µg/ml. The EOG treatment induced micronuclei in a concentration manner at all assessment times and a maximum rise in micronuclei frequency was observed at 30 h post treatment. The comet assay studies revealed that the exposure of KB cells to various concentrations of EOG induced DNA damage in concentration dependent fashion and the greatest damage was observed at 6h post-treatment as indicated by increased migration of DNA into comet tails and rise in Olive tail Moment. The EOG induced apoptosis in KB cells in a concentration dependent manner and the comparison with doxorubicin which was used as positive control revealed that EOG was as effective as doxorubicin in inducing apoptotic death. The present study demonstrates that EOG killed KB cells in a concentration dependent manner and this cell killing effect of EOG was due to induction of DNA damage and apoptosis.

KEYWORDS: Echitamidine-N-Oxide-Glucopyranoside (EOG), Clonogenic assay, Micronuclei, Comet assay.

INTRODUCTION

Cancer is group of diseases which has been treated traditionally using surgery chemotherapy or radiotherapy. The chemotherapy has been highly successful in treating advanced stage of cancers. Several drugs of different molecular structure have been used to control the neoplastic diseases. Some of the important drugs which have formed the basis of chemotherapy include nitrogen mustards, cyclophosphamide, vinca alkaloids, bleomycin, doxorubicin, epipodophyllotoxin and taxols. The nitrogen mustard could be regarded as earliest known chemical agent used to treat lymphomas in the modern medicine.[1] Cyclophosphamide is another important drug which has been used to treat various cancers either alone or in combination with other drugs or radiation.[2] The other important chemotherapeutic agents that are used either alone or in conjunction with other drugs or radiation includes methotrexate, epipodophyllotoxin, cisplatin, beolmycin, doxorubicin and taxol.[3] Methotrexate is used in gestational choriocarcinoma, hydatidiform mole, prophylaxis of meningeal leukemia, breast cancer, epidermoid cancers of the head and neck, advanced mycosis fungoides, and lung cancer, particularly squamous cell and small cell types.[4]

Bleomycin, a radiomimetic agent has been reported to treat head and neck, squamous cell carcinomas, testicular cancer, Hodgkin's lymphoma, various squamous cell carcinomas, and malignant effusions in ovarian and breast cancers.[5] Doxorubicin has been used to treat several human carcinomas of the endothelium, testes, prostrate, cervix, head and neck, plasma cell myeloma.[6] Hodgkin's lymphoma, leukemia and lymphomas acute lymphoblastic leukemia, acute myeloblastic leukemia.[7] The vinca alkaloids like vincrisitne vinblastine and vindesine have been used to treat a variety of tumors including hematological malignancies, Hodgkins and non-Hodgkins lymphomas, Wilm's tumour, neuroblastoma, brain tumours, rhabdomyosarcoma, carcinomas of the breast, bladder and the male and female reproductive systems.[8] Paclitaxel and docetaxel, the microtubule depolymerizing agents are being used to treat ovarian, breast, lung, head and neck, testicular, bladder, esophageal and. gastrointestinal cancers, adenocarcinoma and squamous-cell carcinoma.[9] Topoiomerase inhibitors like topotecan and irenotecan have also found place in chemotherapy of metastatic ovarian carcinoma and small cell lung cancer.[10]

The natural products have been the source for many of the drugs that are used currently for the treatment of various disorders in humans. There has been an increased interest over the past few years to investigate natural materials as a source of potential, new novel chemotherapeutic agents. This approach is pursued in the hope of getting new bioactive molecules through combinatorial chemistry and computerized molecular modeling.[11] The plants have provided raw materials for various chemotherapeutic drugs before their chemical synthesis. Some of the important drugs derived from various plant and natural sources are bleomycins, doxorubicin, vinca alkaloids, epipodophyllotoxins, camptothecins and taxols.[12] Although these drugs have been very successful in treating various kind of neoplasia they induce adverse effects like myelosuppression, gastrointestinal, hair follicle damage, reproductive and nephrotoxicities and even second malignancies. Therefore, it is necessary to identify newer biomolecules that can effectively kill cancerous cells but spare or have negligible toxicity on the normal cells.

Alstonia scholaris R. Br. (family Apocynaceae) commonly known as sapthaparna, has been used in the Indian system of medicine for the treatment of several disorders. Its ripe fruits have been used to treat syphilis, insanity and epilepsy. It is also used as a tonic, antiperiodic and anthelmintic. The milky juice of Alstonia scholaris is able to treat ulcers.[13] It is a bitter tonic, alternative and febrifuge and is reported to be useful in the treatment of malaria, diarrhea and dysentery.[14] Similarly, its stem bark extract has been shown to increase the effect of cyclophosphamide in vivo[15] and reduce tumor occurrence in benzo-a-pyrene-induced chemical carcinogenesis studies.[16] Our earlier study has shown that chloroform extract fraction of 85% ethanol extract of Alstonia scholaris exhibited highest cytotoxic effects than the other extracts.[17] Therefore, present study was undertaken to evaluate the antineoplastic activity of isolated bioactive molecule Echitamidine-N-Oxide-19-0-β-D-Glucopyranoside in cultured KB cells from the chloroform fraction of Alstonia scholaris.

MATERIALS AND METHODS

Chemicals

Eagle's minimum essential medium (MEM), fetal calf serum (FCS), L-glutamine, low melting agarose (LMA, CAS No. A-4718, Lot 111K1532), acridine orange, ethidium bromide, EDTA, Trizma base, RNAase, proteinase K, Triton X-100 and cytochalasin-B were purchased from Sigma Aldrich Chemicals Private Limited, Bangalore, India. The doxorubicin (DOX) was purchased from Dabur Pharmaceuticals-Oncology division, India,

whereas all other chemicals including NaCl, Na_2EDTA, NaOH, formaldehyde, isopropanol, ethanol, DMSO, methanol, acetic acid, K_2HPO_4 and Na_2HPO_4 were procured from Ranbaxy Fine Chemicals, Mumbai, India.

Isolation Of Eog From Alstonia Scholaris

The EOG was isolated from the chloroform fraction of 85% ethanol extract of Alstonia scholaris R. Br. a Medicinal plant of family Apocyanacea. Briefly, the powdered dried bark was extracted with 85% ethanol at room temperature and evaporated in vacuo and the extract was suspended in distilled water, further extracted with hexane, chloroform, ethyl acetate and n-butanol in succession. The solvent was evaporated from chloroform fraction partitioned between 3% HCl and ethanol, the aqueous layer basified to pH 10 by addition of NaOH and extracted once again with $CHCl_3$ so as to give crude alkaloidal fraction. The alkaloidal fraction was subjected to column chromatography, HPTLC, and HPLC for the isolation of indole alkaloids. The glycosidic indole alkaloid obtained was identified as EOG (Echitamidine-N-Oxide-19-0-β-D-Glucopyranoside) by UV, IR, NMR and MASS spectroscopy.[18]

Preparation Of The Drug

The EOG is insoluble in water therefore, a known quantity was dissolved in DMSO and diluted with MEM. The quantity of DMSO did not exceed more than 4 µg/ml.

CELL LINE AND CULTURE

KB (Human carcinoma of buccal mucosa) cells, with a doubling time of 20±2 h, procured from National Centre for Cell Science, Pune, India, were used throughout the study. The cells were routinely grown in 25 cm^2 culture flasks (Techno Plastic Products, Trasadingën, Switzerland) with loosened caps containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 µg/ml gentamicin sulfate in an atmosphere of 5% CO_2 in humidified air in a CO2 incubator at 37°C (NuAir, Plymouth, USA).

Evaluation Of Optimum Treatment Time

A fixed number (5×10^5) exponentially growing cells were seeded into several culture flasks (Techno Plastic Products, Trasadingën, Switzerland) and were allowed to attach for 48 h and the cultures were divided into the following groups:

EOG GROUP

This group of cells was treated with 0, 1, 5, 10, 20, 30, 40, 50 or 100 µg/ml EOG for 1, 2, 4, 6, 8, 12, 16 or 24 h.

DOX GROUP

The cells of this group were treated with 0, 2, 4, 6, 8, 10, 12, 14 or 16 µg/ml doxorubicin for 1, 2, 4, 6, 8, 16 or 24 h and served as a positive control group.

CLONOGENIC ASSAY

The clonogenic potential of cells after EOG or DOX treatment was evaluated as described earlier.[19] Briefly, the drug containing media was removed at 1, 2, 4, 6, 8, 16 or 24 h and the cells were dislodged by trypsin EDTA treatment. 200-300 cells were plated on to several individual culture dishes (Techno Plastic Products, Trasadingën, Switzerland) containing 5 ml drug free medium in triplicate for each drug concentration for each group and incubated until 11 days when the experiment was terminated. The cells were stained with 1 % crystal violet in methanol. The colonies of cells were scored using stereozoom microscope (Ernst Wild M3, Wild Heerbrugg Ltd., Heerbrugg, Switzerland). The clones containing a minimum of 50 or more cells were scored as a colony. Plating efficiency of the cells was determined and surviving fraction (SF) calculated. The data were fitted on to linear quadratic model using Microcal statistical software (Microcal Software Inc., Northempton, MA, USA)

Micronucleus Assay

The cells remaining after clonogenic assay in the group receiving EOG treatment for six hours were used for micronucleus assay, where 3 x 10^5 cells were inoculated in triplicate for each EOG concentration. The micronuclei were prepared according to the modified method. [20] Briefly, the cells were allowed to attach for 6 h and thereafter treated with 3 µg/ml of cytochalasin-B to inhibit cytokinesis. The cells were left undisturbed and allowed to grow for another 20, 30 and 40 h, depending on the assay time. The cell cultures were terminated at 20, 30 and 40 h post-drug-treatment. The media containing cytochalasin-B was removed, the cells were washed with PBS, dislodged by trypsin EDTA treatment and pelleted by centrifugation at 1000 rpm for 5 min. The cell pellet was disturbed and subjected to mild hypotonic treatment (0.75% ammonium oxalate) at 37°C, centrifuged again and the resultant cell pellet was fixed in Carnoy's fixative 3:1 (methanol:acetic acid). After centrifugation, the cells were resuspended in a small volume of fixative and spread on to pre cleaned coded slides to avoid observer's bias. The slides containing cells were stained with 0.125% acridine

orange (BDH, England, Gurr Cat. no. 34001 9704640E) in Sorensen's buffer (pH 6.8) and subsequently washed twice in the buffer to remove excess stain. The slides mounted in Sorensen's buffer were observed under a fluorescent microscope (Carl Zeiss. Photomicroscope III, Oberkochen, Germany) equipped with 450–490 nm BP filter set with excitation at 453 nm using a 40 X Neofluar objective. A minimum of one thousand binucleate cells with well-preserved cytoplasm was scored for each concentration and the frequency of micronucleated binucleate cells (MNBNC) was determined. The micronucleated cells were scored according to the criteria of.[21]

Effect Of Eog On Dna Damage

A separate experiment was conducted to evaluate the effect of EOG on DNA damage, where grouping and other conditions were essentially similar as described above, except that the cells were exposed to EOG for 1 hour and the comet assay was performed at 2, 4, 6 or 8 h post-EOG treatment.

Alkaline Comet Assay

The technique described for alkaline comet assay was followed with minor modification as described by.[22] Briefly, Axiva slides (Axiva Sichem Pvt. Ltd., New Delhi, India, Cat. No. 450100F) were covered with 100 µl of 0.6% LMA prepared in Ca^{++} and Mg^{++}-free PBS at 37°C and the agarose was allowed to solidify under a cover slip on ice after which the cover slips were removed. 1 x 10^5 cells treated with EOG and DMSO in 1 ml MEM were centrifuged at 1500 rpm for 5 min. The slides embedded with cells, were placed in cold lysis buffer containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, pH 10.0 and 1% Triton X-100 (added a fresh) to solubilize cellular proteins leaving DNA as nucleioids, at 4°C for 2 h. After cell lysis, the lysis buffer was drained off from the slides, and the slides were placed into a horizontal gel electrophoresis tank filled with fresh electrophoresis buffer containing 300 mM NaOH, 1 mM Na_2EDTA (pH 13.0) to a level of ~0.25 cm above the slides. Slides were kept in the buffer for 20 min to allow DNA unwinding and then the electrophoresis was carried out for 20 min at 25 V and 300 mA in cold. The slides were drained and flooded slowly with three changes of neutralization buffer (0.4 M Trizma base, pH 7.5) for 5 min each and subsequently stained with 50 µl of ethidium bromide (2 mg/ml) and covered with a cover slip for immediate analysis.

Ethidium bromide stained DNA on each slide was visualized as "comets" with a fluorescent head and a tail at 40 X magnification using epifluorescence microscope. Olympus BX51,

(Olympus Microscopes, Tokyo, Japan) equipped with a 515-535 nm excitation filter, a 590 nm barrier filter, and a CCD camera (Cool SNAP-Proof Digital Color Camera Kit Ver 4.1, Media Cybergenetics, Silver Spring, Maryland, USA) was used to capture the comet images. One hundred comets per slide and a minimum of 400 comets per drug concentration per assay time were analysed to give a representative result of the cell population.[23] The comets thus captured were analysed using Komet Software (Version 5.5, Kinetic Imaging Ltd, Bromborough, UK). The mean olive tail moment (OTM) was selected as the parameter that best reflects DNA damage (defined as the distance between the profile centres of gravity for DNA in the head and tail). The data regarding OTM, head and tail DNA were collected from three independent experiments, each containing quintuplicate measures and presented as mean ± SEM.

APOPTOSIS

The cells were labeled using the nucleic acid-binding dye mix of 100 µg/ml acridine orange and 100 µg/ml ethidium bromide (Sigma Chemical Co.) in PBS. The cells were examined under a fluorescence microscope. The viable cells were recognized by green fluorescent nuclei with organized structure, whereas the early apoptotic cells showed highly condensed or fragmented yellow chromatin in the nuclei. The membrane blebbing was exhibited by Apoptotic cells. The late apoptotic cells had orange chromatin with nuclei that were highly condensed and fragmented. The necrotic cells had bright orange chromatin in round nuclei. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, nonbiased manner. [24] For each sample, at least 10000 cells were counted, and the percentage of apoptotic cells was determined as follows:

$$\text{Apoptotic cells (\%)} = \frac{\text{Number of apoptotic cells scored}}{\text{Total number of cells counted}} \text{ X } 100$$

STATISTICAL ANALYSIS

The statistical significance between the treatments was analysed using one-way ANOVA for SF, micronucleus assay as well as for apoptotic indices. Appropriate post -hoc tests were used for multiple comparisons. The data were confirmed by repetition of the experiments. Test of homogenity was applied to determine any statistical differences between the repeat experiments if any. Since no significant differences were observed between the repeat experiments, data were combined and presented as tables and figures. Solo 4 (BMDP Statistical Software Inc., Los Angeles, CA) was used for data analyses. The comet assay results were analysed using the

regression analysis (Systat Software, Systat, Evanston, IL, USA), where various concentrations of EOG and Olive tail moment (OTM) coefficient were plotted on X and Y axes, respectively.

RESULTS

The results are expressed as surviving fraction (SF), micronuclei frequency per thousand cells, % of apoptotic cells, Olive tail moment (OTM), and head and tail DNA (%), ± SEM (standard error of the mean) in Tables 1-4 and Figures 1-6.

Evaluation of optimum treatment time

Clonogenic assay

An experiment was conducted to evaluate the cytotoxic effect of 4 µg/ml DMSO on KB cells. Briefly, KB cells were treated with 4 µg/ml DMSO for 1, 2, 4, 6, 8, 12, 16 or 24 h. The results of clonogenic assay showed no significant differences between the untreated and DMSO treated cultures (data not shown) therefore all experiments were carried out by dissolving drug into DMSO, where the concentration of DMSO never exceeded 4 µg/ml.

Exposure of KB cells to different concentrations of EOG for 0, 1, 2, 4, 6, 8, 12, 16 or 24 h resulted in a time and concentration dependent decline in the clonogenicity of cells. The cells exposed to different concentrations of EOG showed 50% reduction in the surviving fraction at 32 µg/ml. When the cells were exposed to different concentrations of EOG for 6h, the IC_{50} was found to be 25 µg/ml however, the surviving fraction continued to reduce significantly with increasing EOG concentration and a nadir in cell survival was reached at 100 µg/ml the last concentration evaluated (Fig. 1). The SF also showed a treatment time dependent decline until 6 h, thereafter the cell killing effect plateaued (Fig. 1, Table 1). Since the maximum cell killing effect was observed for 6 h EOG exposure, this treatment time was considered as an optimum treatment time and further studies were undertaken using this time. Likewise, exposure of KB cells to 0, 2, 4, 6, 8, 10, 12 or 14 µg/ml doxorubicin for various times also reduced the surviving fraction in a concentration dependent manner and a highest reduction of cell survival was observed after 8 h treatment for all concentrations of DOX, after that the decrease in the cell survival was not statistically significant (Table 1). The IC_{50} value for DOX was found to be 10 µg/ml when the DOX was available for 8 h to KB cells .

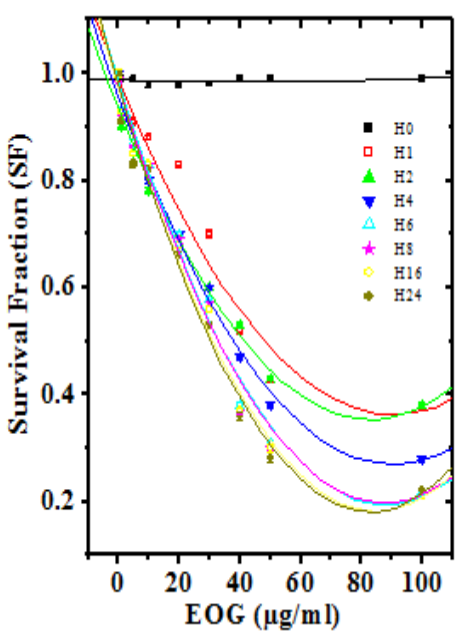
Table:1 Surviving Fraction (Sf) Of Kb Cells Pretreated With Various Concentrations Of Eog Or Dox For Different Times

Treatment (µg/ml)		Surviving fraction ± SEM							
		Treatment duration (hours)							
		0h	1h	2h	4h	6h	8h	16h	24h
EOG	0	0.99±0.006	0.99±0.006	0.99±0.006	1.00±0.005	0.99±0.004	0.99±0.005	1.00±0.006	1.00±0.004
	1	0.99±0.005[a]	0.93±0.005[a]	0.90±0.005[a]	0.92±0.004[a]	0.93±0.005[a]	0.92±0.006[a]	0.93±0.004[a]	0.91±0.005[a]
	5	0.99±0.004[a]	0.91±0.006[a]	0.85±0.004[a]	0.86±0.005[a]	0.87±0.005[a]	0.86±0.005[a]	0.85±0.005[a]	0.83±0.006[a]
	10	0.98±.0006[a]	0.88±0.005[a]	0.78±0.006[a]	0.80±0.004[a]	0.83±0.004[a]	0.83±0.006[a]	0.83±0.005[a]	0.82±0.004[a]
	20	0.98±0.005[a]	0.83±0.006[a]	0.70±0.004[a]	0.70±0.005[a]	0.70±0.005[a]	0.69±0.005[a]	0.68±0.006[a]	0.66±0.006[a]
	30	0.98±0.004[b]	0.70±0.006[b]	0.60±0.005[a]	0.6±0.004[a]	0.58±0.004[b]	0.57±0.004[a]	0.56±0.005[a]	0.53±0.005[a]
	40	0.99±0.006[a]	0.52±0.005[a]	0.53±0.006[a]	0.47±0.005[a]	0.38±0.006[a]	0.37±0.006[a]	0.37±0.004[a]	0.36±0.006[a]
	50	0.99±0.005[a]	0.43±0.006[a]	0.43±0.004[a]	0.38±0.006[a]	0.31±0.004[a]	0.30±0.006[a]	0.30±0.006[a]	0.28±0.007[a]
	100	0.99±0.004[b]	0.38±0.005[a]	0.38±0.005[a]	0.28±0.005[a]	0.22±0.005[a]	0.22±0.005[a]	0.21±0.004[a]	0.22±0.005[a]
DOX	0	1.00±0.003[a]	0.99±0.005[a]	1.00±0.005[a]	1.00±0.005[a]	0.99±0.004[a]	1.00±0.006[a]	0.99±0.005[a]	0.99±0.005[a]
	2	0.98±0.004[a]	0.95±0.006[a]	0.91±0.004[a]	0.89±0.006[a]	0.85±0.005[b]	0.84±0.005[b]	0.83±0.006[a]	0.82±0.004[a]
	4	0.99±0.006[a]	0.91±0.005[a]	0.87±0.005[a]	0.83±0.005[a]	0.80±0.004[a]	0.81±0.006[a]	0.81±0.005[a]	0.81±0.006[a]
	6	0.99±0.005[b]	0.87±0.004[a]	0.78±0.006[a]	0.74±0.006[a]	0.70±0.006[a]	0.69±0.005[a]	0.67±0.006[b]	0.66±0.004[a]
	8	0.99±0.006[a]	0.79±0.005[a]	0.73±0.005[a]	0.69±0.004[a]	0.64±0.004[a]	0.62±0.007[a]	0.62±0.004[a]	0.62±0.005[a]
	10	1.00±0.004[a]	0.71±0.004[a]	0.63±0.006[a]	0.59±0.005[a]	0.53±0.006[a]	0.50±0.006[b]	0.49±0.005[a]	0.46±0.006[a]
	12	0.99±0.005[a]	0.61±0.005[a]	0.54±0.004[b]	0.50±0.006[b]	0.48±0.004[a]	0.45±0.007[a]	0.43±0.006[a]	0.42±0.004[a]
	14	0.99±0.006[b]	0.53±0.006[b]	0.45±0.005[a]	0.42±0.006[a]	0.38±0.005[a]	0.37±0.005[a]	0.34±0.004[a]	0.33±0.006[a]
	16	1.00±0.004[a]	0.49±0.005[a]	0.38±0.006[a]	0.32±0.005[a]	0.29±0.006[a]	0.27±0.006[a]	0.25±0.006[a]	0.24±0.005[b]

EOG = (ECHITAMIDINE-N-OXIDE-GLUCOPYRANOSIDE) DOX=DOXORUBICIN, A POSITIVE CONTROL; AND SEM= STANDARD ERROR OF THE MEAN P < A = 0.05, B = 0.01, C = 0.001, NO SYMBOLS = NOT SIGNIFICANT WHEN COMPARED WITH THE CONCURRENT NON-DRUG TREATED GROUP PLATING EFFICIENCY (PE) AND SURVIVING FRACTION (SF) WERE CALCULATED AS FOLLOWS.

PE = (NUMBER OF COLONIES COUNTED X 100) ÷ (NUMBER OF CELLS SEEDED)

SF = {NUMBER OF COLONIES COUNTED} ÷ {(NUMBER OF CELLS SEEDED) X (MEAN PLATING EFFICIENCY)}



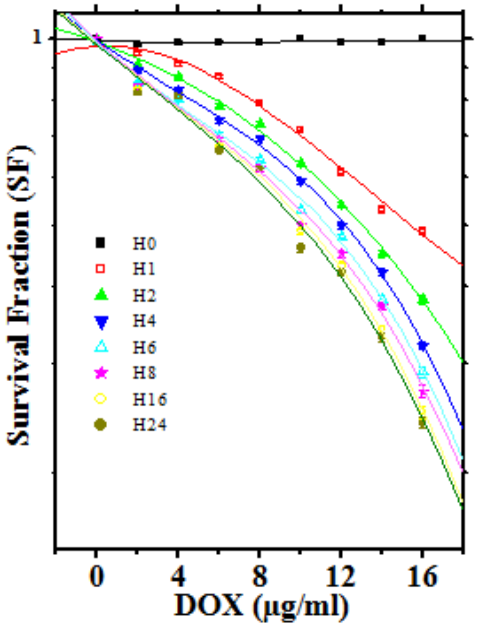


Figure 1a **Figure 1b**

Figure:1 influence of exposure time on the survival of kb cells treated with various concentrations of eog (echitamidine-n-oxide-glucopyranoside) or dox (doxorubicin).

CLOSED SQUARES 0 H; OPEN SQUARES 1 H, CLOSED TRIANGLES 2 H, DOWN CLOSED TRIANGLES 4 H, OPEN TRIANGLES 6 H, CLOSED STAR 8 H, OPEN CIRCLE 16 H, CLOSED CIRCLE 24 H. LEFT EOG AND RIGHT DOX.

Micronucleus assay

Exposure of KB cells to various concentrations of EOG resulted in a concentration-dependent elevation in the frequency of MNBNC at all the post-treatment times (Table 2; Fig 2). The frequency of MNBNC increased significantly at 30 and 40 h post-treatment for all the concentrations of EOG, whereas this increase was nonsignificant for 5 and 10 µg/ml EOG at 20 h post-treatment. The frequency of one MNBNC increased in a concentration dependent manner in cell treated with 10-100 µg/ml EOG at all post-treatment times studied (Fig 2) and this increase in the MNBNC occurrence was significantly greater than non-drug treated control (0 concentration). Similarly, the frequency of cells bearing two MN showed a trend similar to that of one MN except that the frequency of MNBNCs was lower. The frequency of cells with multiple (> two MN) MN elevated significantly in a concentration dependent manner at all post-treatment times evaluated (Fig 2), except at low concentrations, where this rise in multiple MNBNC was statistically non-significant (Table 2).

The increase in the frequencies of cells bearing one, two and multiple MN fluctuated with scoring time. It increased as early as 20 h and reached a peak 30 h post-EOG treatment and declined thereafter (Table 2). Despite the fact that there was a decline in the frequencies of one, two and multiple MN at 40 h, their frequencies were higher when compared with 20 h post-EOG treatment (Fig 2; Table 2). The curve fitting of all MN data showed a non linear response (Fig 2).

Table:2 Effect Of Various Concentrations Of Eog (Echitamidine-N-Oxide-Glucopyranoside) On The Micronuclei Induction In Kb Cells At Different Post-Treatment Times

EOG (µg/ml)	Frequency of micronucleated binucleate cells per 1000 ± SEM											
	20 h				30 h				40 h			
	One MN	Two MN	Multiple MN	Total MN	One MN	Two MN	Multiple MN	Total MN	One MN	Two MN	Multiple MN	Total MN
0	1.78±0.38	0.43±0.04	0.3±0.03	2.51±0.54	2.34±1.45	0.67±0.04	0±0.03	3.01±1.24	2.33±0.54^{a}	0.67±0.03	0.33±0.03	3.33±0.95
1	7.34±0.58^{c}	0.86±0.07^{c}	0.3±0.03^{c}	8.5±0.97^{b}	17.49±0.79^{a}	1.04±0.12^{a}	1.33±0.08^{c}	19.86±1.95^{a}	11.32±0.74^{a}	0.98±0.22^{a}	0.66±0.03^{b}	12.96±1.32^{a}
5	12.43±0.67^{c}	1.38±0.12^{c}	0.82±0.05^{c}	14.63±1.23^{b}	22.48±1.45^{c}	2.24±0.28^{b}	1.66±0.11	26.38±2.18^{a}	15.39±0.38^{a}	1.88±0.17^{a}	1±0.06^{b}	18.27±2.54^{a}
10	14.37±1.34^{a}	2.46±0.26^{c}	1±0.06^{c}	17.83±2.24^{b}	36.28±2.98^{c}	3.87±0.37^{b}	2±0.33^{b}	42.15±3.42^{b}	25.38±1.54^{c}	2.97±0.28^{b}	1.66±0.33^{c}	30.01±3.15^{a}
20	18.34±2.18^{c}	3.62±0.33^{c}	1.66±0.08^{c}	23.62±2.39^{b}	53.24±4.34^{a}	5.45±0.69^{b}	2.66±0.33^{a}	61.35±4.56^{b}	35.64±2.98^{c}	4.27±0.32^{a}	2±0.33^{b}	41.91±3.69^{a}
30	25.35±2.94^{a}	4.78±0.37^{a}	2±0.33^{a}	32.13±2.98^{b}	73.26±4.83^{a}	7.21±0.86^{c}	3.33±0.66^{a}	83.8±5.13^{b}	45.92±2.65^{a}	6.23±0.52^{a}	2.33±0.66^{a}	54.48±4.12^{a}
40	32.36±2.34^{a}	5.78±0.41^{b}	2.33±0.66^{a}	40.47±3.48^{b}	82.88±5.34^{b}	8.67±0.83^{c}	4±0.66^{a}	95.55±5.54^{b}	56.76±3.12^{a}	7.12±0.65^{a}	3.06±0.66^{b}	66.94±4.29^{a}
50	39.54±3.27^{b}	6.98±0.48^{a}	2.88±0.33^{a}	49.4±3.92^{a}	94.23±5.38^{a}	9.98±0.93^{a}	4.66±0.66^{a}	108.87±5.94^{a}	61.24±4.39^{b}	8.45±0.72^{a}	3.33±0.66^{a}	73.02±4.65^{a}
100	48.34±3.54^{a}	7.34±0.63^{a}	3.33±0.66^{a}	59.01±4.23^{a}	104.98±6.23^{a}	12.12±0.98^{a}	5.33±0.66^{a}	122.43±6.13^{a}	73.33±5.82^{a}	9.23±0.84^{a}	4±0.88^{b}	86.56±5.16^{a}
r	0.98	0.98	0.98	0.97	0.97	0.98	0.97	0.96	0.98	0.98	0.97	0.97

P < A = 0.05, B = 0.01, C = 0.001, NO SYMBOLS = NOT SIGNIFICANT WHEN COMPARED TO NON-DRUG TREATED CONTROL. EOG (ECHITAMIDINE-N-OXIDE-GLUCOPYRANOSIDE) SEM = STANDARD ERROR OF THE MEAN; MN = MICRONUCLEI; R^2 = COEFFICIENT OF CORRELATION

A. ONE MN B. TWO MN C. MULTIPLE MN; D. TOTAL MN. CLOSED SQUARES, 20 H; OPEN CIRCLES, 30 H; OPEN TRIANGLES, 40 H POST-TREATMENT TIMES

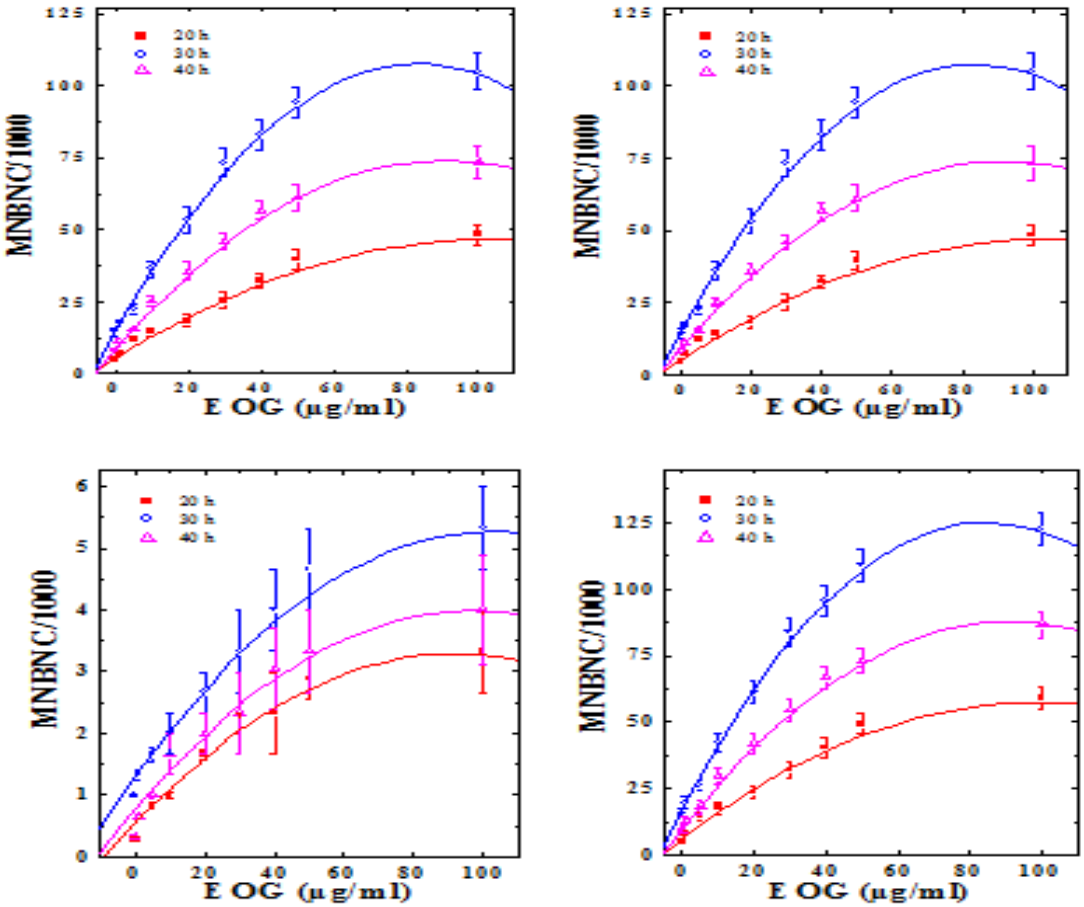


Figure:2 effect of various concentrations of eog (echitamidine-n-oxide-glucopyranoside) on the micronuclei formation in kb cells at different post-treatment times.

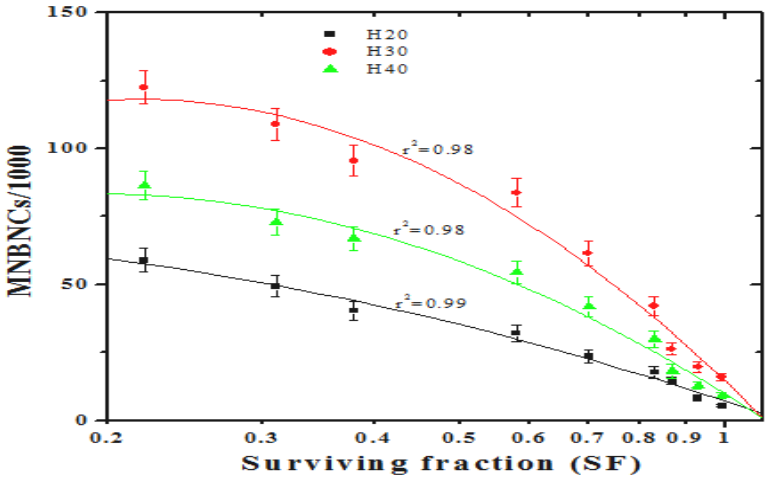


FIGURE 3THE CORRELATION BETWEEN THE SURVIVING FRACTION AND MICRONUCLEI FREQUENCY IN KB CELLS TREATED WITH EOG (ECHITAMIDINE-N-OXIDE-GLUCOPYRANOSIDE).

ASSESSMENT OF DNA DAMAGE

Treatment of KB cells with various concentrations of EOG showed a concentration dependent elevation in the DNA damage expressed as Olive tail moment (OTM, the product of tail length and DNA content in the tail) at various treatment times. The OTM and amount of tail DNA in the KB cells receiving various concentrations of EOG increased in a concentration dependent manner and the maximum DNA damage was induced by 100 µg/ml EOG. Similarly, the DNA damage also increased with assessment time dependent manner, where a significant rise in OTM was observed as early as 2 h post-EOG treatment, which continued to rise with time and a maximum DNA damage was discerned at 6 h post-irradiation for all concentrations of EOG studied (Table 3 and Fig 4). Thereafter, increase in DNA damage was marginal but non-significant as indicated by OTM and tail DNA at 8 h post-EOG treatment (Fig.4).

BIOLOGICAL RESPONSE

The biological response was evaluated by correlating the cell survival and micronuclei induction or olive tail moment, where the surviving fraction was plotted on X-axis and micronuclei frequency or olive tail moment on Y-axis. The dose response relationship was non-linear ($p < 0.001$) for all post-EOG treatment times (Figs 3&5). The cell survival declined with the increasing micronuclei frequency and DNA damage expressed as olive tail moment indicating an inverse correlation between the surviving fraction and micronuclei formation or DNA damage (Figs 3&5)

TABLE 3 ASSESSMENT OF DNA DAMAGE AT VARIOUS POST TREATMENT TIMES IN KB CELLS EXPOSED TO DIFFERENT CONCENTRATION OF EOG (ECHITAMIDINE-N-OXIDE-GLUCOPYRANOSIDE)

EOG (µg/ml)	DNA damage (mean± SEM)														
	Assessment time (hours)														
	0			2			4			6			8		
	Head DNA	Tail DNA	Olive tail moment	Head DNA	Tail DNA	Olive tail moment	Head DNA	Tail DNA	Olive tail moment	Head DNA	Tail DNA	Olive tail moment	Head DNA	Tail DNA	Olive tail moment
0	98.33± 0.078	1.67± 0.087	0.856±0. 043	97.56± 0.129	2.44±0. 21	0.845±0.0 78	98.54± 0.549	1.46±0. 48	0.897±0 .045	97.53 ±0.23	2.47± 0.32	0.878±0 .076	96.49± 0.73[a]	3.51± 0.62[a]	0.856±0 .043[a]
1	98.48± 0.956	1.52± 0.098	0.832±0. 054	94.32± 0.23[a]	5.68±0. 35[a]	1.34±0.08 9[c]	90.43± 0.64[a]	9.57±0. 38[a]	2.67±0. 067[c]	85.12 ±0.92[a]	14.88 ±0.32[a]	3.98±0. 056[a]	74.38± 0.83[a]	25.62 ±0.83[a]	4.12±0. 054[a]
5	97.43± 0.087	2.57± 0.089	0.845±0. 045	93.43± 0.24[a]	6.57±0. 43[a]	2.16±0.06 5[a]	89.78± 0.78[a]	10.22±0 .74[a]	3.98±0. 078[a]	84.39 ±0.74[a]	15.61 ±0.48[a]	5.98±0. 043[c]	73.29± 0.63[a]	26.71 ±0.73[a]	5.99±0. 078[a]
10	97.21± 0.095	2.79± 0.094	0.863±0. 056	92.43± 0.25[a]	7.57±0. 36[a]	3.67±0.07 6[c]	87.67± 0.56[a]	12.33±0 .62[a]	4.63±0. 075[a]	83.29 ±0.56[a]	16.71 ±0.52[a]	7.45±0. 067[b]	72.17± 0.82[a]	27.83 ±0.78[a]	7.67±0. 034[c]
20	96.48± 0.078	3.52± 0.096	0.819±0. 052	91.43± 0.27[a]	8.57±0. 42[a]	4.98±0.06 5[b]	86.49± 0.83[a]	13.51±0 .83[a]	7.38±0. 073[c]	83.11 ±0.47[a]	16.89 ±0.51[a]	9.67±0. 078[a]	71.99± 0.68[a]	28.01 ±0.83[a]	9.89±0. 054[a]
30	97.89± 0.059	2.11± 0.089	0.843±0. 058	90.43± 0.26[a]	9.57±0. 38[a]	7.98±0.08 9[b]	85.49± 0.48[a]	14.51±0 .58[a]	8.98±0. 098[c]	82.19 ±0.63[a]	17.81 ±0.38[a]	15.67±0 .087[c]	70.32± 0.39[a]	29.68 ±0.67[a]	15.86±0 .098[c]
40	97.78± 0.089	2.22± 0.078	0.841±0. 063	89.45± 0.29[a]	10.55± 0.35[a]	9.78±0.09 9[b]	84.39± 0.57[a]	15.61±0 .53[a]	12.65±0 .056[a]	81.54 ±0.49[a]	18.46 ±0.67[a]	18.43±0 .098[b]	69.38± 0.78[a]	30.62 ±0.87[a]	18.65±0 .067[a]
50	98.48± 0.097	1.52± 0.079	0.854±0. 067	87.65± 0.35[a]	12.35± 0.42[a]	13.67±0.0 78[c]	83.28± 0.38[a]	16.72±0 .48[a]	15.84±0 .082[c]	80.43 ±0.83[a]	19.57 ±0.52[a]	23.67±0 .074[a]	68.33± 0.57[a]	31.67 ±0.67[a]	24.54±0 .089[c]
100	97.89± 0.089	2.11± 0.096	0.893±0. 064	86.59± 0.32[a]	13.41± 0.49[a]	16.67±0.0 95[a]	82.19± 0.57[a]	17.81±0 .63[a]	20.54±0 .093[a]	79.32 ±0.28[a]	20.68 ±0.39[a]	31.89±0 .068[c]	67.32± 0.87[a]	32.68 ±0.76[a]	32.11±0 .085[a]

P < A = 0.05, B = 0.01, C = 0.005 (WHEN COMPARED WITH THE CONCURRENT DMSO GROUP); NO SYMBOLS = NOT-SIGNIFICANT

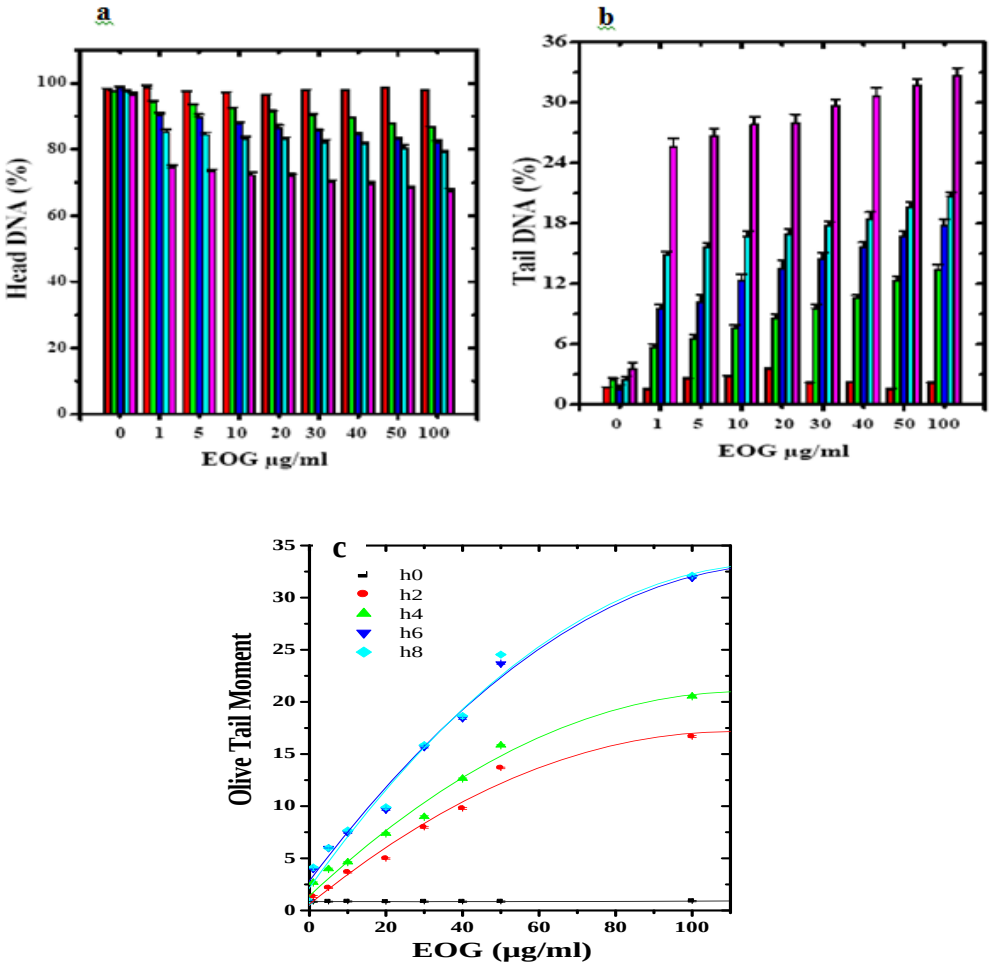


Figure 4 assessment of dna damage in kb cells exposed to different concentrations of eog (echitamidine-n-oxide-glucopyranoside) at various post eog treatment times.

A) HEAD DNA; B) TAIL DNA AND C) OLIVE TAIL MOMENT. RED BARS 0 H; GREEN BARS 2H; BLUE 4 H; CYAN 6 H AND PINK BARS 8 H

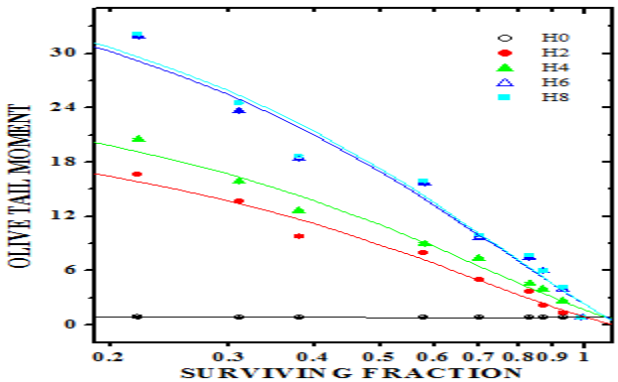


Figure 5the correlation between the surviving fraction and olive tail moment in kb cells treated with eog (echitamidine-n-oxide-glucopyranoside).

OPEN CIRCLES 0 H; CLOSED CIRCLES 2 H; CLOSED TRIANGLES 4 H ;OPEN TRIANGLES 6 H AND CLOSED SQUARES 8 H.

APOPTOSIS

Treatment of KB cells with 0, 1, 5, 10, 20 ,30 ,40 ,50 or 100 µg/ml EOG induced apoptosis in a concentration dependent manner. The microscopic examination of KB cells showed cell shrinkage, membrane blebbing and apoptotic bodies. The induction of apoptosis depended on EOG or DOX concentration. The apoptotic index increased in a concentration dependent manner in cells receiving EOG or DOX and the apoptosis induction was 3 times higher at 20 µg/ml EOG when compared to 1 µg/ml (Table 4). When the concentration of EOG was increased further, the amount of apoptosis induction also elevated and a 6 fold increase in the induction of apoptosis was observed in the cells treated with 100 µg/ml when compared to 1 µg/ml EOG (Fig 6). The induction of apoptosis was greater in EOG treated cells when compared to doxorubicin treated cells (Fig 6). Despite this increase the difference between EOG and DOX treament was statistically non- significant (Table 4).

Table: 4 Induction Of Apoptosis In Kb Cells Exposed To Different Concentrations Of Eog (Echitamidine-N-Oxide-Glucopyranoside) Or Dox (Doxorubicin)

Concentration (µg/ml)	EOG	DOX
0	2.76±0.34	2.45±0.25
1	4.23±0.67[a]	3.78±0.37[a]
5	6.74±0.87[b]	6.32±0.41[b]
10	9.23±0.67[c]	8.56±0.52[a]
20	12.65±0.94[a]	11.43±0.64[a]
30	15.65±0.99[a]	14.23±0.75[a]
40	17.32±1.23[b]	16.76±0.89 [a]
50	20.21±1.43[a]	18.76±1.23[a]
100	27.32±1.65[a]	25.34±1.54[c]

P< A = 0.05, B = 0.01, C = 0.001 WHEN COMPARED WITH NON-DRUG TREATED DRUG CONTROL GROUP.

EOG = (ECHITAMIDINE-N-OXIDE-GLUCOPYRANOSIDE) SEM = STANDARD ERROR OF THE MEAN.

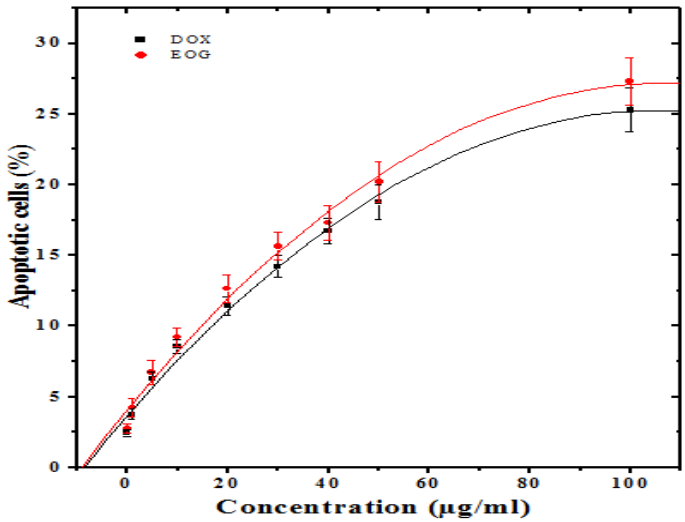


Figure 6 effect of eog (echitamidine-n-oxide-glucopyranoside) and dox (doxorubicin) on the induction of apoptotic cells in kb cells exposed to different concentrations.

CLOSED SQUARES; DOXORUBICIN, CLOSED CIRCLES EOG.

DISCUSSION

A number of active molecules with diverse chemical structures have been isolated from natural sources as anticancer agents.[25] The plant derived chemicals have provided several novel molecules to treat cancer including epipodophyllotoxins, vinca alkaloids, the taxanes and camptothecins. [26] These molecules have played an important role in the treatment of cancer clinically. However, toxicity to normal tissues is of major concern in the patient visiting for cancer treatment. Therefore there is always a need to evaluate newer paradigms to reduce the cytotoxicity to normal cells and increase the therapeutic efficacy in clinical condition. The present study has been undertaken to evaluate the cytotoxic effect of EOG, an indole alkaloid isolated from the bark of Alstonia scholaris R. Br. in cultured KB cells.

Treatment of KB cells with various concentrations of EOG reduced the cell survival in a concentration dependent manner. The literature survey has revealed that there are no reports on the cytotoxic effect of EOG and this is probably the first report on its cytotoxic effects. However, our earlier studies on the ethanol extract of stem bark of Alstonia scholaris and another monoterpene indole alkaloid echitamine chloride have been reported to increase the cytototoxic effect in a concentration dependent manner in invitro and invivo.[27] Cyanidin-3-O-beta-glucopyranoside, a natural compound showed significant cytotoxicity against the HepG2 and CaCo-2 cells. [28] Echitamine an alkaloid, isolated from the bark of Alstonia has

also been reported to have anticancer activity in rodent tumors. [29] Other isolated components from natural sources including doxorubicin, taxol and teniposide (VM-26) have been reported to induce cytotoxicity in V79 cells.[30] An identical effect has been observed with acyclovir and azidothymidine, the nucleoside analogues in the HeLa cells.[31] Sungucine and isosungucine, the two bisindole alkaloids isolated from the roots of the African plant Strychnos icaja Baillon have been reported not only to exhibit antiplasmodial activities but also cytotoxic effects against the human cancer cell lines.[32] [4-demethyl-picropodophyllotoxin - glucoside,induced apoptosis in various human cancer cells in a concentration dependent manner.[33]

I was interested to know how EOG induced its cytotoxic effects in the KB cells and therefore, it was decided to evaluate the DNA damage by studying micronuclei, comet assay and apoptosis. Exposure of KB cells to different doses of EOG showed a concentration dependent rise in the frequency of MNBNCs. EOG treatment not only induced cells bearing one micronuclei but also cells bearing more than one micronuclei (two and more) indicating that it is able to induce complex DNA damage and the observed effect may be due to production of damage to cellular DNA in the form of micronuclei. This induction of DNA damage in the form of micronuclei may have subsequently caused cell death. The dose response was non-linear. Although reports on the micronuclei induction by EOG are not available other plant derived molecules including vinblastine, vindesine, vincristine and taxole have been reported to induce micronuclei in a concentration dependent manner. [34] Similarly, treatment of HeLa cells with doxorubicin has been reported to increase the frequency of micronuclei in a concentration dependent manner. [35] The cells with MN can be regarded therefore, as having suffered damage in their reproductive integrity and may lose clonogenic potential [36] and this may be the one of the reasons of cytotoxic effects of EOG. The frequency of MNBNCs increased with time, and a peak was observed at 30 h post drug treatments and a decline thereafter. This was expected as the doubling time of KB cells 24 h and drug treatment may have caused delay in the progression of cell cycle. A similar effect has been observed earlier in HeLa cells treated with DOX or extract of Tinospora cordifolia.[37] A concentration dependent increase in micronuclei frequency has been reported in human lymphocytes receiving various concentrations of aqueous extract of Physalis angulata.[38]

This type of linear relationship is in good agreement with the earlier report of HeLa cells after treatment with various doses of acyclovir and azidothymidine.[39]

The induction of DNA damage by EOG was further confirmed by comet assay or single cell gel electrophoresis. The single cell gel electrophoresis assay developed by[40] is a useful tool to estimate DNA damage . The comet assay determines the amount of DNA damage (both single- and double-strand breaks and conformational changes), alkali labile sites and cross links in the cell exposed to DNA damaging agents after removing most of the non-DNA material and applying a weak electric field to the remaining DNA embedded in an agarose gel.[41] The whole cells are embedded in an agarose gel, lysed and treated in situ with alkali to render the DNA single stranded prior to running the gel. In an appropriate electrical field, the genomic DNA migrates out of the nucleus into the agarose and is then stained with the intercalating fluorescent dye, ethidium bromide, allowing visualization of the DNA. Viewed microscopically the combination of the DNA that has stayed within the confines of the nucleus and the 'tail' of DNA that has migrated makes individual cells look like comets. Quantitative microscopic evaluation is done by measuring the length and intensity of the comet in relation to the signal of the non migrating nuclear DNA in comparison with standards.[42]

Treatment of KB cells with different concentrations of EOG resulted in a concentration dependent increase in the DNA damage as revealed by a constant rise in the OTM and tail DNA. This DNA damage augumentation was not only concentration dependent but also assessment time dependent as it continued to increase with time and the maximum DNA damage could be observed at 8 h post-EOG treatment, although difference between 6 and 8 h was statistically non-significant. Bleomycin treatment has been reported to increase OTM and tail DNA up to 6 h post-treatment in V79 cells earlier.[43] The cells with damaged DNA show increased migration of fragments or unwound DNA (comet tail) from the nucleus (comet head), which may also be considered as DNA fragmentation with the necrotic/apoptotic death process [79, 80]. Migration of DNA into comet tails (% tail DNA) and increase in OTM has been found to be directly proportional to the DNA damage.[44]

The evaluation of apoptosis in KB cells treated with EOG or DOX showed a concentration dependent rise in the apoptosis and EOG treatment showed greater apoptois than the positive control DOX. This indicates that EOG induces apoptotic cascade to exert its cytotoxic effect. Anthocyanin, cyanidin-3-O-beta-glucopyranoside (Cy-g) has been reported to induce apoptosis in T-lymphoblastoid, as well as HL-60 promyelocytic cells.[45] Oxindole alkaloids from Uncaria tomentosa have also been reported to induce apoptosis in proliferating, G0/G1-arrested and bcl-2-expressing acute lymphoblastic leukaemia cells.[46] Likewise, pyrrolizidine alkaloid,

clivorine induced apoptosis in human normal liver L-02 cells and reduced expression of p53 protein.[47]

CONCLUSION

The cytotoxicity, generally means damage or loss of DNA in the form micronuclei, which could affect the clonogenic potential of cells.[48] The study of biological response indicates that EOG caused DNA damage in the form of strand breaks and micronuclei, which turned out to be lethal events causing cell death. The rise in DNA strand breaks and micronuclei caused a related increase in cell death, indicating an inverse correlation between genomic damage and cell survival. An inverse response between genomic damage in the form of DNA strand breaks and micronuclei has been reported earlier in V79 cells treated with belomycin.[43] The correlation between DNA damage and clonogenicity has been found to be non-linear and an identical response has been observed earlier in different mammalian cell lines earlier.[49] Likewise, A close correlation between cell survival and micronuclei-induction has been observed.[50] Studies on five cell lines revealed that there has been an excellent correlation between the micronuclei induction and cell survival in cell lines such as SHIN-3, DU-145 and CHO-K1, identical to that of the present study, whereas F9 and COLO 320DM cells did not show this correlation.[51] In contrast, there are a few studies, where no correlation between the cell survival and micronuclei formation was found.[52]

The exact mechanism of cytotoxic effects of EOG is not well understood. However, the micronuclei, and comet assay studies indicate that EOG intreacts with cellular DNA causing DNA strand breaks, which are subsequently converted into micronuclei after a subsequent cell division leading to cell death. The induction of apoptosis may be another mechanism by which EOG induced cell death. The apoptosis is an early event and immediate assesmment of apoptosis revealed that EOG efficiently induced apoptosis in a concentration dependent manner indicating that EOG may have also employed this pathway to cause cell death. Although no attempts were made to study the molecular mechanisms of action of EOG, induction of apoptosis and DNA damage indicate that EOG may have employed different molecular mechanisms to inflict its cytotoxic effects. EOG treatment may have inhibited the activation of topoisomerases and polymerase enzymes resulting in damaged DNA as result there has been formation of DNA strand breaks and subsequently micronuclei and cell death. This contention is supported by the presence of glucopyranoside in EOG ring similar to VM-26 or teniposide which is a known topoisomerase inhibitor. It may have inhibited the trasactivation

of NF-κB that may have orchestrated cytochrome-c release, transactivation of p53, PARP cleavage and activation of caspases which may have induced apoptosis and subsequently cell death. The EOG treatment induced DNA strand breaks, micronuclei and apoptosis causing cell death. These events may have been induced by stimulation of a cascade of interrelated molecular events by EOG.

The cytotoxic effect induced by EOG may be due the induction of DNA damage in the form of micronuclei, DNA strand breaks and apoptosis. The cytotoxicity in KB cells may have been induced by damaging the cellular genome by EOG this may have lead to a cascade of events leading to cell death. Although no attempt was made to dissect the molecular mechanisms, it is possible that EOG may have induced genes related to cell death and caused apoptosis of cells and subsequently the cell death.

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