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Assessment of molecular DNA damage by comet assay in HeLa cells exposed to rohituka, *Aphanamixis polystachya* (Wall.) R.N. Parker

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Abstract

The stem bark extract of rohituka, *Aphanamixis polystachya* Wall. Parker (family Meliaceae) used traditionally to treat spleen and liver tumors was evaluated for its DNA damaging ability in the HeLa cells. The cells were treated with various concentrations of chloroform fraction of the stem bark of rohituka (APE) for 2, 4, 6 or 8 h to find out the optimum exposure time. HeLa cells embedded in agarose were microelectrophoresed in alkaline conditions to visualize the DNA damage as comets and expressed as Olive Tail Moment (OTM). Incubation of HeLa cells with APE for 6 or 8 h increased the OTM when compared with 2 or 4 h treatment and no significant difference was observed between 6 or 8 h treatment duration as a result, 6 h was considered as an optimum treatment time. The DNA repair kinetics were studied at different post-treatment times in HeLa cells exposed to 5, 10, 25, 50, 75 or 100 µg/ml APE. APE treatment caused a significant rise in the DNA damage as indicated by increased OTM when compared with the dimethyl sulfoxide treatment ($p<0.01$). An approximate 7, 12, 18, 25, 34, 40-folds increase in baseline DNA damage was recorded in HeLa cells treated with 5, 10, 25, 50, 75 or 100 µg/ml APE in comparison with dimethyl sulfoxide treatment. When the APE treated cells were allowed to repair for various post-treatment times, a concentration-dependent inhibition in repair of DNA damage was observed. The repair of DNA damage was inversely related to APE concentration. A mild but non-significant repair of the damaged DNA was observed up to 2 h post-APE treatment, which remained unaltered thereafter. Exposure of HeLa cells to different concentrations of APE resulted in a concentration-dependent decline in the growth kinetics up to day 5 and 6, respectively followed by a reduction in the clonogenic potential of HeLa cells in a concentration dependent manner. Our study demonstrates that rohituka triggers DNA damage in HeLa cells at molecular level that may be the major cause of reduced clonogenicity and growth kinetics.

Keywords: *Aphanamixis polystachya*; HeLa cells; Comet assay; DNA damage; Growth kinetics;

Introduction

Use of plants and natural products for human healthcare originated when the humans faced with some ailments since the advent of human history. This has evolved into the sophisticated traditional medicine systems in Southeast Asia, China and Japan that have originated thousands of years ago in these regions. The Ayurveda, the healthcare system of India has its origin in Vedic times and it is at least five thousand years old [1]. The humans from different parts of the globe have positive attitude towards medicinal use of plants and natural products as they are time tested, their use is very old and do not possess known adverse side effects. The medicinal plants have been tested for their curing capacity for generations on humans, which also repose faith in their use for human healthcare [2]. This is the reason that an estimate by world health organization shows that approximately 80% of the global population use plant based traditional medicinal systems for healthcare in the era of modern medicine [3].

The plants have a very long history of use to cure cancer, when the cancer was not defined as it is in the modern context. However, many, if not all of such claims for their efficacy for cancer treatment should be viewed with skepticism because cancer, as a specific disease entity, is poorly defined in terms of folklore and traditional medicine. Cancer has been defined by these systems as a hard swelling, tumors, corns, calluses, abscesses, warts, polyps etc., which all may not be cancerous [4]. Despite this, several modern chemotherapeutic agents including, vinca alkaloids, vinblastine and vincristine have been isolated from the Madagascan periwinkle, *Catharanthus roseus* before their chemical synthesis actually begun. Similarly, other molecules like camptothecins, epipodophyllotoxins, taxols etc. have been isolated from plants [5]. Extracts from plants like *Tinospora cordifolia, Alstonia scholaris, Colocasia gigantea, Helicia nilagirica* and *Schima wallichii* have been reported to show a marked tumor inhibitory activity *in vitro* against HeLa cells [6-11]. Therefore, plants still form the source of new drugs for the cancer treatment and continue to attract the attention of the investigators for the development of new molecules for cancer treatment.

Aphanamixis polystachya Wall. Parker also known as *Amoora rohituka* (Roxb.) Wight & Arn. (family: Meliaceae) or rohituka grows throughout India in evergreen forests. Stem bark and

seeds of rohituka have been reported to cure splenomegaly, liver disorders, and tumors [12]. Its stem bark has been reported to treat liver and spleen tumors [13&14]. The alcoholic extract of stem bark of rohituka has been reported to exhibit anticancer activity against Friend's leukemia and Ehrlich ascites carcinoma in mice and increase the effect of radiation [15-17]. Our recent study has indicated that chloroform extract of rohituka had cytotoxic effect on HeLa cells and increased micronuclei in a concentration dependent manner [18]. Its chloroform extract increased the cell killing effect of radiation in cultured HeLa cells recently [19]. Earlier, ethyl acetate fraction of rohituka showed radioprotective action against the radiation-induced chromosome damage [20]. The amoorastatin and 12-hydroxyamoorastatin alkaloids isolated from the stem bark extract of rohituka have been shown cytotoxic and growth inhibitory activities in murine P388 lymphocytic leukaemia cells [21]. Rohituka contains limonoids and these phytochemicals have shown cytotoxic action against various human cancer cell lines [22-24].

Cytotoxic effect of most chemotherapeutic drugs is due to their ability to damage DNA and kill the neoplastic cells. This indicates that it is prudent to evaluate DNA damage at molecular level in neoplastic and normal cells against chemotherapy to determine their effectiveness [25]. Comet assay is one such technique, which is able to estimate the amount of DNA damage (both single and double strand breaks and conformational changes) in individual cells treated with chemical or physical agents that damage DNA after removing most of the non-DNA material in a weak electric field to the remaining DNA embedded in an agarose gel [26]. The cells are lysed and treated *in situ* with alkali to render the DNA single-stranded prior to applying the electric field to the gel, which allows the genomic DNA to migrate out of the nucleus into the agarose, which can be visualized by staining with the intercalating fluorescent dye ethidium bromide under a fluorescent microscope. The combination of the DNA that has stayed within the confines of the nucleus is called head and DNA that migrates out of the nucleus as "tail" of DNA of the individual cells and look like 'celestial comets' [27]. Quantitative microscopic evaluation is done by measuring the length and intensity of the comets in relation to the signal of the non-migrating nuclear DNA in comparison with standards [28]. Our earlier study has shown that chloroform extract of rohituka induced damage to the genome of HeLa cells in the form of micronuclei [26]. However, the induction of DNA damage by the chloroform extract of rohituka at molecular level has not been studied. Therefore, the present study was undertaken to investigate the effect of various concentrations of *chloroform extract of polystachya* on cell proliferation, DNA damage and cell survival in cultured HeLa cells.

Materials and Methods

Chemicals

Low melting agarose (LMA, CAS No. A-4718, Lot 111K1532), Eagle's minimum essential medium (MEM), agarose, dimethyl sulfoxide (DMSO), fetal calf serum, L-glutamine, Ethylenediaminetetraacetic Acid (EDTA), Trichloroacetic Acid (TCA) and ethidium bromide were procured from Sigma Chemical Co. (St. Louis, MO, USA), whereas all other chemicals were requisitioned from Ranbaxy Fine Chemicals, Mumbai, India. Doxorubicin (DOX) was a kind gift from, Dabur Pharmaceuticals, Oncology Division, New Delhi, India.

Preparation of Extract

Aphanamixis polystachya (Wall) R. N. Parker (family-Meliaceae), Rohituka also known as *Aglaia polystachya, Amoora rohituka* and *Andersonia rohituka* was identified and authenticated by Dr. G. K. Bhat (a well-known taxonomist), Department of Botany, Poorna Pragna College, Udupi, India and the herbarium specimen (RB-AP01) has been stored with us. The matured and non-infected stem bark of rohituka was carefully peeled off, washed with clean water, chopped, shade-dried, and coarsely powdered in a ball mill. The powdered bark was extracted sequentially in petroleum ether and chloroform in a Soxhlet apparatus until the solvents became clear. The chloroform extract (APE) was collected and allowed to evaporate at room temperature and the viscous extract was subjected to freeze-drying so as to obtain a fine powder of the extract.

Dissolution of APE

APE was weighed and freshly dissolved in DMSO immediately before use at a concentration of 25 mg/ml and diluted with MEM as required.

Cell line and Culture

HeLa S3 (human cervical carcinoma) cells, having 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) containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 µg/ml gentamicin sulfate at 37°C in an atmosphere of 5% CO_2 in humidified air in a CO_2 incubator (NuAir, Plymouth, USA) with their caps loosened.

Experimental Design

A fixed number (5 x 10^5) of exponentially growing HeLa cells were inoculated into several culture flasks and were allowed to grow for 2 days. The cells from the above cultures were divided into the following groups according to the treatment: -

DMSO treatment: The cells of this group were treated with 2 µl/ml of sterile DMSO.

APE treatment: This group of cells was exposed to 5, 10, 25, 50, 75 or 100 µg/ml of APE.

DOX treatment: The cultures of this group were treated with 10 µg/ml of doxorubicin and served as a positive control.

Optimum Treatment Duration

A separate experiment was conducted to evaluate the effect of APE treatment duration on the DNA damage, where HeLa cells were divided into all the groups listed above and the cells were exposed to APE for 2, 4, 6 or 8 h. The results of this experiment

showed that 6h APE treatment induced maximum DNA damage in HeLa cells as evidenced by a highest OTM. Therefore, 6 h was considered as an optimum APE treatment time and further studies were carried out using this treatment time.

DNA Repair Kinetics

A separate experiment was carried out to assess the APE-induced alteration in the DNA damage with assay time, where the grouping and design of experiment was essentially similar to that described above except that HeLa cells were treated with 5, 10, 25, 50, 75 or 100 µg/ml APE for 6 h (optimum duration). After the elapse of 6 h, the cells were washed twice with PBS and harvested. Thereafter, cells were embedded in Low Melting Agarose (LMA) and layered on to precoated agarose slides and incubated in MEM for 0.5, 1, 2, 4, 6, 10, 12, 16, 18 or 24 h post APE-treatment to study the APE-induced alteration in DNA repair.

Alkaline Comet Assay

The technique described by Singh et al., for alkaline comet assay was followed with minor modification as described earlier [28&29-32]. Briefly, frosted 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 congeal under a cover slip on ice. Thereafter, the cover slips were removed and 1 x 10^5 cells treated with APE or MEM in 1 ml MEM were centrifuged at 1500 rpm for 5 min. The pelleted cells were resuspended in 80 µl of 0.6% LMA, mixed gently, layered onto the precoated agarose slides and allowed to solidify on ice. All the steps described were conducted under dim light to prevent additional DNA damage.

The agarose slides embedded with cells, were kept in cold lysis buffer consisting of 2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Trizma base, pH 10.0 and 1% Triton X-100 (added a fresh) to solubilize cellular proteins leaving DNA as nucleoids, at 4°C for 2 h. After cell lysis, lysis buffer was drained off from the slides that were placed into a horizontal gel electrophoresis tank filled with fresh electrophoresis buffer containing 300 mM NaOH, 1 mM Na_2EDTA (pH 13.0) up to a level of ~0.25 cm above the slides. The slides were kept in the buffer for 20 min to allow DNA unwinding. Electrophoresis was carried out for 20 min at 25 V and 300 mA in the cold condition. The slides were removed from the electrophoresis tank drained and flooded slowly with three changes of neutralization buffer (0.4 M Trizma base, pH7.5) for 5 min each. The slides were stained with 50 µl of ethidium bromide (2 mg/ml) and covered with a coverslip 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 (CoolSNAP-Procf 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 [26&33]. 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). OTM (Olive Tail Moment= (Tail.mean - Head.mean) X Tail%DNA/100) was measured from three independent experiments, each containing quintuplicate measures and presented as mean ± SEM.

Growth Kinetics

A separate experiment was carried out to assess the effect of APE on growth kinetics. Six hours after different treatments, the drug-containing medium was removed and the cells were dislodged from the culture flasks by trypsin-EDTA treatment. The growth kinetic assay was performed as described earlier [35]. Usually, 1 x 10^4 cells were inoculated into 25 cm^2 petridishes in triplicate for each drug concentration. The cells were allowed to grow for 1, 2, 3, 4 and 5 days. The cells from each culture dish were detached at the end of 1, 2, 3, 4 or 5 days, stained with trypan blue and viable cells were scored under an inverted microscope (Ernst Leitz, Wetzlar GmbH, Wetzlar, Germany) using a hemocytometer (American Optical Company, USA).

Clonogenic assay

The reproductive integrity of HeLa cells after treatment with different concentrations of APE was determined as described earlier by setting a separate experiment [34]. Generally, 200-300 log phase cells were plated on to several individual culture dishes (Cellstar, Greiner, Germany) containing 5 ml drug free MEM in triplicate for each drug concentration for each group. The cells were incubated for 24 h and the cells of APE group were exposed to 5, 10, 25, 50, 75 or 100µg/ml APE for 6h as indicated in the earlier section, thereafter APE containing media was removed, the cultures were washed with Phosphate Buffered Saline (PBS) and fed with APE free medium. The cells were transferred back into CO_2 incubator and allowed to grow for next 11 days. Thereafter, the cultures were stained with 1 % crystal violet dissolved in absolute methanol. The cells colonies were scored under a stereozoom microscope (Wild M3, Wild Heerbrugg Ltd., Heerbrugg, Switzerland). The clones containing 50 or more cells were scored as a colony. The plating efficiency was determined and Surviving Fraction (SF) calculated. The data were fitted on to linear quadratic equation:

$$SF = \exp^{-[\alpha D + \beta D^2]}$$

Statistical Analysis

The statistical analyses were performed using GraphPad Prism version 2.01 statistical software (GraphPad Software, San Diego, CA, USA). The comet results were analysed using the regression analysis (Systat Software, Systat, Evanston, IL, USA), where the various concentrations of APE and OTM were plotted on to *X* and *Y* axes, respectively. The statistical significance of various groups in other studies was obtained with one-way ANOVA with application of Tukey's post-hoc test. A p value of <0.05 was considered statistically significant. All data are expressed as mean±standard error of the mean (SEM).

Results

The results of DNA damage as OTM, growth kinetics, and cell survival are expressed as mean ± SEM in Tables 1-3 and Figure 1-5.

Assessment of DNA Damage

Optimum treatment duration

Olive tail moment (OTM, the product of tail length and DNA content in the tail) is recommended and commonly applied in such analyses as the most complex and reliable parameter to describe the degree of DNA damage on a per cell basis [27]. OTM from the mean of 100 comets randomly screened under a microscope for each culture has been presented in tables (1-2). Treatment of HeLa cells with DMSO did not alter the baseline DNA damage significantly at various post-treatment times (Table 1). Treatment of HeLa cells with 0, 5, 10, 25, 50, 75 and 100µg/ml APE increased DNA damage significantly ($p < 0.0001$) and in a concentration dependent manner at all treatment times (Figure 1). The greatest DNA damage was observed for 100 µg/ml APE, whereas the lowest DNA damage was induced by 5 µg/ml APE. Similarly, DNA damage increased with increase in APE treatment duration and a maximum OTM was observed for 8 h APE treatment for all concentrations (Figure 1). APE treatment resulted in a greater than 10 folds rise in DNA damage in HeLa cells treated with 25 µg/ml APE (9.16±0.075), which was significantly ($p<0.005$) higher when compared with concurrent DMSO treatment (Table 1). A further increase in APE concentration resulted in an increased DNA damage and the highest damage was observed for 100 µg/ml APE (63.72±0.077) for 6 h APE treatment duration. Incubation of HeLa cells with APE for 6 h increased the DNA damage further when compared with 2 or 4 h (Figure 1). Since a greater DNA damage was observed for 6 h treatment, further studies were conducted using this APE treatment duration (Table 1). A similar trend was registered for 10 µg/ml doxorubicin, which also showed the maximum DNA damage at 6h treatment duration (Table 1). The amount of DNA damage caused by 10 µg/ml doxorubicin was almost equal to 75µg/ml APE (Figure 1).

Table 1: Effect of treatment time on the DNA damage in HeLa cells exposed to different concentration of chloroform stem bark extract of *Aphanamixis polystachya* or doxorubicin

Post-treatment time (h)	Olive Tail Moment (mean±SEM)							
	DMSO	Doxorubicin (10 µg/ml)	5	10	25	50	75	100
0	0.76±0.035	0.76±0.032	0.75±0.038	0.77±0.034	0.77±0.030	0.75±0.042	0.74±0.033	0.77±0.034
2	0.75±0.057	12.84±0.064^c	2.18±0.026^a	4.53±0.050^b	7.16±0.075^c	9.38±0.067^c	12.44±0.084^c	15.73±0.089^c
4	0.78±0.038	17.55±0.072^c	3.58±0.051^a	6.82±0.063^b	9.64±0.054^c	13.39±0.065^c	16.77±0.075^c	19.86±0.080^c
6	0.82±0.071	24.75±0.066^c	5.68±0.058^b	9.92±0.062^c	14.44±0.065^c	19.47±0.070^c	25.30±0.069^c	30.72±0.077^c
8	0.81±0.057	25.68±0.074^c	5.77±0.062^b	10.11±0.070^c	15.00±0.086^c	19.82±0.079^c	25.92±0.093^c	31.36±0.085^c

$p < a = 0.05$, $b = 0.01$, $c = 0.005$ (when compared with the concurrent DMSO group); no symbols = not-significant

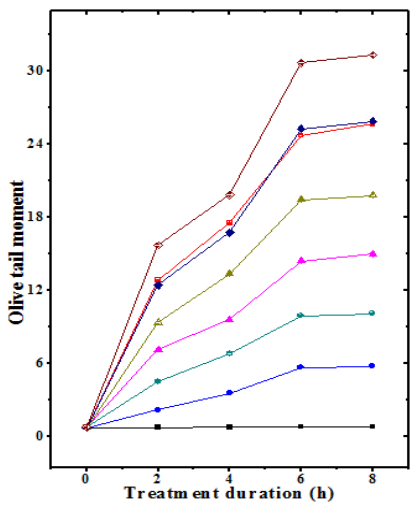

Figure 1: Effect of treatment duration of various concentrations of chloroform stem bark extract of *Aphanamixis polystachya* (APE) or doxorubicin (DOX) on the induction of molecular DNA damage in HeLa cells. Closed squares: DMSO; Open squares: DOX; Closed circles: 5µg/ml APE; Open circles: 10µg/ml APE; Closed triangles: 25µg/ml APE; Open triangles: 50µg/ml APE; Closed diamonds: 75µg/ml APE and Open diamonds: 100µg/ml APE.

DNA Damage Repair Kinetics

Estimation of DNA damage in HeLa cells exposed to 0, 5, 10, 25, 50, 75 and 100 µg/ml showed a concentration-dependent but significant elevation in the DNA damage at various post-treatment assay times (Table 2; Figure 2). The greatest acceleration in DNA damage was observed at 0 h post-treatment for all APE concentrations which declined steadily thereafter with post-treatment assay time, indicating repair of DNA damage (Figure 2). However, control values could not be restored by 24 h even for the lowest concentration of APE (5 µg/ml). The OTM steadily declined with assay time up to 2 h post-APE treatment and remained almost unaltered for subsequent assay times (Table 2; Figure 2), indicating a maximum repair in the damaged DNA by 2 h post-treatment for all APE concentrations. This repair was statistically non-significant when compared with 0 h post-treatment (Table 2; Figure 2). Assessment of DNA damage in HeLa cells exposed to 10 µg/ml doxorubicin also showed a trend similar to that of APE treatment where the maximum DNA damage was recorded at 0 h post treatment, that constantly declined with assay time up to 2 h post-treatment and remained unaltered thereafter (Figure 2). The degree of DNA damage induced after 75µg/ml APE has been equal to that of 10µg/ml doxorubicin treatment at all assay times (Figure 2).

Table 2: Alteration in the DNA repair in HeLa cells exposed to different concentrations of chloroform stem bark extract of *Aphanamixis polystachya* or doxorubicin

Post-treatment time (h)	Olive Tail Moment (mean±SEM)							
	DMSO	Doxorubicin (10 µg/ml)	Chloroform stem bark extract *Aphanamixis polystachya* (µg/ml)					
			5	10	25	50	75	100
0	0.86±0.055	24.26±0.052	5.55±0.029^{b}	9.62±0.059^{c}	14.43±0.057^{c}	19.89±0.078^{c}	25.96±0.087^{c}	31.13±0.096^{c}
0.5	0.91±0.056	23.32±0.05	4.79±0.022^{b}	8.82±0.038^{c}	13.65±0.055^{c}	18.07±0.064^{c}	24.26±0.082^{c}	29.75±0.090^{c}
1	0.92±0.052	21.55±0.043	4.24±0.031^{b}	7.97±0.043^{c}	12.82±0.048^{c}	17.54±0.063^{c}	23.67±0.079^{c}	28.26±0.076^{c}
2	0.92±0.041	20.74±0.043	3.94±0.024^{a}	6.42±0.052^{c}	12.20±0.06^{c}	16.98±0.072^{c}	22.12±0.076^{c}	27.82±0.097^{c}
4	0.92±0.035	20.20±0.05	3.44±0.022^{a}	5.84±0.057^{b}	12.32±0.053^{c}	16.36±0.077^{c}	22.76±0.064^{c}	27.70±0.090^{c}
6	0.92±0.035	20.46±0.047	3.56±0.028^{a}	5.65±0.062^{b*}	12.43±0.055^{c}	16.97±0.081^{c}	22.40±0.077^{c}	27.53±0.110^{c}
8	0.91±0.035	20.56±0.053	3.47±0.030^{a}	5.26±0.06^{b*}	12.34±0.062^{c}	16.13±0.082^{c}	22.23±0.059^{c}	27.46±0.096^{c}
12	0.90±0.056	20.30±0.055	3.73±0.028^{a}	5.30±0.059^{b*}	12.68±0.049^{c}	15.78±0.079^{c}	21.82±0.062^{c}	27.34±0.075^{c}
16	0.89±0.054	20.28±0.052	3.36±0.03^{a}	5.13±0.067^{b*}	12.07±0.052^{c}	15.22±0.070^{c}	21.53±0.072^{c}	26.40±0.088^{c}
24	0.88±0.033	20.33±0.049	3.05±0.028^{a}	5.15±0.062^{b*}	11.86±0.055^{c}	15.16±0.072^{c}	21.36±0.07^{c}	26.42±0.090^{c}

p < a = 0.05, b = 0.01, c = 0.005 (when compared to the concurrent DMSO group); * = 0.05 (in comparison with the 0 h group); no symbols = not-significant

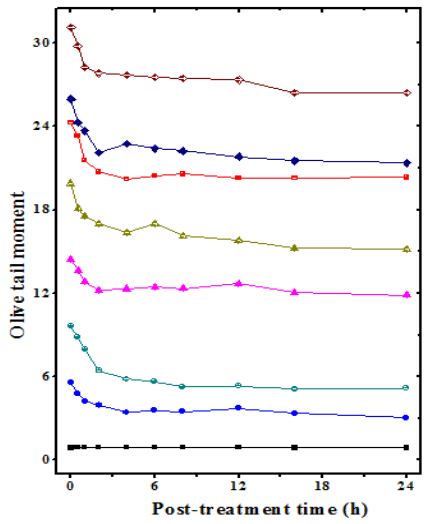

Figure 2: Effect of various concentrations of chloroform stem bark extract of *Aphanamixis polystachya* (APE) or doxorubicin (DOX) on the repair of molecular DNA damage in HeLa cells at different post treatment assay times. Closed squares: DMSO; Open squares: DOX; Closed circles: 5µg/ml APE; Open circles: 10µg/ml APE; Closed triangles: 25µg/ml APE; Open triangles: 50µg/ml APE; Closed diamonds: 75µg/ml APE and Open diamonds: 100µg/ml APE.

Growth Kinetics

The number of viable cells increased with each scoring time in the non-drug-treated group, and a peak number of cells was observed on day 5 (Table 3; Figure 3). Exposure of HeLa cells to 5, 10, 25, 50, 75, and 100 µg/ml APE led to a concentration-dependent decline in cell viability when compared to DMSO treatment (Figure 3). Despite this decline the cell proliferation increased with assay time and a maximum increase in cell number for all concentrations of APE was scored on day 5 post-treatment (Figure 3). The number of viable cells scored was significantly lower in APE-treated group when compared to non-drug treated control ($p<0.01$ for 25-100 µg/ml APE on day 2; $p<0.05$ for 10 and $p<0.01$ for 25-100 µg/ml APE on day 3; $p<0.01$ for 10 and $p<0.005$ for 25-100 µg/ml APE on day 4; $p<0.05$ for 5, $p<0.01$ for 10, and $p<0.005$ for 25-100 µg/ml APE on day 5 post-treatment incubation time). A significant decrease in the number of viable cells was observed on day 5 post-treatment, irrespective of the APE concentration in the APE treated group when compared to concurrent non-drug treated control group (Table 3). Treatment of HeLa cells with 100 µg/ml APE completely inhibited the cell division as there was no appreciable change in the cell number with increasing scoring time (Table 3; Figure 3). An almost identical effect was observed for 10 µg/ml DOX treatment ($p<0.01$; Table 3). The inhibition in the proliferation of HeLa cells by 50 µg/ml APE was equivalent to 10 µg/ml DOX (Figure 3).

Clonogenic Assay

Exposure of HeLa cells with different concentrations of APE resulted in a concentration dependent decline in the survival of HeLa cells (Figure 4). The lowest concentration of APE attenuated the survival by 10%. Increasing APE concentration led to a corresponding decline in the cell survival and 50µg/ml APE reduced the surviving fraction by 0.58 and further increase in APE concentration reduced the surviving fraction by 0.79, when cells were exposed to 100µg/ml resulting in a survival of 21% (Figure 3).

Biological Response

The biological response was determined by plotting survival on X-axis and OTM on Y axis. The increasing DNA damage in the form of OTM resulted in a corresponding decline in the cell survival (Figure 5).

Table 3: Alteration in growth kinetics of HeLa cells exposed to various concentrations of chloroform extract of *Aphanamixis polystachya* (concentrations) or doxorubicin

APE (µg/ml)	Cell count (in 10^4) ± SEM when cells were treated for 6 h with APE and allowed to grow in separate culture dishes for different times (days)				
	1	2	3	4	5
0	2.71±0.017	4.24±0.028	8.23±0.046	16.34±0.086	31.08±0.193
5	2.60±0.014	3.90±0.020	6.30±0.033[b]	10.50±0.064[b]	17.50±0.010[c]
10	2.30±0.015	3.10±0.021[b]	4.50±0.023[c]	6.50±0.051[c]	10.50±0.072[c]
25	1.30±0.014[c]	1.61±0.011[c]	2.10±0.016[c]	3.02±0.024[c]	4.41±0.031[c]
50	1.08±0.012[c]	1.29±0.010[c]	1.41±0.013[c]	1.68±0.013[c]	2.25±0.016[c]
75	0.88±0.009[c]	1.19±0.010[c]	1.27±0.011[c]	1.51±0.012[c]	2.16±0.016[c]
100	0.71±0.006[c]	0.89±0.007[c]	1.14±0.010[c]	1.39±0.011[c]	1.82±0.013[c]
DOX(µg/ml)					
0	2.67±0.016	4.16±0.033	8.21±0.059	16.29±0.136	31.04±0.242
10	1.12±0.010[b]	1.33±0.012[c]	1.54±0.014[c]	1.79±0.015[c]	2.37±0.021[c]

APE = chloroform fraction of the stem bark of *Aphanamixis polystachya*; DOX=doxorubicin; and SEM = standard error of the mean. p < a = 0.05, b = 0.01, c = 0.001, no symbols = not significant calculated using one-way ANOVA.

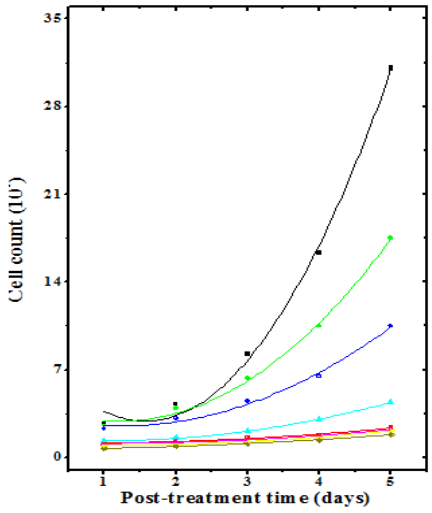

Figure 3: Effect of different concentrations of chloroform stem bark extract of *Aphanamixis polystachya* (APE) or doxorubicin (DOX) on the growth kinetics of HeLa cells. Closed squares: DMSO; Open squares: DOX; Closed circles: 5µg/ml APE; Open circles: 10µg/ml APE; Closed triangles: 25µg/ml APE; Open triangles: 50µg/ml APE; Closed diamonds: 75µg/ml APE and Open diamonds: 100µg/ml APE.

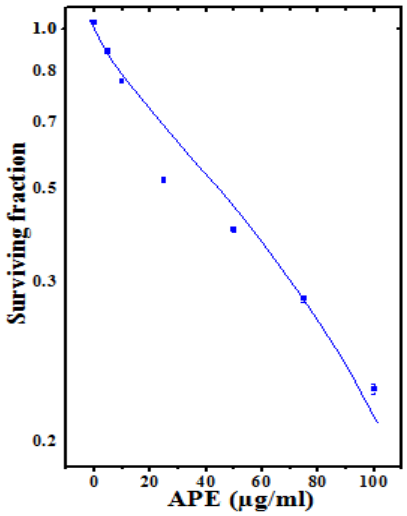

Figure 4: Effect of different concentrations of chloroform stem bark extract of *Aphanamixis polystachya* on the survival of HeLa cells. r2=0.95.

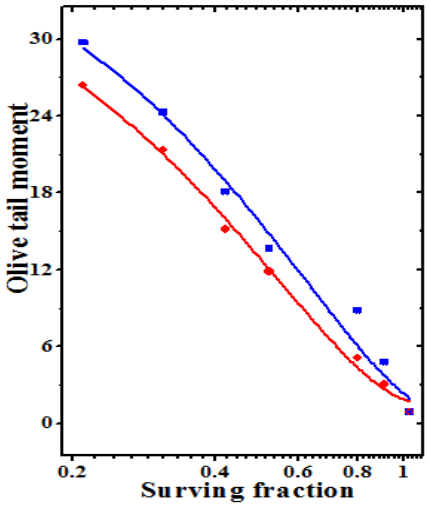

Figure 5: Correlation of DNA damage expressed as Olive tail moment with cell survival in HeLa cells exposed to different concentrations of chloroform stem bark extract of *Aphanamixis polystachya.* Squares: 0.5 h and Circles: 24 h post-treatment. r^2=0.98.

Discussion

The history of use of natural products and plants can be traced to the time when humans faced with some kind of ailments. The use of natural products has been tested for several generations and their safety has been beyond doubt in humans [36]. Development of drug resistance is a major impediment in the success of modern cancer chemotherapy, yet it is generally not possible to predict the degree or timing of the emergence of tumor resistance in most chemotherapy protocols [37]. Recent developments in the single-cell gel electrophoresis or 'comet' assay for estimation of molecular DNA damage at the single-cell level indicate that this technique is able to identify and potentially monitor tumor cell responsiveness to many anticancer agents *in situ* [38]. In principle, this assay could be applied to any accessible tumor being treated with chemotherapeutic agents that cause overt DNA damage and also in the lymphocytes of patients undergoing chemotherapy [39&40]. The comet assay can be easily performed in super coiled genomic cell DNA of any cell line or tissue, which can be made into single cell suspensions including differentiated cells as it does not require cells to undergo division [41]. Therefore, present study was designed to assess the DNA damage in the HeLa cells exposed to different concentrations of chloroform extract of the stem bark of *Aphanamixis polystachya.*

Comet assay of HeLa cells exposed to different concentrations of APE for different exposure times revealed a concentration and time dependent increase in the DNA damage as indicated by increased OTM in the present study up to six hours, where a maximum DNA damage was estimated. A similar effect has been reported earlier in HeLa cells treated with dichloromethane extract of giloe, *Tinospora cordifolia* [30]. Likewise, treatment of HeLa cells with berberine for different times has been found to increase DNA damage in a concentration dependent manner up to a certain time [31]. The time dependent treatment of HCT-116 and HT-29 cells exposed to irinotecan has been found to increase maximum DNA damage after 24h of drug exposure [42]. The study of DNA repair kinetics in HeLa cells exposed to different concentrations showed a maximum DNA damage/OTM at 0 h and a steady decline thereafter indicating that cells were able to undergo repair. However, the rate of DNA repair dwindled with increasing concentrations of APE, especially after 10 µg/ml. The level of DNA damage was far from the control even at 5µg/ml, where it was 3.5 folds higher at 24 hours. *Tinospora cordifolia* has been reported to increase DNA damage consistently up to 10 h post-treatment in HeLa cells in a concentration dependent manner and a marginal repair thereafter [30]. An identical effect was observed in HeLa cells treated with different concentrations of berberine earlier [31]. This variation in the time of maximum damage in the present study may be due to the variation in experimental design. In earlier studies the cells were impregnated in agarose and then exposed to drug/s, whereas in the present study the cells were exposed to drug and then embedded in agarose for comet assay. Bleomycin exposure to V79 cells also continuously increased the OTM with no signs of DNA repair up to six hours earlier [29]. Irinotecan exposure of HCT-116 and HT-29 cells also increased the DNA damage in a concentration dependent manner up to 24h and a repair in the DNA damage thereafter [42]. Doxorubicin treatment has been reported to increase OTM and tail DNA in V79 cells and a repair with time up to 24 h however, the DNA damage did not reach to spontaneous level even by 6 h post-treatment [32]. In contrast, treatment of *Agaricus blazei,* a mushroom in hepatic carcinoma of rat and bladder cancer has been reported to induce DNA repair [43&44].

The APE has been reported to induce DNA damage in the form of micronuclei in our earlier study and the present study supports these observations as the APE triggered DNA damage at molecular level and did now allow it to repair [18]. The extracts of *Ajuga postii* containing an iridoid glucoside, reptoside, and the ethanolic extract of *Stephania dinklagei* containing corydine and atherospemidine alkaloids have been reported to cause DNA damage in RS321NpRAD52 and RS321NpRAD52 yeast cells [45]. Ethyl acetate fractions of *Combretum apiculatum, Combretum hereroense, Combretum molle* and *Combretum mossambicense* reported to have DNA damaging properties in different cells [46]. Similarly, idarubicin has been reported to cause DNA strand breaks in a concentration-dependent manner in promyelocytic leukemia cells, HL-60, murine pro-B lymphoid, BaF3 cell lines earlier as assessed by comet assay [47]. Nitrogen mustards, cisplatin and related platinum compounds have been reported to induce covalent modifications of DNA and induce DNA inter-strand cross-links, resulting in DNA double strand breaks in A2780/100 cells [48&49].

The gross cytotoxicity of any chemical or drug can be easily determined by Pratt and Willis assay and treatment of HeLa cells with different concentrations of chloroform extract of rohituka killed the cells in a concentration dependent manner as there was a constant decline in the growth kinetics of HeLa cells with each assay day after treatment with rohituka. The lowest survival was observed for 100 µg/ml APE. Determination of reproductive

integrity further confirmed these results where, clonogenicity of HeLa cells declined with increasing concentration of APE. Our earlier study has shown a similar effect in HeLa cells treated with different concentrations of APE, where the reproductive capacity of HeLa cells declined with increased APE concentration [18]. Similarly, a reduction in the growth kinetics has been observed in HeLa cells exposed to different concentrations of the dichloromethane extract of *Tinospora cordifolia*, berberine, *Tinospora cordifolia* or berberine and γ-radiation [30,31,50&51]. Amooranin isolated from *Aphanamixis polystachya* (*Amoora rohituka*) stem bark, has been reported to be cytotoxic by inducing DNA damage and apoptosis in HeLa, MCF-7, MCF-7/TH and MCF-10A cells [51]. Amooranin arrested leukemia and colon carcinoma cell cells in G2+M phase [52]. A semi synthetic derivative of rohitukine, the flavopiridol has been reported to target serine/threonine kinases and inhibit cell proliferation [53&54]. An identical effect has been observed in the HeLa cells treated with stem bark extract of *Tinospora cordifolia* or berberine chloride earlier [7&55].

The biological response determination revealed an inverse correlation between DNA damage and cell survival for all the concentrations of APE. The higher the DNA damage lower was the cell survival. Our earlier study on micronuclei has shown that increasing frequency of micronuclei resulted in a rise in the cytotoxicity of HeLa cells [18]. Treatment of HeLa cells with different concentrations of dichloromethane extract of *Tinospora cordifoila* increased the OTM and subsequently reduced the clonogenicity [30]. A similar correlation between increasing OTM and reduced clonogenic survival was reported in HeLa cells treated with different concentrations of berberine and doxorubicin [31,32]. Similarly, treatment of V79 cells with Bleomycin has been found to reduce their clonogenicity with increasing OTM [29]. An identical observation has been reported earlier where increasing DNA damage (Olive tail moment) was accompanied by a corresponding decline in the cell survival [50,51&57-59].

The exact mechanism of action of APE by which it induced DNA damage and led to a decline in cell the survival is not known. The DNA damaging effect of APE may not be due a single mechanism but it may have acted through numerous putative mechanisms. APE may have increased oxidative stress in HeLa cells by triggering formation of free radicals, which may have attacked the cellular genome leading to different types of DNA damages including, DNA adduct formation, base damages, DNA strand breaks or alkali labile sites, which are measured by comet assay and expressed as OTM in the present study. Attrition of glutathione might have caused chromatin dysfunction causing single strand and double strand DNA breaks and internucleosomal DNA fragmentation, whose repair would have been a daunting task, which might have ultimately led the cells to undergo apoptosis and necrosis [60]. DNA damage response triggers the activation of many enzymes including nucleases, topoisomerases, helicases, polymerases, recombinases, ligases, glycosylases, demethylases, kinases and phosphatases to neutralize the DNA damage [61]. The presence of APE seems to suppress the activation of these enzymes leading to inhibition of DNA repair and higher amount of DNA damage in HeLa cells. Inhibition of topoisomerase II by APE in HeLa cells may have also contributed to the increased DNA damage. Quercetin which is present in the stem bark of *Aphanamixis polystachya* has been reported to inhibit topoisomerase II [62&63]. Topoisomerase II acts by passing an intact segment of duplex DNA through a transient double stranded break that it generates in a separate double helix and inhibition of topoisomerase II by APE may cause stabilization of the DNA double strand breaks detected in the present study [64-66]. The APE may have inhibited p53 expression and disallowed DNA repair as indicated by futile attempt by HeLa cells to repair the DNA damage in the present study. p53 is involved in the repair of DNA damage apart from other cellular functions [67]. The increased DNA damage by APE may be due to suppression of NF-κB, and COX-II. Amooranin present in APE has been found to repress NF-κB, and COX-II transcriptional activation [68&69]. The Nrf2 transcription is known to facilitate DNA repair, whereas its inactivation suppresses repair of DNA strand breaks [70]. A similar possibility cannot be ruled out in the present study, where APE might have down modulated Nrf2 expression.

The study was carried out in the homogenous populations of HeLa cells that have less variability than the natural in situ tumor cells therefore, the result of DNA damage may be variable when compared to in vivo study which may be one of the limitations. The use of lesion specific enzymes during comet assay would have given better information about individual specific lesions induced by APE at molecular level in the DNA. However, we were unable to incorporate lesion specific enzymes, hence our study provides indication of the gross DNA damage in the HeLa cells and not the specific DNA lesions induced by APE in HeLa cells. The use of enzymes would have also given the information about the repair of specific DNA lesions with elapse of time. Further in our study it was not possible to pin point specific chemical component that would have been responsible for triggering DNA damage by APE as it is a complex mixture of several chemicals.

Conclusions

APE treatment induced DNA damage in concentration-dependent manner as evident by a corresponding increase in the OTM. This was reflected in reduction in the cell proliferation and clonogenicity of HeLa cells. The induction of DNA damage in HeLa cells by APE may be due to the rise in oxidative stress and repression of the activation of nucleases, topoisomerases, helicases, polymerases, recombinases, ligases, glycosylases, demethylases, kinases and phosphatases. APE may have suppressed the transcriptional activation of p53, NF-κB, COX-II and Nrf2 leading to suppression of DNA repair and higher DNA damage in the present study.

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Conflict of interest statement

Authors don't have any conflict of interest statement to declare.

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