**Evaluation of Cytotoxicity and DNA Damaging Activity of Three Plant Extracts on Cervical Cancer Cell Lines**

Subhabrata Paul, Shuvechha Chakraborty, Anita Mukherjee, Rita Kundu*
Prof in Botany, Centre of Advanced Study, Department of Botany, University of Calcutta, Ballygunge Circular Road, Kolkata, WB, India.
*Corresponding author's E-mail: kundu_rita@yahoo.co.in

**ABSTRACT**

*Solanum nigrum*, *Phyllanthus amarus* and *Heliotropium indicum* are three medicinal plants indigenous to Asia belonging to the family Solanaceae, Euphorbiaceae and Boraginaceae respectively. They have been traditionally used in ancient medicine for treating variety of diseases. In our present study, the extract fractions (aqueous, chloroform and hexane) of these three plants have been tested against three cervical cancer cell lines – HeLa (HPV 18 positive), SiHa (HPV 16 positive) and C33A (HPV negative). The antiproliferative activity of plant extracts were evaluated by standard cytotoxicity measuring assay like 3-(4, 5-dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide (MTT) reduction assay. Genotoxicity was measured by Comet assay or Single Cell Gel Electrophoresis (SCGE) assay. Plant extract fractions showed varied levels of cytotoxicity which depends on cell types and also on the nature of extract fractions. The IC50 doses determined using MTT assay was found to be non-toxic to normal mouse embryonic cell line NIH3T3. However the fractions which showed anti-proliferative activity at low dose level were considered effective. Among the medicinal plant extract chloroform fraction of *Solanum nigrum*, *Phyllanthus amarus* and both chloroform and hexane fraction of *Heliotropium indicum* were found to be most efficient in inhibiting cell growth and inducing apoptosis.

**Keywords:** apoptosis, cancer, cytotoxicity, traditional medicine, phytochemicals, genotoxicity

**INTRODUCTION**

India is a treasure trove of native plant resources, among these, around seven thousand species are considered to have medicinal properties. With the advancement of scientific research it has been possible to acquire numerous compounds for drug discovery through different methods which include isolation of compounds from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modelling. Nevertheless medicinal plants and natural products remain important as a source for drug discovery serving as new drug leads. Although a large number of plant extracts have been used for curing common ailments like, cough & cold, fever, enteric diseases or inflammation, their application on lifestyle diseases like cancer were less explored. Over the last half century several of the plant metabolites were found to play a significant role in combating cancer mainly through induction of pro-apoptotic pathways. Random screening of large number of crude extracts or fractionated plant extract is found to be effective in finding new anticancer compounds.

In our present study we have evaluated the cytotoxic and genotoxic effect of *Phyllanthus amarus*, *Solanum nigrum* and *Heliotropium indicum*, three medicinally important plants on three cervical cancer cell lines-HPV18 positive HeLa cell line, HPV16 positive SiHa cell line and HPV negative but malignant C33A cell line. These three plants have been used traditionally in ancient Indian medicine for treatment of a number of diseases.

*Phyllanthus amarus*, a member of the family Euphorbiaceae is a common pantropical weed that grows well in moist and shady places. The genus *Phyllanthus* has a long history of use in the treatment of liver, kidney and bladder problems, diabetes and intestinal parasites. It has found its conventional use in treatment of jaundice, diabetes, gonorrhoea, irregular menstruation, tachycardia, dysentery, spasmodic cough, itchiness, arthritis, otitis, swelling, skin ulcer etc. Recent findings have shown that only *P.urinaria* and *P.maderaspatensis* exhibited significant hepatoprotective activity against tBH induced cytotoxicity using HepG2 cells. *P.emblica* has shown anticancer activity against cervical cancer cell lines. The Solanaceae family has ethnobotanical importance and is an important source of food, spice and medicine. Steroidal glycosides obtained from solanaceous plants showed potent cytotoxic activity. *Solanum nigrum* is an erect, annual herb seen wild throughout India and traditionally used as emollient, diuretic and antispasmodic.

Ethanolic extracts of *S.nigrum* fruits were reported to have hepatoprotective effect in CCl4 induced liver damage and can inhibit the proliferation of MCF-7 cells by inducing apoptosis. Huang and co-workers have reported that leaf extracts of *S.nigrum* preferentially inhibited the growth of Her2/neu overexpressing breast cancer cell line AU565. *Heliotropium indicum* is a member of the family Boraginaceae is an annual weed of wasteland and native to Asia. In Indian traditional system of medicine it is used to cure wounds, flatulence, inflammation, skin ulcers and
conjunctivitis.\textsuperscript{23} It has been reported to contain tumorigenic pyrrolizidine alkaloids.\textsuperscript{24}

Increasing incidences of cancer is a great concern in the Indian scenario, adding nearly a million cases each year. There is an increase in the incidence rates for some HPV associated cancers and low vaccination coverage among adolescents underscores the need for additional prevention efforts for HPV-associated cancers\textsuperscript{25} such as cervical cancer. At least 70% of the cases worldwide have been found to be associated with HPV16 and HPV18.

The aim of the present study is to find a natural compound, capable of inducing apoptosis in the cancer cells with selective cytotoxicity. Plants extracted with organic solvents and water yield several phytochemicals, such as, flavonoids, terpenoids, phenolics and it was already been reported that these phytochemicals can induce apoptosis. Papademetro and co-workers have reported that catechin flavonoid, isolated from \textit{Ligaria cunifolia} not only reduces cell growth, but can induce apoptosis in murine lymphoma cell line LB02 by down regulating survivin and Bcl-2.\textsuperscript{26} In this regard, three traditional medicinal plants are taken to assess their apoptosis inducing capacity. Though, some sparse report of cytotoxicity of the plant extracts were found, no reports regarding their genotoxicity were available. Therefore the present study reports for the first time the genotoxic potentiality of the organic fractions of these medicinal plants. For this, the methanolic extracts of whole plants have been subjected to fractionation in organic solvents. The cytotoxic activity of plant extract fractions have been assessed by MTT assay and Single cell gel electrophoresis (Comet assay). Agarose gel electrophoresis was deployed to test the apoptosis inducing capability of the extract. The specificity of the extracts for cancerous cell lines has been determined by using normal mouse fibroblast cell line NIH3T3.

**MATERIALS AND METHODS**

**Cell Culture**

HeLa, SiHa and C33A cells were maintained in monolayer cultures in Minimum essential medium (MEM) [HiMedia] supplemented with 0.035% (w/v) Sodium bicarbonate [HiMedia], 0.03% (w/v) L-Glutamine [HiMedia], 10% (v/v) Fetal Bovine Serum [HiMedia], 1% (v/v) Antibiotic antimycotic solution [HiMedia] and incubated at 37°C and 5% CO\textsubscript{2} concentration in a humidified atmosphere. Adherent cells were treated in a humidified atmosphere.

**Collection of Plants**

Plants were collected from the Departmental garden during November-December 2012, identified properly and submitted to Calcutta University Herbarium for accession number.

\textit{Solanum nigrum} (local name-kakmachi, English name-black nightshade, accession number 20014-CUH), \textit{Phyllanthus amarus} (local name-bhun amla, English name-stone breaker, accession number 20015-CUH), \textit{Heliotropium indicum} (local name-hatisur, English name-Indian heliotrope, accession number 20016-CUH) were collected, washed properly and shade dried.

**Preparation of Plant Extracts**

Crude methanolic extracts (50 g of dried whole plant powder extracted in 500ml MeOH of \textit{Solanum nigrum}, \textit{Phyllanthus amarus} and \textit{Heliotropium indicum} were dried to 1/4th of its original volume by a rotary evaporator at 37°C and further fractionated into hexane, chloroform and aqueous fractions.

The respective fractions were dried and dissolved in DMSO according to the required concentration (DMSO concentration not exceeding 0.5%) and further diluted in 10% PBS.

The extracts (aqueous, chloroform and hexane fractions) were sterile filtered through Nylon-66 membrane filter of pore size 0.22μm (HIMEDIA). A total of nine different fractions were obtained – PAA, PAC, PAH; SNA, SNC, SNH; HIA, HIH, HIC (P.amarus, S.nigrum and \textit{H.indicum} - aqueous, chloroform and hexane fractions).

**MTT Assay**

The anti-proliferative potential of the extracted fraction was measured by reduction of MTT [3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide] to formazan crystals by mitochondrial dehydrogenase enzymes present in live cells based on the method described by Charmicheal.\textsuperscript{27} The MTT assay was used to determine drug sensitivity and IC\textsubscript{50} dose of the fractions by comparing the cell viability of the treated cells with that of the control sets (containing no extract). This IC\textsubscript{50} dose is the concentration of plant extract that can inhibit cell proliferation by 50%.

HeLa, SiHa, C33A and NIH3T3 cells were seeded in 96 well plates [HIMEDIA] at an initial density of 2x10\textsuperscript{4} cells per well and incubated overnight in MEM at 37°C and 5% CO\textsubscript{2} in a humidified atmosphere. Adherent cells were treated with extract fraction in an increasing concentration from 20-200 μg/μl with an increment of 20μg/μl from the initial dose.

Treatments were performed in triplicate. Wells containing untreated cells (without addition of any extract) were treated as negative control whereas cells incubated only with DMSO (0.5% v/v) were used as vehicle control.

After 24 hrs incubation period under the above mentioned condition, 20μl of MTT [Sigma] was added to each well and further incubated for 3 hrs.

After removal of medium from each well, formazan was extracted with 100μl of dimethyl sulfoxide (DMSO) and quantitated in an ELISA reader at 595 nm.

The percent inhibition was calculated according to the equation given below:

\[ \% \text{ inhibition} = \left( \frac{\text{Absorbance}_{control} - \text{Absorbance}_{treatment}}{\text{Absorbance}_{control}} \right) \times 100 \]
Single Cell Gel Electrophoresis

The assessment of genotoxicity of the extracts on single cell was monitored by alkaline comet assay or single cell gel electrophoresis. The assay works upon the principle that strand breakage of the supercoiled duplex DNA leads to the reduction of the size of the large molecule and these strands can be stretched out by electrophoresis.

Also, under highly alkaline conditions there is denaturation, unwinding of the duplex DNA and expression of alkali labile sites as single strand breaks. Comets form as the broken ends of the negatively charged DNA molecule become free to migrate in the electric field towards the anode.

Control and treated cell suspension were mixed with 1% low melting point agarose (LMPA) and embedded on microscopic slides base coated with 1% normal melting agarose (NMA) [HIMEDIA]. A third layer of 0.5% LMPA was added above the cell containing layer. It was followed by alkaline lysis (pH 10) and alkaline unwinding and electrophoresis (pH>13) at 25 volts for 30 mins. The slides were neutralized with 0.4M Tris-Cl (pH 7.5), stained with ethidium bromide and scored under fluorescent microscope (Lietzf). The extent of DNA damage in the form of tail DNA was measured by Komet 5.5 software.

DNA Fragmentation Analysis by Agarose Gel Electrophoresis

Internucleosomal DNA fragmentation (180 bp or multiples) detected as a typical ladder by agarose gel electrophoresis is a well established apoptotic marker. Cells (HeLa, SiHa and C33A) were seeded at a density of 5 x 10^5 cells per T-25 flask overnight in MEM at 37°C and 5% CO₂ in a humidified atmosphere.

After incubation period, cells were treated with the IC₅₀ doses of the extract fraction along with a control set each for 24 hrs.

The treated and control cell samples were collected in micro centrifuge tubes, washed with PBS and resuspended in Tris hydroxymethyl aminomethane (Tris)-Ethylenediaminetra-acetic acid(EDTA) buffer (TE) pH 8.0 (10mM Tris, 1mM EDTA). Then they were incubated in ice for 30 mins with lysis buffer (pH 8.0) containing 20mM EDTA, 50mM Tris, 1% Sodium Dodecyl Sulfate (SDS), 1% Nonidet P40 (NP40). Phenol-chloroform-Isoamyl alcohol (25:24:1) extraction was done to purify DNA from protein contamination. DNA was precipitated from aqueous solution with the help of isopropanol and sodium acetate at - 20°C for 30 minutes. The DNA was precipitated by centrifugation at 27000 x g, washed with 70% alcohol and dissolved in TE buffer. The solution was then incubated at 37°C for 30 mins with RibonucleaseA (RNase A). DNA precipitation step was again repeated and the DNA was finally dissolved in TE buffer. The DNA samples were then electrophoresed through a 1.5% agarose (Sigma) gel having Ethidium bromide (EtBr) at a final concentration of 0.5 µg/ml, in 1x Tris Acetate EDTA buffer (TAE) and subsequently visualized under UV.

RESULTS

MTT Assay

The extracts selectively inhibited the growth and proliferation of HeLa, SiHa and C33A compared to untreated control in a dose dependent manner. The IC₅₀ concentration for the three cell lines and the three plant extracts are summarized in Table 1. PAC and SNC showed cytotoxicity for HeLa, SiHa and C33A cells at low dose concentrations. The concentration was found to be non-toxic for normal mouse fibroblast NIH3T3. The highest DMSO concentration for all the six extracts was 0.5% (v/v) which is non-cytotoxic to NIH3T3.

The chloroform fraction of both plants (S. nigrum and P. amarus) showed potent inhibitory concentration at 250 ± 1.01µg/ml, 87.5 ± 0.82µg/ml, 100 ± 0.03µg/ml for SN extracts and 175 ± 2.13µg/ml, 50 ± 1.01µg/ml, 150 ± 3.06µg/ml for PA extracts in HeLa, SiHa and C33A cells respectively. Hexane fraction of the HIH extract is found to be more intense, as the IC₅₀ concentration observed were 100 ± 0.01µg/ml and 50 ± 0.03µg/ml for HeLa and SiHa cells while the chloroform fraction showed inhibitory effect at 375 ± 0.01µg/ml for C33A cells (Table 1.)

These are the doses at which 50% cell death occurred and are taken as the treatment dose for subsequent experiments.

Single Cell Gel Electrophoresis

Different classes of comets were observed which includes comets with intact DNA and no tails to comets with internucleosomal DNA fragmentation (180 bp or multiples) detected as a typical ladder by agarose gel electrophoresis. Different comets form as the broken ends of the negatively charged DNA molecule become free to migrate in the electric field towards the anode.

Table 1: Cytotoxicity in terms of IC50 doses of plant extract fractions on cervical cancer cell lines.

<table>
<thead>
<tr>
<th>IC₅₀ doses (µg/ml)</th>
<th>SNA</th>
<th>SNC</th>
<th>SNH</th>
<th>PAA</th>
<th>PAC</th>
<th>PAH</th>
<th>HIA</th>
<th>HIC</th>
<th>HHF</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>950 ± 34.67</td>
<td>250 ± 0.01</td>
<td>305 ± 4.94</td>
<td>390 ± 11.7</td>
<td>175 ± 10.65</td>
<td>450 ± 8.05</td>
<td>415 ± 0.05</td>
<td>155 ± 0.40</td>
<td>100 ± 0.55</td>
</tr>
<tr>
<td>SiHa</td>
<td>500 ± 2.45</td>
<td>87.5 ± 6.37</td>
<td>200 ± 0.32</td>
<td>350 ± 21.91</td>
<td>50 ± 2.93</td>
<td>250 ± 31.37</td>
<td>650 ± 0.05</td>
<td>125 ± 4.5</td>
<td>50 ± 0.35</td>
</tr>
<tr>
<td>C33A</td>
<td>850 ± 9.43</td>
<td>100 ± 2.12</td>
<td>750 ± 1.40</td>
<td>450 ± 33.3</td>
<td>150 ± 15.3</td>
<td>350 ± 16.35</td>
<td>750 ± 0.10</td>
<td>375 ± 0.05</td>
<td>590 ± 0.05</td>
</tr>
</tbody>
</table>

a IC50 doses in bold characters are considered to have significant cytotoxic effect at concentration below or equal to 100µg/ml.
Relative fluorescence intensity of head and tail, normally expressed as a percentage of DNA in the tail has been considered as the parameter for measuring DNA damage in this case. This parameter is linearly related to the break frequency and covers the widest range of damage.

DNA damage in the form of comet tail has been observed in all three cell lines. In HeLa, the tail DNA percent ranges from 3.50 ± 1.58% to 50.22 ± 0.11% in treated sets. The values for the treatment sets SNH (7.99 ± 1.03%), SNC (11.77 ± 1.48%), PAH (17.19 ± 4.85%), and HIC (50.22 ± 0.11%) increased significantly (P<0.05) with respect to control (4.35 ± 0.38%) [Fig. 2].

The percent tail DNA is least for the treatment set HIA with the value of 7.91 ± 1.55% and highest for the treatment set PAC corresponding to the value 73.01 ± 3.11% for SiHa. PAA (15.37 ± 2.21%), PAC (73.01 ± 3.11%), PAH (30.27 ± 3.45%), SNC (64.38 ± 3.65%), SNH (30.56 ± 4.27%), HIC (24.43 ± 3.02%), HIH (20.35 ± 1.71%) shows significant (P<0.05) difference from control (7.826 ± 1.10%) [Fig. 3].

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The extent of damage in C33A cell line is greater than HeLa and SiHa as evident from the illustration [Fig.4.]. The percent tail DNA values in C33A ranges from 14.81 ± 2.75% to 85.72 ± 5.00%. The values for the treatment sets PAC (85.72 ± 5.00%), PAH (70.27 ± 2.96%), SNC (26.16 ± 2.31%), SNH (37.77 ± 5.10%), HIC (64.74 ± 1.89%), HIH (57.88 ± 4.41%) increased significantly (P<0.05) with respect to control (10.99 ± 0.77%).

DNA Fragmentation Analysis

Non-treated control and vehicle control sets showed clear bands of genomic DNA (L2 and L3), whereas inter nucleosomal fragmentation (180bp and multiples of it) were observed in HeLa (except HIA and HIH sets), SiHa (except SNH set) and C33A cell lines (Figure 5).

**Figure 5:** DNA fragmentation analysis by AGE. A. HeLa; B. SiHa; C. C33A. L1:marker, L2:control, L3:vehicle control, L4-L12: treatment sets SNA, SNC, SNH, PAA, PAC, PAH, HIA, HIC and HIH.

**DISCUSSION AND CONCLUSION**

In the present study it is observed that all the plant fractions have profound effect on cervical cancer cell lines and showed varied degree of response to different cell lines. The striking property of tumour cells is its uncontrolled proliferation. The anti-proliferative property as well as the IC50 concentration of the extract fractions was determined by the MTT assay.

It is generally accepted that any extract with an IC50 value below or equal to 100µg/ml can be considered to have cytotoxic activity. In this regard, it can be observed that SNC for SiHa and C33A, PAC for SiHa, HIH for Hela and SiHa are the most potent fractions (Table 2). However, SNC, PAC, HIH and HIC showed growth inhibitory effect at concentrations lower than the other fractions for the same cell line. It was reported by previous workers that the fruits of *S.nigrum* have shown in vitro cytotoxic activity against HeLa and Vero cell lines. In-vitro cytotoxicity is often associated with DNA damage. Comet assay is one of the most important tool for measuring DNA damage in individual cells. Alkaline comet assay has been performed and the appearance of comet tails indicates the presence of single stranded damage, double stranded damage and alkali labile sites as previously stated in cells treated with plant extract fractions. Treatment of cells with the IC50 doses of the plant fractions shows significant (P<0.05) DNA damage in terms of percent tail DNA which has been considered an important parameter in a number of study. Of all the significant results, HIC for HeLa and PAC for SiHa and C33A showed the presence of higher amount of DNA in tails indicating greater amount of DNA damage. The aqueous fraction of all the plant extracts showed less potency as distinct comets were not observed in all cases and it showed a lot of heterogeneity. Heterogeneity, a condition that explains the presence of a wide range of appearance in comets indicating higher amount of DNA damage in some nuclei while others remained undamaged, was also observed in sets of SNC (HeLa), PAC(HeLa), HIC(SiHa), PAH(HeLa), HIH(HeLa, SiHa). This explains the capacity of drug resistance in cancer cells as already observed in some studies. Well formed comets with increased DNA damage where nearly all the DNA has migrated into the tail showing a distinct head tail separation have been observed.

Treatments with SNC (in SiHa), PAC (in SiHa and C33A), HIC (in HeLa and C33A), SNH, PAH, HIH (in C33A) showed the presence of class 4 hedgehog comets characteristic of apoptotic cells. Comets similar to that of our observation have been termed as apoptotic comets was observed in Colo 320 cells. However this criterion cannot be used as specific indicators of apoptosis. During apoptosis DNA is fragmented into internucleosomal segments and it is less likely that these small pieces can be detected by comet assay.

DNA damage by generation of internucleosomal fragments (180 bp and multiples) is characteristic of apoptotic cell death. Agarose gel electrophoresis of DNA followed by EtBr staining shows a typical ladder formation for all the three cell lines except for a few treatment sets (HIA and HIH fractions in HeLa and SNH fraction in SiHa). DNA damage beyond repair is a necessity for cell death. Apoptosis triggered by various DNA lesions have already been identified. Apoptotic DNA fragmentation has been observed in sets which showed extensive DNA damage in comet assay indicating that genotoxicity has led to cell death. Extensive nuclear DNA damage acts as an intrinsic signal to the mitochondria, inducing release of cytochrome c and tipping the balance in favour of cell death rather than repair and survival. *Phyllanthus* species (*P.amarus, P.niruri and P.watsoni*) were found to be effective in inducing apoptosis in PC3 and MeWo cell line with minimal necrotic effects and suppresses proliferation through multiple signalling pathways.
From this study, it can be said that these three traditionally important plants are capable of inducing cell death in the cervical cancer cells and cause a significant amount of DNA damage to them. The cell lines used for the experiment originated from cervical cancer tissues. (two of them are HPV bearing, HeLa and SiHa and one without HPV, C33A), responded differentially to the plant extracts. C33A cell line which is HPV-ve, showed maximum DNA damage, as is evident by the comet assay and also showed DNA laddering in all the extract treated sets, indicates maximum sensitivity to the plant extracts. Among the fractions, the chloroform fractions of Phyllanthus sp. and Solanum sp., and hexane fraction of Heliotropium sp. were found to be effective at lower concentrations. HPLC analysis of chloroform extracts of Solanum nigrum, Phyllanthus amarus and Heliotropium indicum and hexane fraction of H.indicum showed the presence of phenolic acid, phenols, squalene (precursor of terpenoids), terpenoid and fatty acid respectively (data not shown). Terpenoids are reported to be an inducer of cell death. A recent report shows that ferutinin, a terpenoid isolated from Ferula ovina is capable of inducing apoptosis in MCF7 (breast cancer) and TCC (bladder cancer) cells. In a recent study it was reported that, terpenoid isolated from Zingiber officinale was found to induce apoptosis in endometrial cancer cells.

CONCLUSION

It can be concluded from the experiments that chloroform extracts of the three plants have the potentiality to induce cell death in all the cervical cancer cell lines tested, among the cell lines C33A cell line showed maximum sensitivity. SCGE assay showed a great extent of DNA damage in the extract treated cells, visible as comet tails, indicates that cells may be induced to death due to DNA damage. Presence of DNA laddering indicated that the study surely confirms the ability of these plant extracts in regulating the proliferation of cancer cells mainly by apoptosis. Further work is needed to identify the bioactive compounds present in these traditional medicinal plant fractions.

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