Comparative Analysis of Apoptotic Activity From Extracts of Saponin-rich Indian Medicinal Plants

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ABSTRACT
Natural products have been regarded as an important source that could contribute to potential chemotherapeutic agents. In particular, plant derived compounds have gained importance in anticancer therapy. In our study, we investigated the inhibitory effects of different Indian medicinal plant extracts (Bacopa monerri, Tribulus terrestris, Asparagus racemosus and Nelumbo nucifera) on cancer cells which are known to be highly saponin producing. Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are usually characterized by their structure containing a steroidal or triterpenoid aglycone and one or more sugar chains. In the present investigation, methanolic and ethanolic saponin rich plant extracts were compared for its cytotoxic effect on cancer and normal cell lines. The extracts also have been shown to induce apoptosis and cell cycle arrest in cancer cells which implies its anticancer efficacy. Based on the current findings, it can be concluded that extracts from Nelumbia nucifera and Bacopa monerri have cytotoxic and apoptotic activity towards cancer cells which indicates the need of further investigation of these extracts for cancer therapeutics.

KEYWORDS: Natural products, chemotherapeutic agents, saponins, cytotoxic effect, apoptosis, cell cycle, cancer therapeutics

INTRODUCTION
Natural products are considered powerful sources of novel drug discovery and development. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs1,2; and interest in this area of research has increased in all over countries. Natural remedies derived from herbs3, food or raw materials are used by pharmaceutical and food industries4. In recent years, antitumor drugs from plants have drawn attention because of their low toxicities compared with conventional chemotherapy drugs5. Cancer is the main cause of death worldwide and plant derived drugs, like vinblastine, vincristine, taxol, and camptothecin have improved the chemotherapy of some cancers6,7. Many of these plant-derived anticancer agents have been discovered through large-scale screening programs5. During literature survey, it was observed that majority of the work done (77% of the total work done in 2009-2013) on saponins has followed a characteristic pattern of extraction of phytocompounds from plant source and testing its toxicity on cell lines but there are few reports on the apoptotic activity of these compounds and further cell cycle arrest studies.

Table 1. Summary of work done on saponins in the year 2009-2013

<table>
<thead>
<tr>
<th>Type of study</th>
<th>Percentage Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxicity assay</td>
<td>77</td>
</tr>
<tr>
<td>Cell cycle arrest studies</td>
<td>6</td>
</tr>
<tr>
<td>Anti-invasive/ anti-angiogenic/ anti-metastatic activities</td>
<td>7</td>
</tr>
<tr>
<td>Total mechanism of action (Multiple assays)</td>
<td>10</td>
</tr>
</tbody>
</table>

The present study was conducted to evaluate the anti-cancer activity of crude extracts from four different Indian medicinal plants with the objective of testing the toxicity of the extracts on cancer cells and to study apoptosis for determining cell death and evaluation of effect on cell cycle.

MATERIALS & METHODS

Reagents
HPLC grade methanol and ethanol were purchased from Himedia
Laboratories, India. The cell culture medium and reagents, penicillin, streptomycin, kanamycin, MTI [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and Dimethyl Sulphoxide (DMSO, cell culture grade) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Fetal bovine serum was purchased from Lonza.

**Extraction of plant materials**

Four plants, *Bacopa moneri*, *Tribulus terrestris*, *Asparagus racemosus* and *Nelumbo nucifera* known to be highly saponin producing were collected from different areas of Hyderabad, India. Soxhlet, which has been used for a long time, is a standard technique and the main reference for evaluating the performance of other solid–liquid extraction methods9. The leaves of *Bacopa moneri*, *Tribulus terrestris*, *Asparagus racemosus* and roots of *Nelumbia nucifera* were separated and oven dried at 45 °C overnight. The plants were grounded into powder form using the grinder. Extraction using Soxhlet apparatus with either 95% (v/v) ethanol or methanol as solvent for 40 hours was performed90.

**Preparation of test materials**

The crude extracts were dissolved in dimethyl sulfoxide (DMSO) at 500mg/ml as stock solutions which were then diluted with DMEM to desired concentrations. The final concentrations of the extract dilutions in culture were 10, 50, 75, 100 and 150, µg/mL. The final concentration of DMSO in each sample did not exceed 1% v/v, to keep the cytotoxicity of DMSO at less than 10%. Ethanolic extracts were named as BME, TTE and NNE and methanolic were named as BMM, TTM, STM, NNM for *Bacopa moneri*, *Tribulus terrestris*, *Asparagus racemosus* and roots of *Nelumbia nucifera* respectively.

**Cell lines and cell cultures**

AS49 (human lung cancer cell line), and CHO (chinese hamster ovary cells) were obtained from the National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in DMEM (Dulbecco’s modified eagle medium) supplemented with 10% (v/v) FBS, penicillin 50 mg/Lt, streptomycin 50 mg/Lt, kanamycin acid sulphate 100 mg/Lt and 3.7gm/Lt sodium bi-carbonate solution. The cells were maintained as monolayers in 25 cm² plastic tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO₂ in air.

**Cytotoxicity studies**

The MTT colorimetric assay was used to screen for cytotoxic activity of each plant extract. This colorimetric assay is based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-di Methyl Thiazol-2-yl)-2,5-diphenyl Tetra-zolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells11. This test is a good index of mitochondrial activity and, thus, of cell viability. The cells were seeded in 96-well plates at a density of 1x10⁴ cells/well in 100 µL culture medium and allowed to attach overnight at 37°C. The cells were then incubated with 100 µL of extract at different concentrations of 10 µg/mL- 150 µg/mL for 24 hours. Untreated cultures and blank wells received 100 µL of DMEM medium supplemented with 10% (v/v) FBS. After 24 hours, 10 µL of the MTT reagent was added to each well, and the plate was incubated for 4 hours at 37°C. The MTT solution was removed from the wells by aspiration. After removal of the medium, 0.1 ml of 1:1 DMSO: methanol was added to each well and plates were shaken. The absorbance was measured at 550 nm in an automated plate reader. The percentage of cytotoxicity compared to the untreated cells was determined with the equation:

Cell viability (%) = OD of treated cells/OD of control cell ×100.

The results were generated from three independent experiments; each experiment was performed in triplicate.

**Quantification of Apoptosis Studies**

The annexin V-FITC- labeled apoptosis detection kit (Sigma) was used to detect and quantify apoptosis by flow cytometry as per manufacturer’s protocol. In brief, cells (2x10⁴ cells/well) were seeded in 6-well plates and cultured over night in 10% fetal bovine serum media. After 16-18h cells were either kept untreated or treated with 75µg/mL of different plant extracts for 24 hours. Then the cells were harvested and collected by centrifugation for 5 minutes at 1000 rpm. Cells were then resuspended at a density of 1 x 10⁶ cells/ml in 1X binding buffer and stained simultaneously with FITC- labeled Annexin V (25 ng/ml) and propidium iodide (50 ng/ml). Cells were analyzed using a flow cytometer (FACS Canto II) and data were analyzed with FCS Express V3 software. A minimum of 10,000 events were gated per sample.

**Cell cycle analysis**

Cells were seeded at a density of 8x10⁴ cells/well in a 6-well plate and allowed to attach overnight. Cells were treated with 75µg/ml of each plant extract for 18 hours. Cells were harvested, washed with 1X PBS, and fixed with 70% ethanol and kept at -20°C for at least overnight. Cells were centrifuged to discard ethanol and re-suspended in 1X PBS. After centrifugation cells were suspended in 1ml of PI staining solution (0.05% Triton X-100, 0.1mg/ml RNase A and 50µg/ml of PI from 50X stock solution of 2.5mg/ml) and kept in dark for 45 min at 37°C. After incubation cells were collected by centrifugation, washed with 1X PBS, re-suspended in 1ml PBS, and analyzed using a flow cytometer (FACS Canto II, Becton- Dickinson, San Jose, CA, USA) and data were analyzed with FCS Express V3 software. A minimum of 10,000 events were gated per sample.

**Statistical analysis**

All experiments were repeated three times and then data were shown.
as means ± standard deviation (S.D) of three assays. Student t-test was applied, and p<0.05 was considered as statistically significant.

RESULTS

MTT assay for cytotoxic studies
Initially, cytotoxic studies were carried out for dose optimization. Different extracts were used in a range of 10µg/mL - 200µg/mL of media for A549 and CHO cell lines. A well marked increase in toxicity was observed between 10-100µg/mL, whereas after that cell viability was noticed to be almost negligible. The dose was optimized to be 75µg/mL media and used as optimized dose for carrying out further studies. Upon treatment of normal cells with these extracts, viability remained comparatively high to be around 80%. Also, not much difference was observed in results obtained for ethanolic and methanolic extracts as shown in figure 1 and so, for further studies treatments were carried out with ethanolic extracts.

Apoptotic assay
Cell death was determined in the A549 cell line using FITC-conjugated Annexin-V membrane staining and PI nuclear counterstaining. Cell populations were quantified by flow cytometry analysis after 18 hours of treatment with 75µg/mL of each extract. In Fig 2, the percentage of dead cells is relative to the total cell population, while the percentage of apoptotic cells is presented with regard to the PI, ie, nondead cells. In this experiment, percentage of apoptotic cells was found to be high in those treated with NNE as compared to untreated cells. Percent apoptosis was comparatively low in cells treated with BME and TTE. Negligible apoptosis was reported in STE treated cells (Table 2, Figure 2). While considering MTT data which has shown a good level of toxicity in cancerous cells of each treatment group, it can be pointed out that the extract is toxic to the cells but no apoptosis was observed in STE.
Table 2: Population in terms of percentage showing apoptotic cells in A549 cells: Q1 indicates necrotic cells, Q2- apoptotic cells, Q3- healthy cells & Q4 showing early apoptotic cells

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>% Apoptotic population quadrant wise</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Q1</td>
</tr>
<tr>
<td>UT</td>
<td>3.1</td>
</tr>
<tr>
<td>TTE</td>
<td>1.9</td>
</tr>
<tr>
<td>STE</td>
<td>2.0</td>
</tr>
<tr>
<td>BME</td>
<td>2.8</td>
</tr>
<tr>
<td>NNE</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Cell cycle analysis

The effect of different plant extracts on cell cycle was evaluated using flow cytometric analysis. Human lung cancer cell line A549 was treated with different extracts at a concentration of 75µg/ml for 24 hours and were subjected to flow cytometric analysis after Propidium Iodide staining. These concentrations were chosen based on the results from MTT assay.

Percentage population of S phase after treatment with different extracts was remarkably increased. These results indicate that the extracts induce S phase arrest in A549 cell line. Increase in percentage of cells in pre-phase correlates well with the apoptosis data shown in figure 3.

Discussion

There has been a long standing interest in the identification of medicinal plants and derived natural products for developing cancer therapeutics. Bachran et al. 2008 in their review of saponins playing a role in tumor therapy focused on certain groups of compounds, such as dioscin, saikosaponins, julibrosides, avicins, soy and ginseng saponins, and on combinations of saponins and conventional antitumorigenic drugs.

In the present study, we investigated the inhibitory effects of four different Indian medicinal plant extracts’ on cancer cells. Methanolic and ethanolic plant extracts were compared for its cytotoxic effect on lung cancer cell line, A549 and normal Chinese Hamster ovary (CHO) cells. The results indicated that the extracts caused growth inhibition or cell death in concentration dependent manner. Optimal concentration of the extracts was determined using MTT assay. The concentration used for further experiments is 75µg/ml as the percent cytotoxicity was found to be close to 50. At the same concentration the cytotoxic effect was found to be even more in cancer cell lines. However we could not observe any significant difference between methanolic and ethanolic extracts. Podolak et al., 2010 summarized the cytotoxic activity of extracted saponins isolated from different plants. Patil et al., 2014 reported the phytoextracts from Bacopa moneri to be active against human breast cancer cell lines. Prasad et al., 2008 also reported anticancer property of Bacopa monnieri (L.) Wettst. The high steroid saponins levels in Tribulus terrestris and the data on their cytotoxic activity towards a number of tumor cell lines presumes a high antitumor potential of this medicinal plant. In popular medicine, Nelumbium nucifera is used in the treatment of tissue inflammation, cancer, skin diseases, leprosy and as a poison antidote. Asparagus racemosus is well known for its anti tumor activity. In our study, we have compared the extracts from these four medicinal plants and tested its anti-cancer activity.

The occurrence and development of tumours is closely related to cell apoptosis. The potential to induce apoptosis has therefore become an important topic in the study of antitumor drugs. To prove the data further, apoptosis and cell cycle analysis experiments were carried out. Apoptotic data indicated that NNE has maximum apoptotic effect on cancer cells. The genus Asparagus is rich source of saponins having apoptotic activity against cancerous cells but in
present investigation, *Asparagus* extract, STE has shown no apoptotic effect on cancer cells. Extracts from *Bacopa monnieri* was able to induce apoptosis as shown in Figure 2. Sivakrishna et al., 2005 isolated two triterpenoid glycosides along with 10 known saponins from *Bacopa monnieri* and delineated the chemical compositions of Bacosides A and B by Ji et al., 2012 showed saponin extracts from *Asparagus racemosus* inducing apoptosis on HepG2 cells. We, however, could not observe an inhibiting or apoptotic effect of the extract of *Asparagus racemosus* in our experimental model. This may depend on the method of extraction and different compounds that have been isolated with the extraction method used. Cell cycle was analysed using propidium iodide method. Cell cycle analysis results correlate well with apoptosis data. When comparing untreated and treated cells, we observed a remarkable increase from 6.61 in S-phase of untreated cells. Further studies are being carried out in our laboratory to purify and determine the effect of different groups present in these crude extracts.

**CONCLUSION**

In this work, extracts of four different Indian medicinal plants were screened for their anti-cancer and cytotoxic properties. The findings of the present investigation demonstrate that the ethanolic extracts caused growth inhibition irrespective of the origin of the cell, however we could observe high cytotoxicity (IC$_{50}$ = 75µg/ml) in cancer cells. Screening of the extracts revealed that though all the ethanolic extracts had shown cytotoxicity, BME and NNE could induce apoptosis in cancer cells. Also, these extracts have shown difference in phases of cell cycle using Fluorescence activated cell sorter (FACS). The present study indicates the need of phyto-extract screening for cancer therapeutics from Indian medicinal plants.

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