RESEARCH ARTICLE

**In Vitro Anticancer Activities of Anogeissus latifolia, Terminalia bellerica, Acacia catechu and Moringa oleifera Indian Plants**

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Abstract

The present study was designed to evaluate in vitro anti-proliferative potential of extracts from four Indian medicinal plants, namely Anogeissus latifolia, Terminalia bellerica, Acacia catechu and Moringa oleifera. Their cytotoxicity was tested in nine human cancer cell lines, including cancers of lung (A549), prostate (PC-3), breast (T47D and MCF-7), colon (HCT-16 and Colo-205) and leukemia (THP-1, HL-60 and K562) by using SRB and MTT assays. The findings showed that the selected plant extracts inhibited the cell proliferation of nine human cancer cell lines in a concentration dependent manner. The extracts inhibited cell viability of leukemia HL-60 and K562 cells by blocking G0/G1 phase of the cell cycle. Interestingly, A. catechu extract at 100 μg/mL induced G2/M arrest in K562 cells. DNA fragmentation analysis displayed the appearance of a smear pattern of cell necrosis upon agarose gel electrophoresis after incubation of HL-60 cells with these extracts for 24h.

Keywords: Anogeissus latifolia - terminalia bellerica - acacia catechu - moringa oleifera - cytotoxicity - DNA ladder

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Introduction

There has been a long-standing interest in identification of natural products and medicinal plants for developing new cancer therapeutics. India, represented by rich culture, traditions, and natural biodiversity, offers a unique opportunity for researchers in drug discovery and development (Brusotti et al., 2014). This country has 2 of 18 hotspots of plant biodiversity in the world, namely Eastern Himalaya and Western Ghats (Chitale et al., 2014). In the present study, we selected four medicinal plants namely Anogeissus latifolia, Terminalia bellerica, Acacia catechu and Moringa oleifera to explore their antitumor efficacy in human cancer cell lines. Stem bark of Anogeissus latifolia has been extensively utilized in the treatment of various disorders like skin diseases, snake and scorpion bites, leprosy, diabetes, stomach diseases, colic, cough, and diarrhea (Patil and Gaikwad, 2011). The fruit of Terminalia bellerica has been used for the treatment of anemia, asthma, cancer, colic, constipation, diarrhea, dysuria, headache, hypertension, inflammation, and rheumatism (Rashed et al., 2014). The bark and heartwood of Acacia catechu are widely used for the treatment of chronic fever, ulcer, cough, worm infestation, poisonous bites, obesity, hepatomegaly, splenomegaly, and problems related to skin, throat, tooth, and urinary tract (Stohs and Bagchi, 2015). Various parts of Moringa oleifera such as leaves, roots, seed, bark, fruit, flowers and immature pods are used as cardiac and circulatory stimulants, and have been shown to have diuretic, antitumor, anti-inflammatory, antispasmodic, antibacterial, and antifungal activities (Caceres et al., 1992; Biswas et al., 2012; Krishnamurthy et al., 2015). Considering the vast therapeutic potential of above mentioned medicinal plants, the present study was planned to investigate their anti-proliferative potential in different panel of human cancer cell lines. To get some insight into the cellular mechanism of action of the extracts, the most active concentrations of the plants extracts were also studied for cell cycle arrest and apoptotic potential in human leukemia cell line.

Materials and Methods

**Chemicals and reagents**

The RPMI-1640 medium, Dulbecco’s modified eagle’s medium (DMEM), fetal calf serum (FCS), trypsin, gentamycin, penicillin, and streptomycin, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), tris-base, and phosphate buffered saline (PBS) were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Ethylenediaminetetraacetic acid (EDTA), tris-base, and phosphate buffered saline (PBS) were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Trichloroacetic acid (TCA) was procured from Merck Specialties Pvt. Ltd., (Mumbai, India).

**Extraction Process**

The plants were harvested from herbal garden of Indian Institute of Integrative Medicine (IIIM), Jammu, and...
The plants were identified and authenticated by taxonomist of the IIIM. The extraction process of these plants was procured from the National products Chemistry Division of the IIIM. Dried and powdered plants were placed in a conical glass percolator, submerged in 95% ethanol or 50% ethanol, and kept at room temperature for 20h (Table 1). The extraction procedure was repeated four times and the percolate were collected and filtered. Ethanol was distilled off from pooled percolate using a rotavapour under reduced pressure at 50°C. The final drying was done initially in vacuum desiccators and finally lyophilized. The dried extracts were scrapped off and transferred to a wide mouth glass container. Nitrogen was blown in the container before capping and stored at -20°C in a desiccator.

Cell culture and treatment
Nine human cancer cell lines were obtained from National Cancer Institute, Frederick, USA. The cell lines were derived from different cancers including lung (A549), prostate (PC-3), breast (T47D and MCF-7), colon (HCT-16, Colo-205), and leukemia (THP-1, HL-60 and K562). All cell lines were routinely cultured in RPMI-1640 growth medium except MCF-7 cell line was cultured in DMEM medium. Both growth media (pH 7.2) were supplemented with 10% FCS, 1% penicillin (100 U/mL) and streptomycin (10 mg/mL), in tissue culture flask in an incubator at 37°C with 95% relative humidity and 5% CO₂ gas environment. The cells were harvested either by trypsinization (adherent cultures) or by centrifugation at 1000 rpm for 5 min (suspension cultures). Stock solutions (20 mg/mL) of the extracts were dissolved in DMSO and serially diluted with complete growth medium containing 50 μg/mL of gentamycin to the desired concentrations (10, 50, 100, 200 μg/mL, excepting Colo-205 cells at density 1000 cells/well). Untreated control cultures received only the vehicle (DMSO≤1%).

In vitro cytotoxicity screening
Sulforhodamine blue (SRB) assay: Measurement of the cellular protein content was performed using the SRB assay as described earlier (Vichai and Kirtikara, 2006). Briefly, seven adherent cultures namely A549, PC3, MCF-7, T47D, Colo-205, HCT-16 and THP-1 were harvested in log phase using trypsinization (0.05% trypsin and 0.02% EDTA, in PBS) and the cells were counted using a hemocytometer. The cells were seeded into 96-well plates at density 1000 cells/100μL/well excepting Colo-205 cells were seeded at density 1500 cells/well in 100μL medium into 96 well plate. After 24h, the medium was aspirated and the cells were exposed to 100 μL/well of freshly prepared medium containing test materials at desired concentrations for 48 h. At the end of exposure time, 50 μL of ice-cold 50% TCA was added to each well and left at 4°C for 1h to fix the cells attached to the bottom of the wells. The plates were washed five times with distilled water and then air-dried. The TCA-fixed cells were stained with SRB (0.4% in 1% acetic acid, 100 μL) for 30 min, followed by washing with 1% acetic acid and air-dried. The adsorbed SRB was dissolved by adding 100 μL of 10 mmol/L Tris buffer (pH 10.5) to each well and the plate was gently stirred for 10 min on a shaker platform. The absorbance at wavelength 540 nm was read using a microplate reader (Tecan, Switzerland).

MTT assay
Mitochondrial activity was evaluated by MTT assay as described earlier (Vega-Avila and Pugsley 2011). This assay based on enzymatic reduction of the yellow colored MTT dye to purple colored formazan crystals by a variety of mitochondrial and cytosolic enzymes that are operational in viable cells. Briefly, HL-60 and K562 cells (5000 cells/well) were seeded in 100 μL of medium into 96-well plate and left to settle in a CO₂ incubator. After 60 min of incubation, the test material was added in each well (100 μL/well) and the plate was incubated for 48 h. Four hours before the end of incubation period, 20 μL of MTT solution (2.5 mg/mL in PBS) was added to each well and re-incubated for 4h at 37°C. The plate was centrifuged with rotor for 96-well plate assembly (Beckman GS-6R, USA) at 3000 rpm for 15 min. Then, the supernatant was removed and the pellet was washed twice with 2 mL of PBS. The cells were fixed overnight in chilled 70% ethanol at 4°C and then subjected to RNase digestion (400 μg/mL) at 37°C for 1h. Finally, the cells were stained with PI (10 μg/mL) for 30 min in dark and analyzed immediately for DNA contents on a Flow Cytometer FACS Diva (Becton Dickinson, Franklin Lakes, NJ, USA). The cell cycle histograms were analyzed using the ModFit LT™ 3.2.1 software packages (Verity Software House Inc., Topsham, ME). In this program, debris and single cell populations were gated out using two parameter histogram of FL2-A versus FL2-W.

Cell cycle analysis
Flow cytometry was used to analyze cell cycle distribution according to the standard procedures (Saxena et al., 2010). Briefly, HL-60 and K562 cells (2×10⁶ / mL/6 well plate) were treated with plant extracts at 50 and 100 μg/mL for 24 h. The cells were harvested and centrifuged at 400g for 5 min. The supernatant was discarded and the pellet was washed twice with 2 mL of PBS. The cells were fixed overnight in chilled 70% ethanol at 4°C and then subjected to RNase digestion (400 μg/mL) at 37°C for 1h. Finally, the cells were stained with PI (10 μg/mL) for 30 min in dark and analyzed immediately for DNA contents on a Flow Cytometer FACS Diva (Becton Dickinson, Franklin Lakes, NJ, USA). The cell cycle histograms were analyzed using the ModFit LT™ 3.2.1 software packages (Verity Software House Inc., Topsham, ME). In this program, debris and single cell populations were gated out using two parameter histogram of FL2-A versus FL2-W.

DNA ladder assay
HL-60 cells (2×10⁶ cells/mL in 6-well plate) were harvested after 24h treatment with the selected plant extracts at 50 and 100 μg/mL. The cells were centrifuged at 1000 rpm for 10 min and washed in with PBS containing 20 mmol/L EDTA. The pellet was lysed in 250 μL of lysis buffer (100 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0, and 5% Triton X-100) containing 400 μg/mL DNase-free RNase and incubated at 37°C for 90 min, followed by incubation with proteinase K (200 μg/mL) at 50°C for 2h. The DNA was extracted with phenol-chloroform-isoamyl alcohol (25: 24: 1) for 1 min
and centrifuged at 15000 rpm for 2 min. The aqueous phase was further extracted with chloroform-isooamyl alcohol (24: 1) and centrifuged at 15000 rpm for 2 min. The DNA was allowed to precipitate with 3 volumes of chilled alcohol and 0.3mol/L sodium acetate at 20°C overnight. The precipitate was centrifuged at 15000 rpm for 10 min. The DNA pellet was washed in with 80% alcohol, dried, dissolved in 50 μL of Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.4) and subjected to electrophories in 1.8 % agarose gel at 50 V for 1.5h (Saxena et al., 2012).

**Data analysis**

All experiments were repeated at least three times, and each experimental condition was repeated at least in quadruplicate wells in each experiment. The IC\(_{50}\) values of plant extracts were calculated by linear regression analysis. All the data were analyzed by using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) for comparison between different treatment groups. Differences were considered statistically significant at P<0.05. All computations were made by employing the statistical package for Social Sciences software (SPSS Inc., version 17, Chicago, IL, USA).

**Results**

**In vitro cytotoxicity**

The anti-proliferative activity of the selected plant extracts was measured using the SRB assay for adherent cultures and MTT assay for suspension cultures against a panel of nine human tumor cell lines. The cytotoxicity assays were assessed at 48h following treatment with different concentrations of plant extracts in the range of 10-100 μg/mL. The findings showed that, the extracts inhibited differential growth inhibition against the examined cancer cells in a concentration dependent manner.

The sensitivity of the cell lines to extracts exposure was characterized by IC\(_{50}\) values (Table 2). In the US National Cancer Institute plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC\(_{50}\) value in cancer cells is ≤30 μg/mL following incubation between 48-72h (Boik, 2001). According to this criterion, the extract of *A. Latifolia* was strongly active against A549, PC3, MCF-7, HCT-16, Colo-205, THP-1, and HL-60 cells (IC\(_{50}\)=10.6-28.7 μg/mL), and moderately active against T47D and K562 cells (IC\(_{50}\)=42.2-46.6 μg/mL). The extract of *T. bellerica* exerted strong cytotoxicity against A549, PC3, MCF-7, HCT-16, Colo-205, and THP-1 (IC\(_{50}\)=9.0-28.4 μg/mL) and moderate activity against T47D, HL-60, and K562 cells (IC\(_{50}\)=33.5-50.0 μg/mL). The extract of *A. catechu* was strongly active against A549, PC3, MCF-7, HCT-16, and HL-60 cells (IC\(_{50}\)=9.7-25.9 μg/mL) and moderately active against T47D, Colo-205, THP-1, and K562 cells (IC\(_{50}\)=37.8-42.8 μg/mL). The extract of *M. oleifera* was strongly active against A549, PC3, MCF-7, and HCT-16 cells (IC\(_{50}\)=13.2-28.8 μg/mL) and moderately active against T47D, Colo-205, THP-1, HL-60, and K562 cells (IC\(_{50}\)=33.5-50.0 μg/mL).

**Cell cycle analysis**

Flow cytometry analysis exhibited an increase in the percentage of cells in G\(_1\)/G\(_0\) phase with concomitant decrease in S-phase population in HL-60 and K562 cells treated with plant extracts (Figures 1&2). This implied that the extracts inhibited the proliferation of HL-60 and K562 cells through arresting G\(_1\)/G\(_0\) cell cycle phase progression.

The sub-G\(_1\)/G\(_0\) peak was increased to maximum values 37.21% in HL-60 and 17.49% in K562 cells treated with 100 μg/mL of extracts of *T. bellerica* and *A. Latifolia*, respectively vs 2.08% and 2.97% in the control cells. Further G2/M values were 8.80% in the control cells). Further G2/M values were 8.80% in the control cells). Further G2/M values were 8.80% in the control cells). Further G2/M values were 8.80% in the control cells). Further G2/M values were 8.80% in the control cells).

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**Table 1. Taxonomical Profile of Four Indian Medicinal Plants Used in the Current Study**

| Name of Plants | Family         | Accession No. | Parts used        | Extraction mode |
|---------------|----------------|---------------|-------------------|-----------------|
| Anogeissus Latifolia | Combretaceae | 19909         | Stem and leaves   | 95% EtOH extract |
| Terminalia bellerica  | Combretaceae | 17983         | Stem bark         | 95% EtOH extract |
| Acacia catechu  | Leguminosae   | 2901          | Fruit             | 50% EtOH extract |
| Moringa oleifera | Moringaceae   | 20402         | Leaves            | 50% EtOH extract |

**Table 2. IC\(_{50}\) Values (μg/mL) of Extracts of Four Indian Medicinal Plants Against Human Cancer Cell Lines**

| Cell lines | Anogeissus latifolia | Terminalia bellerica | Acacia catechu | Moringa oleifera |
|------------|----------------------|----------------------|----------------|------------------|
| A549       | 20.0±1.2             | 28.4±2.7             | 25.9±1.5       | 13.2±1.8         |
| PC3        | 10.6±0.4             | 17.7±1.7             | 14.3±1.6       | 22.2±4.9         |
| T74D       | 42.2±1.4             | 43.7±2.2             | 38.5±1.4       | 33.5±2.5         |
| MCF-7      | 20.1±4.8             | 9.0±1.7              | 22.8±4.9       | 26.4±5.7         |
| HCT-16     | 25.8±1.5             | 24.8±1.3             | 20.6±3.9       | 28.8±2.2         |
| Colo-205   | 16.1±0.8             | 22.9±1.1             | 39.2±2.9       | 49.7±0.8         |
| THP-1      | 21.0±2.4             | 9.6±1.9              | 37.8±2.6       | 35.8±1.7         |
| HL-60      | 28.7±2.3             | 33.5±1.0             | 9.7±2.2        | 50.0±1.0         |
| K562       | 46.6±2.4             | 50.0±1.9             | 42.8±2.6       | 49.9±1.7         |

*Human cancer cell lines were treated with different concentrations of plant extracts in 96-well microculture plates for 48h. IC\(_{50}\) values were determined by linear regression analysis using SPSS software*
phase was not affected in HL-60 cells treated with these extracts. Camptothecin was used as a positive control in this experiment and exhibited an increase in S-phase population in both HL-60 cells (59.85% vs 49.35% in the control) and K562 cells (83.76% vs 72.63% in the control)). It is worth noting that camptothecin exhibited an increase in sub-G_0/G_1 of HL-60 (97.47% vs 2.08% in control cells) and K562 (4.90% vs 2.97% in control cells).

DNA ladder assay
To elucidate whether plant extracts decreased cell survival by the induction of DNA fragmentation, genomic DNA was isolated from control and treated HL-60 cells and then subjected to 1.8% agarose gel electrophoresis. In the present study, no DNA ladder was observed in control or treated HL-60 cells with extracts (Figure 3). This implied that, incubation HL-60 treated with the extracts for 24h induced cell death which apparently was accompanied by the formation of a large DNA fragments, represented as smear upon agarose gel electrophoresis. On the other hand, a characteristic ladder of nucleosome-sized DNA fragments was observed in camptothecin-treated HL-60 cells.

Discussion
Our in vitro experiments showed that the selected plant extracts exhibited variety of cytotoxicity against the examined cancer cell lines. Among the examined cell lines, K562 cells were the most resistant toward the extracts with IC_{50} value in the range of 42.8-50 µg/mL. Whereas, PC3, MCF-7 and A459 are the most sensitive cells with

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**Figure 1. Human Cancer Cell Lines were Treated with Different Concentrations of Plant Extracts in 96-well Microculture Plates for 48h.** IC_{50} values were determined by linear regression analysis using SPSS software. The different letters above the bars for each cell cycle phase indicate significant differences (P < 0.05) between treated cells as determined by ANOVA followed by Duncan’s multiple comparison tests.

**Figure 2. The Cell Cycle Phase Distribution of Four Indian Medicinal Plants in K562 Cells Within 24h.** The different letters above the bars for each cell cycle phase indicate significant differences (P<0.05) between treated cells as determined by ANOVA followed by Duncan’s multiple comparison tests.

**Figure 3. DNA Fragmentation Assay in HL-60 Cells.** HL-60 cells (2×10^4/ml/well) were incubated with plant extracts for 24h. Lane 1 (untreated cells), lane 2 (2µM camptothecin), lanes 3-4 (50 & 100µg/ml of A. latifolia), lanes 5-6 (50 & 100µg/ml of T. bellerica), lane 7-8(50 & 100µg/ml of A. catechu), lane 9-10 (50 & 100µg/ml of M. oleifera)
The appearance of the characteristic sub-G1/G0 (sub-­diploid) peak on a DNA histogram is a specific marker of apoptosis (Morgan, 2006). The strongest accumulation of sub-diploid peak was observed in HL-­60 cells treated with 100 µg/mL of *T. bellerica* (18.8-fold higher compared to the control) and in K562 cells treated with 100 µg/mL of *A. latifolia* (5.8-fold higher compared to the control). These data are consistent with findings of Ghate et al. (2014) who demonstrated that flow cytometric analysis of 70% methanol extract of *T. bellerica* at high concentration (100 µg/mL) induced inducing apoptosis in lung A549 and breast MCF-7 cancer cell lines.

Interestingly, the absence of apoptosis in K562 cells by *A. latifolia*, *T. bellerica* and *M. oleifera* at low or high concentrations within this experimental duration suggested that the examined extracts inhibited proliferation of K562 cells in cytostatic manner without killing tumor cells. According to Rixe and Fojo (2007), it is possible to distinguish between the cytostatic and the cytotoxic effect. Cytotoxic agents, at both high and low concentrations delay cell progression in both S and G2/M phase, but lethality occurs only in S-phase. By contrast, a cytostatic agent delays cell progression in G1 phase, without lethality at intermediate drug concentrations. The cytostatic activity of whole plant extract on cancer cells is often much better than effect of their isolated active biological compounds, due to a complex interplay of the composite mixture of compounds present in the whole plant (additive/synergistic and/or antagonistic) rather than constituent single agents alone (Smit et al., 1995; Katiyar et al., 2012).

To confirm whether the sub-G1 peak is due to apoptotic HL-­60 cells, the DNA was examined by DNA gel-electrophoresis. To date, two major mechanisms of eukaryotic cell death have been identified: necrosis and apoptosis. Necrosis is characterized by random DNA fragmentation resulting in a smear on agarose gel. Apoptosis is characterized by cleavage of chromosomal DNA into oligonucleosomal DNA which detected upon agarose gel electrophoresis as distinct DNA laddering (Kim et al., 2005). In our experiments, incubation HL-­60 with the extracts produced a smear pattern of DNA degradation upon agarose gel. A possible mechanism for these extracts was suggested to be necrosis rather than apoptosis, because of the appearance of sub-G1 peak and the non ladder type degradation of nuclear DNA in the early stage of cell death (Qian et al., 1995). This implied that, these extracts exerted their cytostatic activity on HL­-60 cells by accumulation of cells in G1 phase of the cell cycle and reduction of cells in S and G2/M. Therefore, plant extracts could be proposed as adjuvant in cancer chemotherapy. Based upon the initial screening work reported here, further experiments are required to provide a better understanding of their molecular anticancer mechanisms involved in both in vitro and in vivo system.

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