Leaf Extracts of *Azadirachta indica* and *Terminalia arjuna* Induce Death of HeLa cells without DNA Degradation

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ABSTRACT: Cancer is one of the major causes of death all over the world. Different attempts have been taken for the prevention and cure of this devastating disease. Along with various chemically synthesized drugs, extracts from different medicinal plants have been studied to combat cancer. In this study, the effects of leaf extracts from *Azadirachta indica* and *Terminalia arjuna* have been investigated on HeLa cells, a well known human cancer cell line. Both of the extracts were found to cause death of HeLa cells dose-dependently. However, the extract of *T. arjuna* was found more promising than the extract of *A. indica* in mediating cell death. At a concentration of 50 µg/ml, *T. arjuna* extract killed 95% cells, whereas the same concentration of *A. indica* extract killed only 55% cells. In many cases, cell death induced by various chemicals often involves fragmentation of DNA. In this study, the extracts of both plants did not induce any detectable level of HeLa cell DNA fragmentation as demonstrated by agarose gel electrophoresis. This result argued that the cell death might occur before initiation of the process of cellular DNA fragmentation bypassing the apoptotic pathway of cell death.

Key words: Cancer, Cell death, *Azadirachta indica*, *Terminalia arjuna*.

INTRODUCTION

Cancer is a complex disease characterized by uncontrolled growth and spread of transformed cells which, if not controlled, may ultimately lead the patient to death. An alarming number of patients have already been diagnosed with cancer worldwide and most of them awaiting for death due to lack of an effective drug therapy.[1] The management of cancer depends on its types, proper diagnosis and stage of cell growth. Mostly, cancer is treated by surgery, chemotherapy or chemoprevention, hormonal therapy, immunotherapy and radiotherapy.[2] Generally, cancer prevention methods result in delaying or blocking the processes of initiation and progression from pre-cancerous cells into cancers.[3] Cancer chemoprevention includes the use of drugs or other chemical agents that can inhibit, delay or revert the development of cancer.[4] Significant advancements in cancer chemoprevention have lowered the incidence of certain cancer types.[3,4] Secondary metabolites so far been discovered from different plants are considered as promising new source of therapeutic agents to combat this deadly disease, cancer.[5] Extensive research around the globe is going on with various traditional medicinal plants aiming to discover new potential therapeutic agents that are deficient in toxic manifestations.

Azadirachta indica (Family Meliaceae) and *Terminalia arjuna* (Family Combretaceae) are well-known medicinal plants in our country. Different parts of the plants and their extracts have been used for medicinal purposes to fight different diseases

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since the ancient time.[6] Many studies with plant extracts have been reported to induce death of cancer cell. For example, extracts of trichosanthes root tubers have shown to induce apoptosis on Hep2 and HeLa cells.[7] Aqueous extract of cactus pear have been reported to show anticancer activities in immortalized ovarian epithelial cells in mouse model.[8] Moreover, flavonoid-rich extracts from mature roots of *Scutellaria baicalensis* showed effective anticancer activities on human malignant brain tumor cells.[9]

Cancer chemotherapy is one of the wide-ranging approaches in cancer treatment and cure, in which chemical agents are used to heal cancer in patients. Although chemotherapy has shown promise in treating some cancers, available therapeutic agents are still limited and costly, and have adverse side effects. In this study, we determined the anti-cancer activity of two locally important and widely used plants, *A. indica* and *T. arjuna*. Our data demonstrated that the leaf extracts of these plants have significant growth inhibitory effect in HeLa cell, a human cervical carcinoma cell line.

MATERIAL AND METHODS

Preparation of plant extracts. *A. indica* (accession number DABC-33885) and *T. arjuna* (accession number DABC-33886) leaves were air dried in a cool dried place keeping away from direct sunlight. Then the dried leaves were powdered and soaked in 95% ethanol for 24 hrs. After filtration, the filtrate was evaporated using a vacuum rotary evaporator. The gummy substance thus obtained was freeze-dried. The freeze-dried extracts were dissolved in 40% ethanol (v/v) to prepare different concentrations of the extract(s), prior to anticancer assay.

From 125 g dried leaf powder, 12.2 g of *A. indica* and 12.54 g of *T. arjuna* extracts was obtained. Both the extracts were amorphous powder and bitter in taste. *A. indica* extract was little bit gummy and looked dark green in color whereas *T. arjuna* looked dark brown.

Maintenance of HeLa cells: HeLa cells were cultured in DMEM (Dulbecco's modified Eagle's medium, GIBCO, USA) containing 10% fetal calf serum (Sigma, USA) at 37°C in a 5% humidified CO_2 atmosphere.

Effect of the plant extracts on HeLa cell morphology: HeLa cells (2.5×10^4 cells/well) were plated and cultured for 24 hrs to obtain confluent growth. The medium was then discarded and fresh serum-free DMEM medium was added containing various concentrations (5, 10, 25 and 50 µg/ml) of the plant extracts. The cells were incubated for 48 hrs and observed under the microscope for any change in their morphology. The cells were then trypsinized and subjected to stain with trypan blue to count viable and dead cells. A known cytotoxic compound, vincristine sulphate (Sigma, USA) (5 µg/ml) was used as a positive control.

Preparation of genomic DNA and agarose gel electrophoresis: HeLa cells (10^5/ml) were cultured in each well of a 6-well microtitre plate under starved condition (without FCS) for 8 hours with 2 ml of DMEM. Then the solutions of plant extracts were added to respective wells so that the final concentration of extracts become 50 and 100 µg/ml. After 24 hrs, all cells (both floating and attached) were collected, centrifuged and washed twice with phosphate buffered saline (PBS). Cellular DNA was then isolated as described previously.[10] Briefly, the cells were lysed using lysis buffer (50 µl solution consisting 50 mM Tris, 25 mM EDTA, 400 mM NaCl, 10% SDS bringing to pH 8 with HCl) followed by the addition of 2 µl of proteinase K (20 mg/ml) and 6 µl of RNase (10 mg/ml). The resultant mixture was incubated in water bath at 55 ^{0}C for 1 hour. Then 3 µl of 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose was added. The sample was subjected to agarose gel (1.5%) electrophoresis and photographed was taken under UV transilluminator.[10]

RESULTS AND DISCUSSION

Plant extracts showed cytotoxic effect on HeLa cells: Many plant extracts have been reported to induce death of cancer cells.[7-9, 11-14] We examined here whether *A. indica* and *T. arjuna* plant extracts have any effect on HeLa cells. When we examined the plant extracts-treated HeLa cells under microscope, we observed significant morphological changes of the cells. The adherent HeLa cells were detached from the culture plate and become floated resulting in rounding up of cellular shape (data not shown). This degenerative morphological change ultimately led the cells to death. The percentage of dead cells was then calculated by counting the cells after stained with trypan blue. It was observed that both extracts were able to induce death of HeLa cells in a concentration-dependant manner (Figure 1). We observed that *T. arjuna* extract was more effective to induce cell death than *A. indica* extract. Higher concentration (50 µg/ml) of *T. arjuna* extract induced about 95% of cell death, however, the same concentration of *A. indica* extract induced only 55% of cell death. A lower concentration (5 µg/ml) of both extracts could not induce cell death significantly. Vincristine sulphate (5 µg/ml), used as a positive control, induced more than 95% cell death.

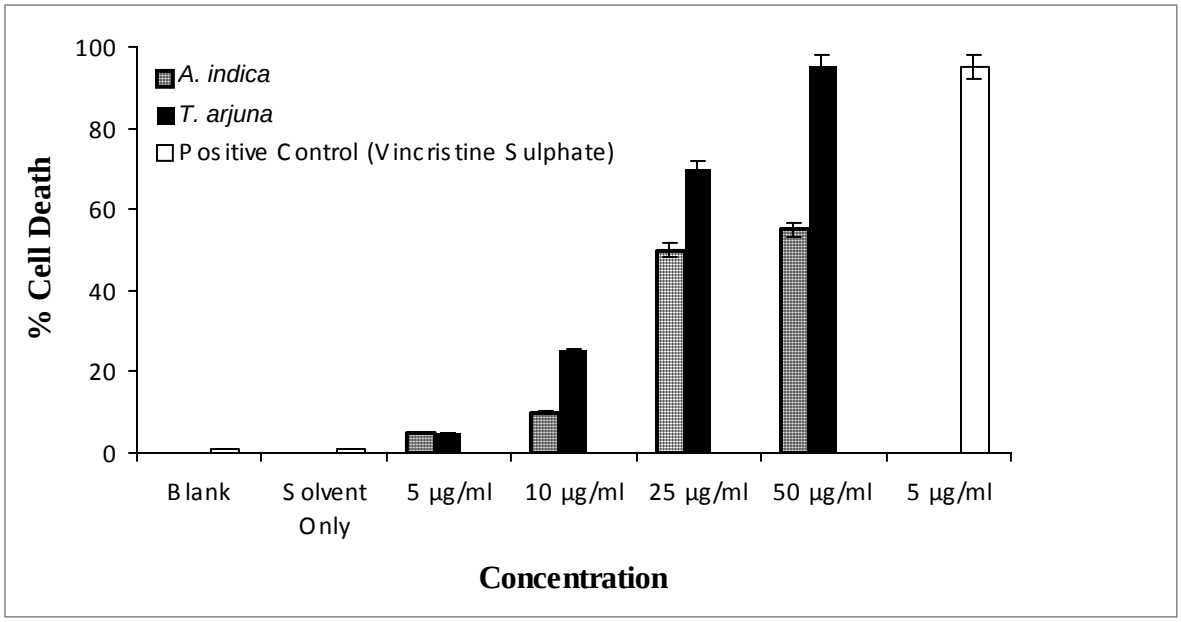

Figure 1. Cytotoxic activity of *A. indica* and *T. arjuna* ethanolic leaf extract on HeLa cells. HeLa cells were grown on DMEM media with different concentrations of plant extracts as indicated. In one group, vincristine sulfate was used as a positive control. In addition to that, media (blank) and solvent were added to another two groups instead of the plant extracts. After 48 hrs of incubation, number of viable and dead cells was counted by trypan blue exclusion method. Percent cell death was plotted against indicated concentrations of the plant extracts. The data shown here is the average of three experiments.

The plant extracts failed to induce DNA fragmentation. Many herbal medicines induced apoptosis of cancer cells involving DNA fragmentation.[7,11-14] We next investigated to find out whether the death of HeLa cells was due to the fragmentation of their chromosomal DNA or not. The DNA isolated from *A. indica* and *T. arjuna* extracts-treated HeLa cells were detected at the same area of the agarose gel at an upper side together with that of the untreated control cells (Figure 2) indicating that the plant extracts did not induce DNA fragmentation. This result might argue that cell death induced by the plant extracts perhaps involved necrotic effect that bypassed fragmentation of chromosomal DNA.[10]

Both *A. indica* and *T. arjuna* are well known and popular medicinal plants in the South Asian region including Bangladesh. In the present study, we observed that the leaf extracts of *A. indica* and *T.*

arjuna could induce death to HeLa cells. As HeLa cells are adherent type of a cancer cell line, live cells normally adhere at the surface of culture plate. When the cells die, they are morphologically changed and are detached from the surface resulting in rounding up of their shape. After treatment with these two plant extracts, most of the HeLa cells were detached from the surface of culture plate and became round in shape which were observed under the microscope (data not shown). However, the control HeLa cells showed confluent growth in monolayer. This result corresponded well with an earlier report of vincristine-induced morphological changes of HeLa cells that ultimately lead the cells to death.[15]

In our study, many of the cells that experienced morphological changes were found dead by trypan blue staining. We then determined the percentage of cell death induced by the plant extracts. It was found that *T. arjuna* extract was more effective than the extract of *A. indica* for inducing cell death. At 50 μg/ml concentration, *T. arjuna* extract killed about 95% and *A. indica* extract killed only 55% of the cells. This discrepancy between their potential in cell killing might be due the presence of higher amount of effective constituents in the former extract than in the latter. The cytotoxic effects of the extracts might be due to reduction in chromatin condensation and formation of cytoplasmic blebs.[14, 15]

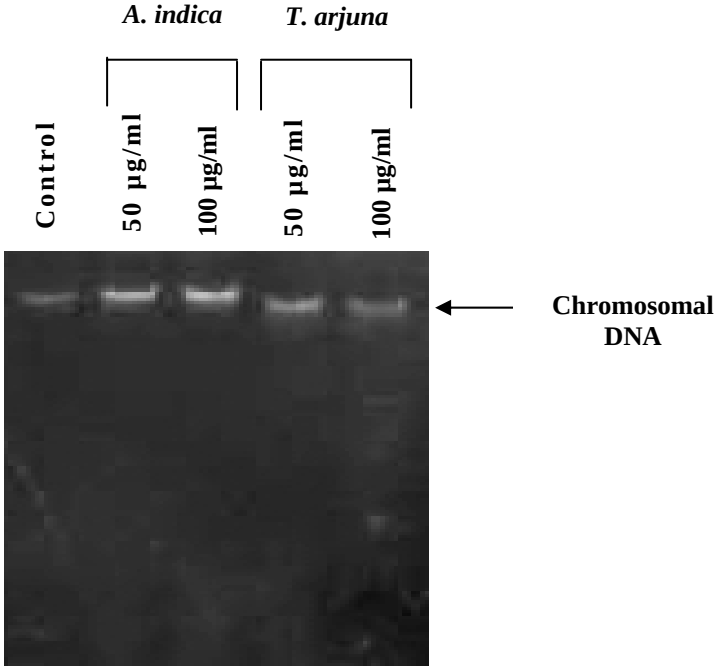

Figure 2. Agarose gel electrophoresis of HeLa cell chromosomal DNA. HeLa cells were cultured with or without indicated concentrations of the plant extracts for 24 hrs. The cells were then lysed to isolate the DNA and the DNA was run on 1.5% agarose gel. DNA bands were visualized under UV light and photograph was taken.

As many cancer drugs induce apoptotic death of cells involving DNA fragmentation, we investigated to find out whether the death of HeLa cells was due to the fragmentation of their chromosomal DNA. We observed that both of the plant extracts failed to induce fragmentation of HeLa cell DNA (Figure 2). It was previously reported that some toxic chemicals or compounds could induce death of murine T-lymphocytes with or without DNA fragmentation. Cell death without DNA fragmentation, which is apparently necrotic, also required a protein phosphorylation-linked signal.[10] This led us to conclude that the plant extracts, used in this study, induced death of the cells bypassing the classical apoptotic pathway that accompany DNA fragmentation. Most probably the cells were dead due

to the direct cytotoxic effects of the plant extracts and the cell death might occur before activation of the DNA fragmentation machinery inside the cells.

The findings of this study clearly indicated that the extracts of *A. indica* and *T. arjuna* have potential against cancer cells. Further works are needed to isolate and characterize the active component(s) of the extracts for future therapeutic application. Also, the genes responsible for the synthesis of the component(s) would be identified for further manipulation of the genes to obtain various biotechnological benefits.

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