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In vitro cytotoxic effect of hydroalcoholic extracts of medicinal plants on Ehrlich's Ascites Carcinoma (EAC)

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Abstract

Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives each year. An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents (alone or in combination) to block the development of cancer in human beings. Plants, vegetables, herbs and spices used in folk and traditional medicine have been accepted currently as one of the main sources of cancer chemopreventive drug discovery. Hydroalcoholic extracts of *Terminalia chebula*, *Terminalia belerica*, *Emblica officinalis*, *Caesalpinia crista*, *Cajanus cajan*, and *Tinospora cordifolia* are found to be variably and selectively cytotoxic to normal and EAC cells. Hydroalcoholic extracts of *Terminalia chebula*, *Terminalia belerica*, *Emblica officinalis*, *Caesalpinia crista*, *Cajanus cajan*, and *Tinospora cordifolia* which have been found to be possible natural antioxidant are evaluated for their selective cytotoxic effect on murine tumor cell Ehrlich's Ascites Carcinoma (EAC) and normal spleenocyte cell. The extracts were subjected to cytotoxicity test by the tetrazolium cell proliferation reagent (WST-1) assay *in vitro*. The results showed that the plant extracts were invariably non toxic for the normal spleenocyte cell, whereas they showed toxicity for EAC cells in different degree. The cell cycle analysis for the EAC cells treated with the extracts of the aforesaid plants showed a variable, yet dose-dependent increasing percentage of apoptosis. The results signify that the plants which have antioxidant property may function as cytotoxic agent for cancer cell.

Keywords: Medicinal plants, hydroalcoholic extract, EAC, cytotoxicity

Introduction

Over the past few years, cancer has remained a major cause of death and the number of individuals living with cancer is continuing to expand. Hence, a major portion of the current pharmacological research is devoted to anticancer drug design customized to fit new molecular targets [1]. Chemotherapy is a major treatment modality for cancer and some plants have provided active principles, which are used to control the advanced stages of malignancies in clinical settings [2,3]. However, most of these

chemotherapeutic agents exhibit severe normal toxicity, and cause undesirable side effects. Many of the potent drugs are highly expensive, mutagenic, carcinogenic and teratogenic. Therefore, a need is felt to find alternative drugs, which are highly effective at non-toxic doses, inexpensive and accessible to common man. This can be achieved by screening plant products with antioxidant property, which may be effective at non-toxic dose levels, since antioxidants are compounds that protect cells from free-radical induced degenerative diseases like cancer. Due to the enormous propensity of plants to act as natural antioxidants by scavenging of reactive oxygen species (ROS), through synthesis of a variety of structurally diverse bioactive compounds, the plant kingdom is a potential source of chemical constituents with antitumor and cytotoxic activities [4-6].

Experimental tumors have great importance in modeling, and Ehrlich ascites carcinoma (EAC) is one of the commonest tumors. EAC is referred to as an undifferentiated carcinoma and is originally hyperdiploid, has high transplantable capability, rapid proliferation, shorter life span, 100% malignancy and also does not have tumor-specific transplantation antigen. EAC resembles human tumors which are the most sensitive to chemotherapy due to the fact that they are undifferentiated and that they have a rapid growth rate. The ideal drug being ineffective or minimally effective for normal cells have been focused on, and at this point, the usage of natural sources as an alternative cancer therapy is thought to have a great value for cancer control.

Of the six plants studied here, *Terminalia chebula* Retz. has been reported for its anticancer activity [7] and along with two other Myrobalans, viz., *Terminalia belerica* Roxb. and *Emblica officinalis* Gaertn. has produced an Ayurvedic polyherbal formula, Triphala which has been established as potent anticancer agent [8,9] for quite some time. These plants have already been reported to have antioxidant property [10]. The other plants used in the study, viz., *Caesalpinia crista* Linn. and *Tinospora cordifolia* (Thunb.) Miers have already been reported as a natural antioxidant [11,12], along with antineoplastic effect of the methanol extract of the former and dichloromethane extract of the latter plant on EAC bearing mice [13,14]. Lastly, antioxidative and anticancer properties of *Cajanus cajan* (L.) Millsp. has already been reported [15-17]. However, no earlier study has been done regarding the cytotoxic effect of the hydroalcoholic extract of the aforementioned plants on EAC cell *in vitro*, thus initiating the effort for the study.

Materials and Methods

Collection & Authenticity of medicinal plants

The plant materials were collected from Bankura district of West Bengal, India and the genus and species of the medicinal plants are authenticated through the Central Research Institute (Ayurveda), Kolkata, India, where specimens for each plant were deposited. A list of the specimen numbers for the plants is provided below:

Serial No.	Name of the plant	CRI (Ayurveda) specimen No.
1	*Terminalia chebula*	CRHS 113/08
2	*Terminalia belerica*	CRHS 114/08
3	*Emblica officinalis*	CRHS 115/08
4	*Caesalpinia crista*	CRHS 121/08
5	*Cajanus cajan*	CRHS 119/08

Preparation of Plant Extract

The powder (100 g) of the dried fruits of *T. chebula, T. belerica* and *E. officinalis*, leaves of *C. crista* and *C. cajan* or stem of *T. cordifolia* was stirred using a magnetic stirrer with 500 ml mixture of ethanol:water (6:4) (hydro-alcoholic extract) for 15 hours; then the mixture was centrifuged at 2850 x *g* and the supernatant decanted. The process was repeated again with the precipitated pellet. The supernatants were collected, concentrated in a rotary evaporator at room temperature and freeze dried in a

lyophilizer. The dried extract was stored at -20°C until use.

Animals

The Animal Ethical Clearance Committee of the Institute (Registration number: 95/1999/ CPCSEA) approved the use of adult male Swiss Albino mice, weighing 20-25 gm for experimentation. The animals were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. They were housed in standard environmental conditions of temperature (25 ± 2°C), humidity (60 ± 5%) and under light and dark cycles of 12-h.The mice were fed standard laboratory diet and were given sterilized water *ad libitum.*

Isolation of Cancer cells (EAC)

The transplantable cancer cell namely Ehrlich's Ascites Carcinoma (EAC) cells were used in the present study. The EAC cells were maintained *in vivo* in Swiss albino mice, by interperitoneal (ip) transplantation of 2 x 10^6 cells /mouse after every 10 days. EAC cells 9 days old were used for the experiment. These tumors in the ascites form are fast growing and kill the host animals within the period of three-four weeks approximately after tumor transplantation with 2 x 10^6 cells. The tumor uptake by the host was manifested by a very high initial growth rate followed by exponential growth up to a period of 10^{th} day post transplantation (log phase) followed by gradual decline in this growth rate with progressive accumulation of ascites fluid (lag phase). By the 21^{st} day post transplantation, the tumor volume reaches its maximum.

The mice were killed by cervical dislocation (acceptable method of euthanasia), dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000 rpm for 5 min. It was then resuspended in excess RPMI-1640 medium (with 10% HI FBS) and taken in sterile Petri dishes and incubated in a CO_2 incubator (37°C; 5% CO_2) for 1 h. The cells of macrophage lineage adhered to the bottom of the Petri dishes. The non-adherent population was aspirated out gently and washed repeatedly with PBS. The cells were centrifuged at 3000 rpm for 5 min, resuspended in medium and counted in a haemocytometer.

Isolation of Spleenocyte

Spleens from normal healthy mice were dissected after sacrifice of the mice and washed twice with sterilized PBS. The spleen was then taken in a Petri dish containing RPMI-1640 medium (with 10% HI FBS) and incubated in a CO_2 incubator (37°C; 5% CO_2) for 1 h. The non-adherent spleenocyte population was aspirated out gently, centrifuged at 3000 rpm for 5 min, resuspended in medium and counted in a haemocytometer.

Cytotoxicity and Cell Viability

For the estimation of the cytotoxic activities of the plant extracts, a colorimetric assay was employed for quantification of cell proliferation, cell viability, and cytotoxicity using a kit (Cell Proliferation Reagent WST-1) from Roche Diagnostics GmbH. The cytotoxicity of the various concentrations of hydroalcoholic crude of the different plants were evaluated in both EAC and Spleenocyte cells, where separate experiments were set up for each cell line. Briefly, 1 × 10^4 viable cells from each cell line were seeded into a 96-well flat bottomed plate with requisite amount of RPMI-1640 medium. Thereafter, the cells were treated with various concentrations of each plant extract (in triplicates) and were left undisturbed and allowed to grow for 16 h. Then, 10 µl/well Cell Proliferation Reagent WST-1 was added and again incubated for one and a half hour. It was shaken thoroughly for 1 min on a shaker. The absorbance of the samples was measured against a background control as blank using a microplate (ELISA) reader at 450 nm.

Anticancer activity based on quantification of apoptotic cell by flowcytometry

For the determination of cell cycle phase distribution, around 1.5-2 × 10^6 cells were cultured in several 24-well plates containing RPMI-1640 medium (with 10% HI FBS) and treated with various concentrations of each plant extract (in triplicates) and were left undisturbed and allowed to grow for 16 h in a CO_2 incubator (37°C; 5% CO_2). Post treatment, the cells were centrifuged to discard any medium, fixed with chilled methanol and diluted with sterilized PBS. After dilution, Triton X-100 and RNAse A were added to permeate the cell wall and digest the RNA during incubation for 3 hrs at 37°C. Nuclear DNA of EAC cells was labeled with ethidium bromide and phase distribution was determined on FACS, fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using CellQuest software (Becton Dickinson). A total of 10,000 events were acquired and analysis of flowcytometric data was performed using ModFit software. A histogram of DNA content (*x*-axis, red fluorescence) versus counts (*y*-axis) has been displayed.

Statistical analysis

All data are reported as the mean ± SD of six measurements. Statistical analysis was performed using KyPlot version 2.0 beta 15 (32 bit). The IC_{50} values were calculated using the formula Y = 100*A1/(X + A1) where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC_{50} values were compared by paired t-Test. The results with a value of $p < 0.05$ were considered significant.

Results & Discussion

Cancer is a disease of misguided cells that have high potential of excess proliferation without apparent relation to the physiological demand of the process. It is the second largest cause of death in the world. Of all the available anticancer drugs during 1940 – 2002, 40% were natural products per se or natural product derived, with another 8% being natural product mimics [18]. The greatest recent impact of plant derived drugs is observed in the area of antitumor research, where compounds such as taxol, vinblastine, vincristine, and camptothecin have dramatically improved the effectiveness of chemotherapy against some of the dreaded cancers [19]. Hence, there is a great potential for the development of anticancer drugs from the essentially untapped reservoir of the plant kingdom. A large number of plants possessing anticancer properties have been documented [4,20-22].

Cytotoxicity and Cell Viability

Biological activity of any plant is most certainly depends on its cytotoxic property. For that purpose, the cytotoxicity effect of the hydroalcoholic extracts of the plants were studied on both normal cells (spleenocyte) and cancer cell (EAC).

The results (Figure 1) showed that all the 6 plant extracts showed no or very low cytotoxic activity against healthy normal spleenocyte cell lines tested. The low cytotoxic potential of the extracts is of great significance for their traditional use in the treatment of various disorders along with cancer.

Moderate or highly selective cytotoxic activity was observed for all the plant extracts against EAC cell lines (Figure 2) as is also evident from their IC_{50} values (Table 1). The extracts of *Terminalia belerica* and *Emblica officinalis* displayed moderate cytotoxicity (IC_{50} 564.06 ± 77.03 µg/ml and 542.53 ± 44.51 µg/ml, respectively, whereas the hydroalcoholic extracts from *Terminalia chebula* fruits and *Tinospora cordifolia* bark showed significantly lower IC_{50} values of 344.06 ± 22.09 µg/ml and 306.34 ± 44.75 µg/ml, respectively. However, the extracts from *Caesalpinia crista* and *Cajanus cajan* leaves showed the highest cytotoxicity (IC_{50} 188.42 ± 18.74 µg/ml and 168.42 ± 29.49 µg/ml) against EAc cell lines among all extracts tested in this study. The cytotoxic activities of the hydroalcoholic extracts of the plants as summarized in Table 1, is worth noting since the IC_{50} values for some plants, viz., *Terminalia belerica* and *Emblica officinalis*, while somewhat high, still point subtly towards selective activity.

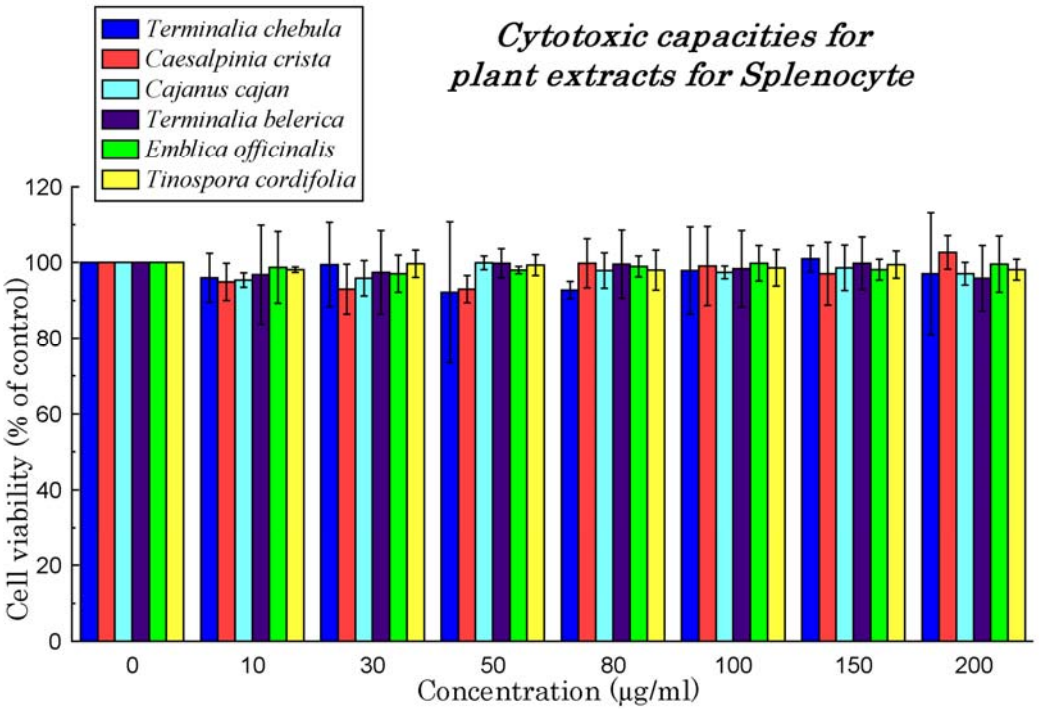


Figure 1: Cytotoxicity for normal spleenocytes

Effect of the plant extracts on normal spleenocyte cells. The results were expressed as mean ± S.D. (n=6). All the data were found to be highly significant with respect to control.

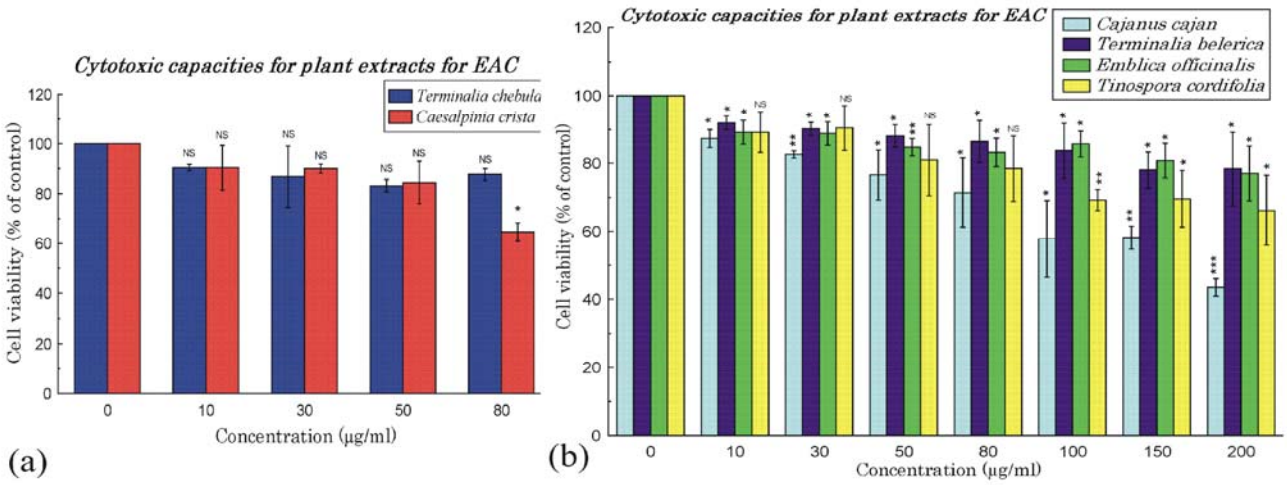


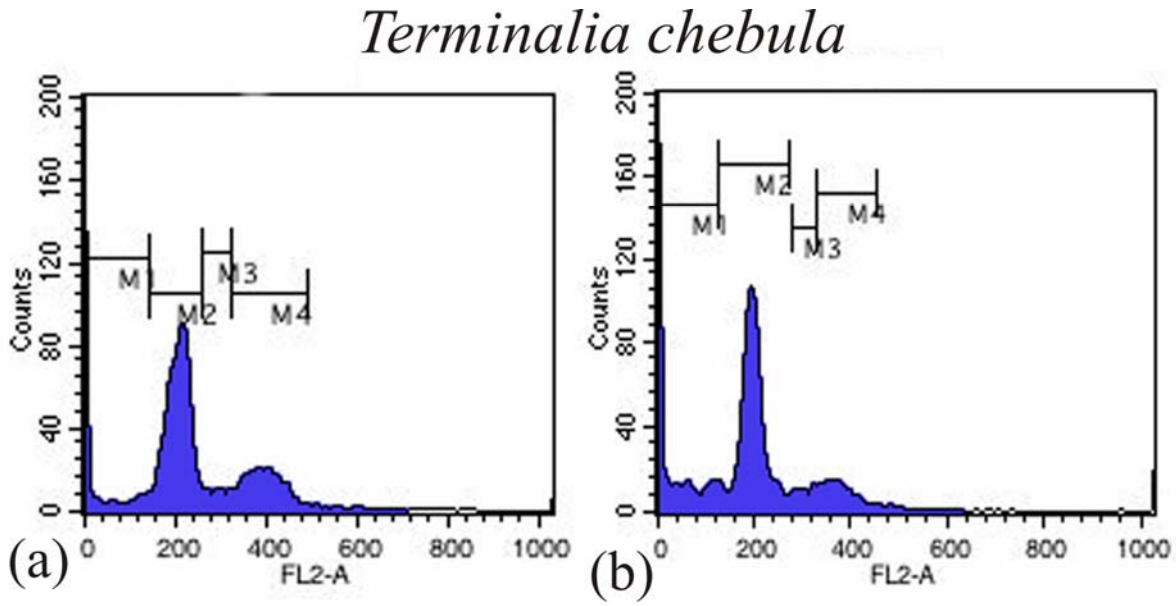
Figure 2: Cytotoxicity for Ehrlich's Ascites Carcinoma

Effect of the plant extracts on normal spleenocyte cells. (a) The extracts of *Terminalia chebula* and *Caesalpinia crista* showed significant toxicity at lower levels (0-80 µg/ml), whereas (b) a higher concentration range was to be observed for the extracts of *Cajanus cajan, Terminalia belerica, Emblica officinalis* and *Tinospora cordifolia* (0-200 µg/ml). All data are expressed as mean ± S.D. (n=6). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. 0 µg/ml.

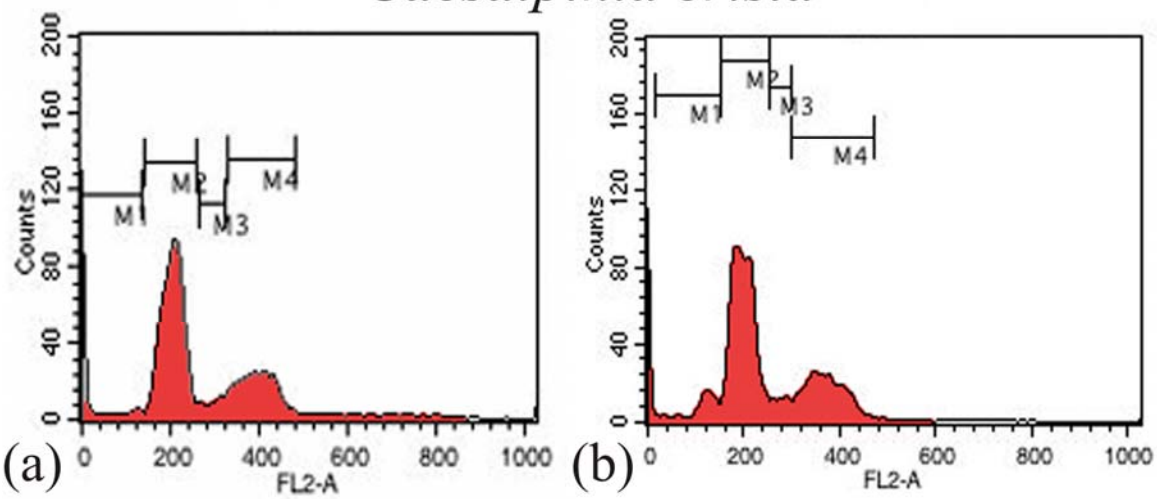
Table 1: IC_{50} values for the cytotoxic capacities of hydroalcoholic extracts of the studied plants

Serial No.	Name of the plant	IC_{50} values (in µg/ml) for the cytotoxic effect on EAC cell
1.	*Terminalia chebula*	344.06 ± 22.09***
2.	*Caesalpinia crista*	188.42 ± 18.74***
3.	*Cajanus cajan*	168.42 ± 29.49**
4.	*Terminalia belerica*	564.06 ± 77.03^{NS}
5.	*Emblica officinalis*	542.53 ± 44.51**
6.	*Tinospora cordifolia*	306.34 ± 44.75*

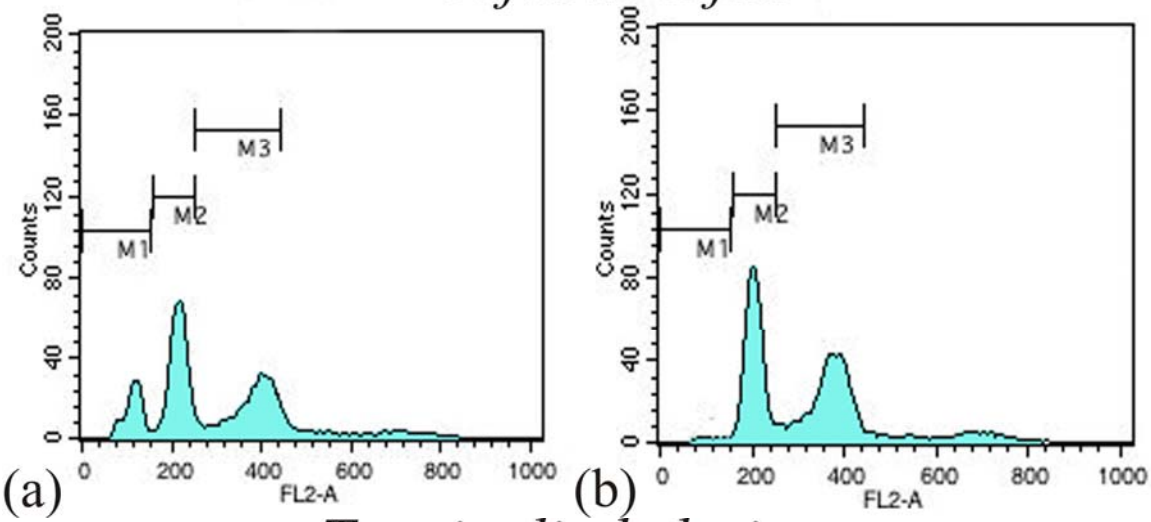
IC_{50} values of the extracts are represented as mean ± SD (n=6). * p< 0.05; ** p< 0.01;*** p< 0.001; NS = Non significant



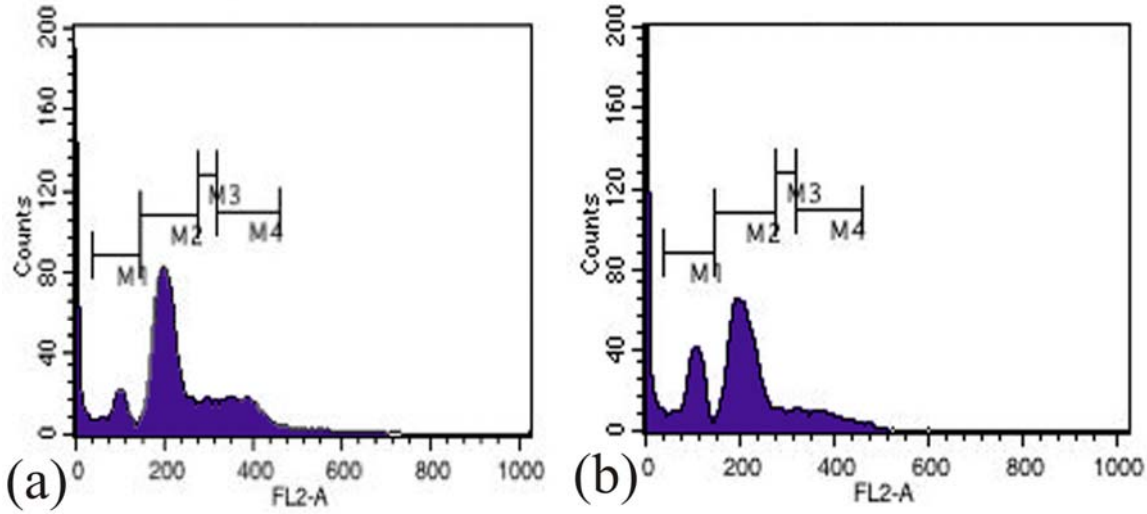
Caesalpinia crista



Cajanus cajan



Terminalia belerica



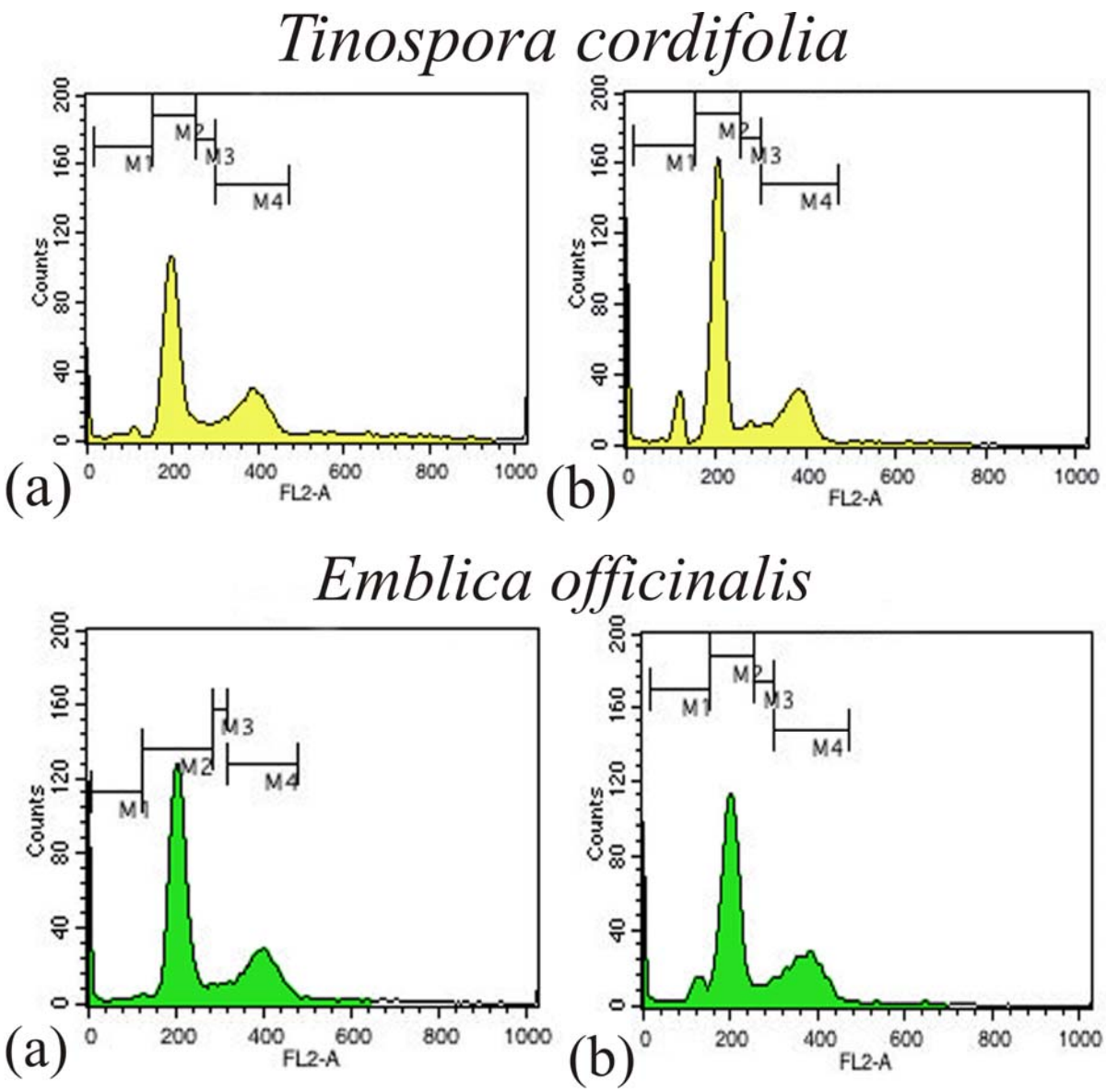


Figure 3: Cell cycle analysis for EAC treated with plant extracts

Induction of apoptosis by the plant extracts on Ehrlich's Ascites Carcinoma (EAC) cells. The histogram analysis of the cell cycle for each plant has been depicted in different colors. The subgroupings of each picture signifies (a) cell cycle for the EAC treated with no dose and (b) highest dose of the mentioned plant extracts. Areas marked in each histogram signifies: M1 – G_0 phase; M2 – G_1phase; M3 – S phase; and M4 – G_2 phase for each cell cycle. M3 for *Cajanus cajan* combines the S phase and the G_2 phase.

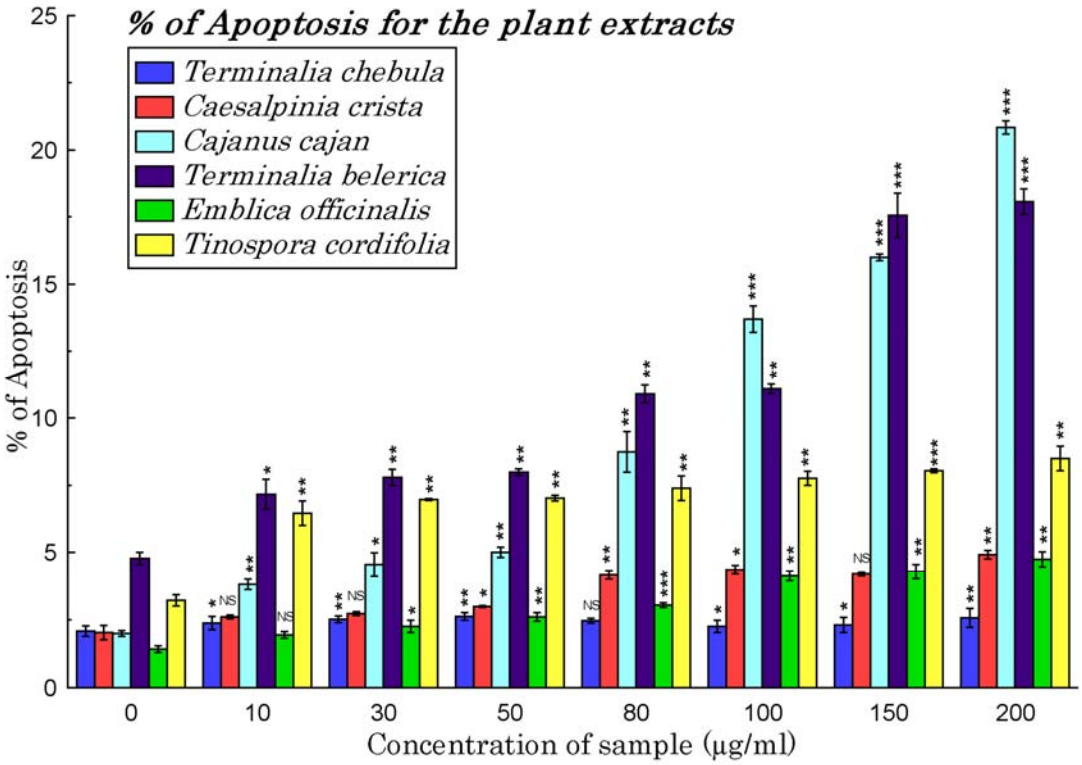


Figure 4: Comparison of apoptosis in EAC cells among various medicinal plants

Induction of apoptosis by the plant extracts on Ehrlich's Ascites Carcinoma (EAC) cells. All data are expressed as mean ± S.D. (n=6). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. 0 µg/ml

These "high" values are likely due to very low concentrations of compounds of interest, which would be considerably enriched upon bioactivity-guided fractionation.

Anticancer activity based on induction of apoptotic cell:

Since anticancer activity of any compound should induce apoptosis of cancer cell, our *in vitro* studies have shown that the plant extracts were effective in imparting growth inhibition, cell cycle deregulation and apoptosis in EAC cells. Apoptotic cell death was measured as the percent of cells with hypodiploid DNA.

The histogram analyses of the interphase cycle of EAC cells treated with plant extracts as shown in Figure 3 indicate that the hydroalcoholic extracts of three plants, viz., *Terminalia belerica*, *Cajanus cajan* and *Tinospora cordifolia* selectively induced apoptosis of cancerous EAC cell in a dose dependent manner. The variation of apoptosis of EAC cells as induced by the plant extracts is furthermore quantified to get a better picture of the comparative activity of plant extracts as has been shown in the Figure 4. The figure as can be found undoubtedly corroborates with the histogram analysis of the cell cycle for the plant extract treated EAC cells. The qualitative indication that the plant extracts of *Terminalia belerica*, *Cajanus cajan* and *Tinospora cordifolia* are more effective than the other three has been quantitatively confirmed in this comparison of percentage of apoptosis.

Conclusions

In the Ayurvedic system of treatment, dry powder or crude extracts of plants are used to treat various disorders including cancer. Many such compounds are known to possess potent antitumor properties [23]. The observed effect is attributed to not only a single compound but also the other components present in the crude extract/s. The rationale for this type of treatment is that the toxicity of an active component may be counteracted by another component, which may not have the desired therapeutic property.

This study supports the traditional uses of *Caesalpinia crista* and the reported cytotoxic activities of *Terminalia chebula*, *Cajanus cajan*, and *Tinospora cordifolia*. Some of the plant extracts, such as *Terminalia belerica* and *Emblica officinalis* exerted selective cytotoxic activity, but neither cytotoxic activity had been reported previously. This study provides an important basis for further investigation into the isolation, characterisation and mechanism of cytotoxic compounds from some of the screened Bangladeshi medicinal plants. Thus, these plants could be used as a source for new lead structures in drug design to combat cancer.

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