Antitumor Potential of Total Alkaloid Extract from *Tabebuia rosea* (Bertol.) DC. Leaves on MOLT-4 Cells *In Vitro*

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Abstract: Currently there is considerable scientific and commercial interest in the continuing discovery of new anticancer agents from plants. Here the *in vitro* antitumor potential of the total alkaloid extract from *Tabebuia rosea* (Bertol.) DC. leaves was evaluated by MTT (3-(4,5-dimethythiazol-2yl)2,5-diphenyl tetrazolium bromide) based cytotoxicity test using the human leukemic cells (MOLT-4) and the genotoxic potential of the extract was also tested using cytokinesis block *in vitro* micronucleus assay. Simultaneously, the cytotoxic and genotoxic potential of the extract were compared with mitogen stimulated T-lymphocyte cultures derived from peripheral blood of healthy volunteers. The MTT test revealed that the extract exhibited comparatively higher toxicity towards the cancer cells than the normal cells and the GI_{50} values at 24, 48 and 72 h exposure were found to be 46.95, 36.05, and 25.75 µg/ml/1×10^6 cells against cancer cells. The micronucleus assay showed that in both cultures the number of micronuclei obtained even at the highest exposure concentration tested was very low than that of the positive control mitomycin-C. The results of the present investigation demonstrate that the alkaloid extract from *Tabebuia rosea* leaves is preferentially cytotoxic to human T-cell leukemia (MOLT-4) cells in a dose and time dependent manner with the absence of genotoxicity. [Nature and Science 2010;8(9):77-85]. (ISSN: 1545-0740).

Key words: *Tabebuia rosea*; total alkaloid extract; MOLT-4 cells; MTT test; micronucleus assay.

List of Abbreviations: TAE - total alkaloid extract; MTT - 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide; MN – micronucleus; BNC - binucleated cell; GI_{50} - concentration of total alkaloid extract (µg/ml) required to induce 50 % cytotoxicity in the respective time point.

1. Introduction

Tabebuia rosea (Bertol.) DC. is commonly known as "Pink Trumpet Tree" often grown as an ornamental tree for its grand and majestic pink or purple flowers which offer different shades of colours. The graceful beauty is a treat for the eyes, but the tree has medical uses as well (Gentry, 1992). Recently there is considerable scientific and commercial interest in the continuing discovery of new anticancer agents from plant products (Cragg and Newman, 2005) and such investigations about plant products have recently regained prominence with the increasing understanding of their biological significance and increasing recognition of the origin and function of their structural diversity (Conforti et al., 2008). Recently several herbals have been screened for anticancer activity and many patients with cancer take plant extracts in addition to chemotherapy (Dholwani et al., 2008). Nowadays the safety of natural products use has been questioned due to the reports of illness and fatalities (Veiga-Junior et al., 2005). Considering the complexity and their inherent biological variation, it is now necessary to evaluate their safety, efficacy and quality (WHO, 2002). Thus, an assessment of their mutagenic potential is necessary to ensure the relatively safe use of plant-derived medicines (Castrol et al., 2009).

To the best of our knowledge, the effect of *T. rosea* on human cancer cell lines *in vitro* or *in vivo* has hitherto not been reported. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells (Sharma et al., 2009). With this background, the present research is thus motivated by the need to find new substances of natural origin which possess antitumor activities with a low degree of toxicity for humans and an attempt has been made to screen the cytotoxic effect of the total alkaloid extract from its leaves against the human T-cell leukemia (MOLT-4) cells as well as on mitogen stimulated T-lymphocyte cultures derived from the venous blood of healthy volunteers *in vitro* using the standard MTT (3-(4,5-dimethythiazol-2yl)2,5-diphenyl tetrazolium bromide) test. Simultaneously the extracts were subjected to genotoxic assessment using cytokines block *in vitro* micronucleus assay on cancer and normal cells to evaluate their safety as it has been used as an indicator of genotoxicity in mammalian cells (Andrews et al., 1990; Kayani et al., 2009).

2. Materials and Methods

2.1. Chemicals and Reagents

All reagents and solvents used in the study were of analytical grade. Histopaque 1077, L-

glutamine, mitomycin-C and cytochalasin-B were purchased from Sigma, St Louis, USA. RPMI-1640 medium, MTT, streptomycin and penicillin were purchased from Himedia, Mumbai, India. Phytohemeagglutinin and fetal calf serum were obtained from Gibco, New York, USA. All the other chemicals were purchased from SD Fine Chemicals, Mumbai, India.

2.2. Preparation of Total Alkaloid Extract

Leaves of *Tabebuia rosea* were harvested from the Centre for Biodiversity and Forest Studies of Madurai Kamaraj University. The total alkaloids were extracted by the method of Ott-Longoni et al. (1980) with slight modifications (Kandasamy et al., 1989). The leaves were dried, powdered and defated by cold hexane extraction. The defated powder was subjected to repeated methanol extraction and the combined extracts were concentrated in vacuum. The concentrated residue was stirred with 0.2 N HCl and filtered after 16 h. The aqueous solution was shaken with methylene chloride to remove the non basic material and they were made alkaline with ammonium hydroxide in cold. The alkaline solution was extracted with methylene chloride and the organic extract was passed though anhydrous sodium sulphate. The resulting solution was evaporated to dryness to yield total alkaloids of its leaves. The alkaoids freed from other components like flavonoids, glycosides and saponins were tested for their purity using their respective identification test by adopting the method of Peach and Tracey (1959). The yield of TAE was 11.5 % (w/w) with reference to the dried leaves.

2.3. Cells, Media and Culture Conditions

Human T-cell leukemia (MOLT-4) cells procured from National Centre for Cell Sciences, Pune, India were used for evaluating anticancer potential. Mitogen stimulated T-lymphocyte cultures derived from venous blood of healthy persons were used for normal cells. Both the cells were maintained in RPMI-1640 media supplemented with 10 % heat inactivated fetal calf serum, 2 mM L-glutamine, 0.4 % sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 % phytohemeagglutinin in a humidified atmosphere containing 5 % CO_2.

Peripheral whole blood (15 ml) from 3 healthy, 22-25 years old female volunteers was collected with informed consent in heparinized vacutainer tubes (Becton Dickinson Lab ware, USA). Donors were nonsmokers and had not been exposed to chemicals, drugs or X rays in the last 6 months before blood sampling. Lymphocytes were separated using histopaque-1077 (a leukocyte separation technique by Sigma Diagnostics) by density gradient centrifugation at 500 g for 10 min. After recovering the buffy coat, lymphocytes were washed twice with phosphate buffered saline and resuspended in complete media. Lymphocytes were counted using heamocytometer and adjusted to a density of 1×10^6 cells/ml for cell culture studies.

2.4. Determination of Cell Viability and Selection of Exposure Concentration

The extract was dissolved in phosphate buffered saline and diluted with RPMI-1640 medium to obtain 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 µg/ml medium for cell viability assay. Lymphocytes were isolated from the venous blood of healthy volunteers using lymphocyte separation medium and about 1×10^6 cells were seeded in 96-well plates. 150 µl of culture medium containing different concentrations of extract was added to each well and incubated for 3 h at 37 ºC in an atmosphere of 5 % CO_2. After 3 h, 50 µl of 0.4 % erythrosine-B in phosphate buffered saline was added to each well and again incubated for 30 minutes. The number of stained (dead) and unstained (live) cells were counted using hemocytometer under the microscope. The viability was expressed as viable cells in percent of the total cells (absolute viability), and a cell viability below 70 % that of control (relative viability) was considered as a sign of excessive cytotoxicity (Tice et al., 2000).

2.5. Screening of anticancer Potential Using MTT Test

The cytotoxic potential of the total alkaloid extract from *T. rosea* leaves against cancer cells was tested using MTT assay which is based on the respiratory ability of the mitochondrial succinate-tetrazolium reductase system, which converts the yellow tetrazolium salt to a purple formazan dye. The amount of formazan produced by the dehydrogenase enzymes was directly proportional to the number of living cells in culture. About 1×10^6 cancer (MOLT-4) and normal (lymphocytes from healthy donors) cells were seeded in 96-well plates, treated with 10 µg, 20 µg, 30 µg, 40 µg and 50 µg of TAE/ ml medium and incubated at 37 ºC in an atmosphere of 5 % CO_2 for 24, 48 and 72 hours. Cells incubated in complete medium without TAE served as control. At the end of incubation, medium was removed and 50 µl MTT (5 mg/ml) was added and the cells were further incubated for 4 h. After the incubation, the MTT solution covering the cells was removed. 100 µl of dimethylsulfoxide was added to the wells and the cell viability determined by measuring the absorbance in a microplate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm (Mosmann, 1983). The experiment was repeated thrice. Cell viability was calculated using the following formula and from that the percentage of cytotoxicity and GI_{50} values of leaf

extracts were calculated for the different time points studied.

% of cell viability = [Mean OD of experimental wells/Mean OD of control wells] × 100.

2.6. Genotoxic Assessment Using Cytokinesis-block Micronucleus assay

The micronucleus assay was performed according to Matsuoka et al. (1993) with modifications (Bonacker et al., 2004). About 1×10^6 cells/ml medium were exposed to increasing concentrations of leaf extracts in 5 ml culture medium and incubated at 37 °C in an atmosphere of 5 % CO_2. Mitomycin-C (6 µg/ml medium) and complete media were treated as positive and negative controls respectively. After 44 h, cytochalasin-B (6 µg/ml) was added and further incubated for 28 h. At the end of incubation the cells were harvested by low centrifugation, treated with a hypotonic solution of KCl (0.075 M) and fixed in methanol: acetic acid (3:1) for 3-4 h. Two to three drops of the fixed cell suspension were dispensed onto the surface of cold microslides, air dried and stained with 3 % Giemsa solution in Sorenson phosphate buffer (pH 6.8) for 5-7 min. The slides were coded and for each treatment at least 2000 binucleated cells (BNC) per concentration were scored blind for micronucleus frequency in each treatment. The criteria employed for the analysis of micronuclei and binucleated cells were established by Fenech (2000).

2.7. Statistical analysis

All the experiments were independently preformed thrice with three replicates for each treatment. The results were expressed as mean ± SEM. and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test and $p < 0.05$ was considered statistically significant.

3. Results

The results of cell viability assay based on the erythrosine-B dye exclusion potential of living cells treated with logarithmically increasing concentration of TAE are presented in Figure 1. It is very much clear that the optimum viability (70 and above) lies within 100 µg level for all the extract provided a way to the determination of concentration range suitable for cell culture studies.

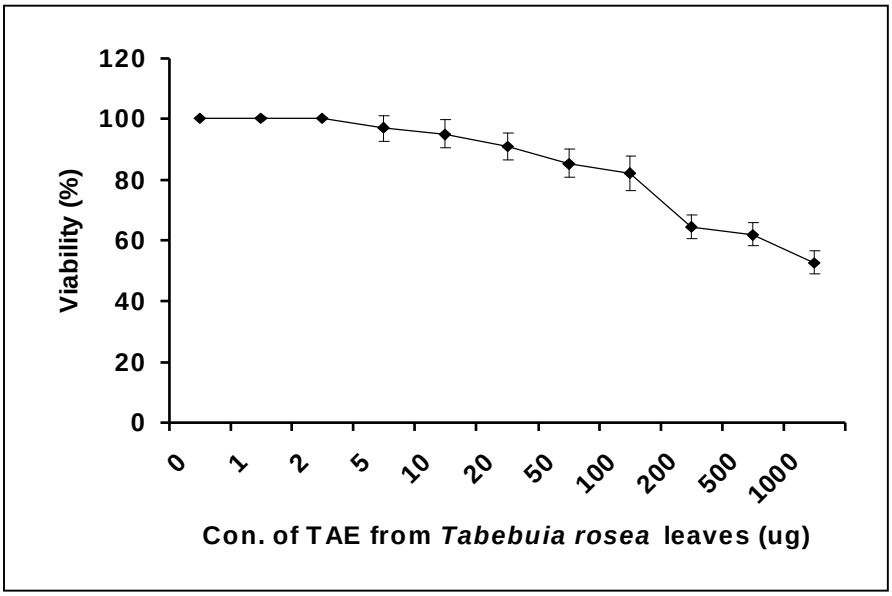

Figure 1. Viability of peripheral blood monocytes exposed to the total alkaloid extract of *Tabebuia rosea* leaves. Peripheral blood monocytes were exposed to increasingly logarithmic concentration of TAE for 3 h and the percentage of viable cells was determined. Error bars represent SEM.

The anticancer potential of TAE was tested by MTT assay and the results are presented in Figure 2. From the results it can be observed that all the three extracts exhibited significantly higher cytotoxicity on cancer cells than in normal cells by exhibiting the GI_{50} values of 46.95 ± 2.5 µg, 36.05 ± 1.75 and 25.75 ± 0.95 µg at 24, 48 & 72 h exposure durations, for cancer cells (Figure 3). When the same dosage was added to normal cells determination of GI_{50} values was not possible at all the time points studied which showed the growth inhibition by TAE was significantly lesser in normal cells than in cancer cells.

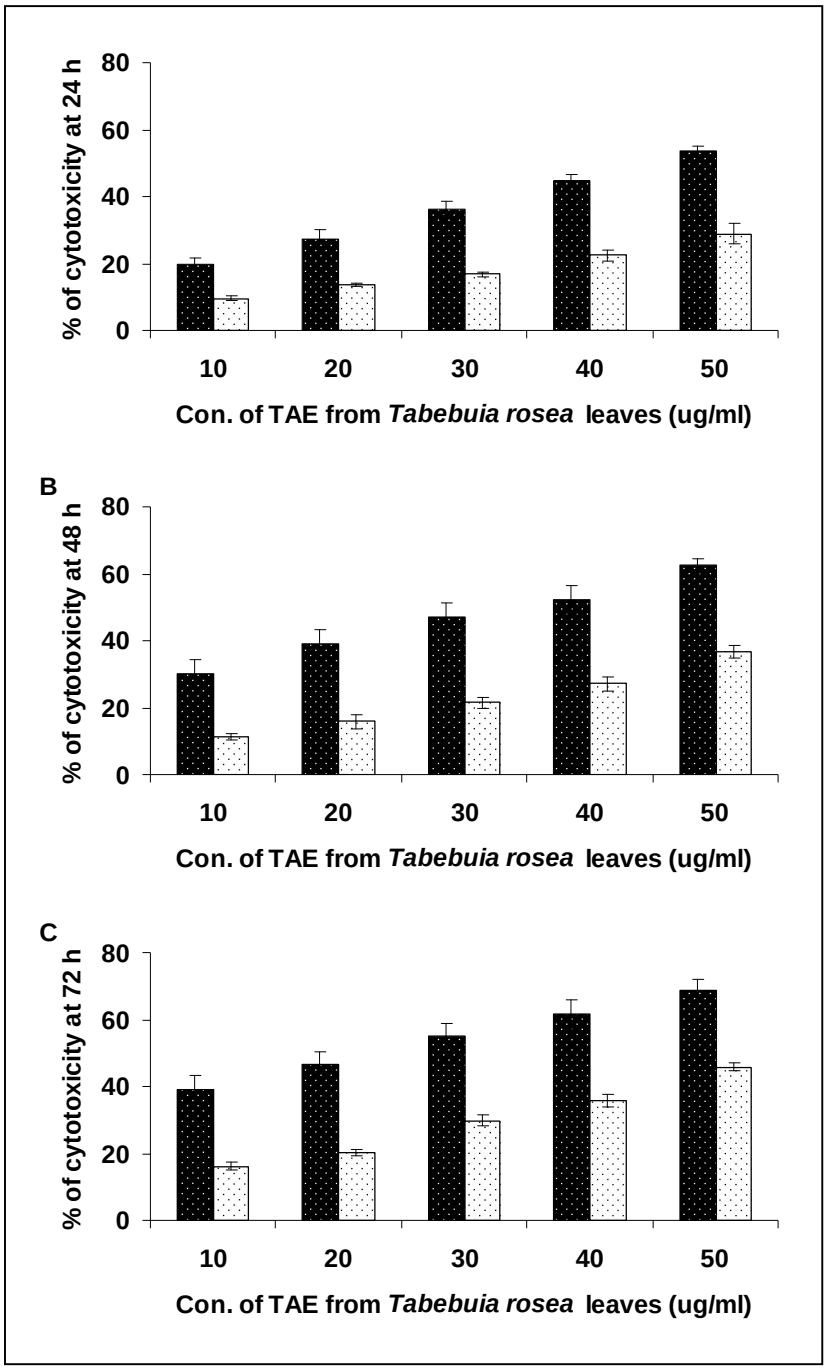

Figure 2. Cytotoxic effect of of total alkaloid extract of *Tabebuia rosea* leaves against cancer (black bars) and normal (white bars) cells at 24, 48 and 72 h incubation. Cancer (Molt-4) and normal (mitogen stimulated T-lymphocytes from the venous blood of healthy volunteers) cells were treated with different dosage of TAE for 24 (A), 48 (B) and 72 hours and the cyotoxicity was measured using MTT test. Error bars represent SEM.

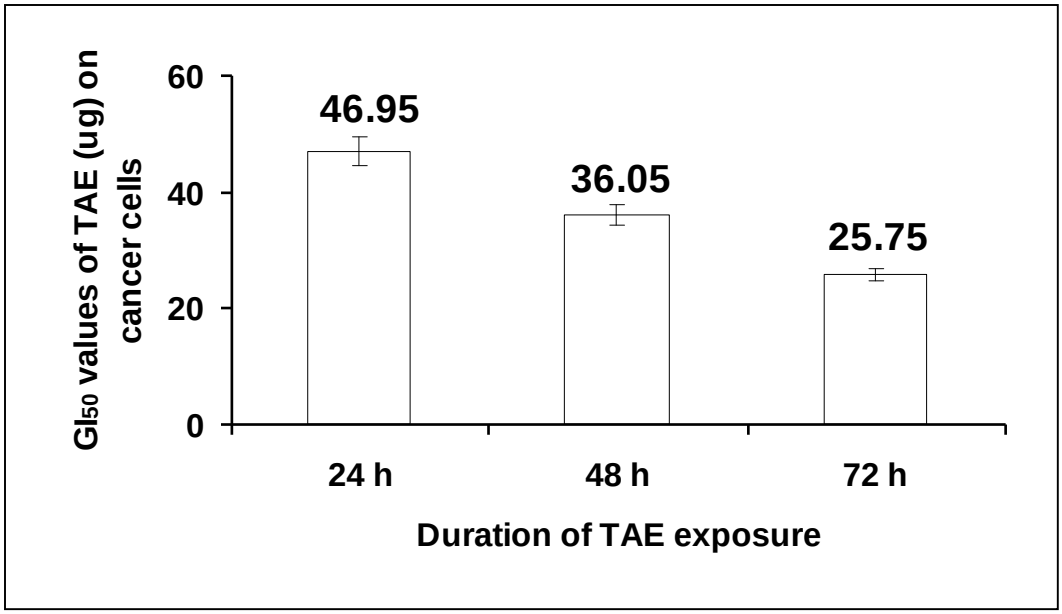

Figure 3. GI_{50} values of total alkaloid extract of *Tabebuia rosea* leaves on Molt-4 cells. The GI_{50} was calculated from the percentage of cytotoxicity as determined by MTT test by linear extrapolation of the data using Microsoft Excel. Error bars represent SEM.

The percentage of cytotoxicity increased with increasing concentration as well as exposure duration. The cytotoxicity observed at the highest exposure concentration (100 µg/ml medium) was 53.44, 62.46 and 68.66 % at 24, 48 and 72 h incubation for cancer cells where the same was 28.88, 36.78 and 45.77 % for normal cells. Similarly the cytotoxicity observed at the lowest exposure concentration at 24, 48 and 72 h incubation was found to be 19.95, 30.15 and 39.07 % in cancer cells and was found to be 9.55, 11.44 and 16.09 % for normal cells.

The cytokinesis-block *in vitro* micronucleus test provides a very accurate and efficient tool to detect chromosomal aberrations as micronuclei in divided verses non-divided cells. The results of micronucleus assay have been presented in Figure 4. As a measure of cell proliferation the number binucleated cells (BNC) obtained in each treatment were counted. BNC yield was inversely proportional to the concentration of extract in both cultures and was expressed as percentage. In both cells, maximum BNC was observed with the negative control. The BNC observed in cancer cells were of 70.34, 66.54, 63.32, 51.24, 47.56, 41.79 and 32.17 % for the negative control, 10 µg, 20 µg, 30 µg, 40 µg, 50 µg TAE and positive control respectively. Where the BNC observed in normal cells were of 72.43, 68.78, 65.99, 61.56, 60.28, 56.99 and 52.47 % for the negative control, 10 µg, 20 µg, 30 µg, 40 µg, 50 µg TAE and positive control respectively. Like wise the total number of micronuclei counted in cancer cells were of 9.4, 9.06, 12.35, 14.33, 20.12, 21.44 and 52.52 % for the negative control, 10 µg, 20 µg, 30 µg, 40 µg, 50 µg TAE and positive control respectively. Where as the total number of micronuclei counted in normal cells were of 6.5, 7.93, 13.65, 15.65, 19.68, 23.57 and 48.79 % for the negative control, 10 µg, 20 µg, 30 µg, 40 µg, 50 µg TAE and positive control respectively. In both cultures the number of micronulclei obtained showed a gradually increasing pattern with increasing concentration of the extract. But, the total number of micronuclei obtained even at the maximum concentration was significantly ($p< 0.01$) lower than that of the positive control. In both the cultures there was no significant difference between the number of micronulclei observed in lowest concentration tested and the negative control indicating the possible absence of genotoxicity in its use.

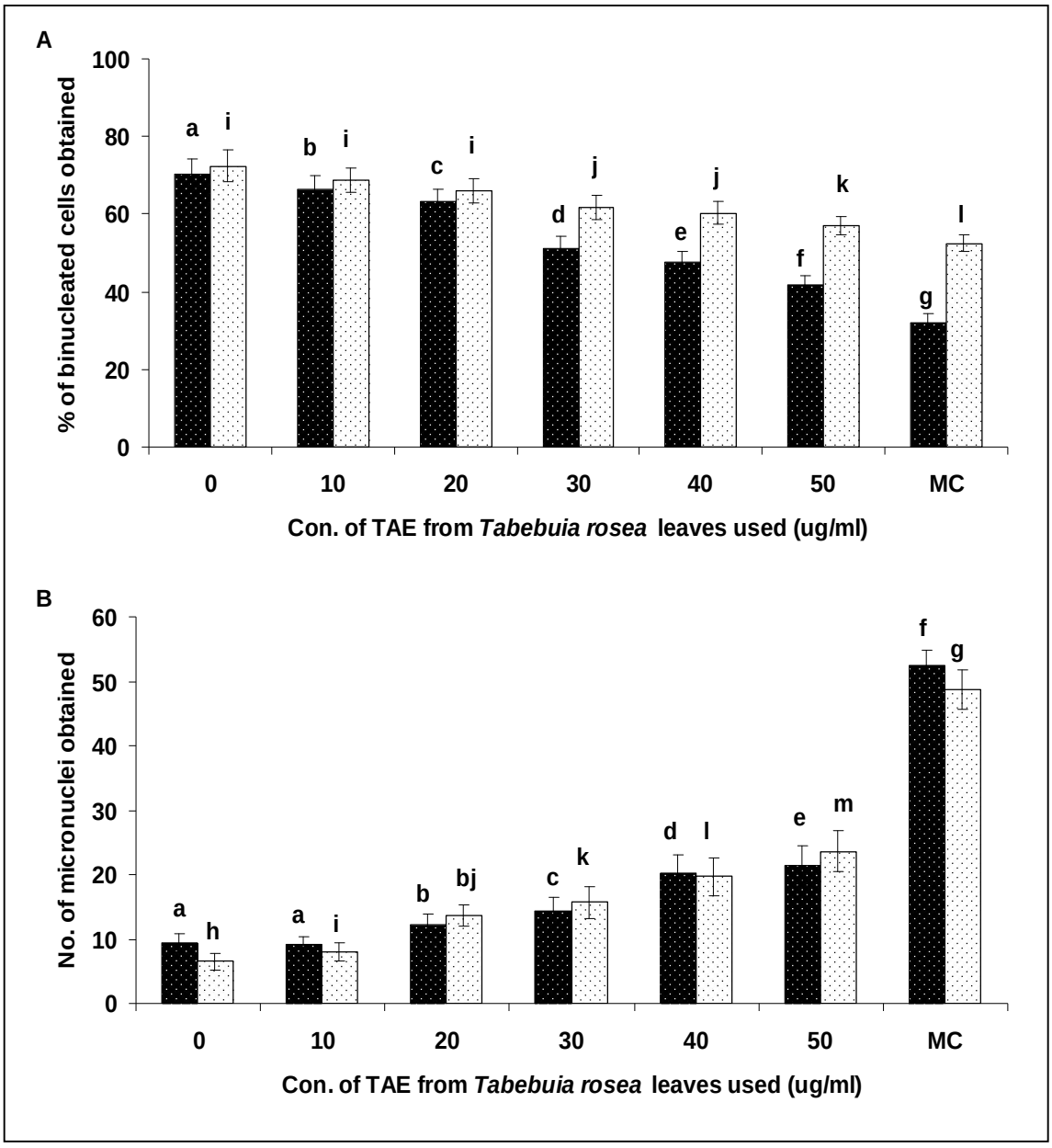

Figure 4. Genotoxic evaluation of total alkaloid extract of *Tabebuia rosea* leaves against cancer (black bars) and normal (white bars) cells. Cancer (Molt-4) and normal (mitogen stimulated T-lymphocytes from the venous blood of healthy volunteers) cells were treated with different dosage of TAE and the cells were harvested at binucleated stage by the addition of cytochalsin-B. Binulceated cells were counted for 1000 cells per treatment. The micronuclei were counted from 2000 BNC per treatment. Error bars represent SEM and the dissimilar letters indicate statistical significance at $p< 0.05$.

4. Discussion

Cultures of cancer (MOLT-4) and normal (T lymphocytes from healthy volunteers) were exposed to different concentrations of TAE isolated from *T. rosea* leaves such as 10, 20, 30, 40 and 50 µg/1×10^6 cells/ml medium, to study the effect on cell proliferation. As determined by MTT assay the maximum cytotoxicity observed with cancer and normal cells were found to be 68.66 % and 45.77 % at the highest dosage and longest exposure duration studied and the minimal observations were about 19.95 % and 9.55 % on cancer and respectively at the lowest dosage and exposure

duration tested. The cleavage of tetrazolium ring in MTT involves the mictochondrial succinate dehydrogenase and depends on the activity of the respiratory chain and the redox state of the mitochondria (Mosman, 1983; Shearman et al., 1995) responsible for the production of cell energy. The exposure of cancer cells to TAE induced a significant reduction in the converstion of MTT, which means a cellular disintegration and cytotoxicity reflected from the parallel dose and time dependent decrease of the absorbance measured.

Recently several plant extracts have been evaluated and shown to have cytotoxic or cytostatic effects in cancer cell lines. They include those of *Solanum lyratum* tested on human colon adenocarcinoma cell line (colo 205) (Hsu et al., 2008), *Annona glabra* on human leukemia cell lines (CEM/VLB) (Cochrane et al., 2008), *Gynostemma pentaphyllum* on human lung cancer (A549) (Lu et al., 2008) and *Blumea balsamifera* on rat and human hepatocellular carcinoma cells (McA-RH7777 and HepG2) (Norikura et al., 2008), *Artocarpus altilis* on human breast cancer (T47D) cells (Arung et al., 2009) and *Lindackeria paludosa* on human colon and lung carcinoma and murine melanoma and fibrosarcoma (Fazio et al., 2010).

The scientific evaluation of traditional medicinal plants would assure the credibility of their use (Garcia-Alvarado et al., 2001; Gomez-Flores et al., 2009) and this has led to the discoveries of many important drugs and their secondary metabolites also show promise for the cancer chemoprevention, which has been defined as “the use of non-cytotoxic nutrients or pharmacological agents to enhance physiological mechanisms that protect the organism against mutant clones of malignant cells” (Morse and Stoner, 1993).

It is important to determine the potential genetic hazards of compounds present in medicinal plants aligned with their beneficial effects to the human body and the verification of the possible mutagenic and/or anti-mutagenic effects of medicinal plants infusion/extracts is another important factor in scientific evaluation studies. Such effects have been elucidated in some plant species by using various test systems (Roncada et al., 2004). However, biological data on the medicinal properties associated with plant extracts with phytopharmacological activities are relatively few, especially regarding mutagenic potential (Lohman et al., 2001).

Hence, the biological activity of the alkaloid extract was measured in order to test the possible clestogenic or aneugenic effect through the induction of micronuclei which reflects its genotoxic potential. There is a vast difference between the number of micro nuclei observed in cultures exposed to TAE and the positive control i.e. mitomycin-C. The maximum number of micronuclei induced by TAE at the highest tested concentration was found to be 26.05 and 24.80 for cancer and normal cells respectively and it was 52.52 and 48.79 by the mitomycin-C. The two fold rise in micronuclei formed by mitomycin-C attributed with the highly significant difference between the values.

From the results of MTT test and micronucleus assay it has been observed that the extract has significantly higher anti-proliferative effect on cancer cells than that of the normal cells in a dose and time dependent manner and the number of micronuclei formed even at the highest concentration tested was insignificant with that of the positive control thus indicating the possibility of the lack of genotoxicity associated with its antitumor potential.

5. Conclusion

Taken together the above results it is clear that the total alkaloid extract of this plant have anti proliferative potential against the MOLT-4 cells in vitro. Although the TAE has shown some encouraging results but they need to undergo carefully controlled trials in order to evaluate their usefulness and safety against wide range of cancer and normal cell lines and elaborate studies are required to determine intracellular pathway(s) involved in the mechanism of cytotoxicity. Further studies in this plant with its pure compounds may lead to the development of newer and safer anticancer drug with higher therapeutic potential.

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