

Full Length Research Paper

Anti-tumor potential of total alkaloid extract of *Prosopis juliflora* DC. leaves against Molt-4 cells *in vitro*

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The total alkaloid extract from *Prosopis juliflora* DC. leaves was obtained using acid/base modified extraction method. The *in vitro* anti-tumor potential of the extract was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) based cytotoxicity monitoring after 24, 48 and 72 h exposure of the MOLT-4 cells (1×10^6 cells/ml medium) to different concentration of the extract ranging from 10 to 100 $\mu\text{g/ml}$ medium. 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 at all the tested concentrations and at all the time points studied. The IC_{50} values of the extract at 24, 48 and 72 h were found to be 90.5, 42.5 and 20.0 $\mu\text{g/ml}/1 \times 10^6$ cells against cancer cells. The genotoxic assay showed that, in both cultures, the number of micronuclei obtained even at the highest exposure concentration tested was very low when compared with that of the micronuclei obtained with the positive control mitomycin-C. The results of the present investigation demonstrate that, *P. juliflora* leaf alkaloids are preferentially cytotoxic to human T-cell leukemia (Molt-4) cells in a dose and time dependent manner with the absence of genotoxicity.

Key words: *Prosopis juliflora*, alkaloids, molt-4 cells, MTT test, micronucleus assay.

INTRODUCTION

Cancer is the second leading cause of death in the world (Madhusudan and Middleton, 2005). Currently over 60% of the drugs are derived in one or the other way from natural sources including plant, marine organisms and micro-organisms (Newman et al., 2003). Among many recent advances in cancer chemotherapy, plant natural products have played an important role in contributing to approximately 60 cancer chemotherapeutic drugs on the market including *Catharanthus* (*Vinca*) alkaloids (vinblastine, vincristine, vinorelbine), the pipodophyllotoxins

(etoposide, etoposide phosphate, teniposide), the taxanes (paclitaxel and docetaxel) and the camptothecin derivatives (irinotecan and topotecan) (Cragg et al., 1997; Kinghorn, 2000; Conforti et al., 2008; Dholwani et al., 2008; Bhuvan et al., 2009). Recently, there is considerable scientific and commercial interest in the continuing discovery of new anticancer agents from plant products (Kinghorn et al., 2003; 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). In large number of medicinal plants, the therapeutic value is due to the presence of alkaloids, which in certain respects, ranks among the most interesting of the naturally occurring substances (Ahmad et al., 1995).

Prosopis juliflora DC., mesquite tree is one of the major invasive alien species of India and it has been used to treat eye problems, open wounds, dermatological ailments

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Abbreviations: RPMI, Roswell Park Memorial Institute medium; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; BNC, binucleated cells; IC_{50} , concentration of the extract required for 50% of reduction in cell proliferation.

and digestive problems by the native tribes of many countries (Kirtikar and Basu, 1935; Davidow, 1999). It has soothing, astringent, antiseptic, antibacterial and antifungal properties (Ahmad et al., 1995; Kay, 1996). The extracts of *P. juliflora* seeds and leaves were well studied for several *in vitro* pharmacological effects such as antibacterial (Aqeel et al., 1989; Kanthasamy et al., 1989; Caceres et al., 1995; Satish et al., 1999), anti-fungal (Ahmad et al., 1989a,b; Kaushik et al., 2002) and anti-inflammatory properties (Ahmad et al., 1989a,b). These properties have been attributed to piperidine alkaloids (Ahmad et al., 1986; Batatinha, 1997). The main alkaloids including the juliflorine and julifloricine of *P. juliflora* were isolated by Ahmad et al. (1978) and their antibacterial and antifungal activities have also been reported (Khan et al., 1986; Ragavendra et al., 2009). The flavonoid patulitrin isolated from its flowers and fruits showed significant activity against lung carcinoma *in vivo* (Wassel et al., 1972).

To the best of our knowledge, the effect of *P. juliflora* alkaloids 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 anti-tumor 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 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 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.

MATERIALS AND METHODS

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. Phytohemagglutinin and fetal calf serum were obtained from Gibco, New York, USA. All the other chemicals were purchased from SD Fine Chemicals, Mumbai, India.

Collection of leaves and extraction of alkaloids

Leaves of *P. juliflora* 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 defatted by cold hexane extraction. The defatted 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 through anhydrous sodium sulphate. The resulting solution was evaporated to dryness to yield total alkaloids of *P. juliflora*. The alkaloids 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).

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 cells were maintained in Roswell Park Memorial Institute medium (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% phytohemagglutinin in a humidified atmosphere containing 5% CO₂.

Peripheral whole blood (15 ml) from 3 healthy, 22 to 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 hemocytometer and adjusted to a density of 1×10^6 cells/ml for cell culture studies.

Determination of cell viability and selection of exposure concentration

The extracts were dissolved in 100 µl of 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 24 h at 37°C in an atmosphere of 5% CO₂. After 24 h incubation, 50 µl of 0.4% erythrosine-B in phosphate buffered saline was added to each well and again incubated for 30 min. The number of stained (dead) and unstained (live) cells were counted using hemocytometer under the microscope. From that, the cell viability was calculated for each concentration. Experimental dosages which resulted in viability of 70% and above were decided as optimum for cell culture studies.

Screening of anticancer potential using MTT test

The cytotoxic potential of *P. juliflora* leaf alkaloids 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 1×10^5 cancer (Molt-4) and normal (lymphocytes from healthy

donors) cells were seeded in 96 well plates, treated with 10, 20, 50 and 100 µg of TAE/ ml medium and incubated at 37°C in an atmosphere of 5% CO₂ for 24, 48 and 72 h. 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 dimethyl sulphoxide was added to the wells and the cell viability was determined by measuring the absorbance in a micro plate reader with a test wave length of 570 nm and a reference wave length 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 IC₅₀ 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.

Genotoxic assessment using cytokinesis-block micronucleus assay

The micronucleus assay was performed according to Matsuoka et al. (1992) with modifications (Bonacker et al., 2004). About 1 × 10⁶ 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% CO₂. 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 to 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 to 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).

Statistical analysis

All the experiments were independently performed 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.

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 of *P. juliflora* 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 which provided a way to determine the concentration range suitable for cell culture studies.

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, the extract exhibited significantly higher cytotoxicity on cancer cells than in

normal cells by exhibiting IC₅₀ values of 90.5 ± 2.5, 42.5 ± 1.75 and 20 ± 0.5 µg after 24, 48 and 72 h exposure durations, for cancer cells (Table 1). When the same extracts were exposed to normal cells for the same duration at similar concentration, determination of IC₅₀ values to normal cells was not possible at all the time points studied which showed that, the growth inhibition by TAE was significantly lesser in normal cells than in cancer cells.

The percentage of cytotoxicity increased with increasing concentration as well as exposure duration. The cytotoxicity observed in cancer cells at the highest exposure concentration (100 µg/ml medium) was found to be 53.86, 66.74 and 72.65% after 24, 48 and 72 h incubation, where as, the same for normal cells was found to be 29.24, 38.37 and 46.51%, respectively for the same durations. Similarly, the cytotoxicity observed in cancer cells at the lowest exposure concentration was found to be 53.86, 36.55 and 46.71% after 24, 48 and 72 h incubation, where as, the same for normal cells was found to be 8.31, 11.27 and 18.43%, respectively, at the same durations.

The cytokinesis-block *in vitro* micronucleus test provides a very accurate and efficient tool to detect chromosomal aberrations as micronuclei in divided versus non-divided cells. The results of micronucleus assay have been presented in Figure 3. As a measure of cell proliferation, the number binucleated cells obtained in each treatment was 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 in the positive control. The BNC observed in cancer cells were of 68.22, 53.35, 47.59, 43.83, 37.74 and 32.17% for the negative control; 10, 20, 50, 100 µg TAE for the positive control, respectively. The BNC observed in normal cells were of 76.48, 67.35, 62.16, 59.26, 54.37 and 52.47% for the negative control, and 10, 20, 50, 100 µg TAE for positive control, respectively. Like wise the total number of micronuclei counted in cancer cells were of 9.4, 16.6, 19.33, 22.48, 25.65 and 52.52% for the negative control, 10, 20, 50, 100 µg TAE for positive control, respectively. Where as, the total number of micronuclei counted in normal cells were of 6.5, 15.78, 16.86, 20.46, 23.75 and 48.79% for the negative control, 10, 20, 50, 100 µg TAE for positive control, respectively. The number of micronuclei obtained, showed a gradually increasing pattern with increasing concentration of the extract in both cultures. But, the micronuclei obtained even at the maximum concentration were significantly lower than that of the positive control. In both cultures, there was no significant difference between the number of micronuclei observed in lowest concentration tested and the negative control, indicating the possible absence of genotoxicity at this concentration.

DISCUSSION

Cultures of cancer (Molt-4) and normal (T lymphocytes

Table 1. IC₅₀ values of total alkaloid extract of *P. juliflora* leaves against the human leukemia cells (Molt-4) after 24, 48 and 72 h incubation periods as determined from the results of MTT based cytotoxicity test.

Exposure duration (h)	IC ₅₀ values of TAE ($\mu\text{g}/1 \times 10^6$ cells/ml medium)
24	90.5 \pm 2.5
48	42.5 \pm 1.75
72	20 \pm 0.5

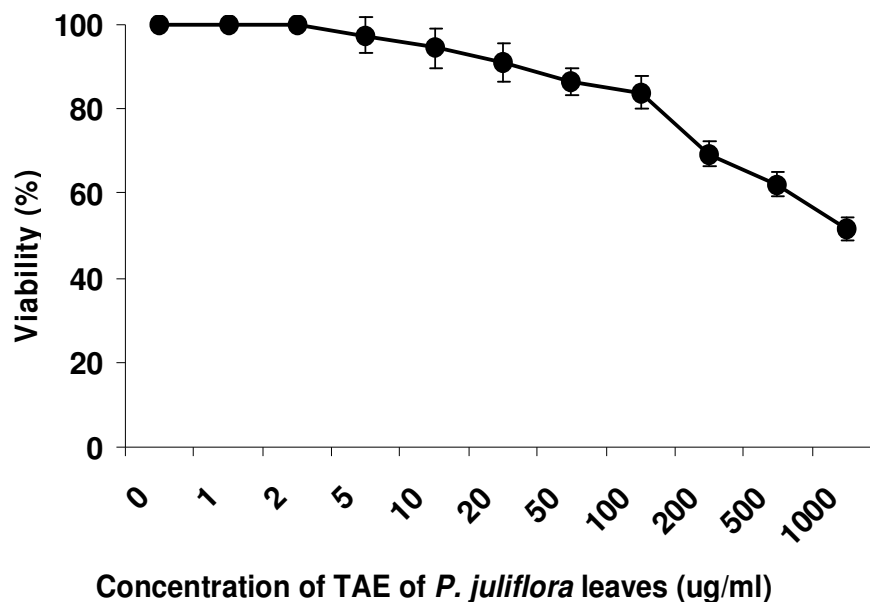


Figure 1. Viability of normal lymphocytes exposed to increasingly logarithmic concentration of total alkaloid extract of *P. juliflora* leaves.

from healthy volunteers) cells were exposed to different concentrations of TAE isolated from *P. juliflora* leaves such as 10, 20, 50, 100 $\mu\text{g}/1 \times 10^6$ 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 72.65 and 46.51% at the highest dosage and longest exposure duration studied and the minimal observations were about 21.71 and 8.31% on cancer and normal cells, respectively, at the lowest dosage and exposure duration tested. The cleavage of tetrazolium ring in MTT involves the mitochondrial 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 conversation of MTT, which means a cellular disintegration and cytotoxicity reflected from the parallel dose and time dependent decrease of the absorbance measured.

Several plant extracts and their isolated compounds

have been shown to possess antioxidant, anti-inflammatory and anti-tumor properties (D' Alessandro et al., 2003; Kim and Lee, 2004; Arts and Hollman, 2005; Lambert et al., 2005). The scientific evaluation of traditional medicinal plants would assure the credibility of their uses (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 and has also show promises 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).

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)

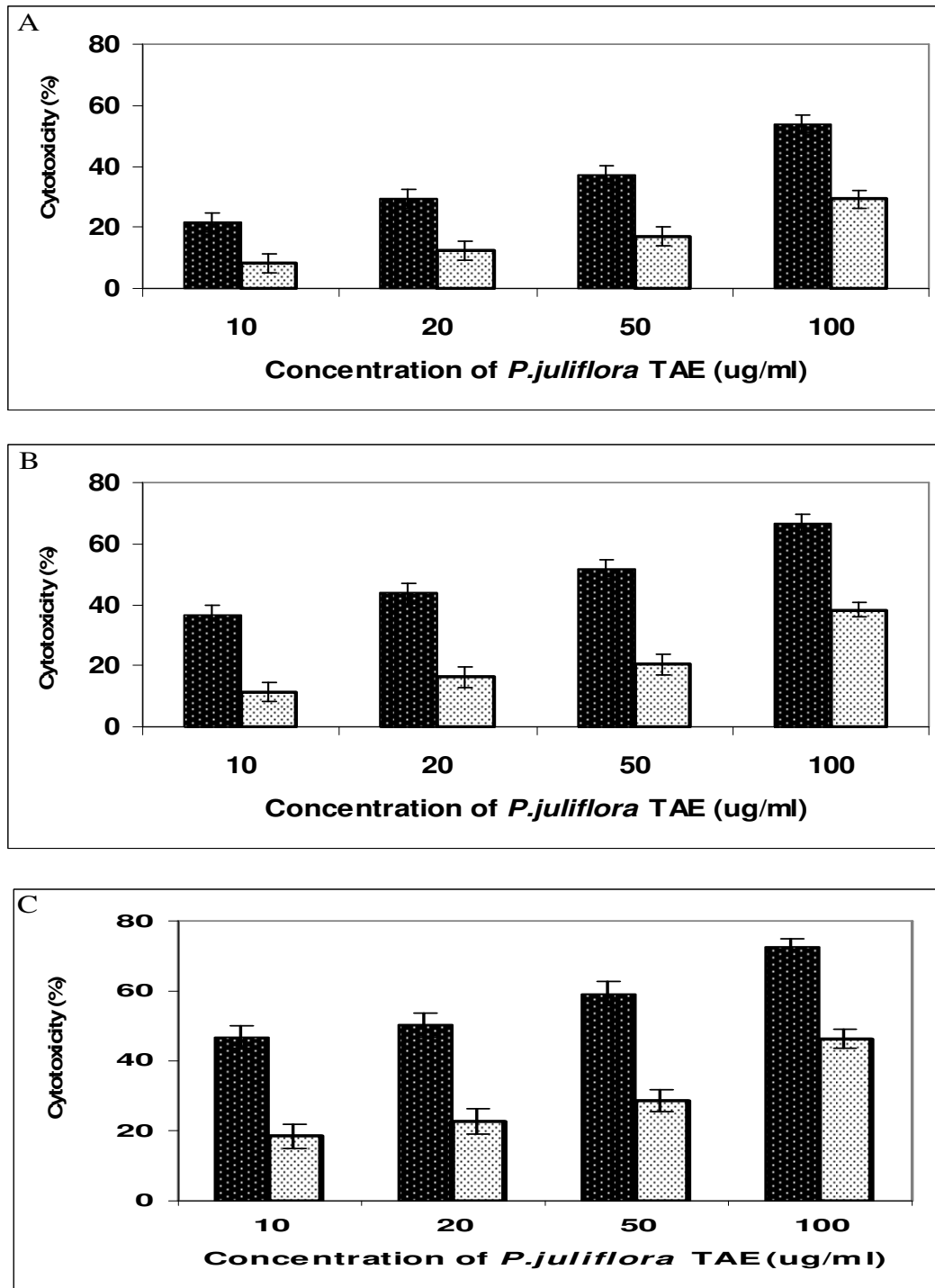


Figure 2. Cytotoxic effect of total alkaloid extract of *P. juliflora* leaves against cancer (black bars) (Molt-4 cells) and normal (white bars) (mitogen stimulated T-lymphocytes from the venous blood of healthy volunteers) cells after 24 (A), 48 (B) and (c) 72 h incubation period. Each determination was performed from three replicates of three independent experiments.

(Lu et al., 2008), *Blumea balsamifera* on rat and human hepatocellular carcinoma cells (McA-RH7777 and HepG2) (Norikura et al., 2008) and *Artocarpus altilis* on human breast cancer (T47D) cells (Arung et al., 2009).

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-

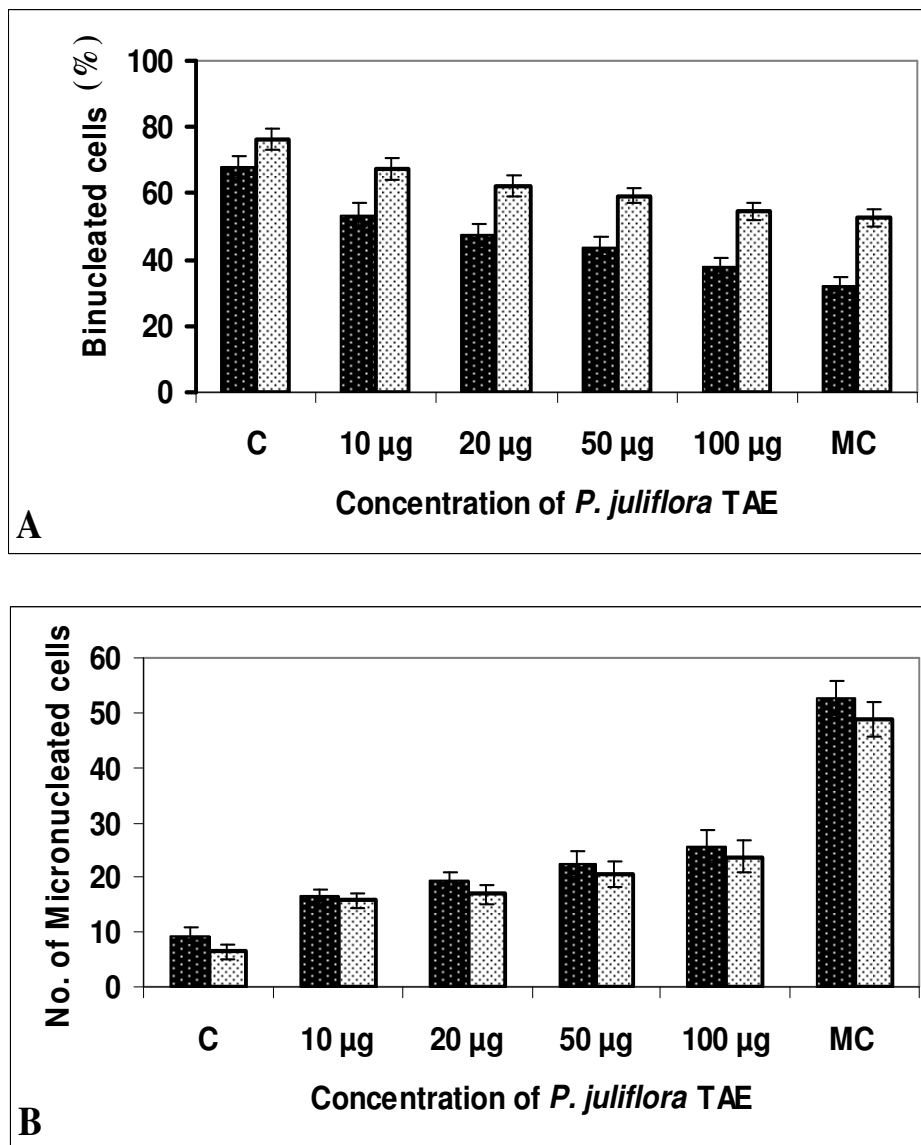


Figure 3. Genotoxic evaluation of total alkaloid extract of *P. juliflora* leaves against cancer (black bars) and normal (white bars) cells as measured by cytokines block *in vitro* micronucleus assay. Each determination was performed from three replicates of three independent.

mutagenic effects of medicinal plants infusion/extracts is another important factor in scientific evaluation studies. Such effects have been elucidated in some plant species using various test systems (Roncadi 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 clastogenic or aneugenic effect through the induction of micronuclei which reflects its genotoxic potential. There is a vast difference between the number of micronuclei observed

in cultures exposed to TAE and the positive control that is, mitomycin-C. The maximum number of micronuclei induced by TAE at the highest tested concentration was found to be 25.65 and 23.75 for cancer and normal cells, respectively and it was 52.52 and 48.79 by the mitomycin-C. This two fold rise in the number of micronuclei formed by mitomycin-C attributed with the highly significant difference between the values. The results of the cytokinesis block *in vitro* micronucleus assay revealed the lack of genotoxic potential in these cells which are corroborative with the previous findings of mutagenicity study through Ames test using some of the alkaloids from this plant that proved their non-mutagenicity up to 500 µg/

plate (Ahmad et al., 1991).

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 anti-tumor potential.

Taking together the stated results, it is clear that the TAE of this plant have anti proliferative potential against the Molt-4 cells *in vitro*. Although the TAE has shown some encouraging results, they need to undergo careful 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 on this plant with its pure compounds may lead to the development of newer and safer anticancer drugs with higher therapeutic potential. Hence, this can open a new avenue for the effective management of this menace invasive alien species in a useful manner.

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