EVALUATION OF ANTIOXIDANT AND ANTITUMOR POTENTIALS OF *PROSOPIS JULIFLORA* DC. LEAVES *IN VITRO*

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Summary

This work was conducted with the purpose to evaluate the antioxidant and antitumor potential of total phenolic extract of *Prosopis juliflora* leaves through standard methods. Antioxidant potential was tested by DPPH (2, 2-diphenyl-1-picryl-hydrazyl) free radical scavenging assay. The antiproliferative effect of the extract was tested against three normal (T-lymphocytes, Tig-7 and CHO) and three cancer (MOLT-4, oral (KB) and HeLa) cell lines using MTT (3-(4,5-dimethythiazol-2yl) 2,5-diphenyl tetrazolium bromide) test and as a measure of gentoxic assessment cytokinesis block *in vitro* micronucleus assay was also done in cell cultures incubated with the extract. The DPPH scavenging effect of the extract was increased with the increasing dosage and the IC_{50} estimations for the extract and the ascorbic standard were found to be 33.43 µg and 1.51 µg respectively. The cytotoxic potential of the extract was significantly higher on the cancer cells than that of the normal cells at all the tested doses and time points studied. The GI_{50} estimations of the extract against the cancer cell lines (MOLT-4, oral (KB) and HeLa) were found to be 46.44, 46.12 and 45.61 µg/ml at 24 h exposure; 32.35, 32.25, 32.08 µg/ml at 48 h exposure and 23.75, 23.51 and 22.65 µg/ml at 72 h exposure respectively. In all the cultures there was only a slight difference between the number of micronuclei observed in the negative control and the highest tested concentration which may be due to the absence of genotoxicity associated with its antiproliferative effects. The results indicate that more elaborate study in this plant and deeper investigations extended to other cell lines may lead to the discovery of new therapeutic agents.

Key words: *Prosopis juliflora*, Total phenolic extract, Antioxidant potential, Antitumor, micronucleus assay.

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Introduction

Antioxidants play an efficient protective role against degenerative diseases by inhibiting the oxidation process through blocking the initiation or propagation of oxidizing chain reactions and they may function as free radical scavengers, complexers of pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation (1-2). Many researchers have focused on natural antioxidants, and in the plant kingdom numerous crude extracts and pure natural compounds were previously reported to have antioxidant properties and play an important role in human health because of their defense mechanisms against oxidative stresses (3-5). Therefore, the development and utilization of more effective antioxidants of natural origin are desired and nowadays much attention has been paid to plants and other organisms as resources of natural antioxidants (2). Diphenyl picryl hydrazyl is a useful reagent for investigating the free radical scavenging activities of phenolic compounds and a substrate to evaluate the antioxidative activity of antioxidants (6).

Plants are known to be the source of effective and versatile therapeutic agents against various diseases and the surge of interest in phytochemicals has thus led to the identification of many pharmaceutical agents (7). Recently there is considerable scientific and commercial interest in the continuing discovery of new anticancer agents from plant products (8-9). Chemopreventive properties have long been attributed to polyphenolic compounds present in the diet as they are potential sources of anticancer compounds with minimal debilitating toxicity and side effects (10).

Nowadays the safety of natural products use has been questioned due to the reports of illness and fatalities (11). Considering the complexity and their inherent biological variation, it is now necessary to evaluate their safety, efficacy and quality. Hence, an assessment of their mutagenic potential is necessary to ensure the relatively safe use of plant-derived medicines and the cytokinesis-block *in vitro* micronucleus test has been used as an indicator of genotoxicity in mammalian cells (12-13). It provides a very accurate and efficient tool to detect chromosomal aberrations as micronuclei in divided verses non-divided cells (14). The present research is thus motivated by the need to find new substances of natural origin which possess antioxidant and antitumour activities with a low degree of toxicity as it has been reported that a successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells (15).

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 and digestive problems by the native tribes of many countries (16). It has soothing, astringent, antiseptic, antibacterial and antifungal properties (17-18). The extracts of *P. juliflora* seeds and leaves were well studied for several *in vitro* pharmacological effects such as antibacterial, antifungal and anti-inflammatory properties (19-22). The flavonoid, patulitrin isolated from its flowers and fruits showed significant activity against lung carcinoma *in vivo* (23).

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. Hence, the antiproliferative effect of total phenolic extract (TPE) of *P. juliflora* leaves on human cancer cell lines *in vitro* has been studied with parallel monitoring of its growth inhibitory potential on normal cells and an attempt has been made to screen the antioxidant and anitumoural effects of the total phenolic extract from *P. juliflora* leaves by DPPH (2, 2-diphenyl-1-picryl-hydrazyl) free radical scavenging assay and MTT (3-(4,5-dimethythiazol-2yl)2,5-diphenyl tetrazolium bromide) test using cultured cancer and normal cells along with the simultaneous genotoxicity monitoring using cytokines block *in vitro* micronucleus assay on cancer and normal cells to evaluate their safety.

Materials and methods

Chemicals and reagents

All reagents used in the study were of analytical grade. DPPH, histopaque 1077, L-glutamine, mitomycin-C and cytochalasin-B were purchased from Sigma, St Louis, USA. Cell culture media, 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. The solvents used were of analytical grade.

Extraction of phenolics

Leaves of *P. juliflora* were harvested from the Centre for Biodiversity and Forest Studies, Madurai Kamaraj University, Madurai. The total phenolics were extracted by the method of Yu et al. (24). Briefly, the leaves were dried, powdered and extracted with 60 % ethanol with a ratio of 1:20 (w/v) for 2 h at 50 °C. The extract was then filtered through muslin, evaporated under reduced pressure and vacuum dried to get the total phenolic extract. The yield of TPE was 13.75 % (w/w) with reference to the dried leaves.

Determination of total phenolic content

The total phenolic content was determined by the Folin-Ciocalteau method as described by Singleton et al. (25). Briefly, the extract was dissolved in methanol and 0.5 ml of this solution was mixed with equal amount of Folin-Ciocalteau reagent. After 3 min, 0.5 ml of 1 % Na_2CO_3 was added and allowed to stand for 2 h with intermittent shaking at room temperature. The absorbance was measured at 760 nm and the total phenolic content was expressed as mg of gallic-acid equivalents (GAE) on dry weight (DW) basis. In this study the total phenolic content of *P. juliflora* leaves was estimated as 103.22 mg GCE/ g DW of the extract.

Screening for antioxidant activity using DPPH free radical scavenging assay

The extract was dissolved in dimethyl sulfoxide and methanol was added to obtain 20 µg, 200 µg, 400 µg, 600 µg, 800 µg, 1000 µg, 1200 µg, 1400 µg, 1600 µg, 1800 µg, 2000 µg/ ml. From each dilution 50 µl of the sample solution was taken in micro titre plate and 200 µl of DPPH solution (10 mM of 2, 2-diphenyl-1-picryl-hydrazyl prepared in methanol) was added to each well. The plate was incubated in dark for half an hour and the absorbance was measured at 540 nm using microplate reader. Methanol blank and DPPH blank were used for calculating the percentage of antioxidant activity. Ascorbic acid in methanol ranging from 1-10 µg was used as positive control.

The antioxidant activity in percentage was calculated by the formula: 1-(Absorbance of sample/ Absorbance of DPPH) × 100 (26).

Cells, media and culture conditions

The cell lines such as human T-cell leukemia (MOLT-4), oral carcinoma (Oral (KB)), cervical carcinoma (HeLa), oral fibroblast (Tig-7) and Chinese hamster ovary (CHO) were procured from National Centre for Cell Sciences, Pune, India and mitogen stimulated T-lymphocyte cultures derived from venous blood of healthy persons were used for this study. For culturing normal T-lymphocytes, peripheral blood monocytes were obtained from the venous blood of healthy volunteers using histopaque-1077 (a leukocyte separation technique by Sigma Diagnostics) by density gradient centrifugation at 500 g for 10 min. Both T-lymphocytes and MOLT-4 cells were maintained in RPMI-1640 media supplemented with 10 % heat inactivated fetal calf serum, 2 mM L-glutamine, 0.4 % sodium bicarbonate, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 % phytohemeagglutinin in a humidified atmosphere containing 5 % CO_2. Oral (KB) HeLa and Tig-7 cells were maintained in Dulbecco's modified minimum essential medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine and gentamycin sulphate (50 µg/ml) at 37 °C in an incubator containing 5 % CO_2. The CHO cells were maintained in Eagle's modified minimum essential medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine and gentamycin sulphate (50 µg/ml).

Determination of cell viability and selection of exposure concentration

The extracts were dissolved in 100 µl of phosphate buffered saline and diluted with culture medium to obtain 1, 2, 5, 10, 20, 50, 100, 200, 500 and 1000 µg/ml medium for cell viability assay. About 1×10^6 cells were seeded in 96-well plates along with 150 µl of culture medium containing different concentrations of extract added to each well and incubated for 24 hours at 37 ºC in an atmosphere of 5 % CO_2. 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 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 (27).

Screening of anticancer potential using MTT test

The cytotoxic potential of *P. juliflora* leaf phenolics against cancer and normal cells was tested using MTT (3-(4,5-dimethythiazol-2yl)2,5-diphenyl tetrazolium bromide) assay (28). About 1×10^6 cancer and normal cells were seeded in 96-well plates, treated with 10 µg, 20 µg, 30 µg, 40 µg and 50 µg of TPE/ ml medium and incubated at 37 ºC in an atmosphere of 5 % CO_2 for 24, 48 and 72 h. Cells incubated in complete medium without TPE 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 determined by measuring the absorbance in a microplate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm. 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.

Genotoxic assessment using cytokinesis-block micronucleus assay
The micronucleus assay was performed according to Matsuoka et al. (29) with modifications (30). About 1×10^6 cells/ml medium were exposed to 50 µg of phenolic extract 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 (31).

Statistical analysis
All the experiments were independently preformed thrice with three replicates for each treatment. The results were expressed as mean ± S.E.M. and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test using the software programme Sigma Plot (11.0). Statistical significance was confirmed at $p < 0.01$.

Results

From the results of antioxidant activity test based on the DPPH free radical scavenging potential, it has been observed that the free radical scavenging activity was increased in a concentration dependent manner (Table 1). At 10 µg level the free radical scavenging potential of the extract and ascorbic standard were of 41.57 and 91.44 % respectively. The IC_{50} values observed were 33.43 µg for the former and 1.51 µg for the later that indicates a moderately high antioxidant potential of the extract.

Con. of phenolic extract (µg)	DPPH scavenging (%)	Con. of ascorbic acid standard	DPPH scavenging (%)
10	41.57 ± 1.25	**1**	46.57 ± 1.01
20	44.48 ± 1.35	**2**	52.09 ± 1.17
30	48.07 ± 1.31	**3**	57.18 ± 1.32
40	52.46 ± 1.27	**4**	63.55 ± 1.22
50	57.15 ± 1.64	**5**	68.71 ± 1.04
60	63.74 ± 1.57	**6**	72.59 ± 1.56
70	66.45 ± 1.72	**7**	77.25 ± 1.27
80	68.93 ± 1.86	**8**	82.68 ± 1.04
90	72.85 ± 1.83	**9**	88.27 ± 1.36
100	76.43 ± 1.77	**10**	91.44 ± 1.84
IC_{50}	33.43 µg	**IC_{50}**	1.51 µg

Table 1. DPPH free radical scavenging activity (%) of total phenolic extract of *P. juliflora* leaves and ascorbic acid standard
Results are expressed as mean ± S.E.M. of three experiments, performed in triplicates.

The results of cell viability assay are presented in Fig. 1. It is very much clear that the optimum viability (70 and above) lies within 100 µg extract for all the three normal cells provided a way to the determination of concentration range suitable for cell culture studies.

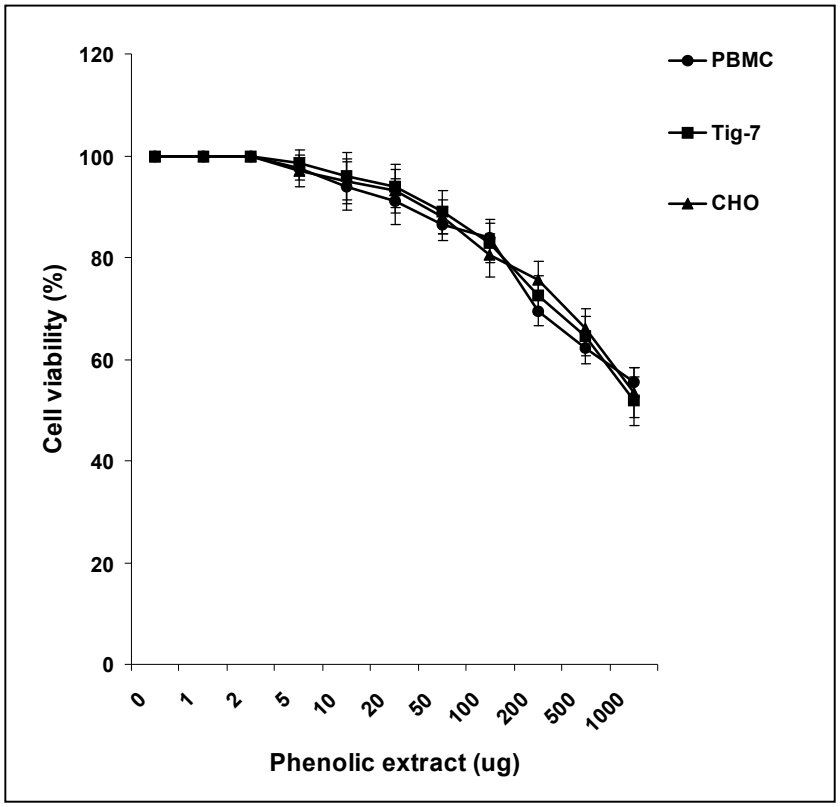

Fig.1. Viability of normal cells treated with logarithmically increasing dosages of total phenolic extract of *P. juliflora* leaves for 24 h as tested by erythrosine-B dye exclusion assay. Error bars represent S.E.M. Each determination was obtained from three replicates of three independent experiments.

The antiproliferative effect of the TPE on cancer and normal cell lines have been presented in Figs 2 & 3. From the results it can be observed that all the extract exhibited significantly higher cytotoxicity on cancer cells than in normal cells and the estimated The GI_{50} estimations of the extract against the cancer cell lines (MOLT-4, oral (KB) and HeLa) were found to be 46.44, 46.12 and 45.61 µg/ml at 24 h exposure; 32.35, 32.75, 32.08 µg/ml at 48 h exposure and 23.75, 23.51 and 22.65 µg/ml at 72 h exposure respectively (Fig. 4). When the same dosage was applied to normal cells for the same duration, determination of GI_{50} values were not possible at all the time points studied which showed the growth inhibition by the extract was relatively lesser in normal cells than in cancer cells.

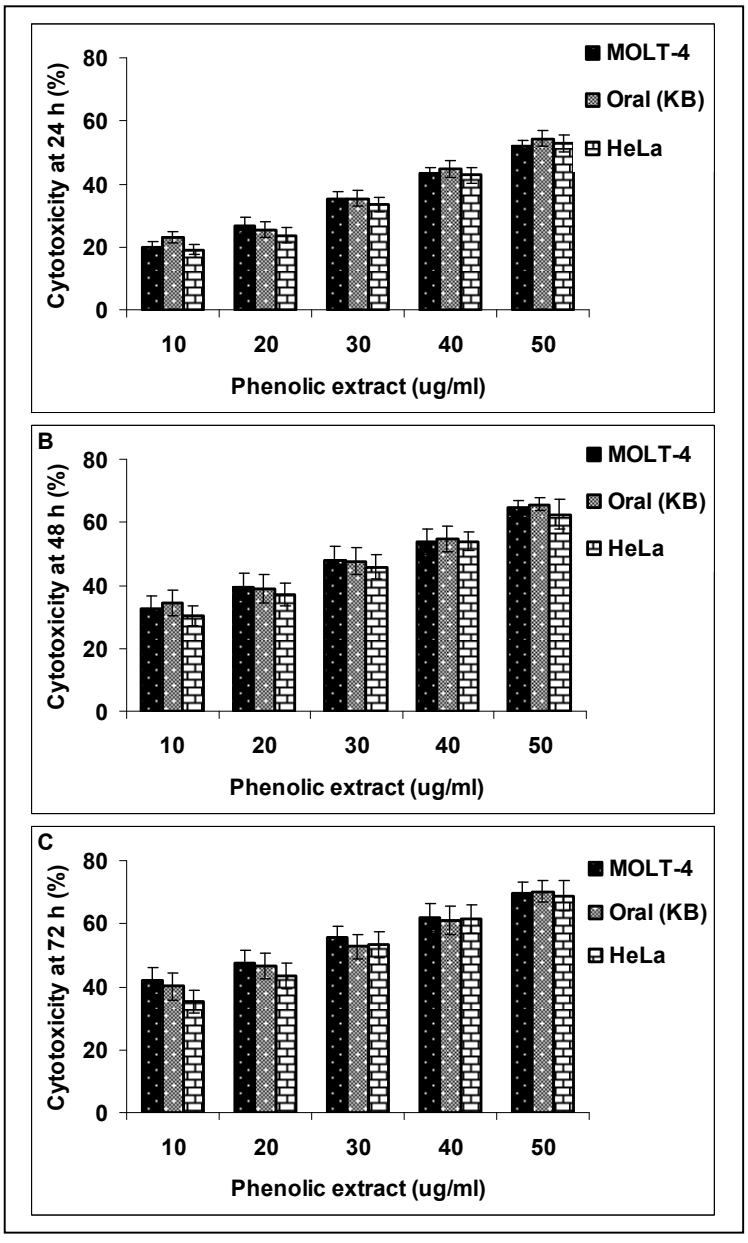

Fig. 2. Cytotoxic potential of *P. juliflora* leaf phenolic extract on cancer cells at 24 (A), 48 (B) and 72 (C) hour treatments. MOLT-4, oral (KB) and HeLa cells were cultured with indicated concentration of phenolic extract for 24, 48 and 72 h and subjected to MTT test. Error bars represent S.E.M. Each determination was obtained from three replicates of three independent experiments.

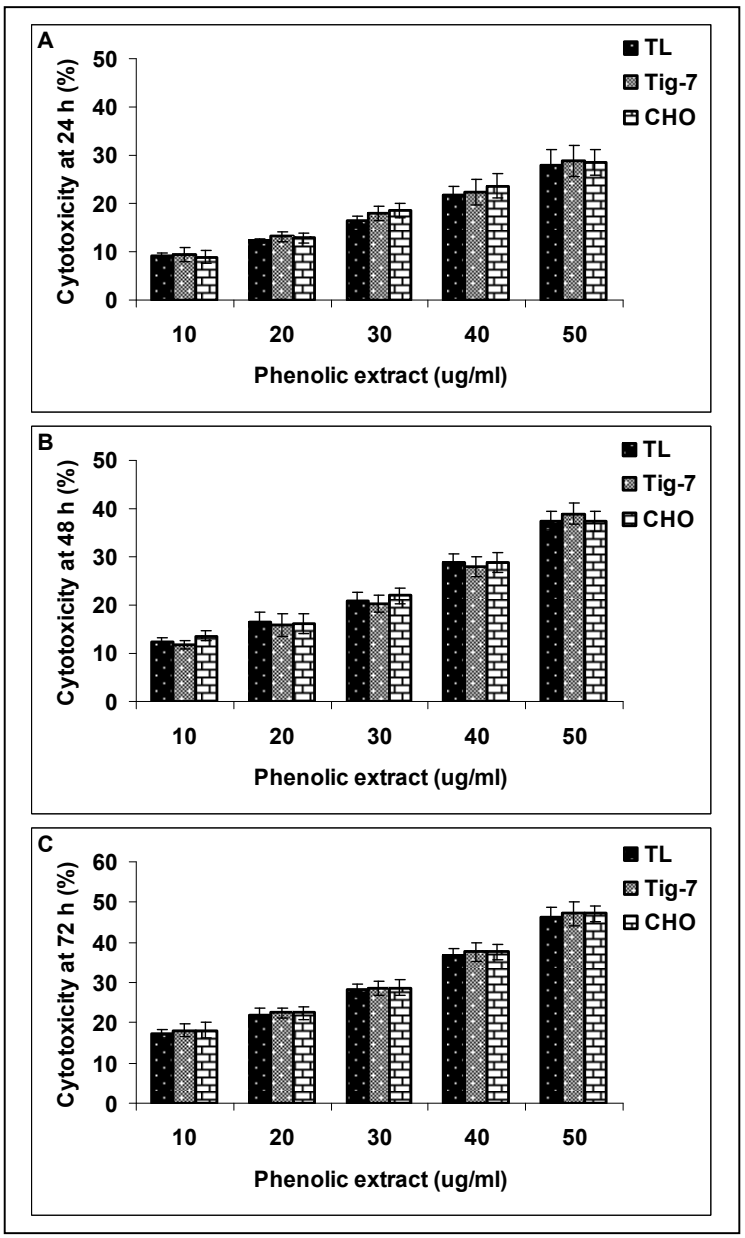

Fig. 3. Cytotoxic potential of *P. juliflora* leaf phenolic extract on normal cells at 24 (A), 48 (B) and 72 (C) hour treatments. T-lymphocytes, Tig-7 and CHO cells were cultured with indicated concentration of phenolic extract for 24, 48 and 72 h and subjected to MTT test. Error bars represent S.E.M. Each determination was obtained from three replicates of three independent experiments.

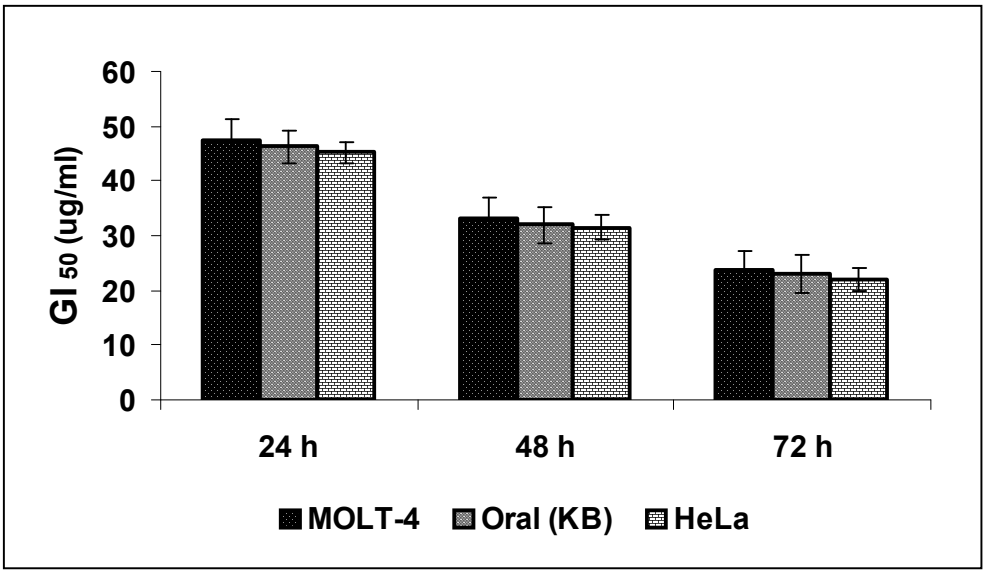

Fig. 4. GI_{50} estimation of phenolic extract of *P. juliflora* leaves on cancer cell lines at 24, 48 and 72 h incubation. GI_{50} values were obtained using an in-house programme of Microsoft Excel. Error bars represent S.E.M. Each determination was obtained from three replicates of three independent experiments.

The percentage of cytotoxicity observed shows an increasing pattern with increasing dosage and exposure duration and the rate of this increase observed with cancer cells was very high with that of the normal cells. As seen in the Fig. 2, at 24, 48 and 72 h incubations and at the highest exposure dosage (50 µg) the inhibitory potentials of the extract against cancer cells were found to be 53.01, 64.54 and 68.82 % respectively for MOLT-4 cells; 54.37, 65.01 and 70.85 % respectively for oral (KB) cells; 52.09, 62.65 and 68.17 % respectively for HeLa cells. Where as the same observed at the lowest exposure dosage were found to be 21.05, 32.41 and 41.04 % respectively for MOLT-4 cells; 23.43, 34.45 and 40.72 % respectively for oral (KB) cells; 19.49, 30.42 and 35.25 % respectively for HeLa cells. Similarly, from the Fig. 3, it can be observed that the inhibitory potentials of the extract against the normal cells at the highest exposure dosage and at 24, 48 and 72 h incubation were found to be 27.08, 37.54 and 46.61 % respectively for T-lymphocytes; 28.91, 38.14 and 47.34 % respectively for Tig-7; 28.37, 37.59 and 47.87 % respectively for CHO cells. Where the same observed at the lowest exposure dosage were found to be 8.29, 12.38 and 17.83 % respectively for the T-lymphocytes; 9.53, 11.69 and 18.75 % respectively for Tig-7; 8.18, 13.16 and 18.55 % respectively for CHO cells.

The results of micronucleus assay have been presented in Figs 5 & 6. As a measure of cell proliferation the number binucleated cells (BNC) obtained in each treatment were counted and was expressed as percentage (Fig. 5). In all the cultures, maximum BNC was observed with that of the negative control and when treated with the extract, the BNC yield in cancer cells were lower than that of the normal cells. The percentage yield of

BNC estimated with the negative control, extract and the positive control (mitomycin-C) were found to be 70.44, 42.22 and 32.66 % respectively for MOLT-4 cells; 68.43, 35.84 and 31.68 % respectively for oral (KB) cells; 74.93, 32.25 and 29.54 % respectively for HeLa cells. Where as the same for the normal cells were found to be 75.31, 60.45 and 52.97 % respectively for T-lymphocytes; 69.91, 43.95 and 54.48 % respectively for Tig-7; 72.11, 50.49 and 56.42 % respectively for CHO cells.

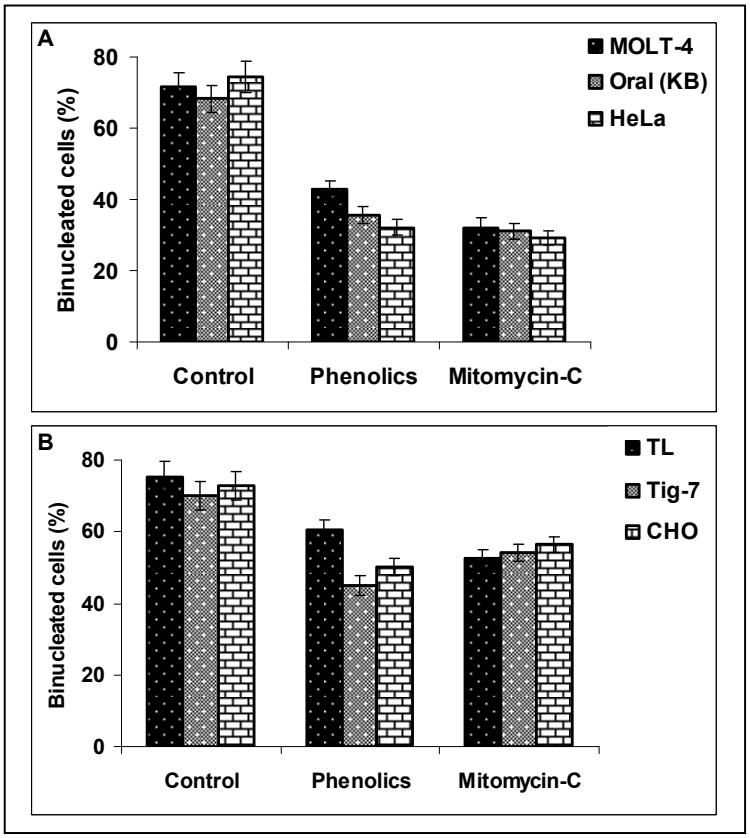

Fig. 5. Percentage of binucleated cells obtained in cancer (A) and normal (B) cells treated with total phenolic extract (50µg/ml) and mitomycin-C (6µg/ml). Cells were arrested at binucleated stage by the addition of cytochalsin-B and the number of binulceated cells was counted for 1000 cells per treatment. Error bars represent S.E.M.

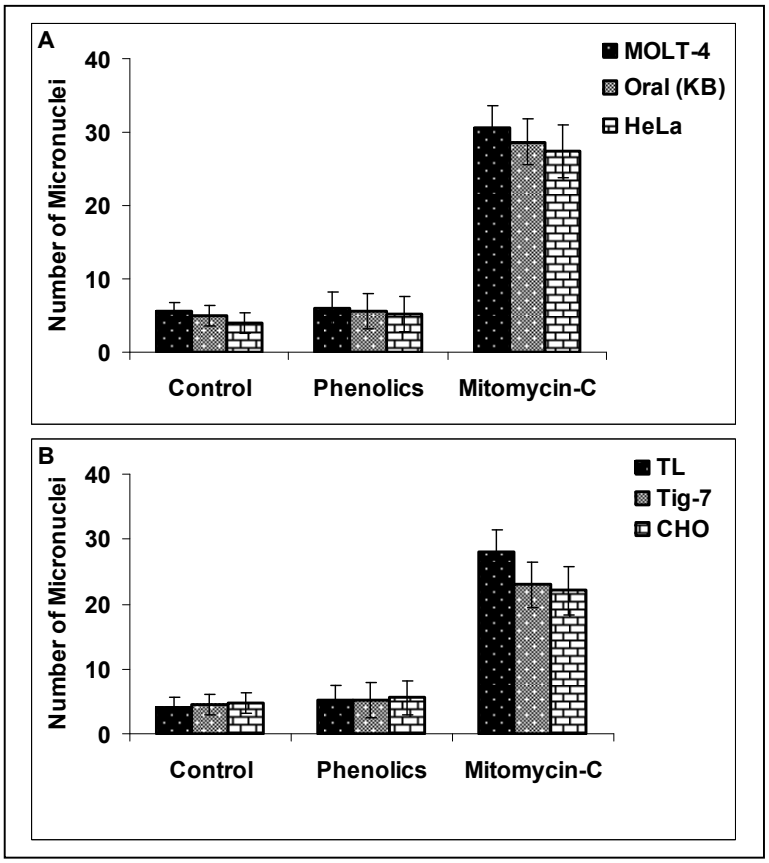

Fig. 6. Number of micronuclei cells obtained in cancer (A) and normal (A) cells treated with total phenolic extract (50µg/ml) and mitomycin-C (6µg/ml). Cells were arrested at binucleated stage by the addition of cytochalsin-B. The number of micronuclei was counted from 2000 binucleated cells per treatment. Error bars represent S.E.M.

The total number of micronuclei observed per 2000 binucleated cells were presented in Fig. 6 and in all the cultures the maximum number of micronuclei were observed in the positive control. The total number of micronuclei observed with the negative control, extract and the positive control (mitomycin-C) were found to be 5.56, 5.95 and 30.65 respectively for MOLT-4 cells; 4.98, 5.57 and 31.67 respectively for oral (KB) cells; 4.02, 5.21 and 27.44 respectively for HeLa cells. Where as the same for the normal cells were found to be 4.01, 5.09 and 28.05 respectively for T-lymphocytes; 4.54, 5.26 and 23.04 respectively for Tig-7; 4.77, 5.59 and 22.05 respectively for CHO cells. In all the cultures there was only a slight difference between the number of micronuclei formed in extract treated cells and the negative control and there was a very high difference observed between the number of micronuclei formed in response to the extract and the positive control indicating the possible absence of genotoxicity at this concentration.

Discussion

Recently, a greater emphasis has been given towards the researches on complementary and alternative medicine that deals with cancer management. Phenols and polyphenols, the flavonoids and their derivatives, are ubiquitous in plants and more than 8,000 different compounds are included in this group where many of them are antioxidants. They have been associated with the inhibition of atherosclerosis, cancer and other biological activities (32). These biological activities of the plants are due to vitamins, flavonoids and polyphenolic compounds which possess antioxidant activities (33).

A positive correlation between the antioxidant potential and antitumor potential has been reported and shown that the high content of antioxidants is responsible for the inhibition of tumour cell proliferation (34-35). The present study of the *P. juliflora* phenolic extract showed that the leaf possesses strong anti-proliferative properties against the tested human tumor cell lines, and also showed antioxidant effects at certain concentrations. In this sense, new studies on this fraction are necessary for a better characterization of its possible biological application.

Nowadays antioxidants have been at the centre of focus in chronic disease prevention research. The reduction of DPPH absorption is indicative of the capacity of the phenolic extract to scavenge free radicals, independently of any enzymatic activity and our results are in agreement with earlier reports on the ability of phenolic compounds to scavenge free radicals and active oxygen species (6,36-38). Therefore, we have evaluated the antioxidant potency through free radical scavenging with the phenolic extract or ascorbic acid standard and the results indicated that the free radical-scavenging activity of the extract enhanced with increasing concentration. This could be because of the antioxidant mechanisms associated with phenolic compounds, which are able to donate a hydrogen ion or an electron or stabilize or scavenge radicals (39).

The cytotoxicity screening models provide important preliminary data to help select plant extracts with potential antitumoural properties for future studies (40). Various 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 (41), *Annona glabra* on human leukemia cell lines (42) *Gynostemma pentaphyllum* on human lung cancer (43), *Blumeabalsamifera* on rat and human hepatocellular carcinoma cells (44), *Artocarpus altilis* on human breast cancer cells (45) and *Atrocarpus heterophyllus* on Hep2 cells (46).

We demonstrate for the first time that *P. juliflora* phenolic extract has a strong dose- and time- dependent antiproliferative activity on MOLT-4, oral (KB) and HeLa cells as observed from the results of MTT test (Fig. 2). All though the extent of the growth inhibition exerted by the extract was similar in all the three tested cancer cell lines a slight variation among the cell lines was observed through MTT test and the MOLT-4 cells showed more sensitivity followed by oral (KB) and HeLa cells as reflected from the GI_{50} estimations (Fig. 4). The growth arrest of cancer cells by the extract provides an opportunity for cells to undergo apoptosis (10) and the variable sensitivity of the cancer cell lines to the extract suggest that *P. juliflora* phenolic extract has a general function in suppressing cancerous cell growth but may act through multiple pathways (47).

A number of herbal preparations are traditionally used in the treatment of cancer therapy but there may be bioactivated components that may be responsible to promote cancer. In India, the majority of population uses traditional natural preparation derived from the plant material for the treatment of various diseases, and for that reason it becomes necessary to assess the mutagenic potential. Studies of genotoxicity and of natural plant extracts can help us to evaluate the safety and effectiveness of herbal health products (48). The cytokinesis block *in vitro* micronucleus assay has been used as an indicator of genotoxicity in mammalian cells (12-13). It is suggested that dietary polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (49).The antigenotoxic potential of the plant extracts have been attributed to their total phenolic content (50). The results of the study reveal that the phenolic extract from this plant leaves is not genotoxic and suggests that the compounds present in the extract are not mutagenic on their own.

In conclusion, our present *in vitro* study of the extract showed that *Prosopis juliflora* possesses strong anti-proliferative effect against the tested human tumour cell lines, and also showed strong antioxidant potential through free radical scavenging ability in a concentration dependent manner. These observations also suggest that at least some of the bioactive components of this plant can be found in its leaf phenolic extract and it can not be ruled out that the overall reported effects were contributed by the interaction between the various compounds in the extract. More elaborative study in this plant with its pure compounds may lead to the development of natural antioxidant and alternative anticancer agent of clinical significance.

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