

Evaluation of Apoptosis Inducing Ability of *Parkia javanica* Seed Extract in Cancer Cells

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Khangembam *et al.*: *Parkia javanica* induces Apoptosis in Cancer Cells

Bioactive compounds present in *Parkia javanica* seeds are reported to have anticancer effect. The present study was taken up to evaluate the apoptosis inducing ability of methanol extract of *Parkia javanica* seeds in cancer cells. The extract was found to cause 50 % cell death in HeLa and MCF-7 cells at 0.54 and 0.74 mg/ml, respectively, which is lower than that of normal healthy cells. In haematoxylin-eosin and fluorescent staining of the extract-treated cancer cells, chromatin condensation and nuclear fragmentation were observed. Fluorescent-activated cell sorting analysis using Annexin V-fluorescein isothiocyanate/propidium iodide also revealed the presence of apoptotic and secondary necrotic cell populations in the extract-treated cancer cells. In gel electrophoresis, DNA fragmentation, a biochemical hallmark of apoptosis was also observed. Caspase-3, an enzyme activated both in extrinsic and intrinsic pathways of apoptosis was detected in extract-treated HeLa cells by immunocytochemistry. The results of the present study suggested that *Parkia javanica* seed has the ability to induce apoptosis in cancer cells and could be considered in future endeavours for development of safer natural anticancer agent.

Key words: *Parkia javanica*, methanolic extract, apoptosis, cancer cells

In recent years, considerable efforts have been made to identify naturally occurring substances for development of alternative therapeutics against cancer. Phytochemicals like flavonoids from fruits and vegetables have shown to exert varied beneficial biological functions including antitumor activities^[1]. Other plant-derived chemical compounds like terpenoids, anthraquinones and saponins are also reported to have anticancer effect^[2-4]. Another good example of phytochemical used in treatment of cancer is alkaloids^[5]. Such health beneficial bioactive compounds can also be sourced from plants, which are used in traditional medicine.

In the present work, *Parkia javanica*, a common vegetable which is reported to possess medicinal properties was evaluated for its anticancer activity against cancer cells. Various parts of this plant are edible right from the inflorescence and tender pods to mature seeds. The seeds are a good source of protein and minerals^[6]. The matured seeds with or without the pod can be dried and stored ensuring its availability throughout the year. Traditional use of this plant includes

treatment of diabetes, intestinal disorder, bleeding piles, diarrhoea and dysentery^[7,8]. Such medicinal values of plant to protect against diseases may be associated with their antioxidant properties^[9]. Previous *in vitro* antioxidant assay had shown that *P. javanica* seed extracts are rich in antioxidant activity^[10]. *Parkia* species are known to have bioactive compounds such as cyclic polysulphides, which are used for treatment of kidney, ureter and urinary bladder infections due to their antibacterial activity^[11]. Another compound, a cyclic sulphur containing amino acid, thioproline, which is responsible for the pungent smell in seeds had been identified in *P. javanica*^[12]. This compound, thioproline produced anticarcinogenic effect against squamous cell carcinoma of rats^[13,14]. Though the anticarcinogenic effect of phytochemicals present in

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P. javanica is long known, only few studies were reported on the anticancer activity of *P. javanica* seed against human cancer cells. Therefore, the present study was undertaken to evaluate the anticancer activity of *P. javanica* seed extract and its ability to induce apoptosis in cancer cells.

MATERIALS AND METHODS

All the reagents used in the study were of analytical and cell culture grade and have been mentioned elsewhere in the text. The glassware used in the study were procured from Borosil, India. All the plastic wares including microfuge tubes, microtips were obtained from Tarson, India. Cover slips were obtained from Nunc, Denmark. Cell culture plate, tissue culture flask, cell scraper were from TPP (Switzerland) and 0.2 µm filter was purchased from Nalgene (USA). Cervical cancer cell line, HeLa and breast cancer cell line, MCF-7 were received as gifts from the Department of Pathology, All India Institute of Medical Sciences, New Delhi, India.

Preparation of plant extract:

Dry *P. javanica* seeds were collected from Manipur, India and were identified in the Department of Botany under the identification number, 2011030657610A. The seeds were powdered and then extracted with methanol in a Soxhlet apparatus (Macro Scientific Works, Delhi). Evaporation of the solvent followed by freeze drying (Heto Power Dry LL3000 Freeze Dryer) yielded the crude dry extract (MPJ). The extract was dissolved in 1 % dimethyl sulfoxide (DMSO, in cell culture media), filtered and used for further tests.

Determination of IC₅₀ of the extract in HeLa and MCF-7 cells:

Cancer cells, HeLa and MCF-7 were suspended at a density of 1 00 000 cells/ml in Dulbecco's modified Eagle's medium (DMEM; Mediatech Inc, VA) and incubated for 24 h. The cells were then treated with various concentrations of MPJ ranging from 0.1 to 1 mg/ml. Negative control cells were treated with vehicle (1 % DMSO in DMEM). Cells treated with Fluracil (Biochem Pharmaceutical Industrial Ltd., Mumbai), a known anticancer drug was used as positive control for cell death. The viability of the cells was assessed by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Amresco, USA) uptake method^[15]. The assay was based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. The amount of

formazan was determined by measuring the absorbance at 570 nm using ELISA plate reader (BioRad, Model 680). Results were expressed as the percent growth inhibition with respect to vehicle-treated cells and IC₅₀ of MPJ was determined.

Cytotoxicity assay of MPJ:

For cytotoxicity assay of MPJ on normal healthy cells, lymphocytes were isolated by density gradient centrifugation according to the method of Baker and Knoblock^[16] from a healthy donor. Lymphocytes were counted using the trypan blue exclusion method and diluted to the appropriate concentration (10⁶/ml) with Roswell Park Memorial Institute medium containing 10 % fetal bovine serum and 1 % penicillin (10 000 IU) and streptomycin (100 µg/ml). One hundred microlitres of the cell suspension was dispensed into each well of a 96-well plate. The cells were treated with different concentrations of MPJ. The cytotoxic effect of MPJ on healthy lymphocytes was determined by MTT assay.

Haematoxylin and Eosin staining of cells:

HeLa and MCF-7 cells were prepared for morphological examination^[17]. Cells were grown on cover slips at a density of 1 00 000 cells/ml in six well plate and allowed to attach for 24 h and then treated with the IC₅₀ of MPJ. The cells were then incubated for 24, 48 and 72 h. The cover slips were then removed from the wells and processed for haematoxylin and eosin staining^[18]. The cover slips were then mounted on a clean glass slide and observed under microscope (Nikon Eclipse E600) fitted with camera (Olympus, DP71).

Fluorescent staining of nuclei:

HeLa and MCF-7 cells (100 000 cells/ml) were seeded in six well plates containing cover slips and incubated for 24 h. The growth media was removed and the cells were then treated with MPJ and incubated for 24 h. Media was removed from the well, washed once with serum free media and 1 ml of phosphate-buffered saline (PBS) containing 0.2 µg of Hoechst S769121 (Invitrogen) was added and kept for 5 min in dark at room temperature. The cover slip was then mounted on a clean glass slide with aqueous mountant (Vector Laboratories, Inc. Burlingame, CA) and observed under microscope.

Assessment of cell populations by fluorescence-activated cell sorting (FACS):

The apoptotic and necrotic cell population in the treated cancer cells was analysed by FACS (BD FACS

Calibur, USA) using Annexin V-FITC (Calbiochem, EMD chemicals Inc. CA) and propidium iodide (PI; Sigma Chemicals Co., St. Louis, USA) as per the protocols of the kit. After exposing to the extract for 12 h, cells were harvested and the cell suspension was adjusted to a concentration to 1 00 000 cells/ml. The harvested cells were washed with PBS and suspended in 100 µl binding buffer (1X). Cells were incubated with 5 µl Annexin V-FITC and 5 µl PI for 15 min at room temperature in dark and 400 µl binding buffer (1X) was added to each microtubes. The FITC and PI fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm), respectively and more than 50 000 events were acquired. Annexin V/PI dot plots was positioned into quadrants to distinguish live cells (Annexin V-/PI-), early apoptotic/primary apoptotic cells (Annexin V+/PI-), late apoptotic/secondary apoptotic cells (Annexin V+/PI+) and Annexin V-/PI+ necrotic cells^[19].

DNA fragmentation analysis by gel electrophoresis

DNA fragmentation in the MPJ-treated cancer cells was assessed by agarose gel electrophoresis^[20]. Following treatment with MPJ for 24 h, the cells (both adherent and non-adherent) were washed with cold PBS and pelleted by centrifugation. Cell pellets were then lysed with 50 µl of lysis buffer (1 % Triton X-100 in 20 mM ethylenediaminetetraacetic acid, 50 mM TrisHCl, pH 7.5) for 20 s and centrifuged at 1600×g for 5 min. The supernatant was removed and extraction from the pellet was repeated with the same amount of lysis buffer. Supernatants containing DNA fragments was brought to 1 % sodium dodecyl sulfate and treated with 5 µg/µl RNase A for 2 h at 56° followed by protein digestion with proteinase K (2.5 µg/µl) for 2 h at 37°. DNA was precipitated with 0.5 volume of 10 M ammonium acetate and 2.5 volume of cold absolute ethanol. The resulting DNA was collected by centrifugation, washed with cold 75 % v/v ethanol. The DNA pellet was dissolved in 30 µl Tris-EDTA buffer. The extracted DNA was subjected to electrophoresis through a 1.5 % agarose gel containing ethidium bromide. DNA bands were visualized under ultraviolet illumination (Alpha Imager Gel Documentation System, Alpha Inno Tech Corporation, USA).

Immunocytochemistry for detection of activated caspase-3 in HeLa cells

Immunocytochemistry for detection of activated caspase-3 in HeLa cells was done using caspase-3 polyclonal antibody (Cayman Chemical Company).

The cells were grown on cover slips and treated with MPJ for 12 h. The cells were washed with PBS and then fixed by adding 4 % paraformaldehyde (PFA) followed by incubation for 15 min. The cells were then washed three times with Tween-20 in PBS (PBT, 0.05 %) and incubated for 30 min with 1 % bovine serum albumin (BSA). After washing with PBT thrice, the cells were incubated with 0.2 ml caspase-3 polyclonal antibody diluted in 0.1 % BSA (1:1000) for 60 min at room temperature. Washing was done again with PBT three times for 5 min each followed by incubation with secondary antibody (PK-8800, Vectastain Universal Quick Kit) for 40 min at room temperature. After another round of washing to remove the unbound secondary antibody, the cells were incubated with three drops of Streptavidin-HRP for 40 min at room temperature followed by washing again. The cells were then incubated with chromogenic substrate 3,3' diaminobenzidine and haematoxylin (used as counterstain) for 5 min at room temperature, washed with PBT and fixed again with 0.5 ml of 4 % PFA. The coverslip was mounted on glass slide using aqueous mountant and observed under microscope.

RESULTS AND DISCUSSION

In *in vitro* evaluation, viability of both HeLa and MCF-7 cells treated with MPJ was found to be reduced with increase in treatment period. IC₅₀ of MPJ on HeLa and MCF-7 cells at 24 h were found to be 0.54 and 0.74 mg/ml, respectively (fig. 1A and B). IC₅₀ of Fluracil at 24 h was found to be 46 µg/ml and 25.7 µg/ml for HeLa and MCF-7 cells, respectively (fig. 1C). At 72 h of treatment with Fluracil, almost all HeLa and MCF-7 cells were found to be dead. In lymphocytes, MPJ was found to cause 50 % cell death at a concentration of 1.41 mg/ml (fig. 2). This concentration is much higher as compared to IC₅₀ of MPJ in HeLa and MCF-7 cells. In the present study, further assays were carried out by treating the cancer cells with the IC₅₀ of MPJ.

After treatment of both HeLa and MCF-7 cells with MPJ for 24, 48 and 72 h, morphological changes were observed. Clear nuclear fragmentation could be seen in HeLa cells from 24 h onwards (fig. 3A). MCF-7 cells also showed nuclear fragmentation at 24 h of treatment however the changes were not as prominent as that of HeLa cells (fig. 3B). Cytoplasmic vacuolation was observed in both control (treated only with vehicle) and MPJ-treated HeLa cells at 72 h. In fluorescent staining of nuclei with Hoechst S769121, control cells showed

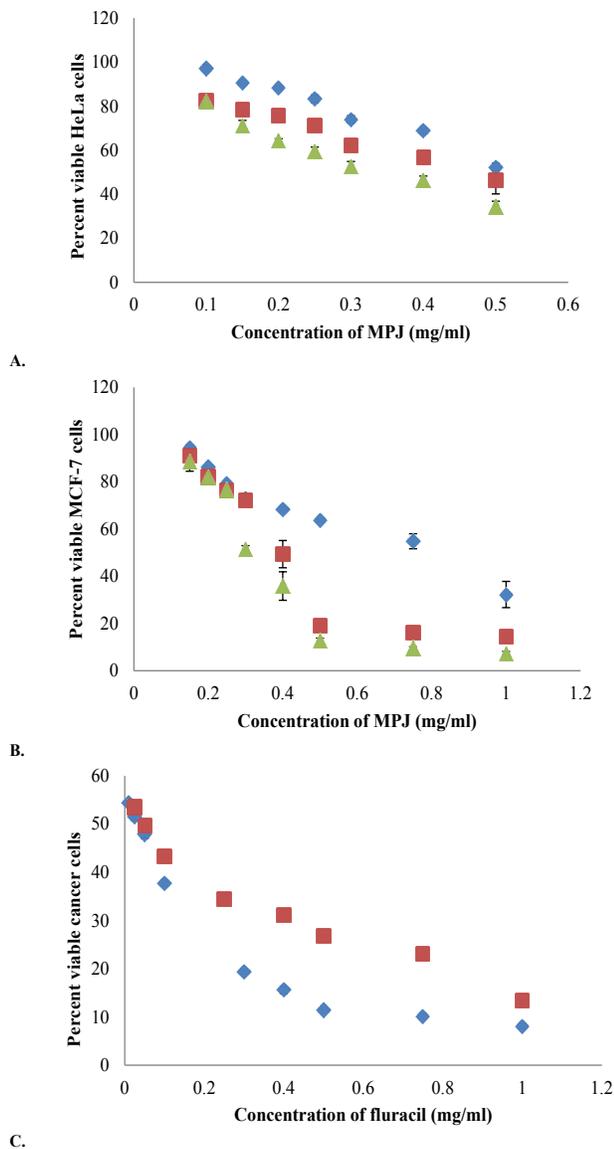


Fig. 1: Effect of MPJ and fluracil on HeLa cells and MCF-7 cells

Effect of (A, B) methanol extract of *Parkia javanica* (MPJ) and (C) Fluracil on viability of HeLa cells and MCF-7 cells at different concentration and treatment period A. HeLa cells, B. MCF-7, ♦ 24 h; ■ 48 h; ▲ 72 h. C. ♦ MCF-7 at 24 h; ■ HeLa at 24 h. Values are presented as mean±SE, n=3

round or oval greenish-yellow nucleus whereas MPJ-treated HeLa cells showed many fragmented nuclei (fig. 4). Fragmentation of nucleus was also observed in MPJ-treated MCF-7 cells but was not as clear as observed in HeLa cells.

Apoptotic and necrotic cell populations in both control and MPJ-treated cancer cells were analysed by FACS using Annexin V-FITC and PI (fig. 5). Positioning of quadrants on Annexin V/PI dot plots showed that the control HeLa cells was 95 % Annexin V-/PI- and 3.74 % positive for Annexin V-FITC. MPJ-treated HeLa cells showed 15.7 % Annexin V+/PI- and

5.63 % Annexin V+/PI+ with 3.03 % Annexin V-/PI+. Control MCF-7 cells were 91.6 % Annexin V-/PI- with around 4 % Annexin V-/PI+. Treated MCF-7 cells showed 75.4 % Annexin V-/PI-, 5.19 % Annexin V+/PI-, 7.65 % Annexin V+/PI+ and 6.38 % Annexin V-/PI+ cell populations.

In gel electrophoresis, DNA isolated from control cells did not exhibit ladder formation. DNA isolated from MPJ-treated HeLa cells showed smearing away from the well while MCF-7 cells showed smearing near the well (fig. 6).

Caspase-3, a key enzyme of apoptosis was detected in the MPJ-treated HeLa cells using polyclonal caspase-3 antibody by immunocytochemistry (fig. 7). Brown spots were observed in the cytosol of the treated cells indicating the presence of caspase-3. Nucleus of treated HeLa cells took deep hematoxylin stain and showed typical condensed chromatin pulled away from the nuclear envelope.

Apoptosis is a popular target of many cancer treatment strategies^[21]. Non-surgical approach for treatment of cancers targets either rectification of a defective apoptotic pathway or activation of apoptotic mechanism to induce apoptosis in malignant cells^[22]. Several medicines are available in the market for treatment of cancer however toxicity of the established drugs is a major issue^[23]. With increase concern about side effects of chemotherapy, many researches are nowadays turning towards plants or plant-derived natural products leading to identification of various plants with anticancer property. In the present study, apoptosis inducing ability of *P. javanica* seed extract

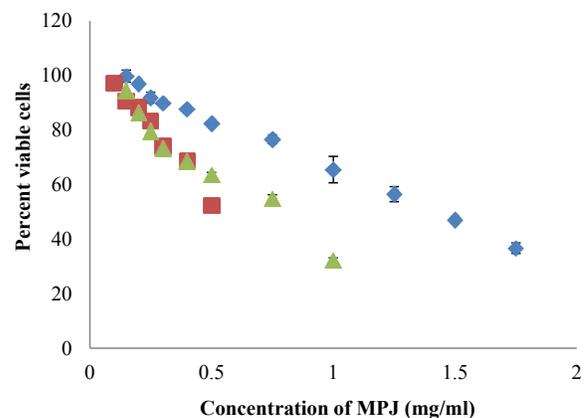


Fig. 2: Effect of MPJ on viability of normal lymphocytes, HeLa and MCF-7 cells

Comparison between the effect of methanol extract of *Parkia javanica* (MPJ) on viability of normal lymphocytes, HeLa and MCF-7 cells after 24 h of treatment. Values expressed as mean±SE, n=3. ♦ Lymphocytes; ■ HeLa; ▲ MCF-7

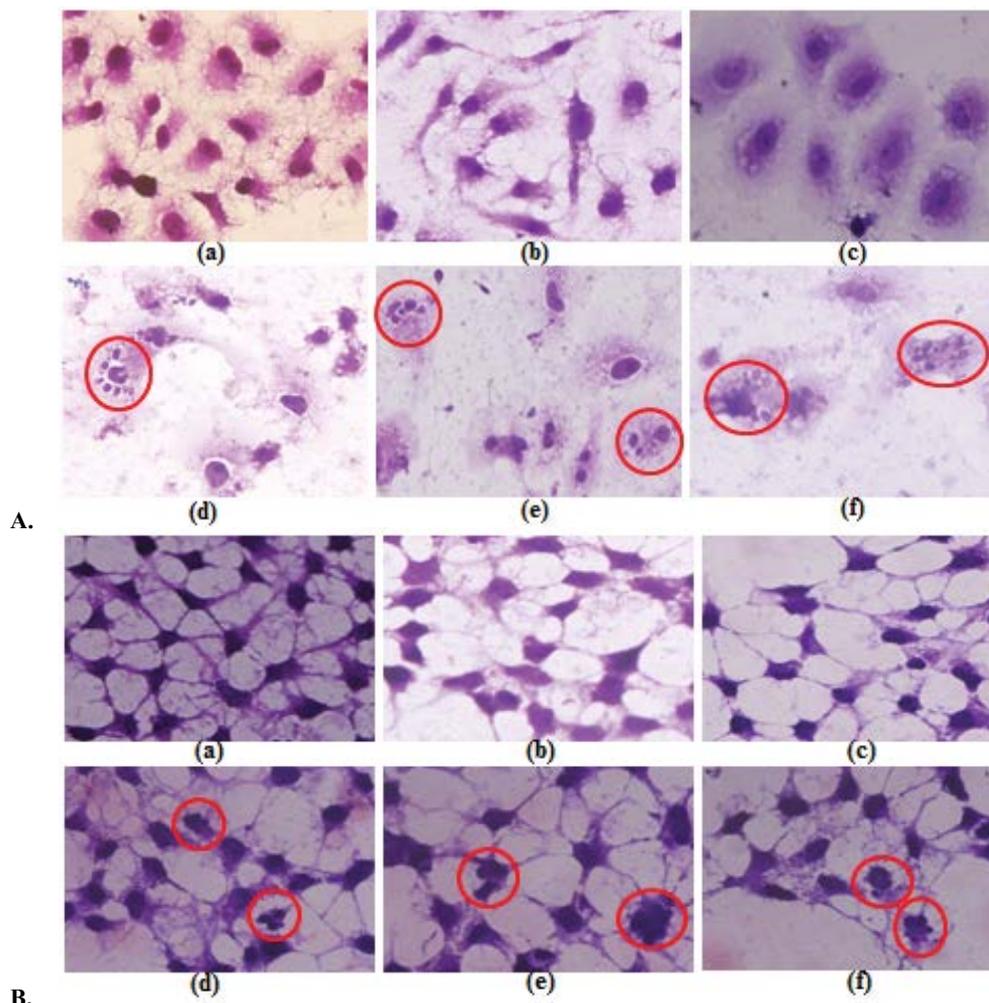


Fig. 3: Haematoxylin and Eosin staining of (A) HeLa cells and (B) MCF-7 cells (40 X)
Control cells treated with vehicle for (a) 24 h (b) 48 h (c) 72 h. Cells treated with MPJ showed fragmentation of nucleus (encircled in red) at (d) 24 h (e) 48 h (f) 72 h

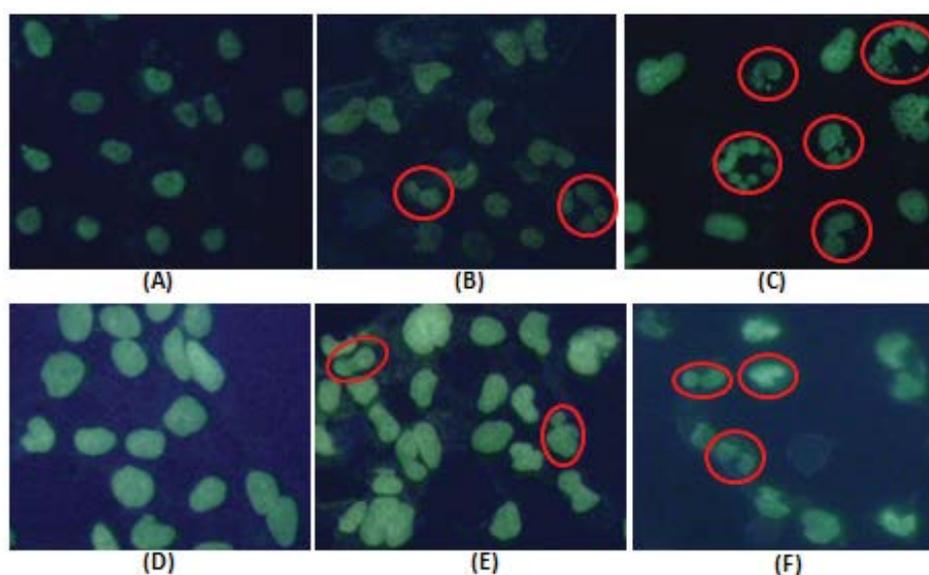


Fig. 4: Fluorescent staining of nuclei with Hoechst S769121 (40 X)
(A) Control HeLa cells (B) MPJ-treated HeLa cells at 24 h (C) MPJ-treated HeLa cells at 48 h (D) control MCF-7 cells (E) MPJ-treated MCF-7 cells at 24 h (F) MPJ-treated MCF-7 cells at 48 h. Some of the fragmented nuclei in the treated group are encircled red

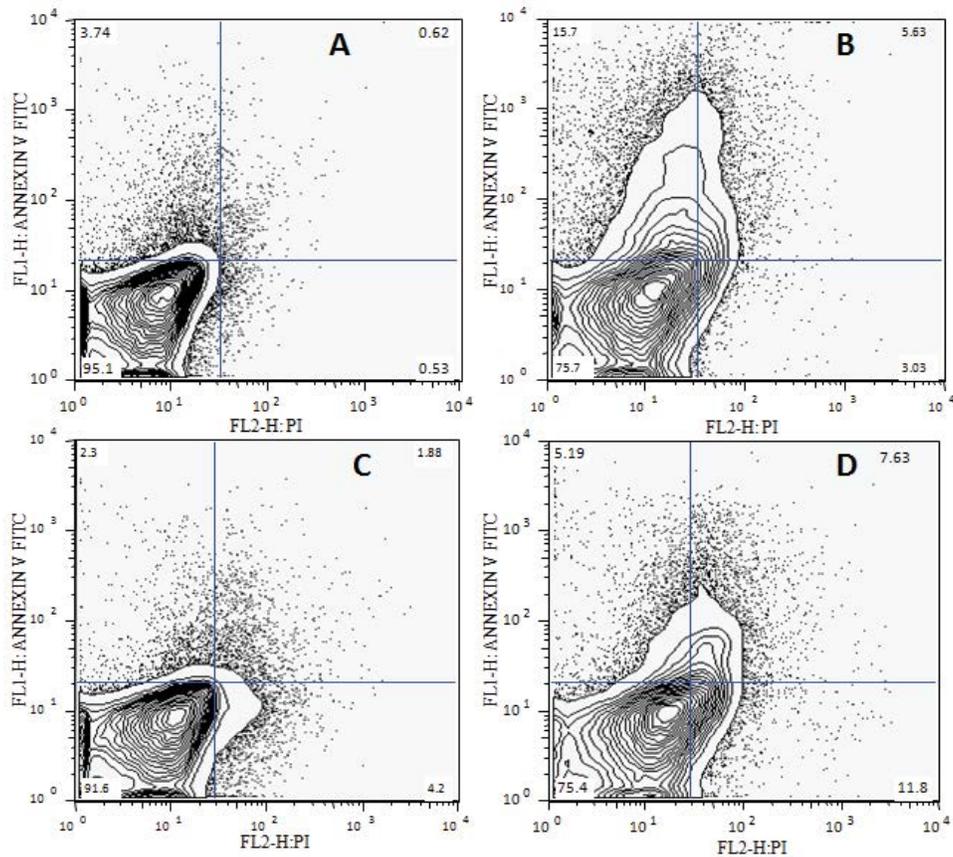


Fig. 5: FACS analysis of apoptotic and necrotic cell population using Annexin V-FITC and PI
 (A) Control HeLa cells (B) MPJ treated HeLa cells (C) Control MCF-7 cells (D) MPJ treated MCF-7 cells

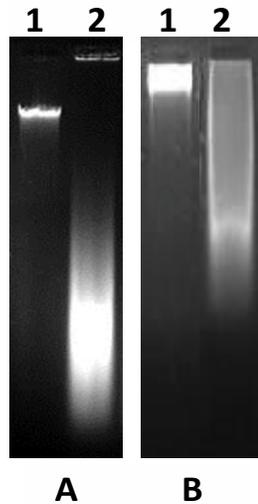
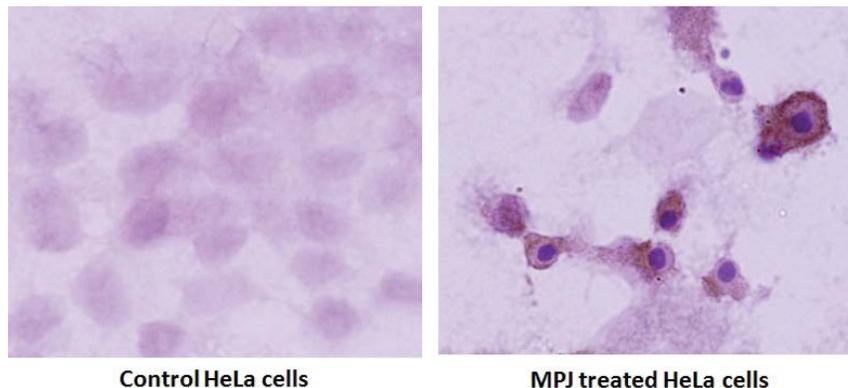


Fig. 6: DNA fragmentation analysis by agarose gel electrophoresis
 (A) HeLa cells (B) MCF-7 cells. Lane 1-control cells, Lane 2-MPJ treated cells

was evaluated in cancer cells. Very recently, the cytotoxicity of aqueous methanol extract of *P. javanica* fruit in sarcoma-180 (S-180), A549, AGS, and MDA-MB435S cells had been reported^[24]. In that study, green fruits of *P. javanica* available only seasonally were used. Moreover, the whole fruit is not edible

and a layer of the skin needs to be removed before consumption as vegetable. So, the present study was taken up to evaluate the anticancer property of dry seeds of *P. javanica*, which are edible as well as available throughout the year.

As a preliminary study, phytochemical screening of aqueous, ethanol and methanol extract of *P. javanica* seeds was carried out^[25]. Based on the results of phytochemical analysis, methanol extract of *P. javanica* seeds was selected for evaluation of anticancer activity. MPJ was found to cause death of HeLa and MCF-7 cells at a non-toxic concentration to normal cells. IC₅₀ of MPJ on normal healthy lymphocytes was around 2.6 and 1.9 times higher than that of HeLa and MCF-7 cells, respectively. It indicates that the extract has comparatively higher specificity towards cancer cells. A drug with high specificity and potency to kill cancer cells without fatal effect on normal cells would be ideal^[26]. Commercially available anticancer drug Fluracil was found to inhibit 50 % of HeLa and MCF-7 cells at 46 µg/ml and 25.7 µg/ml, respectively. 5-Fluorouracil (5-FU) causing 50 % cell death in HeLa at 36 µg/ml when treated for 48 h and MCF-7 at 31.2 µg/ml when used as 5-FU encapsulated CS/Au



Control HeLa cells

MPJ treated HeLa cells

Fig. 7: Detection of caspase-3 using anticaspase-3 polyclonal antibody in immunocytochemistry (40 X)

Brown spots of DAB (3,3'-diaminobenzidine) in the MPJ-treated cells indicates caspase-3. Dark haematoxylin stained nucleus are also visible in the treated cells

nanocomposite had been reported^[27,28]. As compared to Fluracil, the extract produced similar effects on the cancer cells at a higher dose. Requirement of higher dose of the extract to cause 50 % death in cancer cells may be due to the antagonistic relationship among phytochemicals present in the crude extract. It had been reported that co-occurrence of alkaloids and saponins significantly reduced antioxidant activity and these compounds should be kept exclusive to each other in drug formulations^[29].

In haematoxylin and eosin staining, morphological changes particularly in the nucleus of MPJ-treated cells were observed. There was chromatin condensation starting from periphery of the nuclear membrane leading to nuclear condensation. These are common feature of apoptosis during late stages and the condensed nucleus finally break up inside an intact cell membrane^[30]. Fragmentation of nucleus was clearly visible in the MPJ-treated HeLa cells. In fluorescent staining with Hoechst S769121, similar picture of nuclear fragmentation was observed in MPJ-treated cells while control cells showed rounded intact nuclei. Nuclear morphological changes in apoptosis such as convoluted nuclei, and clumps of chromatin may occur before or simultaneously with other hallmarks of apoptosis^[31]. Cells undergoing apoptosis *in vitro* ultimately go through secondary necrosis^[32]. To discern the presence of apoptotic and necrotic cell populations, both control as well as MPJ-treated cells were stained with Annexin V-FITC/PI and analysed by FACS. Annexin V binds to phosphatidylserine, which is exposed on the external leaflet of plasma membrane during early stages of apoptosis and FITC gives fluorescence for detection^[33,34]. PI was used as a counter stain to detect necrotic cells because it is a membrane impermeant nucleic acid stain generally excluded by viable cells

and a commonly used fluorescent dye in multicolour fluorescent techniques^[35,36]. When Annexin V-FITC/PI dots were positioned into quadrants, different cell populations consisting of live (Annexin V-FITC-/PI-), early apoptotic (Annexin V-FITC+/PI-), late apoptotic (Annexin V-FITC+/PI+) and necrotic cells (Annexin V-FITC-/PI+) were observed. HeLa cells showed higher percent of early apoptotic than late apoptotic and necrotic cells. MCF-7 cells showed higher percent of necrotic cell populations followed by late apoptotic and early apoptotic cells, which suggest that the cells were undergoing secondary necrosis.

A biochemical hallmark of apoptosis is cleavage of chromosomal DNA into oligonucleosomal size fragments^[37]. DNA isolated from MPJ-treated HeLa cells showed DNA fragments smearing away from the wells in agarose gel electrophoresis. However, DNA isolated from MPJ-treated MCF-7 cells showed smearing near the wells, which was indicative of formation of high molecular weight DNA, which precedes fragmentation into oligonucleosomal length fragments during apoptosis^[38,31]. It had been documented that irregular size genomic DNA fragments of necrotic cells can also be observed as smear in gel electrophoresis^[39]. In apoptosis, there is activation of caspases (a family of endoproteases), which result in either inactivation or activation of substrates generating a series of signalling molecules that participate in ordered processes of cell death^[40]. In our study, activated caspase-3, the main effector caspase of apoptosis was detected in MPJ-treated HeLa cells by immunocytochemistry. Additionally, MPJ-treated cells showed deep haematoxylin stained condensed nuclei, which is a typical feature of apoptotic cell with condensed chromatin pulled away from the nuclear envelope^[31]. Since MCF-7 doesn't express caspase-3

due to the functional deletion in the CASP-3 gene^[41], immunocytochemistry for detection of caspase-3 was not done in this cell line.

In the present study, the features of apoptosis observed in HeLa and MCF-7 cells were different. Fragmentation of nucleus was found to be more in MPJ-treated HeLa cells as compared to MCF-7 cells. The difference may be due to the activation of caspase-3 in HeLa cells as this enzyme causes DNA fragmentation and other structural changes associated with apoptosis^[41], HeLa cells might have undergone caspase-3 pathway while MCF-7 cells might have adopted other pathways of apoptosis. From the study, it was found that MPJ-induced apoptosis in the cancer cells and causes cell death at a lower concentration as compared to that of normal cells. Future work on *P. javanica* may be focussed on the identification of active compounds in the seeds responsible for the specificity and potency of killing the cancer cells. Further, it would be worth finding out whether the inclusion of *P. javanica* seeds in diet lead to chemoprevention.

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Conflict of interest:

There are no conflicts of interest.

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