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Research Article

Anti-colon Cancer Activity of *Parkia javanica* (Lamk.) Merr. Bark Extract: An *In-vitro* Study

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ABSTRACT

The Methanolic extract of *Parkia javanica* bark (MEPJB) was examined *in vitro* to see whether it has any cytotoxic or chemotherapeutic potential against the colon cancer cell lines HCT116 and SW480. Cell viability or proliferation assay and migration assay were performed, which showed significant results. The bark extract exhibited significant cytotoxic potential against both the colon cancer cell lines, and it also inhibited cell migration in a dose-dependent and time-dependent experiment. The IC_{50} values of MEPJB on the HCT116 cell line for 24 hours, 48 hours, and 72 hours were 83.33 µg/mL, 37.5 µg/mL, and 34.37 µg/mL, respectively, whereas on the SW480 cell line, the IC_{50} values were 87.5 µg/mL, 40 µg/mL and 31.25 µg/mL for 24 hours, 48 hours, and 72 hours respectively. However, IC_{50} doses of MEPJB were inactive against the normal, healthy human fibroblast cell line. Cytotoxicity screening of 5-fluorouracil (5-FU), a commonly used drug in colon cancer treatment, was also done on both the colon cancer cell lines HCT116 and SW480, which showed much higher IC_{50} than MEPJB after 24 hours, 48 hours, and 72 hours of incubation. MEPJB changed the cell morphology and induced apoptosis in colon cancer cell lines, confirmed by a morphological study of treated and untreated colon cancer cell lines and the acridine orange/ethidium bromide (AO/EB) dual staining procedure, respectively. After 48 hours of incubation with respective IC_{50} concentrations of MEPJB, the treated colon cancer cells showed early and late apoptotic features. So this current research work indicates that the methanolic extract of *P. javanica* bark has chemotherapeutic potential against colon cancer cells and promotes apoptosis in cancer cells, which results in cell death.

Introduction

Colorectal adenocarcinoma or colorectal cancer (CRC) is a kind of colon and/or rectum cancer.[1] Precancerous polyp formation on the inner surface of the rectum and colon is the initial feature of CRC. Depending on the site of origination, CRC is known as colon cancer or rectal cancer.[2] Colon cancer is the fourth most common cancer incident globally, whereas carcinoma of the rectum is the eighth most common.[3] Together, colorectal adenocarcinoma (CRC) cases represent the third most frequently diagnosed form of cancer, and it is the second most deadly cancer in the world.[3] The chance of developing CRC is greater in males than females.[4] The incidence of CRC is still emerging in developing countries, but effective therapy is still challenging at advanced stages.[5] Various chemotherapeutic drugs for the treatment of advanced stages of colorectal cancer are also available, like 5-fluorouracil (usually administered into the vein with vitamin leucovorin, which makes it more effective),[6] oxaliplatin, irinotecan, etc.[7-9] However, it has some severe toxic effects on normal tissues.[10,11] In humans, 5-fluorouracil, a commonly used chemotherapeutic agent, causes myelotoxicity,[12] cardiotoxicity,[13] and in rare cases, vasospasm.[14]

Therefore, herbal medicines extracted from various plants have been used for many years as an alternative method of cancer treatment to reduce risk factors associated with chemotherapeutic drugs in developing countries.[15-20]

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P. javanica is a leguminous plant with an ethnomedicinal history and has been used to a great extent by various tribes in northeastern India to cure stomach aches, cholera, diarrhea, dysentery, and food poisoning.[21-23] As some studies have already revealed the anticancer activity of methanolic extract of *P. javanica* fruit[24] and seed[25] on various human cancer cell lines, this study has been designed to look into the *in-vitro* activity of methanolic extract of *P. javanica* bark (MEPJB) on human colon cancer cell lines.

Materials and Methods

Plant Material

The fresh bark of *P. javanica* was collected from Suryamaninagar, Tripura, India. At first, Dr. B. K. Dutta, Taxonomist, Department of Botany, Tripura University, identified the plant, although the plant was further cross-checked and authenticated by Dr. H. J. Chowdhery, the Joint Director, Central National Herbarium, Botanical Survey of India, Shibpur, Howrah, West Bengal. The voucher for specimen No. ≠ BD-01/06 has been deposited in the Herbarium.

Extraction of Plant Extract

P. javanica bark was cleaned with water, then chopped into tiny pieces, and shaded dry for 3-4 weeks. The dried bark was then ground into a powder with the aid of a mortar and pestle. Then 200 gm of powdered bark was mixed with 600 mL of methanol solvent to prepare the methanolic extract of *P. javanica* bark (MEPJB) and kept in a shaker at room temperature for 48 hours. After the predicted time, the solution was filtered at least 3-4 times with Whatman filter paper-1. Then the filtered solution was concentrated by evaporating the pure methanol solvent from the solution with the help of a rotary evaporator at 60°C. Finally, the concentrated solution was lyophilized at 4°C to eliminate the methanol from the extract and stored at -20°C.[23]

Primary Phytochemical Analysis

Methanolic extract of *P. javanica* used for primary phytochemical analysis using a standard procedure.[26] Phytochemical tests were done for alkaloids, flavonoids, phenolic compounds, terpenoids, quinones, saponins, tannins, and oxalate.

Cell Lines and Reagents

The human colon carcinoma (HCT 116 and SW480) and the normal human skin fibroblast cell lines were purchased from the National Centre for Cell Science (NCCS), Pune, India. Both the cell lines were maintained in DMEM supplemented with 10% (v/v) FBS, 100 µg/mL streptomycin, and 100 IU/mL penicillin, under 5 % CO_2 at 37°C.

Cell Viability Assay

Cell viability was estimated by using the colorimetric assay, which is known as the MTT assay.[27] In 96 well microplates, colon cancer cells and normal human fibroblast cells were distributed at 10,000 cells/well. After incubating for 24 hours under 5% CO_2 at 37°C, the cells were treated with or without MEPJB in a dose-dependent manner and incubated (37°C and 5% CO_2) for 24, 48, and 72 hours. To prepare a 5 mg/mL concentrated solution of MTT, 5 mg of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was dissolved in 1 mL of phosphate-buffered saline (PBS). This MTT solution (5 mg/mL of stock concentration) was then diluted 10 times with PBS to prepare the working solution. 100 µL of this diluted solution was added to each well after removing the media from each well. The plates were then incubated (under 5% CO_2; at 37°C) for 3 hours. After incubation, the MTT medium was removed from the wells, and 100 µL of DMSO was added to each well. Finally, using a microplate reader, absorbance was measured at 570 nm.

Cell Migration Assay or Wound-Healing Assay

Colon cancer cells (HCT116 and SW480) were seeded on culture plates, and after ideal confluency, a 0.5 mm wide scratch was manually created on each culture plate with a microtip. Then the cells were treated with or without MEPJB. Microscopic images were taken of different periods from 0 to 72 hours of treatment. The activity of the MEPJB was measured by comparing the gap size of control (without MEPJB) with treated (with MEPJB) scratch.[28,29]

Apoptotic Assay

We used a dual acridine orange/ethidium bromide (AO/EB) fluorescent staining method to determine the morphological changes of the nucleus of colon cancer cells associated with apoptosis.[30,31] 5×10^5 cells were seeded on a 6-well plate for this method, and the cells were allowed to attach to each well. After attachment, the cells were treated with MEPJB with respective IC_{50} doses and incubated for 48 hours. After incubation, treated cells were trypsinized, resuspended in cold PBS, and ultimately subjected to acridine orange (100 µg/mL) / ethidium bromide (100 µg/mL) staining. 10 µL of each stained treated and control cell suspension was put on a glass slide each time, mounted with a cover-slip, and observed under a fluorescent microscope.

Statistical Analysis

All the assays were repeated three times, and the results were represented as the mean ± SEM of three sets of data. The p-value was evaluated by one-way ANOVA, which indicates the significant differences among different groups of experimental data. $P < 0.05$, $P < 0.01$ and $P < 0.001$ are represented as *, **, *** respectively.

Results

Results of Phytochemical Screening

The primary phytochemical analysis revealed the existence of a significant amount of phenolic compound in MEPJB.

Except for phenolic components, phytochemical analysis also showed the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, and quinones in the methanolic extract in limited amounts (Table 1).

MEPJB Inhibits the Proliferation of HCT116 and SW480 Cells

The cytotoxic activity or anti-proliferative activity of MEPJB on the colon cancer cell lines (HCT116 and SW480) and human normal fibroblast cell line were determined by treating the cell lines with different concentrations of MEPJB for 24, 48, and 72 hours (Fig. 1). All the three cell lines were further treated with the positive control, 5-Fluorouracil (5-FU). The cytotoxic activity of 5-FU was compared with the MEPJB. An MTT assay determined the cell viability in each of the cases. The half-maximal inhibitory concentrations (IC_{50}) for MEPJB on the HCT116 cell line were found to be 83.33 µg/mL (after 24 hours), 37.5 µg/mL (after 48 hours), and 34.37 µg/mL (after 72 hours), whereas SW480 showed IC_{50} values of 87.5 µg/mL, 40 µg/mL, and 31.25 µg/mL for 24, 48, and 72 hours respectively (Table 2). However, 5-FU showed a

Table 1: Primary phytochemical screening of Methanolic Extract of *P. javanica* Bark (MEPJB).

Plant Constituent	*Methanolic extract of P. javanica bark (MEPJB)*	*Name of the test*
1. Alkaloids	++	Wagner's test
2. Flavonoids	+	Lead Acetate Test
3. Phenolic compounds	++++	Ferric Chloride Test
4. Terpenoids	++	Salkowski Test
5. Quinones	++	Borntrager's Test
6. Saponins	+++	Honey Comb Test
7. Tannins	+	Ferric Chloride Test
8. Oxalate	-	Ethyl Acetate Test

"+" sign indicates presence of phytoconstituents; "-" sign indicates the absence of phytoconstituents.

Table 2: IC_{50} values of Methanolic Extract of *P. javanica* Bark (MEPJB) on HCT116 and SW480 colon cancer cell lines.

	IC_{50} (µg/mL) Colon cancer cell lines	
	HCT116	SW480
24 h	83.33	87.5
48 h	37.5	40
72 h	34.37	31.25

Human colon cancer cell lines were treated with different concentrations of methanol extract of *Parkia javanica* bark in 96-well microculture plates for 24, 48, and 72 hours. The dose-dependent and time-dependent MTT assay for each cell line was repeated three times and here the results represent the mean of three sets of experimental data. h, hour.

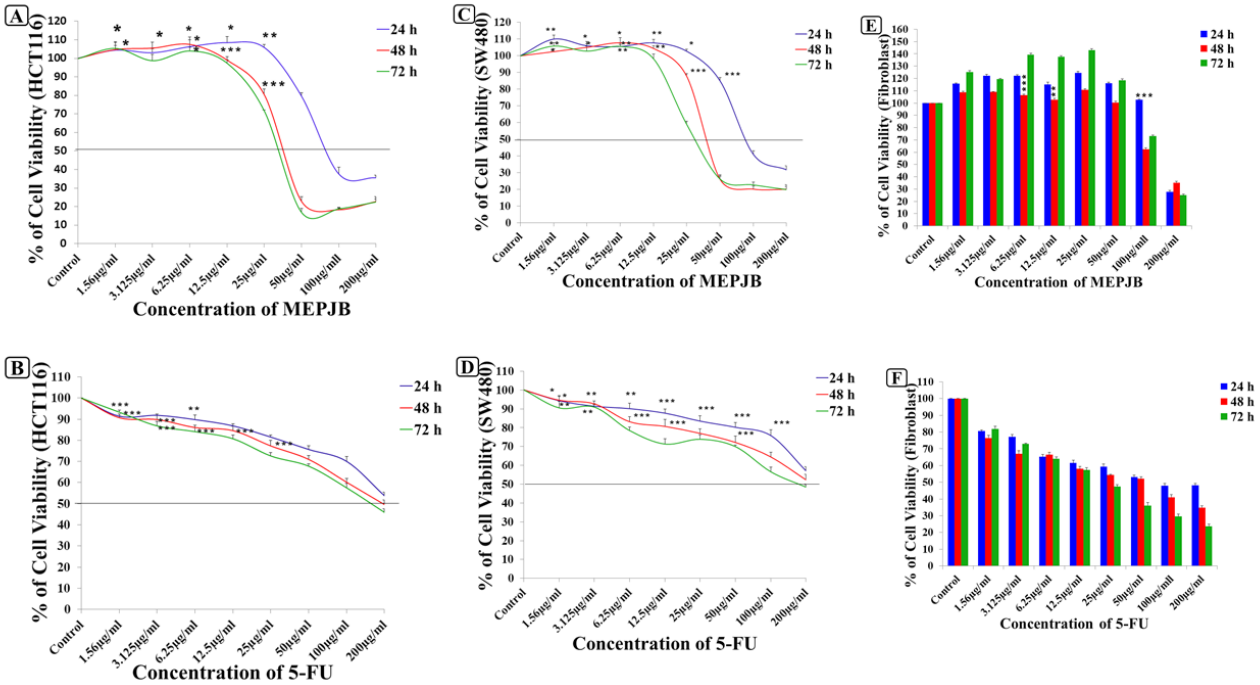

Fig. 1: Cell viability assay: HCT116, SW480, and normal fibroblast cell lines were treated with various concentrations (the range was 1.56 µg/mL to 200 µg/mL) of MEPJB and a positive control 5-FU for 24 hours, 48 hours, and 72 hours. A and B indicate the percentage of HCT116 cell proliferation at different concentrations of MEPJB and 5-FU respectively. C and D indicate the percentage of SW480 cell proliferation at different concentrations of MEPJB and 5-FU, respectively. E and F indicate the percentage of normal fibroblast cell proliferation at different concentrations of MEPJB and 5-FU respectively. Each data point represents average values from three independent experiments (n = 3). The error bar shows mean ± SEM. * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$) represent the level of significant deviation of the values of each data point (treated) from the control (without MEPJB), which was determined by one-way ANOVA ($\alpha = 0.05$). h, hour.

higher IC_{50} (>160 µg/mL) on both the colon cancer cell lines compared to MEPJB and the IC_{50} concentrations of MEPJB on both colon cancer cell lines were completely inactive in the case of the normal human fibroblast cell line (Fig. 1).

MEPJB Affects the Cell Motility of HCT116 and SW480 Cells

A wound-healing assay was performed to determine the effect of MEPJB on cell motility or cell migration of both the colon cancer cell lines. After scratching a wound on a monolayer of cancer cells with a microtip, the cells were treated with MEPJB, and microscopic images of the scratched area were taken between 0 and 72 hours. The gap size of the treated and untreated wounds were compared, which showed that the MEPJB inhibits cell motility as, after 24, 48, and 72 hours, the gap size of the untreated (without MEPJB) wounds of HCT116 and SW480 colon cancer cells was decreased but the gap size of the treated (with MEPJB) wounds was gradually increased (Fig. 2).

Morphological Changes in HCT116 and SW480 Cell Lines Exposed to MEPJB

Cancer cells were incubated with MEPJB, and the morphological changes were observed after 24, 48, and 72 hours, respectively to investigate the morphological changes of HCT116 and SW480 cell lines in the presence or absence of MEPJB. Colon cancer cells showed significant morphological changes in the presence of MEPJB compared to the control. The colon cancer cells began to round up after 24 hours of incubation and completely rounded up after 48, or 72 hours of incubation (Fig. 3).

Apoptosis Induction by MEPJB on HCT116 and SW480 Colon Cancer Cells

It was observed that MEPJB inhibits cell proliferation and cell motility and induces morphological changes in the HCT116 and SW480 colon cancer cell lines, which could be associated with apoptosis. In order to determine cell death and identify whether any apoptosis-associated changes occurred in the cell membrane, the acridine orange/ethidium bromide (AO/EB) fluorescent staining procedure (1:1 ratio) was used, and results were observed after 48 hours of incubation. Acridine orange can easily enter viable cells through the cell membrane and bind to the DNA, whereas ethidium bromide can penetrate through the membrane of nonviable cells and bind to the DNA. After the AO/EB fluorescent staining process, nonviable cells fluoresce red and viable cells fluoresce green under fluorescence microscopy. After 48 hours of incubation, most of the control group cells fluoresce light green color, but cells of both the experimental group (HCT116 and SW480 treated with respective IC_{50} doses of MEPJB extract) fluoresce yellow-green or orange-green color that represents the early and late apoptotic phase, respectively. Membrane blebbing and chromatin condensation, which indicate early apoptosis and the granular nucleus, apoptotic bodies, ring condensation, and necklace condensation of the nucleus, which represent late apoptosis, were observed among the experimental group of cells (Fig. 4).

Discussion

The northeastern region of India has an enormous diversity of plant species,[32] and it accounts for about 50 % of the total plant species in India.[33] Plants with different medicinal values are abundant in this region.[34] Tripura is a state that belongs to northeast India. The state has a large number of medicinal plants.[35] *P. javanica* is an ethnomedicinal leguminous endemic plant in northeastern India. Different tribes in northeast India traditionally use this plant to cure various diseases.[36,37] Several studies have reported the antibacterial, anti-inflammatory, and wound healing activities of *P. javanica* bark.[38-41] The methanolic extract of *P. javanica* bark (MEPJB) has also been reported to inhibit various blood cell malignancies.[42] However, there is no scientific study on the anti-colon cancer activity of *P. javanica* bark. Therefore, the present *in vitro* study was designed to determine the cytotoxic and anti-proliferative activity of MEPJB and its effect on cell migration and morphology. Our current *in vitro* study showed that MEPJB has a strong and higher cytotoxic effect on both the human colon cancer cell lines HCT116 (IC_{50}: 83.33 µg/mL for 24 hours, 37.5 µg/mL for 48 hours, and 34.37 µg/mL for 72 hours) and SW480 (IC_{50}: 87.5 µg/mL

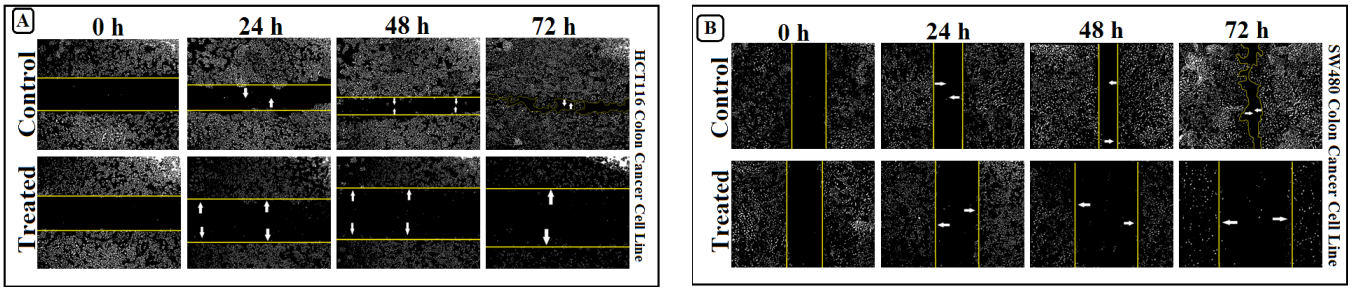

Fig. 2: A and B represent the wound healing assay of HCT116 and SW480 colon cancer cell lines respectively: control (without MEPJB) and treated (with MEPJB) colon cancer cells were incubated for 24 hours, 48 hours, and 72 hours. Control shows a gradual decrease in gap size in both the cases of colon cancer cell lines. Whether treated, microscopic images show a gradual increase in gap size after 24, 48 and 72 h of incubation. h, hour.

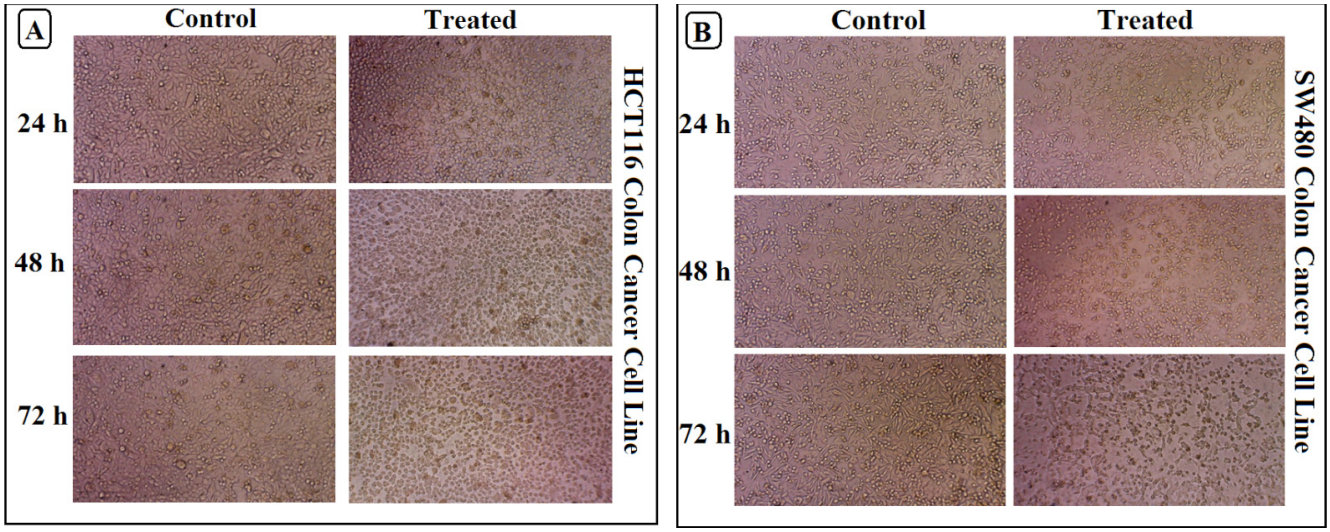

Fig. 3: Morphological changes in treated (with MEPJB) and controlled (without MEPJB) human colon cancer cell lines (A. HCT 116, B. SW480). Control and treated experimental groups of cells were monitored for 24, 48, and 72 hours and the images were captured by an inverted microscope. h, hour.

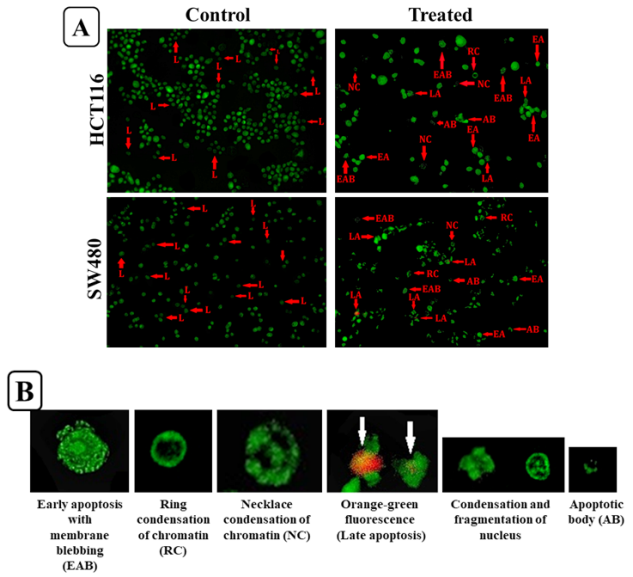

Fig. 4: Detection of Apoptotic morphology by acridine orange-ethidium bromide (AO/EB; 1:1 ratio) fluorescent staining procedure. A. represents treated and untreated (control) human colon cancer cells incubated for 48 hours. Untreated cells for 48 hours (negative control groups): uniformly green stained live cells (L) with a uniformly distributed nucleus in the center. Treated cells for 48 hours: some cells show early apoptosis (EA) with membrane blebbing (EAB) and a concentrated yellow-green nucleus on one side of the cell. Some cells represent features of late apoptosis (LA), like orange-green fluorescence, apoptotic bodies (AB), ring condensation of chromatin (RC), and necklace condensation of chromatin (NC). B. represents an enlarged image of membrane blebbing, ring condensation of chromatin, necklace condensation of chromatin, orange-green fluorescence, nuclear condensation as well as fragmentation and apoptotic bodies, found among the experimental group of cells in Figure A.

for 24 hours, 40 µg/mL for 48 hours, and 31.25 µg/mL for 72 hours) compared to 5-fluorouracil (IC_{50}: > 160 µg/mL for 24, 48 and 72 hours on both the cancer cell lines), the drug which is commonly used in colon cancer treatment. Notably, the IC_{50} doses of MEPJB had almost no toxicity to the normal human fibroblast cell line in a dose-dependent and time-dependent experiment. As uncontrolled proliferation is the main characteristic of cancer cells, we designed our study to induce cancer cell death by using cytotoxic compounds. Migration of cancer cells is an important step that facilitates metastasis and the wound healing assay is the classic method to evaluate the effect of any cytotoxic agent on cell migration and metastasis.[43-45] The MEPJB inhibited the migration of colon cancer cells and reduced the cell density in a time-dependent experiment of wound healing assay, compared to untreated control cells. The wound-healing assay results suggest that MEPJB could be a factor that changes cell morphology, affects the actin cytoskeleton, initiates cancer cell detachment, and ultimately leads to cancer cell death. MEPJB showed positive results in inhibiting the motility of cancer cells and it also showed a great potential to change cell morphology. In a time-dependent experiment, the MEPJB treated cells showed a higher potential to change cell morphology than the positive control 5-FU. But there were no significant morphological changes in the normal human fibroblast cell line when treated with IC_{50} concentrations of cancer cells. Apoptosis induction is the primary and critical mechanism by which chemotherapeutic agents kill cancer cells. During this type of cell death, the cancer cell undergoes various biochemical and morphological changes, like chromatin condensation, DNA damage, ring condensation and necklace condensation of the nucleus, membrane blebbing and budding, etc.[46,47] The early and late stages of apoptosis can be detected by the AO/EB fluorescent staining procedure. After 48 hours of incubation, most of the MEPJB-treated colon cancer cells (with respective IC_{50} doses) showed early and late apoptotic features. Phytochemical analysis indicates the presence of a good

proportion of phenolic compounds in MEPJB. Saponin, alkaloids, flavonoids, terpenoids, quinone, and tannin are also present in MEPJB. All of these compounds have antimetastatic, anti-proliferative, and apoptosis-inducing activity against various cancer cells.[18,48-53] This study opens the doors for further research to identify if there is any active compound present in MEPJB that may exhibit more specific cytotoxic activity on the colon cancer cell line.

Conclusion

This current study suggests that the methanolic extract of *P. javanica* bark (MEPJB) has more cytotoxic potential against the colon cancer cell line than 5-fluorouracil, the drug generally used for colon cancer treatment. The IC_{50} concentrations of MEPJB can inhibit the proliferation and migration of colon cancer cells HCT116 and SW480 in a time-dependent experiment, but the same IC_{50} concentrations of MEPJB are inactive in the normal healthy human fibroblast cell line. The colon cancer cell shows morphological changes, early and late apoptotic features in the presence of respective IC_{50} doses of MEPJB. So it indicates that MEPJB induces apoptosis in the colon cancer cell line and leads to cell death. Further fractionation of MEPJB and chemical analysis of the active fractions could be the future research interest to evaluate the biological significance of *P. javanica* bark as a chemotherapeutic agent.

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