Research Article (Open access)

Anticancer effect of *Mesua ferrea* extracts on Human Pancreatic Cancer Cell line

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ABSTRACT- Plants have been used for medical purposes since the beginning of human history and are the basis of modern medicine. Most chemotherapeutic drugs for cancer treatment are molecules identified and isolated from plants or their synthetic derivatives. In this study, we examined the anticancer effect of different extracts from the leaves of *Mesua ferrea* plant. We found that Di-chloromethane (DCM) extract inhibited the proliferation of different cancer cell lines with most significant effect on pancreatic cancer cell line (Panc-1). Moreover, killing activity was specific for tumor cells, as the leaf extracts had no growth inhibitory effect on normal lung fibroblast cell line (WI-38). Cell death caused by the DCM extracts is by p53 mediated cell cycle arrest leading to caspase 3 activation and apoptosis.

Key Words- Panc-1, p53, Caspase3, Apoptosis, DCM, *Mesua ferrea*

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INTRODUCTION

There is a constant demand to develop new, effective, and affordable anticancer drugs [1,2]. From the dawn of ancient medicine, chemical compounds derived from plants have been used to treat human diseases. Natural products have received increasing attention over the past 30 years for their potential as novel cancer preventive and therapeutic agents in parallel, there is increasing evidence for the potential of plant-derived compounds as inhibitors of various stages of tumor genesis and associated inflammatory processes, underlining the importance of these products in cancer prevention and therapy [3,4].

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Received: 30 Jan 2016/Revised: 19 Feb 2016/Accepted: 29 Feb 2016

Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products and among that the plant kingdom has been the most significant source [5-7]. These include vinca alkaloids, Taxus diterpenes, Camptotheca alkaloids, and Podophyllum lignans. Currently, of 16 new plant-derived compounds being tested in clinical trials, 13 are in phase I or II and three are in phase III. Among these compounds, flavopiridol, isolated from the Indian tree Dysoxylum binectariferum, and meisoindigo, isolated from the Chinese plant *Indigofera tinctoria*, have been shown to exhibit anticancer effects with lesser toxicity than conventional drugs [8-10]. At this time, more than 3000 plants worldwide have been reported to have anticancer properties. Globally, the incidence of plant-derived products for cancer treatment is from 10% to 40% with this rate reaching 50% in Asiatic patients [11,12].

In the current study, we screened leaves extract with the hypothesis that extracts might contain multiple molecules with antitumor activities and would be very effective in

killing human cancer cells. We initially examined the effects of leaves extracts (*M. ferrea*) on a panel of different human tumor cell lines as well as human primary non cancer cultures. The most effective leave extract, obtained from Di-chloromethane (DCM) showed a selective toxicity on pancreatic adenocarcinoma cell line (Panc-1) without much toxicity on normal lung fibroblast cell lines (WI-38). Furthermore, DCM extract evaluated to identify the mechanism behind the toxicity. Cell cycle analysis indicates the cell arrest with DCM extract. Immunoblot studies confirm DCM extract to induce apoptosis via activation of p53, and caspae3.

MATERIALS AND METHODS

Chemicals

Chemicals used in all the experiments were analytical grade. The cell culture media and fetal bovine serum (FBS) were respectively obtained from Sigma (St. Louis, MO, USA) and Gibco (Paisley, Scotland, UK). For the immunoblot analysis, mouse monoclonal anti-p53 antibody (sc-56180), and mouse monoclonal anti-caspase3 antibody (sc7272) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were of analytical grade and were purchased from Sigma unless otherwise specified.

Cell lines and cell culture

A panel of five cancer cell lines representing multiple solid cancers of clinical relevance were selected, namely; ACHN (human renal cell carcinoma, ATCC, CRL-1611), Panc-1 (human pancreatic adenocarcinoma, ATCC, CRL-1469) cultured on MEM media with 2mM L-glutamine and 10% FBS, H460 (human non-small cell lung carcinoma, ATCC, HTB-177), Calu-1 (human lung carcinoma, ATCC, HTB-54), cultured on RPMI, 2 mM L-glutamine and 10% FBS, MDA-MB231 (human breast adenocarcinoma, ATCC, HTB-26) cultured in McCoy's 5a medium and 10% FBS and MCF10A (normal breast epithelium cells) cultured on MEM with 2 mM L-glutamine and 10% FBS.

Extraction

Preparation of Petroleum ether Extract: ~500 gm of leaf crude was taken in clean round bottom flask (5litres) soaked in petroleum ether (2X2.5) LR grade and stirred at room temperature for overnight. The mixture was filtered through Whatman filter paper and filtrate was concentrated at 45°C on rotavapor (Buchi). The 3 gm dark green crude extract was obtained and used for further studies.

Preparation of Dichloromethane Extract: Residue obtained from petroleum ether was further soaked in dichloromethane (2X2.5 lit) LR grade and stirred at room temperature for overnight. The mixture was filtered through Whatman filter paper and the filtrate was concentrated at 45°C on rotavapor. 3.9 gm dark green crude extract was obtained and used for further studies.

Preparation of Ethyl Acetate Extract: Residue obtained from dichloromethane was further soaked in ethyl acetate (2X2.5lit) LR grade and stirred at room temperature for overnight. The mixture was filtered through Whatman filter paper and the filtrate was concentrated at 45°C on rotavapor. 3gm dark green crude extract was obtained and used for further studies.

Preparation of Methanol Extract: Residue obtained from ethyl acetate extraction was further soaked in methanol (2x2.5 lit) LR grade and stirred at room temperature for overnight. The mixture was filtered through Whatman filter paper and filtrate was concentrated at 45°C on rotavapor. The 3.48 gm dark green crude extract was obtained and used for further studies.

Preparation of Aqueous Extract: Residue obtained from methanol extraction was further soaked in distilled water (2X2lit) and stirred at room temperature for overnight. The mixture was filtered through Whatman filter paper and lyophilized to get 6.28 gm brown colored material crude extract was obtained and used for further studies.

Cell proliferation assay

Cell growth was measured using the 3-(4-5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) method [13]. Cells were seeded at the appropriate numbers to prevent confluence throughout the experiment. After a 24 h incubation period, the cells were treated with single or multiple concentrations of the extract. An equal concentration of the vehicle (DMSO; never exceeding 0.1%) was used as a control. At the end of incubation period (24 h), 10 µl aliquots of MTT (final concentration 0.5 mg/ml) were added to each well, and the plates were incubated for 4 h at 37ºC. Formazan crystals formed after addition of MTT were solubilized by adding 100 µl of 20% sodium dodecyl sulfate (SDS) in H_2O followed by incubation overnight at 37ºC. The extent of MTT reduction was measured using a Tecan Sapphire multi-fluorescence microplate reader (Tecan Germany GmbH, Carlsheim, Germany) at 595 nm. DMSO-treated control cells were considered to have a cell viability of 100%. The average number of dead cells at different extracts concentrations was expressed as a percentage of the control.

Protein estimation by High Content Array Scan

The cells were seeded in 96-well plates at a density of 10000 cells per well. 24 h post seeding, the medium was replaced with a fresh medium and the cells were treated with DCM leaves extract (0.5 µg/ml, final concentration), and further incubated for 12 h. To determine the protein expression, the cells were fixed with 3.7% formaldehyde (Sigma, St. Louis, MO) in Phosphate Buffer Saline for 10 min at room temperature, followed by permeabilization with 0.15% Triton X-100 (Sigma St. Louis, MO) for 10 min. After permeabilization, the cells were incubated with primary antibody for 1 h. Following primary antibody incubation, the nucleus was stained with Hoechst 3342 (blue), and primary antibody for different proteins was localized by secondary antibody labeled with Dye Light 549 (red). Immuno-fluorescence of the protein of interest was determined by scanning the plates on Cellomics Array Scan® VTI HCS Reader (Thermo Fisher Scientific, Inc, Waltham, MA) [14]. All the data points were analyzed using the Target Activation Bio-algorithm, Cellomics, and the quantitative data was expressed as fold change in protein expression compared to control cells. Twenty fields were counted for each replicate well and the results were presented as an average±SD.

Cell Cycle Analysis by Propidium Iodide (PI) Staining

Cell cycle analysis was performed as per [15,16] method. Briefly 2×10^6/well of 6 well plate, Panc-1 cells were seeded. After overnight culture in 5% humidified CO_2 incubator, Panc-1 cells were treated with the DCM leaves extract (0.5 µg/ml, final concentration), for 24 h. After incubation, cells were scrapped in ice-cold PBS and were centrifuged at 500g for 6 min at 4°C. The cell pellet was washed with ice-cold PBS and re-suspended in 0.5ml of PI hypotonic solution (50 µg/ml PI; 0.1% sodium citrate; 0.1% triton X100). After overnight incubation at 4°C, cell cycle analysis of the cells was performed using by Fluorescence-Activated Cell Sorting (FACS) scan and the Cell Quest program (Becton Dickinson).

Statistical analysis

Data are presented as the mean±SD of the results from three independent experiments. The ANOVA test was performed using GraphPad Prism 3.03 (GraphPad Software, Inc, CA, USA). $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The leaves of *Mesua ferrea* have been reported to have anti oxidant activity, antibacterial and anticancer activity in breast cancer [17]. Although some compounds have been identified as possessing medicinal properties, none of these compounds has ever reached clinical trials.

The anticancer activity of different extracts was tested with a single test concentration of 10 µg/ml, in a panel of five different cancer cell lines, and the normal lung fibroblast cell line (WI-38). The most significant growth inhibition was observed with DCM extract in pancreatic cancer cell lines (Fig.1A). None of the extracts were toxic to normal cell line (WI-38), indicating a good toxicity window among cancerous and normal cell lines. Furthermore, we performed the dose-dependent inhibition with DCM extract on Panc-1 cells (Fig. 1B). The IC_{50} values, defined as the drug concentration at which 50% of the cells are viable, were calculated after 24 h of incubation with various concentrations of the DCM extract. The IC_{50} of the DCM extract on Panc1 cells was ~0.6 µg/ml post 24 hrs treatment whereas, DCM extract was not toxicity to the WI-38 cell line.

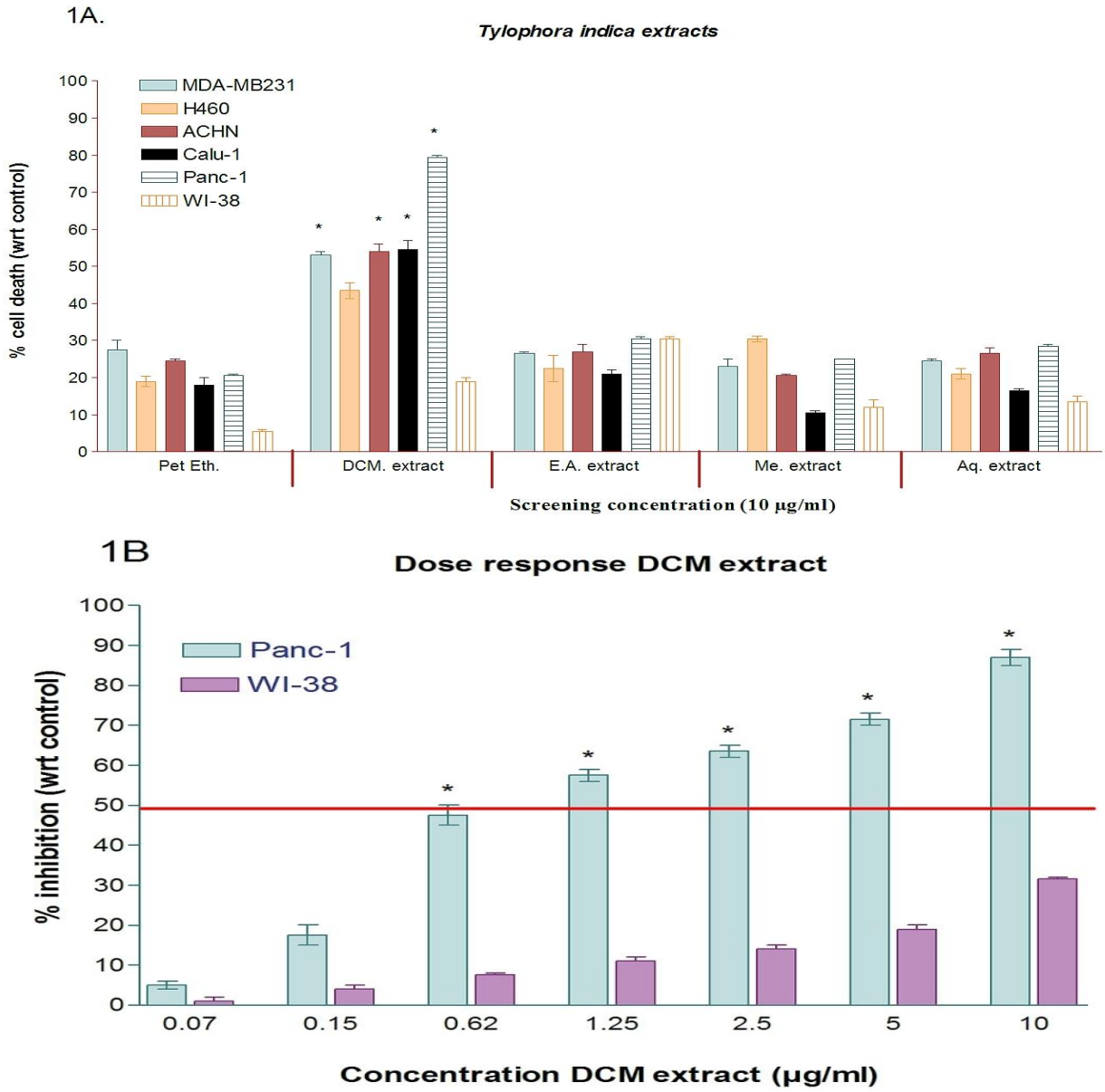
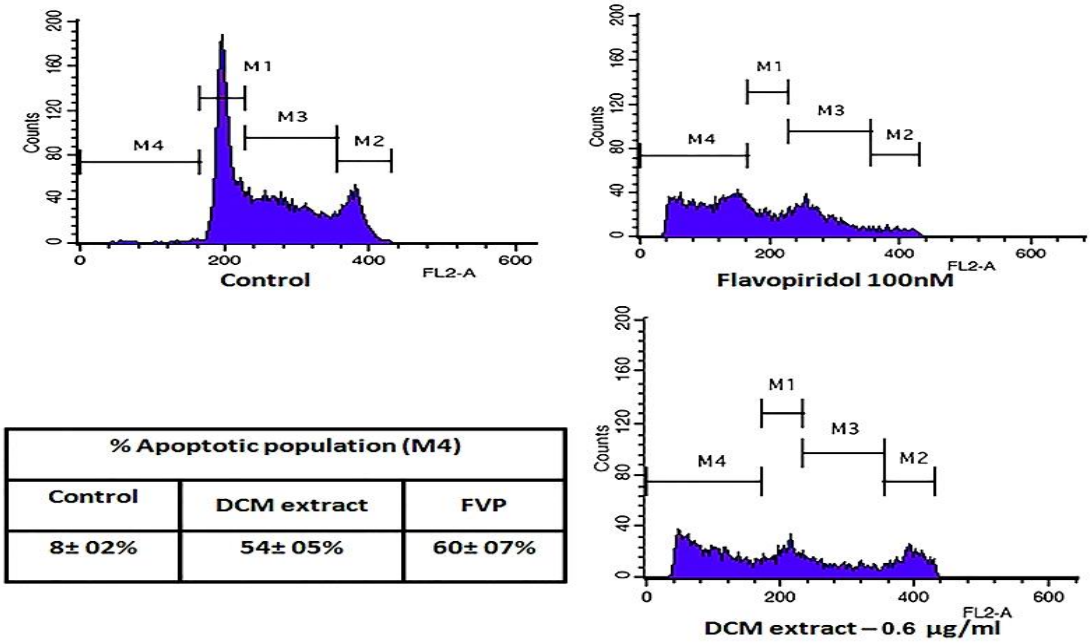


Fig. 1: DCM leaves extract of *M. ferrea* showed inhibition in cell proliferation tested at 10 µg/ml concentration. The most pronounced effect was observed in Panc-1 cells. 1B) Does response activity shows IC_{50} of DCM extract on Panc-1 around 0.6 µg/ml. Primary screening and dose response study was performed in three independent experimental setups using MTT assay. Data are presented as the mean±SD of the results from three independent experiments

In order to demonstrate the effect of DCM extract on the cell cycle, Panc-1 cells were treated with the extract (0.6 µg/ml, final concentration) for 24 hrs and analyzed by FACS. Cells treated with DCM extract showed ~54% cell arrest in the G0/G1 cell phase (M4) (Fig. 2). These results indicate that DCM extracts induce cell death by apoptosis.

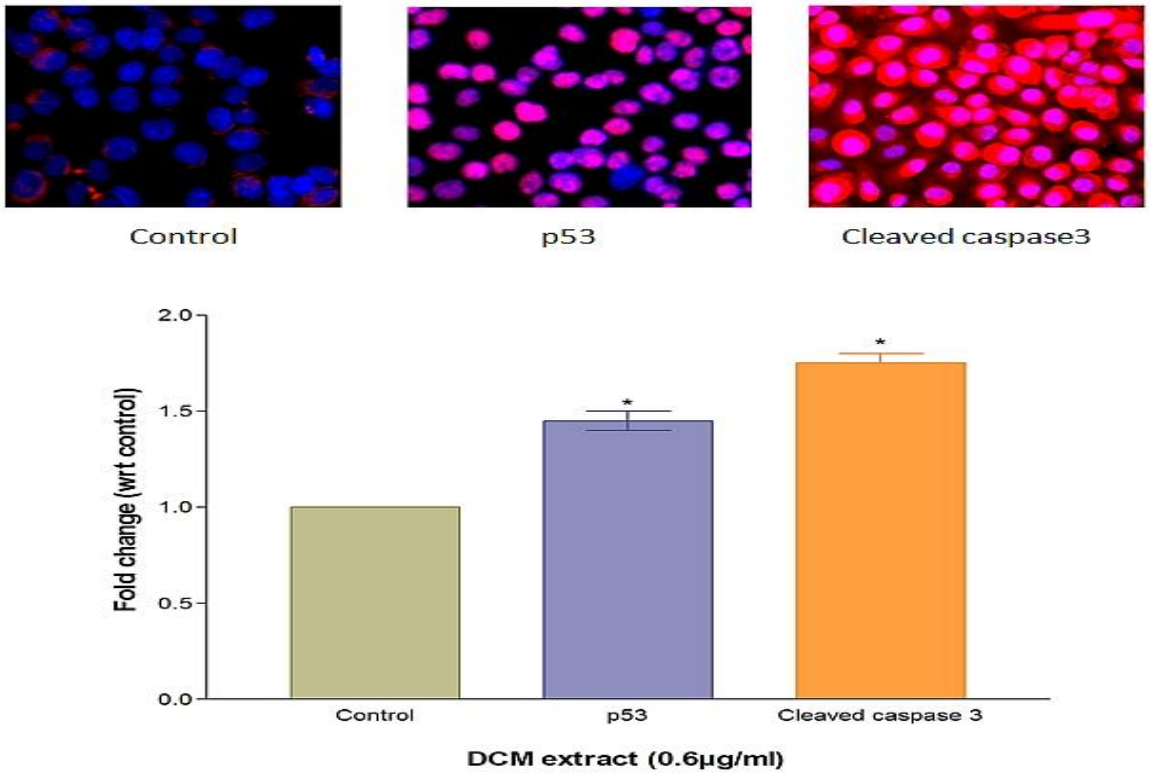
Figure.2



% Apoptotic population (M4)		
Control	DCM extract	FVP
8± 02%	54± 05%	60± 07%

Fig. 2: Effect of DCM leaves extracts on the cell cycle analysis of Panc-1 cells. These graphs illustrate the effect of leaves extract on the cell cycle. Panc-1 cells were treated with 0.6 µg/ml DCM extract for 24 hrs. The cells were stained with 50µg/ml PI, and the DNA content was analyzed by FACS analysis, as described in the materials and methods. Positive control (Flavopiridol) 100 nM showed 60% apoptotic population. Data are presented as the mean±SD of the results from three independent experiments

Figure. 3



p53 is a transcription factor that activates vital damage containment procedures to restrict aberrant cell growth in response to DNA damage [18,19]. It restricts cellular growth by inducing senescence, cell cycle arrest or apoptosis [20]. Further, we looked into the change in protein expression of p53 & cleaved caspase3 in Panc-1 cells treated with DCM extract. Panc-1 cells were treated with 0.6 µg/ml of DCM extract for 12 h and the change in protein expression was determined using Cellomics High Content Screening (HCS) array scan platform. Significant induction of p53 and cleaved caspase3 protein levels was observed with respect to control (Fig. 3). Caspase3 activation is a classical marker for induction of apoptosis [21-23].

Flow cytometric analysis of the DNA content of *M. ferrea* leaves extract-treated cells has shown that the DCM extract cause induction of the sub-G0/G1 population. Our results also showed that the total DCM extract of *M. ferrea* induce apoptosis when analyzed for p53 and caspase3 activation. A concentration-dependent increase in the percentage of dead cells was observed with increasing concentrations of extract. IC_{50} of the extract on Panc-1 cells found to be ~0.6µg/ml, however, the extract is not toxic to the normal cell line. The results from the present study clearly indicated the anticancer potential of *M. ferrea* extract on pancreatic adinocarcinoma cell line, validating its CAM (Complementary and Alternative Medicine) use. Although each extract may contain several compounds, it certainly has potent anticancer compounds which could be isolated and characterized further.

CONCLUSIONS

Five leaves extract of *M. ferrea* have been tested on different cancer cell lines and DCM extract found to be active against pancreatic adenocarcinoma cell line. However, DCM extract specifically did not show the toxicity against normal cell line, suggesting the specificity of the extract on aberrantly proliferating cell lines. Underlying mechanism needs to be investigated further and responsible active principle by which this activity was exhibited needs to be identified. Furthermore these extracts need to be screened in other cell lines also.

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