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Original article

Selective anti-proliferative activities of *Carica papaya* leaf juice extracts against prostate cancer

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ABSTRACT

Background: Prostate cancer (PCa) is the leading cause of cancer related deaths in men. *Carica papaya* is a popular tropical plant that has been traditionally used for its nutritional and medicinal properties.
Methods: We investigated the anti-proliferative responses of papaya leaf juice (LJP) and its various extracts ("biological"- in vitro digested, "physical"- size exclusion, and "chemical"-solvent extraction) on a range of cell lines representing benign hyperplasia, tumorigenic and normal cells of prostate origin.
Results: Time course analysis (by 24 h, 48 h and 72 h) of LJP (1–0.1 mg/mL) before and after in vitro digestion, and of molecular weight based fractions of LJP showed anti-proliferative responses. The medium polarity fraction of LJP (0.03–0.003 mg/mL) after 72 h exposure showed potent growth inhibitory (IC_{50} = 0.02–0.07 mg/mL) and cytotoxic activities on all prostate cells, with the exception of the normal (RWPE-1 and WPMY-1) cells. Flow cytometry analysis showed S phase cell cycle arrest and apoptosis as a possible mechanism for these activities. Medium polar fraction of LJP also inhibited migration and adhesion of metastatic PC-3 cells.
Conclusion: This is the first report suggesting selective anti-proliferative and anti-metastatic attributes of LJP extract against prostatic diseases, including PCa.

1. Introduction

Diseases of the prostate are more likely to occur in men with increasing age. Benign prostatic hyperplasia (BPH) and prostate cancer (PCa) are the most common prostatic conditions. BPH (non-cancerous tumour of the prostate) occurs in men over 40 years of age. It affects the quality of life by enhancing lower urinary tract symptoms, and may double the risk of prostate cancer (PCa) [1,2]. PCa is the second most frequently diagnosed malignancy and the fifth leading cause of cancer related death in men worldwide [3]. In the USA, a total of 220,800 new prostate cancer cases and 27,540 deaths were projected to occur by the close of 2015 [4]. PCa is most lethal when it metastasises to other parts of body (mainly bone, lung and liver) [5]. The five-year survival rate of PCa patients with distant metastasis is approximately 30% in comparison to 100% of localised PCa [6]. PCa is a slow growing disease, and initially surveillance (active surveillance and watchful waiting) strategies used to reduce complications associated with surgery and radiotherapy. Some patients that experience tumour progression are usually treated with invasive or radical procedures such as radical prostatectomy and radiotherapy [7]. Since PCa is hormone dependent in nature, androgen deprivation therapy (ADT) is the first line therapy for the metastatic stage of PCa. Within two years of treatment, however, many patients develop resistance to ADT [8]. Chemotherapy is an important tool for the treatment of cancer. The results from current trials indicated greater role of early chemotherapy to improve survival of PCa patients [9]. Although chemotherapeutics drugs alone or in combination with ADT improved PCa treatment responses [10], their general role in the generation of secondary malignancies and toxicity to cells other than diseased cells limit their use for initial therapy [11]. Therefore, therapies that have potential to inhibit proliferation and/or show cytotoxicity of tumorigenic cells selectively over healthy tissue are highly warranted.

Plants, their constituent phytochemicals and other extracts have a long history in the treatment of diseases including cancer. Phytotherapy is widely used in the treatment of BPH symptoms, and the search for selective anticancer phytochemicals with minimal

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side effects has motivated researchers to seek anticancer drugs of plant origin [12,13]. The US National Cancer Institute (NCI) commenced the pre-screening of plant extracts using major human cancer cell lines in 1990 [14]. It is noteworthy that of the 3000 plants identified by the NCI as having anti-cancer properties, 70% are of tropical origin [15]. *Carica papaya* (also known as pawpaw or papaya) is one of the most popular and widely distributed tropical plant in the family of *Caricaceae* (comprising of six genera and 43 species) [16]. It is used traditionally for its nutritional and medicinal properties [17]. Literature shows the medicinal use of papaya tree (fruit, seed and leaf) in the treatment of cardiovascular diseases, dengue fever, digestion disorders, inflammatory disorders, wound healing, malaria, hypoglycaemia, hyperlipidaemia, bacterial and fungal diseases, and as a male contraceptive [18–28]. Numerous case studies have also demonstrated its protective effects against the cancers of colorectal [29], prostate [30], cervical [31], breast [32], and gall bladder [33]. The fruit and seed extracts of papaya displayed significant cytotoxic and anti-proliferative activities over breast (MCF-7), liver (HepG2) and leukaemia (HL-60) cancer cells [34–36]. Recently, a patent has described the use of a product containing green papaya fruit and other ingredients for the treatment of various tumours (brain, colorectal, prostate and benign) [37].

Papaya leaf extract has been reported to be traditionally consumed by Australian aboriginal people for its anti-cancer activity. A patent by Morimoto et al. reported several case studies, where patients suffering from different cancers (stomach, pancreatic, lung, liver and blood) after consumption of aqueous papaya leaf extract have resulted in an increased long-term survival [38]. Cell lines represent a widely available model for the high-throughput initial screening of newer anti-cancer drugs and a number of *in vitro* cancer cell studies indicated anticancer activities (via different Mechanisms: cytotoxicity or growth inhibition or immunomodulation) of papaya leaf extracts on pancreas, colon, ovary, stomach, breast, cervix, liver, leukaemia, lymphoma, mesothelioma and oral squamous cell carcinoma (SCC25) cell lines [34,35,39–46]. Despite promising anti-cancer attributes of papaya leaf extracts, no scientific study has yet been undertaken to validate the activities of papaya leaf extracts against BPH and PCa cells. Although an evaluation of the overall toxicity, in animal model, of any promising drug candidate is required, their general toxicity at the level of normal epithelial and other cell types provides essential safety information [47]. A recent study has demonstrated the selective cytotoxic activities of lyophilised papaya leaf juice extract (LJP) on an oral squamous cell carcinoma (SCC25) in comparison to non-cancerous keratinocytes (HaCaT) [43]. However, additional studies are warranted to explore and identify bioactive compounds in LJP that are responsible for the selective anti-cancer activity by using a range of cell lines.

Given that the genetic and epigenetic constitution of different cells, even from the same tissue differ noticeably, it is important to explore the bioactivities on numerous cancer cell lines, including cells derived from normal tissue [47]. The use of a range of extraction methods and techniques ensures the release of a wide range of compounds from the plant matrix and therefore represents a valid route to identify the bioactive compounds of plants [48]. For the first time, we report the selective anti-proliferative potential of LJP extracts using a range of extraction methods: ("biological"-*in vitro* digestion, "physical"-size exclusion, and "chemical"-polarity based extraction methods), and a panel of cell lines representing non-tumorigenic (benign, BPH-1), tumorigenic (malignant, RWPE-2; neoplastic, HPR-1; cancer, PC-3, DU145 & LNCaP), and normal (epithelial, RWPE-1; & stromal fibroblast, WPMY-1) cells of prostate origin. LJP extracts with selective growth inhibitory activities were further studied to delineate the mechanism(s) underlying the anti-proliferative activities. The impacts of the medium polar fraction of LJP on various hallmark features of metastatic disease such as migration and adhesion of PCa cells were also analysed.

2. Materials and methods

2.1. Materials

Phosphate buffer saline (PBS), dimethyl sulfoxide (DMSO), paraformaldehyde, Triton X-100, propidium iodide (PI), α-amylase from human saliva, casein, porcine pepsin and RNAse A were purchased from Sigma (MO, USA). Porcine pancreatin enzymes and porcine bile extract were from Applichem GmbH (Darmstadt, Germany). $CaCl_2$ and $MgSO_4{\cdot}7H_2O$ were from Chem-supply (SA, Australia), and HCl, KH_2PO4 and $NaHCO_3$ were from Ajax Finecham (NSW, Australia). LC grade hexane and LC grade ethyl acetate were from Merck KGaA. Milli Q water (Millipore, MA, USA) was used for all experiments.

2.2. Plant material

Papaya leaf samples were collected in September 2013 from the Tropical Fruit World, Duranbah, NSW, Australia (28°17′15″ S and 153°31′347″E). The papaya plants grown in this facility were confirmed to not have been sprayed with any synthetic chemicals such as pesticides. The leaves were rinsed gently with tap water and then Milli Q water. After air-drying at room temperature, the leaf veins were removed and the remaining leaf portions were processed using mortar and a pestle. Juice resulting from this was filtered through a clean muslin cloth by a hand-pressing method, and lyophilised at 0.1 mbar, at −70 °C using Christ Alpha 2–4 LD (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The freeze drying was performed in dark conditions (chamber covered with aluminium foil) to protect any potentially light-sensitive phytochemicals. The freeze dried LJP was stored at −80 °C until further use.

2.3. LJP extraction

2.3.1. In vitro digestion of LJP

The LJP was digested as for previous methods described elsewhere [49,50]. In brief, digestion was performed in three successive steps i.e. oral, gastric and intestinal at 37 °C and 55 rpm over an IKA RCT hot plate (Selangor, Malaysia). Artificial saliva was prepared in PBS (diluted 1:5), containing 1.336 mM $CaCl_2$, 0.174 mM MgSO4, 12.8 mM KH_2PO_4, 23.8 mM NaHCO3 and food casein (2 g/L). Papaya leaf juice (10 mL) was digested in 5 mL of prepared artificial human saliva (containing 1000−1500 units/mL of α-amylase) in a 50 mL beaker. After 10 min, the gastric phase of digestion was performed at pH 2 for 1 h by adding 0.5 mL of pepsin (40 μg/L). The pH was adjusted using 0.1 N HCl using Seven Compact digital pH meter (Lab Tek, QLD, Australia). This was followed by the intestinal phase of digestion with 2.5 mL intestinal juice (below) for 2 h at pH 6.5. The pH was adjusted using 0.1 N $NaHCO_3$. Intestinal juice was prepared by adding 1.4 μg/L porcine pancreatin and 8.6 μg/L porcine bile to 0.1 N $NaHCO_3$. The resultant *in vitro* digested leaf juice extract was freeze-dried, and the powder (IVD-LJP) stored at −80 °C until further analysis. A digested control sample of (DC) was obtained using the same digestion procedure mentioned above without the addition of LJP. Sterile filtered IVD-LJP (equivalent to 1 mg/mL of LJP), and DC (equivalent to 1 mg/mL of IVD-LJP) as negative control, were dissolved in media for cell assay.

2.3.2. Molecular weight (MW) cut-off fractionation

Lyophilised LJP was solubilised in appropriate cell media (1 mg/mL), and filtered using Amicon Ultra-15 Centrifugal Filter Units

(Merck KGaA, UFC900324) at 5000 rpm for 30 min. The resulting MW cut-off fractions of above and below 3000 Da were reconstituted using media to a concentration of 1 mg/mL equivalent LJP for cell assay.

2.3.3. Polarity based fractionation

Papaya life juice (45 mL) was successively partitioned in a separating funnel using hexane and then ethyl acetate (EA), triplicate fractionations (15 mL x 3) of each to obtain a 45 mL fraction for each solvent. The two fractions were dried under vacuum at 40 °C. The remaining aqueous residue of LJP (the polar fraction) was freeze dried. All fractions were stored at −80 °C until further analysis. For cell assays, the dry hexane (nonpolar) and EA (medium polar) fractions were dissolved in DMSO resulting concentration 10 mg/mL, and the polar fraction dissolved in cell media resulting concentration 1 mg/mL.

2.4. Cell culture and conditions

Cells RWPE-1, WPMY-1, RWPE-2, HPR-1, BPH-1, PC-3, DU145 and LNCaP of human origin were obtained from ATCC. The RWPE-1, RWPE-2 and HPR-1 cells were cultured in keratinocyte-SFM media as recommended (Life technologies, NY, USA). Other cells were cultured in phenol red free RPMI1640 (1X) (Life technologies, NY, USA) media supplemented with 5% heat-inactivated foetal bovine serum and 1% penicillin-streptomycin (Sigma, MO, USA). All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in CO_2 incubator.

2.5. Cell proliferation assay

A cell proliferation assay using the CyQUANT NF cell proliferation kit (Molecular Probes, OR, USA) was performed to examine the anti-proliferative activity of LJP extracts. The cell numbers for assay were determined by seeding tests to achieve 70–80% confluence by 96 h. Briefly, RWPE-1 (5×10^3 cells/well), WPMY-1 (1.5×10^2 cells/well), RWPE-2 (3×10^3 cells/well), HPR-1 (3.5×10^2 cells/well), BPH-1 (1×10^3 cells/well), PC-3 (2×10^3 cells/well), DU145 (3×10^3 cells/well) and LNCaP (4×10^3 cells/well) cells were plated in 96-well black fluorescence micro-titre plates (Perkin Elmer, VIC, Australia). At 24 h after plating, triplicate wells were treated with the sterile filtered LJP (1-0.01 mg/mL) and equivalent concentrations of LJP extracts. At the same time, cells were treated with positive control (Paclitaxel, 100 nM), and appropriate negative controls (media only; and media containing equivalent to amounts of vehicles present in highest concentration of extract tested). As per manufacturers protocol, CyQUANT dye was added to monitor growth of cells by 24, 48 and 72-h intervals after treatment. Fluorescence was measured using a 96-well microplate reader FLUOstar Omega (BMG Labtech, Offenburg, Germany) with filters set at 480 nm excitation and 520 nm emission. The effects of LJP extracts on cell growth were calculated as fold changes in growth of extract treated cells vs untreated (or vehicle treated) groups. Log concentration (inhibitor) vs. response (% growth change) curves were plotted, and nonlinear regression analysis was performed to estimate half maximal growth inhibitory concentration (IC_{50}) values. Simultaneously efficacy of treatments was reported as percent maximal growth inhibition (I_{max}) after 24 h, 48 h and 72 h of exposure.

2.6. Cell viability assay – trypan blue dye exclusion assay

Cells were seeded in 96-wells with cell densities as mentioned above. Following treatment with the respective LJP extracts and vehicle, the supernatant solutions containing dead cells and –previously adhered live cells were collected following trypsin EDTA (Ethylenediaminetetraacetic acid) incubation. Collected dead and live cells were mixed, and centrifuged. The media was aspirated and cell pellet was resuspended in 1 mL media. Equal volumes of cell suspension and trypan blue solution (0.4% v/v) (Sigma, MO, USA) were mixed, and live/dead cells counted using a haemocytometer under light microscope (average of 10 fields).

2.7. Cell and nuclear morphology

The morphologies of treated cells were observed using light microscopy. 4′,6-Diamidino-2-phenylindole (DAPI) (Molecular probes, OR, USA) staining was performed to decide the impact of treatments on impelling morphological changes in the nucleus of cancer cells (PC-3 & LNCaP) that may be experiencing apoptosis. The PC-3 and LNCaP cells were treated with the medium polar fraction (0.01 & 0.03 mg/mL), paclitaxel and vehicle (0.3% DMSO) for 72 h in 96-well plates. After treatment, the cells were rinsed with 100 μL PBS (1X), fixed with 4% v/v formaldehyde, permeabilised using 50 μL Triton-X (10% v/v) and washed with 100 μL PBS (1X). Cell nuclei were stained with 50 nM DAPI (Molecular Probes, OR, USA) for 15 min in dark. After washing extra dye with 100 μL PBS (1X), 50 μL of PBS was added in each well and the images were acquired using an ImageXpress micro-automated epifluorescence microscope (Molecular Devices Corporation, CA, USA) at 10× magnification.

2.8. DNA content by flow cytometry analysis

Cell cycle analysis at flow cytometer was performed by propidium iodide (PI) based measurements of DNA content of the cells. The PC-3 and LNCaP cells were seeded in triplicate into 6-well plates. After 48 h treatment with medium polar fraction (0.01 mg/mL and 0.03 mg/mL) and vehicle control, cells were collected for DNA content analysis. The cells were trypsinised, and centrifuged; the cell pellets were resuspended in PBS (300 μL), and fixed in 700 μL of ice-cold absolute ethanol by overnight incubation at −20 °C. Prior to analysis, the cells were centrifuged and the ethanol aspirated. The cell pellet was resuspended in 500 μL of PBS containing RNAse A (50 μg/mL) for 30 min, and stained with PI solution (50 μg/mL) for 15 min in dark conditions. The fluorescence of PI, a measure of DNA content in a cell population, was assessed using BD Accuri C6 software (Accuri® C6 Flow Cytometer, MI, USA). A total of 10,000 gated events were acquired, and data were analysed using FlowJo software (version 7.6.5).

2.9. In vitro *scratch/wound healing assay*

Cell migration inhibitory activity was assessed using a monolayer wound scratch assay and the IncuCyte ZOOM™ live cell imaging system (Essen BioScience, MI, USA). PC-3 cells at density of 5×10^4 cells/well were seeded in 96-well ImageLock plates (Essen Bioscience, MI, USA) and incubated for 24 h. Mitomycin C (Sigma, MI, USA) at a final concentration of 10 μg/mL was added and incubated for 2 h. Scratches were made using an Essen 96-well wound maker and cells were treated with medium polarity fraction of LJP (MP-LJP) (0.003 mg/mL and 0.01 mg/mL). Plates were incubated in an Essen IncuCyte Live Cell Imaging System contained within a 36 °C incubator with a 7% CO_2 atmosphere. Images of each scratched well under phase-contrast were recorded every 2 h for 36 h, and measurements of the percent of wound closure were calculated using IncuCyte software. All treatments were performed three times in triplicate wells.

2.10. Extra cellular matrix (ECM) related adhesion assay

The effects of the medium polar fraction of LJP on the adhesion of PC-3 cells was examined in 96-well plates. Followed by coating

of wells with type I collagen (Sigma, MO, USA) and fibronectin (Sigma, MO, USA), plates were incubated for 2 h at 37 °C. The plates were washed with 50 μL PBS (two times), then 100 μL of 1% BSA (Bovine serum albumin) solution added to each well for 1 h incubation. Following washing of the plates with PBS (50 μL), serum-starved cells at a density of 2×10^4 cells/well were seeded in serum-free media. The cells were treated with range of concentrations of the MP-LJP (3–30 μg/mL) and respective control for 1 h. The total number of attached cells was estimated by measuring the fluorescence intensity of the CyQUANT reagent. The fold changes in the adhesion observed of the treated cells compared with the vehicle treated cells were calculated.

2.11. Statistical analysis

Data were expressed as mean ± standard error mean (SEM, n = 3). Statistical differences compared between multiple groups of the LJP extracts treated groups and appropriate negative controls were analysed by two-way analysis of variance (ANOVA) and followed by Bonferroni multiple comparison test. GraphPad Prism software (version 6.07) was used for all analysis.

3. Results

3.1. Anti-proliferative responses of LJP extracts

Typically, about 25 mg lyophilised LJP was collected from 1 g of fresh papaya leaf (i.e.1 mg/mL LJP ≈ 40 mg/mL papaya leaf). Anti-proliferative responses of LJP before and after treatment with digestive juices/extract was examined on various cell lines using CyQUANT assay. As shown in Fig. 1A, LJP displayed non-selective dose dependent growth inhibition of all cells tested by 24 h, 48 h and 72 h post treatment. With the highest concentration tested, RWPE-1, BPH-1 & PC-3 post 24 h exposure showed I_{max} values 71.1%, 21.8% and 12.9%, respectively. However, LNCaP cells responded slower, with I_{max} values 27.3% and 35.85% at 48 h and 72 h after treatment, respectively. LJP displayed potent anti-proliferative effects after 72 h treatment on RWPE-1, BPH-1, PC-3 and LNCaP cells with IC_{50} values of 0.22, 0.79, 0.95 and 0.96 mg/mL, respectively. Paclitaxel, an established cytotoxic drug was used as a positive control, displayed strong anti-proliferative effects on all cells tested. There were no significant differences between the anti-proliferative activities of LJP (≥0.5 mg/mL) and paclitaxel over all cells tested after 72 h exposure. Similar to LJP, *in vitro* digested LJP (IVD-LJP) also yielded significant ($p < 0.05$) decrease in growth of all the cells tested; but displayed less potency (high IC_{50} values) and less efficacy (less I_{max} values) compared to the LJP (Fig. 1B). IC_{50} values by 72 h post treatment on RWPE-1, BPH-1, PC-3 and LNCaP cells were 1.46, 1.32, 2.27 and 4.24 mg/mL, respectively.

Considering the promising growth inhibitory activities of papaya leaf, we attempted to identify functional MW cut-off fraction responsible for anti-proliferative activity. Below and above 3000 Da MW cut-off fractions of LJP displayed significant anti-proliferative activities on RWPE-1, BPH-1, PC-3 & LNCaP with comparison to untreated cells (Fig. S1). After 72 h of treatment, >3000 Da fraction displayed better growth inhibitory effect vs <3000 Da fraction over RWPE-1 (IC_{50} = 0.41 vs 1.32 mg/mL), BPH-1 (IC_{50} = 0.97 vs 1.23 mg/mL) and PC-3 (IC_{50} = 0.99 vs 1.23 mg/mL) cells, except LNCaP (IC_{50} = 1.15 vs 1.12 mg/mL) cells.

Polarity based fractionation of LJP was carried out to separate nonpolar (hexane extract), medium polar (ethyl acetate extract), and polar (remaining fraction) fractions resulting 3%, 3% and 84% dry weight yield, respectively. For extraction, and to enhance solubility of plant compounds organic solvents are required. Therefore, selection of solvents and use of proper negative controls plays a significant role in *in vitro* cell studies. To estimate growth inhibitory effects of polarity based LJP fractions, firstly responses of equivalent amount of vehicles (DMSO, DMSO-hexane and DMSO-EA) on proliferation of cells were studied. No significant differences were observed among proliferation responses of vehicle treated cells. Therefore, anti-proliferative responses of non-polar and MP-LJP fractions were analysed with comparison to DMSO (0.3%) treated cells. Following 72 h of treatment, non-polar fraction significantly inhibited the growth of BPH-1 (IC_{50} = 0.02 mg/mL), RWPE-2 (IC_{50} = 0.01 mg/mL), HPR-1 (IC_{50} = 0.01 mg/mL), PC-3 (IC_{50} = 0.02 mg/mL), DU145 (IC_{50} = 0.02 mg/mL), LNCaP (IC_{50} = 0.06 mg/mL) and WPMY-1 (IC_{50} = 0.01 mg/mL) cells; but did not show a significant growth inhibition of normal epithelial RWPE-1 cells (IC_{50} = 0.22 mg/mL) except at highest concentration tested (0.03 mg/mL, I_{max} = 13.6%) (Fig. S2).

Similar to non-polar fraction, the MP-LJP fraction yielded potent anti-proliferative activities over BPH-1, HPR-1 and PC-3 cells with

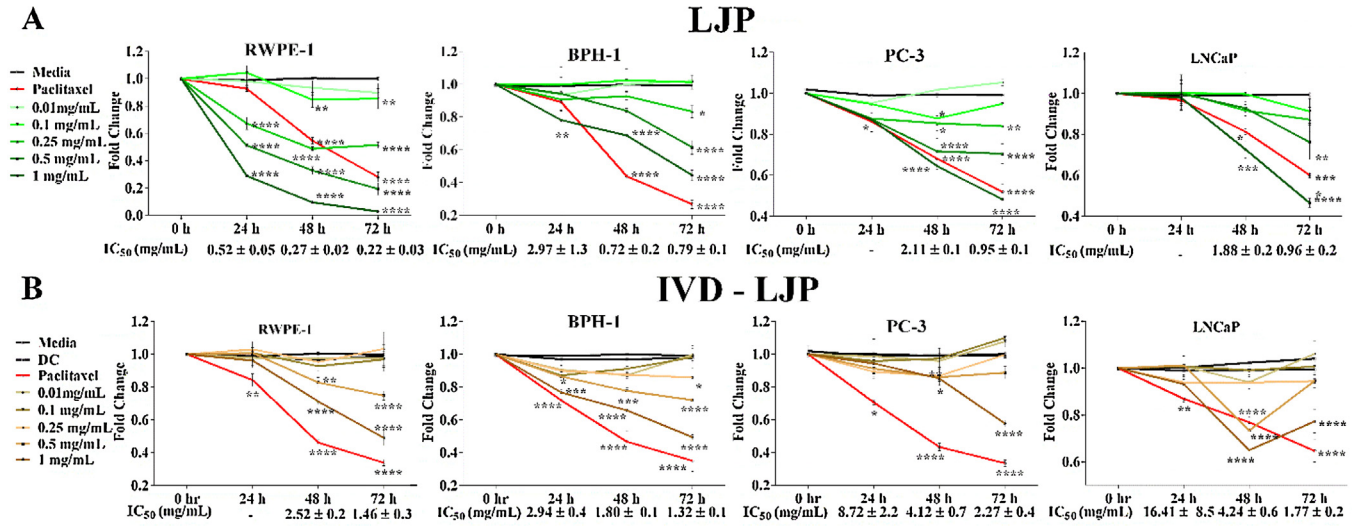

Fig. 1. Papaya leaf juice before and after *in vitro* digestion inhibits the proliferation of cells of prostate origin. Prostate epithelial (RWPE-1), benign tumour (BPH-1) and PCa (PC-3 & LNCaP) cell lines were treated with equivalent amount of LJP & IVD-LJP (0.01–1 mg/mL) for 24–72 h. Fold change in cell growth was measured by CyQUANT NF proliferation assay and presented as fluorescence intensity at 480 nm excitation and 520 nm emission. The data shown are mean ± SEM from three independent sets of three replicates. IC_{50} (half maximal inhibitory concentration) values were reported for individual exposure time. Statistical analysis was done using two way ANOVA followed by Bonferroni multiple comparison test with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$, statistically different from control cells.

IC_{50} values ≤ 0.02 mg/mL; RWPE-2 cells with IC_{50} = 0.03 mg/mL; DU145 cells with IC_{50} = 0.04 mg/mL and LNCaP cells with IC_{50} = 0.07 mg/mL cells at 72 h post treatment. Nevertheless, MP-LJP (0.0003–0.03 mg/mL) showed no significant growth inhibitory effects on both non-tumorigenic normal cells RWPE-1 and WPMY-1 (IC_{50} ≤ 0.27 mg/mL) (Fig. 2A). The remaining polar fraction of LJP after 72 h of exposure, also displayed significant ($p < 0.05$) anti-proliferative activities on RWPE-1, BPH-1, PC-3 and LNCaP cells with I_{max} and IC_{50} values of 52% & 0.76 mg/mL, 50% & 0.77 mg/mL, 50% & 0.76 mg/mL, and 41% & 0.8 mg/mL, respectively (Fig. S3).

Both non-polar and medium polar fraction were found to be the most potent, similar to paclitaxel, in inducing anti-proliferative effects against BPH & PCa cells. Further, from results on noncancerous normal cells, medium polar fraction can be considered to be a better selective anticancer agent compared to nonpolar. Most strikingly, with comparison to paclitaxel, medium polar fraction of LJP displayed similar anti-proliferative response on diseased cells, with minimal growth inhibitory effects on normal cells, making it a promising anticancer agent. Therefore, subsequent studies were focussed only on the MP-LJP fraction.

3.2. Cytotoxic activities of medium polarity LJP fraction

Trypan blue dye exclusion assay was used to check whether cytotoxicity could be a reason behind the anti-proliferative responses seen with the medium polar fraction. Following 72 h of exposure, medium polar fraction (0.003–0.03 mg/mL) was found to be safe (i.e. minimal toxicity) for both RWPE-1 and WPMY-1 cells while at concentrations 0.01 and 0.03 mg/mL, statistically significant ($p < 0.05$) cytotoxic effects were observed on PC-3 and LNCaP cells (Fig. S4). Further, after treatment, alteration in cellular morphology including loss of cell-to-cell contacts, shrinkage and cell rounding were observed (Fig. 2B). In order to analyse possibility of programmed cell death, nuclear morphology of DAPI stained cells were investigated. The MP-LJP fraction altered nuclear morphology of PC-3 and LNCaP cells in comparison to vehicle treated cells (Fig. 2C). As an indication of apoptosis, fragmented nuclei and reduced nuclear size were observed following treatment with MP-LJP fraction and paclitaxel individually.

3.3. Cell cycle arrest and apoptosis in PCa cells

To confirm mechanism behind anticancer activity, medium polar fraction treated cells were stained with propidium iodide (PI), and cell cycle was analysed by flow cytometry. Representative histograms and graph displaying cell cycle distribution data obtained for the PC-3 and LNCaP cells are shown in Fig. 2D. Medium polar fraction yielded in an increase of cells in the S phase, and a corresponding decrease of cells in the G1 and G2-M phases. The proportion of PC-3 cells in S phase increased significantly ($p < 0.0001$) from 24.5 ± 0.7% in control to 38.5 ± 0.8% in cultures treated with MP-LJP fraction (30 μg/mL). Similarly, an increase in the proportion of LNCaP cells in S phase was observed from 24.4 ± 1.9% in control to 37.5 ± 0.4% in MP-LJP fraction (0.03 mg/mL) treated cells. In addition, we observed a significant ($p < 0.001$) sub-G1 apoptotic peak with approximately 24% and 13%

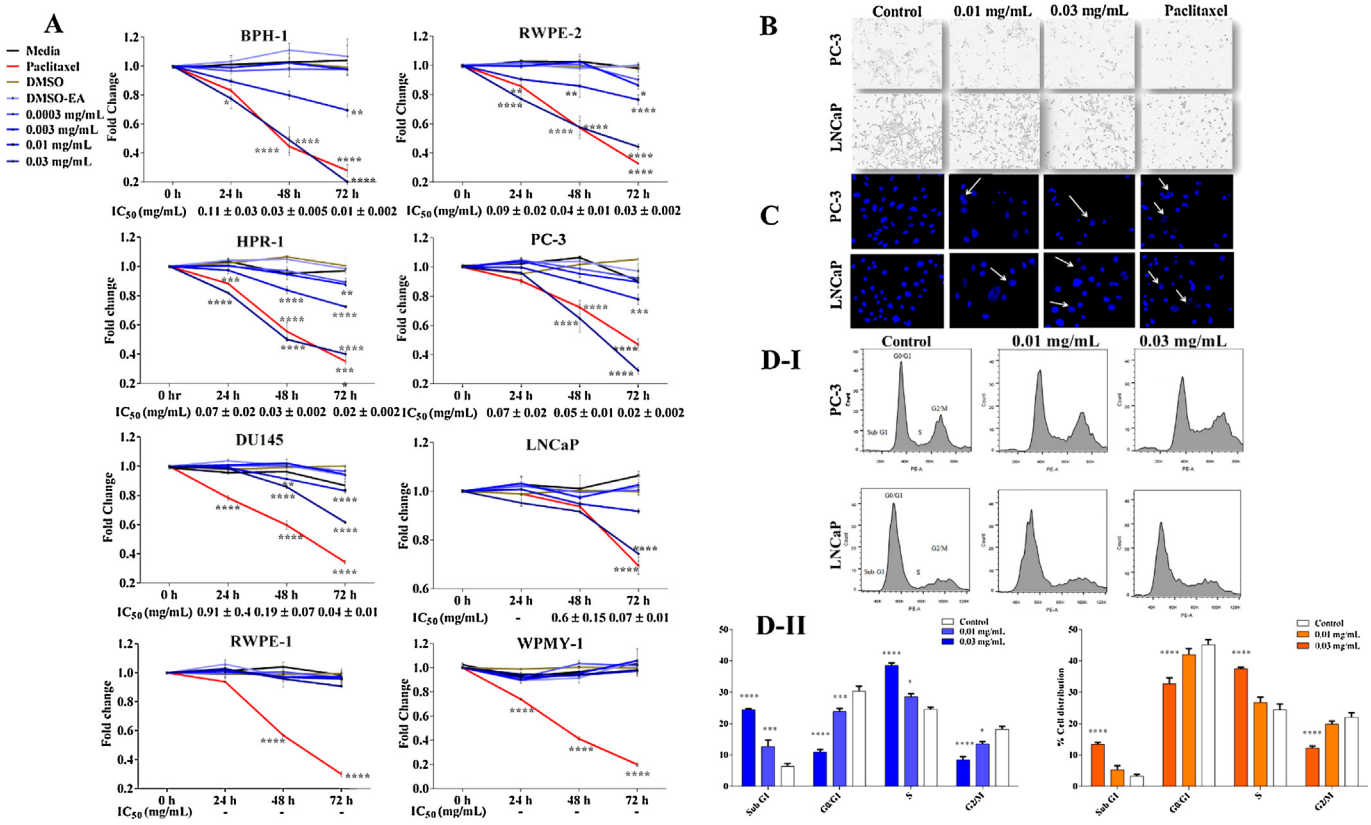

Fig. 2. Medium polarity fraction of LJP (MP-LJP) illustrated selective anti-proliferative activity and cell cycle arrest in PCa cells. **(A)** MP-LJP fraction (0.03–0.003 mg/mL) of LJP displayed selective and potent anti-proliferative effects over BPH-1, malignant (RWPE-2), neoplastic (HPR-1) and cancer (PC-3, DU145 and LNCaP) cells relative to normal epithelial (RWPE-1) and stromal fibroblast (WPMY-1) cells of prostate origin. Followed by 72 h exposure, MP-LJP fraction altered cellular **(B)**, and nuclear morphology **(C)**. MP-LJP fraction induced S phase cell cycle arrest and apoptosis in PCa cells **(D)**. Change in DNA content distribution after 48 h treatment with MP-LJP fraction of LJP (0.01 and 0.03 mg/mL) and vehicle (DMSO) illustrated as **(D-I)** flow histograms and **(D-II)** graph showing % cell distribution.

population of PC-3 and LNCaP cells post medium polar fraction (0.03 mg/mL) exposure, respectively.

3.4. Anti-metastatic effects of medium polarity fraction of LJP

In vitro scratch wound healing assay was performed to examine the effect of medium polar fraction of LJP on migration ability of metastatic prostate cancer PC-3 cell line. To avoid the effects of extract on proliferation and to delineate the effect of treatments on cell migration only, MP-LJP fraction below its IC_{50} value (0.02 mg/mL) was used following exposure of mitomycin C. The MP-LJP fraction at 0.01 mg/mL significantly suppressed migration ability of PC-3 cells compared to the cells treated with vehicle only ($p < 0.05$), whereby the wound was completely healed after 36 h (Fig. 3A).

The effect of MP-LJP fraction (0.0003 mg/mL–0.03 mg/mL) against adhesion capabilities of PC-3 cells in the presence of type-I collagen & fibronectin was studied. Fig. 3B illustrates significant ($p < 0.001$) dose dependent anti-adhesion effect of MP-LJP fraction at all concentrations tested (except 0.0003 mg/mL) in the presence of type-I collagen & fibronectin.

4. Discussion

Traditional therapy involves the use of plant extracts and/or their active principles for health benefits. The selection of the most appropriate extraction method, based on physico-chemical information, is critical to ensure maximum release of bioactive compounds [48]. Therefore, we embarked on a study of various extraction methods for papaya leaf, and the effects of these extracts on proliferation of both cancer cells and non-cancerous cells of prostate origin [51]. Uncontrolled cell proliferation plays a major role in the development and progression of BPH and PCa [52]. Therefore, to investigate the bioactivity of LJP extracts, a proliferation assay was performed at multiple time points. Quantity of cellular DNA is highly regulated and represents a proportional value to cell number. We used a CyQUANT NF proliferation assay, which is based on the measurement of cellular DNA content via fluorescent dye binding.

The first extraction process used was that of cold juicing (using mortar and pestle), a method that has already been reported to release bioactive compounds possessing strong cytotoxic effects with comparison to its aqueous and ethanol extracts [42,43]. Results from proliferation assay indicated that crude LJP (0.25–1 mg/mL) has anti-proliferative property against benign, cancer and normal cells of prostate. *In vitro* testing of plant extracts and plant bioactives over various cell lines have contributed to the discovery of new therapeutic agents (anticancer, antioxidant, anti-inflammatory and immunomodulatory, *etc.*) [53–55]. However, if plant or its extract is administered orally then the *in vivo* effects may not necessarily reflect the observed *in vitro* effects. Biological extraction of plant extract by passing through different stages of human digestion is proposed to be an efficient way of providing results that are closer to the *in vivo* situation [56]. Considering reports where papaya leaf (including root and fruit) is prescribed by natural healers of Cameroon for the treatment of prostate ailments [57,58], LJP was digested *in vitro* to predict its systemic response against proliferation of prostatic diseased cells. We passed LJP through key steps that may be responsible for physiochemical changes during human digestion [59]. However, our extraction did not include some components of human digestion such as mastication, transit time, intestinal metabolic

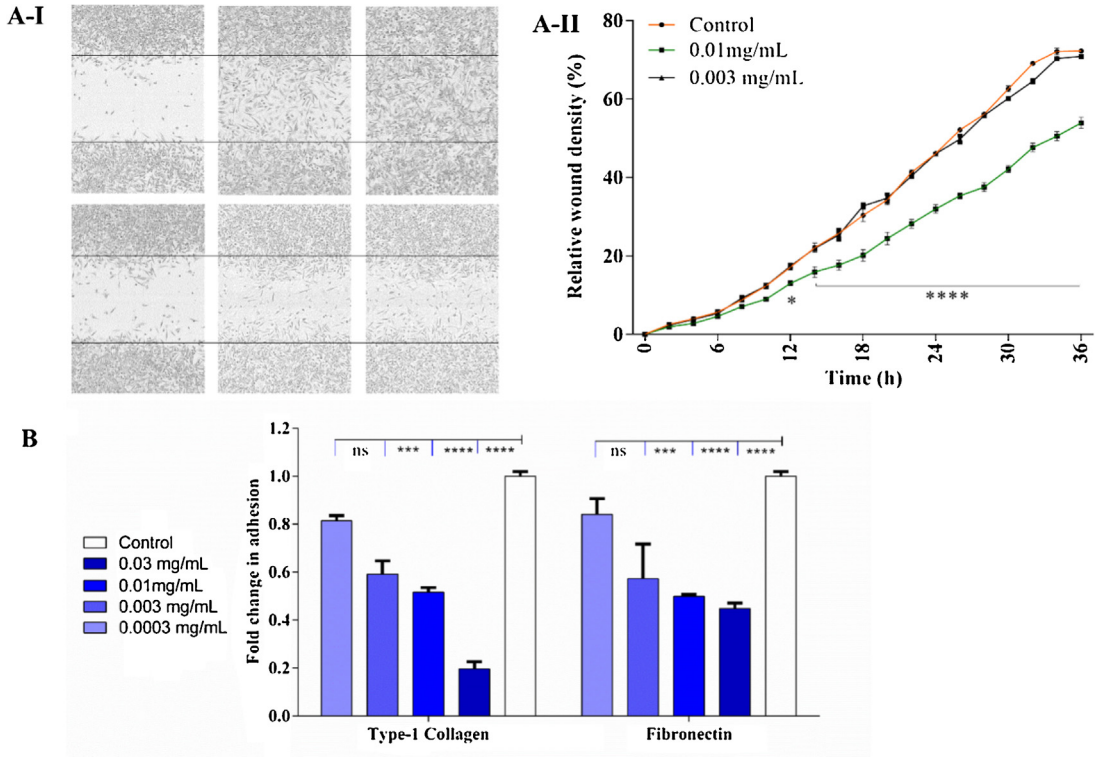

Fig. 3. MP-LJP fraction of LJP has property to inhibit migration and adhesion of PC-3 cells. *In vitro* scratch wound assay was done to investigate effect of MP-LJP fraction (0.003 & 0.01 mg/mL) on migration of PC-3 cells. Scratch in PC-3 cells was made using 96 well wound maker, and images were taken at every 2 h for 36 h with or without treatment using IncuCyte Live Cell Imaging, Scale bar = 20 μm (**A-I**), and quantification of relative wound density (%) was observed and graph was plotted (**A-II**). (**B**) *in vitro* adhesion assay was done after plating PC-3 cells on two different ECM (Collagen-1 and fibronectin) in presence of MP-LJP fraction (0.0003–0.03 mg/mL) for 1 h. Number of adhered cells obtained by 1 h incubation with CyQUANT dye. The values are expressed as fold change with respect to untreated cultures, mean ± SEM (n = 3).

enzymes, absorption and microflora. After digestion, anti-proliferative response of LJP was retained, but with comparison to crude LJP, reduction in potency and efficacy was observed, that may be due to chemical changes in bioactive phytochemicals during the digestion process [60]. IVD-LJP displayed toxic effects on normal cells. While, various previous studies indicated oral safety of LJP with animal model as well as in dengue fever patients [19,61–66]. Our study points towards further investigation on extended utilization of oral LJP to endorse its therapeutic criticalness.

Otsuki et al. examined anti-proliferative responses of brewed aqueous papaya leaf extract [67]. They found fractions of MW <3000 Da, and further <1000 Da were most active in inhibiting tumour cells growth and in stimulating anti-tumour effects. We investigated response of MW based fractions of LJP. Our results suggest LJP compounds responsible for growth inhibitory activities were divided between < & > 3000 Da MW fractions of LJP. Given that the brewing process used in Otsuki et al. study alters papaya leaf compound profile [43], that would be the main factor behind the contrasting results between this study and ours (using cold juicing).

To release a comprehensive range of phytochemicals from plant matrix, sequential fractionation of plant materials using a gradient of polarities is necessary [48]. Anti-proliferative responses of nonpolar, medium polar and polar fractions of LJP were investigated. According to US National Cancer Institute, the plant extract having IC_{50} values <20 μg/mL on cancer cell lines are considered promising anticancer agents [68]. However, for selective anticancer therapy, anticancer agents are expected to have minimum effects on normal healthy tissue. Considering importance of epithelial and stromal cells of prostate in growth and development of normal prostate [69], anti-proliferative effect of polarity based LJP fractions were investigated using diseased cells in parallel to normal cells of prostate. Among fractions, the MP-LJP fraction displayed promising anti-neoplastic effect by reducing proliferation and viability of BPH and PCa cells in a time- and dose-dependent manner, whereby showing minimal effects on normal cells. Among possible reasons, induction of DNA damage (resulting cell cycle arrest) and programmed cell death is the main way for anti-proliferative and cytotoxic activity of plant derived anticancer compounds [70]. Other studies showed that the anti-proliferative and cytotoxic effect of papaya leaf extract (aqueous and protein fraction) is due to induction of apoptosis (via activation of caspase-3/7 and p53-dependent mitochondrial pathway) [39,67]. For the first time, our data revealed S phase cell cycle arrest that is accompanied by apoptosis as a mechanism behind anticancer activity of MP-LJP fraction. These findings along with nuclear fragmentation of DAPI stained PCa cells and alteration in cellular morphology (illustrated in Fig. 2B and 2C) support cytotoxic and apoptosis inducing property of MP-LJP fraction of LJP.

In advanced stage of PCa, cells from primary tumour have tendency to migrate, settle and attach (via various physiological alterations involved in tissue-specific ECM) to other part of body, especially bone of hip, spine or pelvis region. Major treatment strategy during metastatic PCa is to inhibit the migration and adhesion abilities of cancer cells. Although compounds found in papaya leaf (that are not restricted to papaya only) have been shown to reduce hallmarks of metastatic cancer (migration, adhesion and invasion) [71], no study reported the beneficial role of papaya leaf extract against metastatic cancer. In addition to its anti-proliferative and cytotoxic effects, MP-LJP fraction of LJP appeared to be a potent inhibitor of metastasis at non-toxic concentration (Fig. 3A). It has been postulated that extra cellular matrix (ECM) act as a chemoattractants for migration and adhesion of PC-3 cells [72]. Given that the bone matrix is composed of 95% of type- I collagen and 5% of remaining non-collagen proteins (such as fibronectin, osteocalcin *etc.*) [73], effect of MP-LJP fraction on adhesion property of PC-3 cells in presence of type- I collagen and fibronectin was examined. Our data revealed significant anti-adhesion effect of MP-LJP fraction. This is the first report indicating direct beneficial role of papaya leaf extract tested against migration and adhesion of PCa cell. However, thorough investigation is required to explore papaya leaf extracts (including medium polar fraction) and its compound(s) for its anti-metastatic potential against different cancers.

5. Conclusions

For the first time LJP and its various extracts were screened for selective and potent anti-proliferative activity over panel of cells (benign, malignant, neoplastic & cancer) with comparison to normal cells of prostate origin. By the *in vitro* CyQUANT NF proliferation assay, LJP and its size exclusion fractions (<and> 3 kD MW) displayed dose and time dependent inhibition in proliferation of all cells tested. This activity even exists after biological extraction of LJP. Among polarity-based fractions, MP-LJP fraction showed the best selectivity and potent growth inhibitory activity against all cells tested, except normal cells. It also displayed selective cytotoxic property, which was evident from alteration in cellular and nuclear morphology. Flow cytometry data indicated cell cycle arrest of S phase and apoptosis, which could be the main reason for anti-proliferative and cytotoxic property of MP-LJP fraction. MP-LJP fraction also yielded significant inhibitory effects over migration and adhesion attributes of PC-3 cells. In summary, medium polar fraction of LJP showed broad-spectrum efficacy (comparable to paclitaxel) and selective anti-proliferative activity against cells representing various phases of prostatic diseases, including PCa. These anticancer benefits of LJP fraction could have a valuable role in amelioration of patient's suffering from non-tumorigenic (BPH) and tumourigenic (PCa) lesions of the prostate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2017.02.050 .

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