

Original Research Article

Cytotoxicity and Apoptotic Activity of *Ficus pseudopalma* Blanco Leaf Extracts Against Human Prostate Cancer Cell Lines

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Abstract

Purpose: To investigate the cytotoxic and apoptotic activities of *Ficus pseudopalma* (FP) Blanco leaf extracts against normal human FSE cells and human prostate PRST2 cancer cell line.

Methods: FP leaves were extracted with 95 % ethanol, and partitioned with chloroform, ethylacetate, and water. The presence of terpenoid lupeol and flavonoid quercetin was determined through high performance liquid chromatography (HPLC). The cytotoxic and apoptotic effects of different concentrations of FP extracts on PRST2 cells and on non-cancerous human foreskin surface epithelial (hFSE) cells were determined by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay, trypan blue exclusion assay, Live/Dead viability assay and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay.

Results: Ethylacetate, chloroform, and crude ethanol extracts were significantly cytotoxic to PRST2 cell lines ($p < 0.001$) in a concentration-dependent manner with a 50 % cell growth inhibitory concentration of 4.16, 4.83 and 44.53 $\mu\text{g/mL}$, respectively. A significant decrease in the viability of PRST2-treated cells ($p < 0.001$) in a concentration-dependent manner was observed in the trypan blue exclusion assay and in the Live/Dead® viability assay. Using APO-BrdU TUNEL assay, apoptotic activities of the extracts increased in a concentration-dependent manner. All the extracts did not show significant cytotoxic effect on hFSE cells ($p = 0.064$).

Conclusion: The cytotoxic and apoptotic activities of FP extracts may be due to the presence of lupeol and quercetin. This study suggests that apoptotic mechanisms may be involved in the growth inhibitory activity of FP leaf extracts against human PRST 2 cell lines.

Keywords: *Ficus pseudopalma*, Cytotoxicity, Apoptotic, human prostate PRST2 cancer cell, Lupeol, Quercetin.

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INTRODUCTION

Cancer is the second leading cause of mortality in the Philippines [1]. Next to lung carcinomas, prostate cancer is the most common malignancy in Filipino men with an estimated occurrence of 19 out of every 100,000 Filipinos [2]. Like other cancer types, the pathogenesis of carcinomas involves the deregulation of multiple signaling

pathways. Chemotherapy of carcinomas often involves the use of synthetic and/or natural products that control deregulated cell signaling pathways. While there are several plant-derived drugs in clinical use for the treatment of cancer, the discovery of new chemotherapeutic drugs that may regulate multiple cellular signaling pathways in a highly specific and sensitive

manner to control cancer cell growth, proliferation and apoptosis, is still needed.

Ficus pseudopalma (FP) Blanco (Moraceae) is an endemic medicinal and ornamental tree found throughout the Philippines. The use of fig leaves for the treatment of kidney stones and diabetes in folkloric medicine [3] has been recently validated in a study where the anti-urolithiatic and antioxidant properties of the leaves of the plant were demonstrated by promoting free radical scavenging activity, inhibition of lipid peroxidation, and reduction of calcium oxalate crystal deposit in renal tissues [4]. Phytochemical screening of air-dried leaves of FP revealed the presence of ursenone and lupeol [5].

Ursenic acid has cytotoxic activities against cancer cell lines [6]. It has been postulated that lupeol may be a potential agent against prostate cancer through growth inhibition of tumors, cell cycle arrest, and induction of apoptosis [7].

Bioassay studies on the chemopreventive properties of FP has been limited to the cytotoxic effect of its ethanol extract on HepG2 [8]. In this study, we investigated the cytotoxic, antiproliferative, and apoptotic activities of FP extracts.

EXPERIMENTAL

Herbal material and extraction

The mature leaves of *F. pseudopalma* Blanco were collected on May 22, 2012 from Umali Subd., Los Baños, Laguna, Philippines. The plant was authenticated by Mr. Noe Gapas of the Philippine National Museum, Manila, Philippines. Voucher specimen no. 2013.001.001 was kept in the AdDU Herbarium, Ateneo de Davao University for future reference. The leaves were air-dried at room temperature, pulverized using Wiley Mill grinder, and sieved in a 22 mm wire mesh. Approximately 2.5 kilograms of air-dried leaves were completely submerged and extracted twice with 12.5 L of 95 % ethanol (technical grade) at room temperature for 48 hours and evaporated to dryness by reduced-pressure at 40 °C in a rotary evaporator (Heidolph WB 2000) to yield the concentrated crude ethanolic (CE) extract. Fifty grams of CE extract was dissolved in 500 mL distilled water and successively partitioned to exhaustion with chloroform (CF) and ethyl acetate (EF). The remaining water soluble fraction was designated as WF. The resulting fractions were evaporated to dryness by reduced-pressure evaporation at 40 °C in a rotary evaporator (Heidolph WB

2000). The extracts were stored in tightly-sealed amber tubes at 0 – 5 °C [9].

Salkowski's test for terpenoid

To their assigned test tubes, 1 mL of the CE, CF, EF, and WF was added with 1 mL of chloroform. Then, about 1 mL of concentrated sulphuric acid was introduced gently on the test tube wall. A reddish brown coloration of the interface indicates the presence of terpenoid. Lupeol was used for positive control [10].

High performance liquid chromatography (HPLC)

The fractions which confirmed the presence of terpenoids were further screened for the presence of lupeol by HPLC (Agilent 1200 Series, Quaternary HPLC System). One mg of the compound was diluted with 5 mL methanol, and 10 µL of test solution was injected to a C18 column (150 x 4.6, 5 µm) as stationary phase with a mobile phase comprising methanol : acetonitrile (30:70 v/v). The sample was run at a flow rate of 1.0 mL/min and UV detection at 210nm with a run time of 12.0 min [11]. The fractions were also screened for the presence of quercetin by HPLC. One mg of the compound was diluted with 5 mL methanol. For quercetin, 10 µL of test solution was injected to a C18 column (150 x 4.6, 5 µm). The mobile phase was methanol : acetonitrile : water (60:20:20 v/v/v). Flow rate was 1.1 mL / min and UV detection at 262 nm. [12]. A standard curve of the different concentrations of lupeol and quercetin at 5, 25, 50 and 100 ppm were prepared. The concentration of lupeol and quercetin in the fractions were determined by linear regression.

Cell culture

The strain of human prostate PRST2 cancer cell lines was kindly provided by Dr. Samuel Bernal (GlobeTek Professionals, Incorporated). Non-cancerous (healthy) human foreskin surface epithelial cells hFSE were also used in the study. All cells were routinely maintained at 37 °C in a 5 % CO₂-enriched humidified air atmosphere as monolayer cultures in 25 cm² plastic Corning flasks (T-25) in RPMI-1640 (Gibco, USA) containing 10 % fetal bovine serum (FBS). Confluent were harvested with 0.05% trypsin-EDTA (GIBCO, USA). The trypsin was neutralized by addition of RPMI-1640 supplemented with 10 % FBS (1:1). One mL of this suspension was diluted in 4 mL of RPMI-1640 with 10 % FBS into new flasks every five days.

Cytotoxicity assay

Serum-free RPMI-1640 (GIBCO, USA) was used to dilute the human cell lines to a concentration of 5×10^6 cells mL⁻¹. From this cell suspension, 100 μ L was pipetted into designated wells of the 96-well microtiter plate, except for the three wells that contained only the culture medium and served as the blank control group. The plate was incubated for 24 h in a 5% CO₂ incubator at 37°C. After incubation, the spent culture medium was discarded and 100 μ L of serum-free culture medium containing the plant extracts at different concentrations ranging from 25 to 500 μ g/mL were distributed into designated wells in triplicates. Paclitaxel (10 μ g/mL, Sigma, USA) was used as positive control. The plate was incubated in a 5 % CO₂ incubator at 37°C for 48 h. Then, 20 μ L of MTT reagent was added into each well. This plate was further incubated for 4 h in CO₂ incubator at 37 °C until dye was visible. Subsequently, the supernatant was discarded and 100 μ L of DMSO was added unto each well. The absorbance was read with the Microplate ELISA reader (Corona Microplate Reader SH-1000, Hitachi) at 540 nm. Growth inhibition (GI, %) was computed as in Eq 1.

$$GI (\%) = 1 - \{(As - Ab)/(Ac - Ab)\}100 \dots\dots (1)$$

where As, Ab and Ac are the absorbance of sample, blank and negative control, respectively. The medium lethal concentration (IC₅₀) for each extract was determined by interpolation of the graph on mortality percentages versus the concentration of the extracts through linear regression analysis. This was performed in triplicate with three independent experiments.

Trypan blue exclusion assay

PRST2 cells grown to 80 % confluence on 12-well plates were treated with different concentrations of CE, CF, EF, and WF extracts for 48 hours. Treated cells were trypsinized, pelleted, and resuspended in PBS. The viability of cells was determined by staining with an equal volume of 0.08 % trypan blue (Invitrogen). This preparation was transferred on to the edge of the hemocytometer until suspension spreads through capillary action. The cells were counted within the 1 mm area of the hemocytometer under the low power objective of compound microscope. Cells stained blue were counted as dead cells, while unstained cells were counted as viable cells. Percent cell viability was calculated by dividing the total viable cells with total cells (viable and dead cells), and then multiplying this result with 100.

Live/dead viability assay

Briefly, PRST2 cells grown to 80% confluence on 6 mm coverslips, were treated with different concentrations of CE and CF extracts for 48 hours, followed by incubation with 40 μ L of combined LIVE/DEAD® assay reagents (Molecular Probes, Invitrogen) for 30 - 45 min at room temperature protected from light. The cells exposed to UV for 30 min served as positive control. Fluorescence images were captured with Delta Vision System and analyzed using softwoRX software.

Apoptosis assay

PRST2 cells grown to 80 % confluence on 12-well plates were treated with different concentrations of CE, CF, EF and WF extracts for 48 h. The treated cells were trypsinized, pelleted, and re-suspended in 500 μ L of PBS. The cells were fixed with 1 mL of 4 %w/v paraformaldehyde in PBS on ice for 15 min. Cells were transferred into a centrifuge tube and centrifuged for 5 min at 300 x g. The supernatant was discarded by aspiration. Cells were washed twice in 5 mL PBS, and were pelleted through centrifugation. Cells were re-suspended in 500 μ L of PBS and 2 mL of ice-cold 70% ethanol and kept in a -20 °C freezer overnight. After fixation, the cells were washed and incubated in 50 μ L DNA-labeling solution for 60 min at 37 °C. The cells were then rinsed with 1 mL of rinse buffer and centrifuged at 300 x g. The supernatant was removed by aspiration. The cell pellet was resuspended in 100 μ L of Alexa Fluor 488-conjugated anti-BrdU antibody solution for 30 min at room temperature in the dark. Few drops of cells were deposited on a glass slide and counterstained with propidium iodide for another 30 min in the dark. The cells were observed using a fluorescence microscope.

Statistical analysis

Results of the study were based on three independent experiments that were performed in triplicate. Data was expressed as mean \pm standard error of mean (SEM). Four-parameter logistic regression analysis was used to determine the medium Inhibitory Concentration (IC₅₀) of the extracts. The data were analyzed using two-way ANOVA followed by Tukey post-hoc test for multiple comparisons. *P* < 0.05 was set to be the limit of significance. Statistical analyses were carried out using GraphPad Prism, version 5.04.

RESULTS

FP extracts contain triterpenoids and quercetin

From the 2.5 kilograms of dried mature FP leaves used during extraction, the yield CE was 3.48 %w/w. The percentage yields of the fractionated extracts were 37.55, CF, 3.89 EF, and 39.64 % WF.

The presence of terpenoids were detected through Salkowsky's test in CE, CF, and EF. Lupeol and quercetin were quantitatively determined by HPLC method whereby good linearity was achieved in the range 25-150 ppm. The regression equations and correlation coefficient for the reference were $Y = 10.35 * X + 458.8$, $r^2 = 0.9916$ for lupeol and $Y = 87.72 * X + 476.8$, $r^2 = 0.9898$ for quercetin. The concentration of lupeol in CE, CF, and EF were found to be 1591.74 ppm, 1170.76 ppm and 5237.26 ppm, respectively. Furthermore, HPLC chromatogram revealed CE and CF to contain peak nearest to the standard quercetin with a concentration of 355.01 ppm and 457.50 ppm, respectively (Figure 1).

FP extracts induced changes in PRST2 cell ultrastructure

Microscopic analysis of control untreated PRST2 cells showed regular polygonal cells with short extensions and few rounded cells. In contrast, cells treated with CE and CF showed changes such as rounding of cells, loss of cell adhesion, sporadic distribution and decrease in cell density in a concentration-dependent manner.

FP extracts inhibited proliferation of PRST2 cells but not in hFSE

MTT cytotoxicity results showed that chloroform (CF) and ethylacetate (EF) fractions have the highest inhibitory activities followed by crude ethanol (CE) extract in a concentration-dependent manner (Fig 2A). On the other hand, water fraction (WF) exhibited low inhibitory activities against PRST2 cells (Fig 2A). The median inhibitory concentration (IC_{50}) values were estimated to be 4.16 μ g/mL, 4.83 μ g/mL, 44.53 μ g/mL and 708.1 μ g/mL for EF, CF, CE and WF, respectively. Tukey's HSD post hoc analysis indicated that there is no significant difference in the growth inhibitory activities of EF and CF ($p = 0.691$) against PRST2 cells but both have significantly greater inhibitory activities compared to CE ($p = 0.038$) and WF ($p < 0.001$). There is also a significant difference in the mean

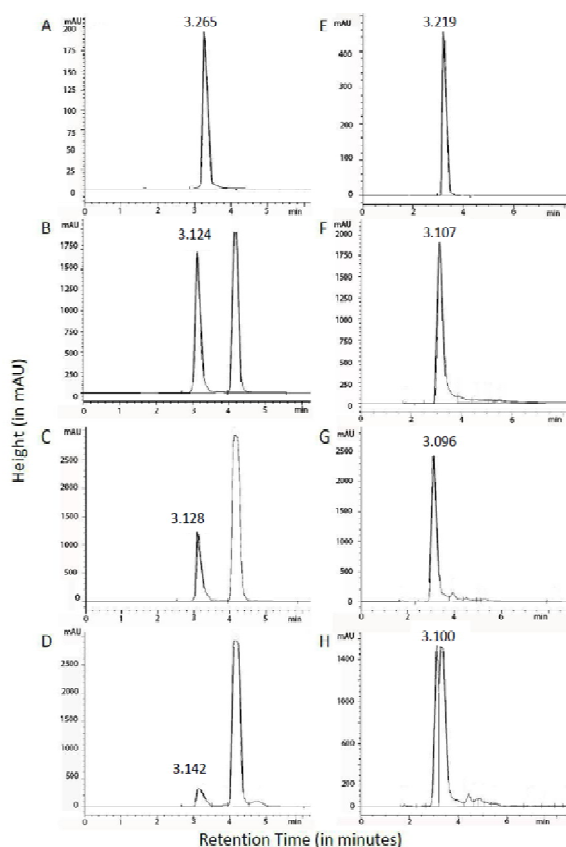


Figure 1: HPLC chromatograms of FP extracts and standards lupeol and quercetin. Ten μ L of extracts CE, CF, and EF as well as standards lupeol and quercetin were injected to a C18 column as standard phase. Mobile phase consist of methanol:acetonitrile (30:70 v/v) and methanol:acetonitrile:water (60:20:20 v/v/v) for lupeol and quercetin, respectively. UV detection at 210 nm and 262 nm for lupeol and quercetin, respectively. (A-D). Chromatograms for detecting the presence of lupeol. (A) Chromatogram of standard lupeol, (B) CE, (C) CF, and (D) EF. (E-H) Chromatograms for quercetin. (E) Standard quercetin, (F) CE, (G) CF, and (H) EF. Results revealed the ethylacetate fraction to contain the peak nearest to the standard lupeol, while CE had the peak nearest to the standard quercetin. HPLC= high performance liquid chromatography, CE = crude ethanol extract, CF=chloroform fraction, and EF= ethylacetate fraction.

percentage inhibition of the seven concentrations ($p < 0.001$), in which Tukey's HSD post hoc analysis indicated that the 25 μ g/mL has significantly the least mean percentage inhibition. The 50 and 100 μ g/mL do not differ ($p = 0.770$) but has significantly higher mean percentage inhibition as compared to the 25 μ g/mL ($p < 0.001$). The 200, 300, 400 and 500 do not differ ($p = 0.436$) but are significantly higher as compared to the other concentrations (all p -values < 0.05). However, the PaclitaxelTM positive control has the highest mean percentage inhibition ($p < 0.05$) as compared to the other concentrations (Figure 2A). The four extracts have no significant inhibitory activity against non-

cancerous normal hFSE cells ($p = 0.064$) (Figure 2B).

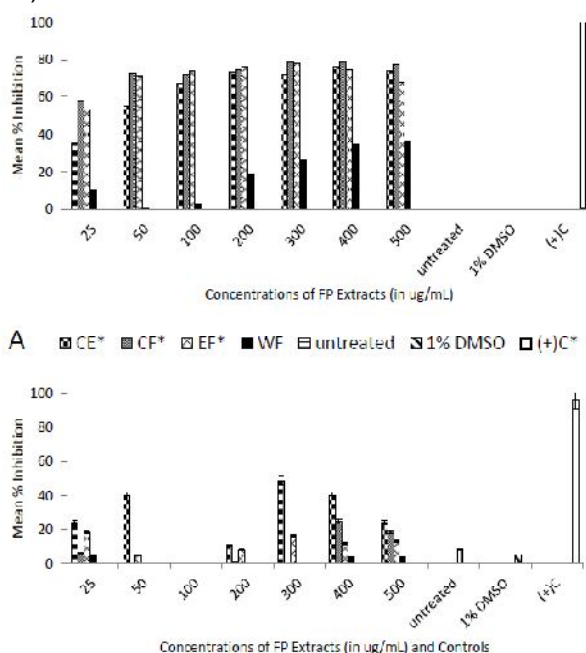


Figure 2: Growth inhibition of FP extracts on PRST2 cells and normal hFSE cells. Cells were seeded at 5×10^6 cells/well and were treated with different concentrations of FP extracts, and growth inhibition (%) was determined by MTT assay 48 h post-treatment. Results are mean values \pm SEM of three independent experiments performed in triplicate with $p < 0.05$. (A) Growth inhibition of FP on PRST2 cells. Data show a concentration-dependent inhibition was observed at concentrations ranging from 25 to 500 $\mu\text{g/mL}$ for PRST2-treated cells. The effects of CE, CF, and EF are significantly different from the untreated control and 1% DMSO control, but is not significantly different from the positive Paclitaxel control. (B) Growth inhibition of FP on normal human Foreskin surface epithelial cells. Results did not show any significant effect of FP extracts on the cell viability of hFSE. CE = crude ethanol extract, CF=chloroform fraction, EF= ethylacetate fraction, WF= water fraction, and (+)C=positive control.

FP extracts decreased the viability of PRST2 cells

There was a significant difference in the mean percentage cell viability of the four extracts ($p < 0.001$), in which Tukey's HSD post hoc analysis indicated that CF exerted the least mean percentage viability, while the WF did not differ significantly with 1% DMSO control and negative control ($p = 0.247$). Furthermore, the positive control did not differ ($p = 0.555$) with CE, CF, and EF. The effect of CE, CF, and EF extracts exerted a concentration dependent decrease in the viability of PRST2 cells (Figure 3).

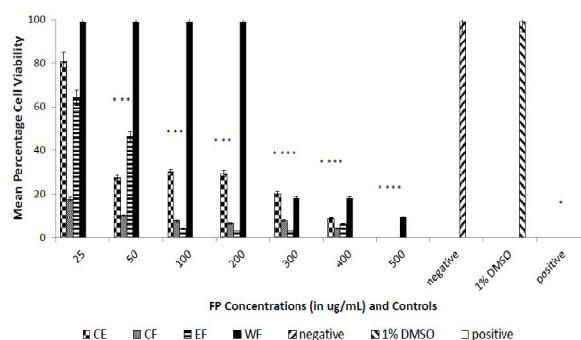


Figure 3: Viability (%) of PRST2 cells 48 h post-treatment with different concentrations of *Ficus pseudopalma* extracts. Cells were subjected to the Trypan blue exclusion assay. Note that water fraction and 1% DMSO do not differ significantly with the untreated PRST2 control ($p = 0.247$). Beginning with 50 $\mu\text{g/mL}$, CE, CF, and EF significantly decreased the viability of PRST2 cells compared to untreated control ($*p < 0.05$), while WF is significantly different beginning at 300 $\mu\text{g/mL}$. However, the positive control do not differ significantly with extracts CE, CF, and EF at the concentrations ranging from 50 to 500 $\mu\text{g/mL}$ ($p = 0.247$). CE = crude ethanol extract, CF = chloroform fraction, EF = ethylacetate fraction, and WF = water fraction.

The effects of CE and CF at 100 $\mu\text{g/mL}$ and 300 $\mu\text{g/mL}$ on cell viability were further confirmed by two-color fluorescence LIVE/DEAD® Assay that measures the intracellular esterase activity and plasma membrane integrity of cells. The green fluorescence intensity observed in PRST2 cells were lower in the cells treated with 300 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ of CE and CF compared to untreated controls. In contrast, the red fluorescence intensity observed in PRST2 cells were higher in the cells treated with 300 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ of CE and CF compared to untreated controls (Figure 4).

FP extracts induced apoptosis in PRST2 cells

The apoptotic activity of FP extracts on PRST2 cells are shown in Figure 5. There was an increase in the number of apoptotic-labeled cells as the concentration of the extract increased.

DISCUSSION

The chemo-preventive activity of *Ficus* species against renal carcinogenesis, human glioma and human hepatocellular carcinoma, HeLa cells, breast cancer cells, human nasopharyngeal carcinoma, oral epidermoid carcinoma KB and colorectal HT29 cancer cell lines have been reported [6,14-17]. However, the study on the chemo-preventive property of the endemic Philippine tree *Ficus pseudopalma* (FP) has been limited to its effect on hepatocellular carcinoma HepG2 cell [8]. Results from this

study confirms that the crude ethanolic extract, chloroform fraction and ethylacetate fractions prepared from the leaves of FP have strong concentration-dependent growth inhibitory activity against PRST2 cells and low cytotoxicity against non-cancerous hFSE cells (Fig 2). Our results show that CE has greater cytotoxicity against prostate cancer (IC_{50} of 44.53 $\mu\text{g/mL}$), compared to HepG2 cells (IC_{50} = 300 $\mu\text{g/mL}$) [8].

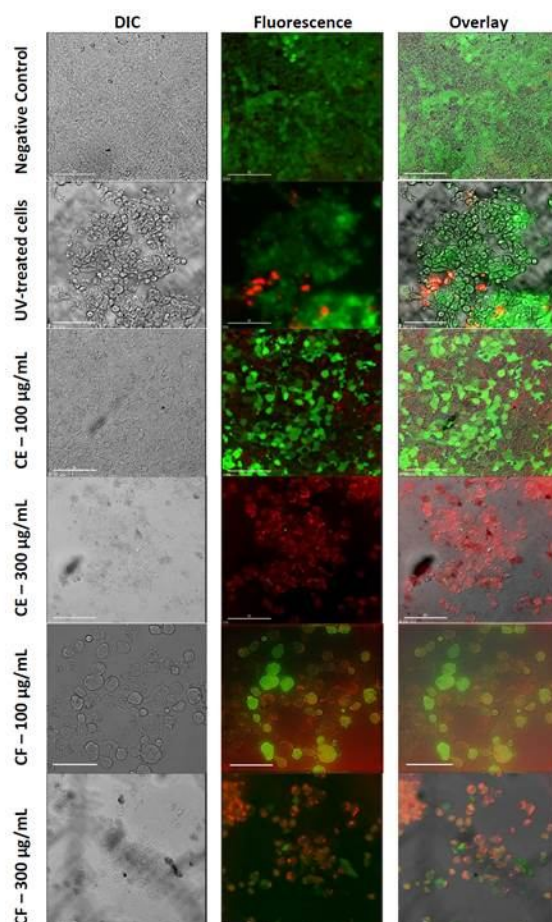


Figure 4: Micrographs of PRST2 cells 48 hours post-treatment with different concentrations of *Ficus pseudopalma* extracts. Cells were subjected to the LIVE/DEAD Cell Viability/Cytotoxicity Assay (Invitrogen). Green fluorescence (λ_{em} = 490 nm) show live cells while red fluorescence (λ_{em} = 555 nm) show dead cells. Note the increasing number of dead cells as the concentration of the extract increases. CE = crude ethanol extract and CF = chloroform fraction; scale bars represent 90 μm . **Magnification:** 40x.

Visible cytotoxic effects of CE and CF shown in the microscopic analysis of treated PRST2 cells revealed changes in cell shape from polygonal to round cells, loss of adherence which is characteristic of dying or dead cells, increase in cellular debris, and decrease in cell density. A decrease in green fluorescence and increase in red fluorescence in the LIVE/DEAD® Assay as the concentration of CE and CF extract increases reinforces the concentration-dependent effect of the extracts on PRST2 cells due to increasing

concentration of phytochemicals that have growth inhibitory activities and/or have disruptive effects on cell membrane integrity. In living cells, non-fluorescent calcein AM is enzymatically converted by ubiquitous intracellular esterase activity into a brightly green fluorescent calcein (excitation/emission at 495/515 nm). On the otherhand, ethidium homodimer 1 (which is excluded from live cells with intact cell membranes) gives-off red fluorescence once intercalated into the nucleus of dead cells (excitation/ emission at 495/635 nm).

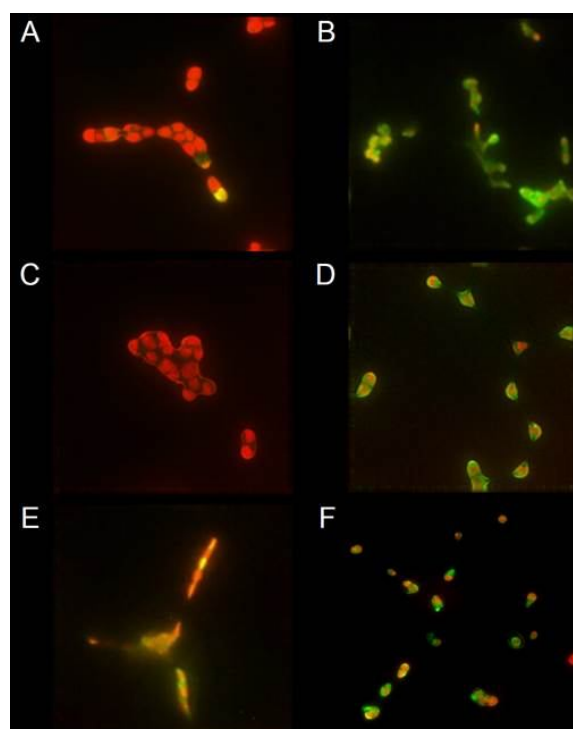


Figure 5: Micrographs of apoptotic PRST2 cells 48 hours post-treatment with different concentrations of *Ficus pseudopalma* extracts. Cells were subjected to the APO-BrdU TUNEL assay (Invitrogen). Green fluorescence (λ_{em} = 490 nm) show apoptotic cells while red fluorescence (λ_{em} = 555 nm) show necrotic cells. Note the increasing number of apoptotic cells as the concentration of the extract increases. (A) Untreated PRST2 cells, (B) Positive control from TUNEL kit, (C) (D) treated with 100 and 300 $\mu\text{g/mL}$ of CE, and (E) (F) treated with 100 and 300 $\mu\text{g/mL}$ of CF. CE=crude ethanol and CF = chloroform fraction. **Magnification:** 40X

The anticancer activity of *Ficus* species have been attributed to its naturally occurring compounds such as terpenoids and flavonoids that may prevent oxidative stress-related diseases like cancer [18]. Earlier studies have confirmed the presence of the triterpenoid lupeol in FP leaves [4-5], as well as its antioxidant properties [4] [8]. In this study, phytochemical screenings of the extracts lead to the identification of compounds such as lupeol and

quercetin which may be responsible for its cytotoxic activity. The anticancer property of lupeol, a dietary triterpene found in certain fruits, vegetables, and medicinal plants, has been demonstrated by its ability to suppress the growth of hepatocellular carcinoma and melanoma cell lines [19]. A decrease in prostate-specific antigen secretion in athymic nude mice implanted with CWR22Rnu1 cells after treatment with lupeol, as well as inhibition of tumor growth suggest that lupeol may have a potential to be an effective agent against prostate cancer [17].

A previous study showed that the dry roots of *Ficus hirta* Vahl inhibited the growth of HeLa cells through induction of apoptosis [16]. In this study, a concentration-dependent increase in apoptotic PRST2-treated cells as shown by the incorporation of BrdU was observed 48 hours post-treatment with CE and CF extracts. The flavonol quercetin has been reported to induce apoptosis in HepG2 cells and MCF-7 cells [20], while lupeol is known to induce G2/M cell cycle arrest by modulating microtubule assembly followed by apoptotic death of prostate cancer cells [7].

The increase in the concentration of lupeol as the concentration of the extract was increased may account for the concentration-dependent cytotoxic and apoptotic activities of CE, CF, and EF extracts. In another study, lupeol induced apoptosis through Fas-receptor mediated pathway in a dose-dependent mechanism [7]. In addition, high concentrations of quercetin were found to have cytotoxic activity against MCF-7 cells [21].

FP was found to have little inhibitory activity against non-cancerous hFSE cells. In another study, FP ethanolic extracts enhanced the proliferation of normal non-cancerous Peripheral Blood Mononuclear Cells (PBMC) [8]. This indicates the protective effect of FP extracts on normal cells, while being cytotoxic to cancer cells.

CONCLUSION

The present study established the cytotoxic and apoptotic activities of FP extracts against PRST2 cells while having no adverse effect on normal hFSE cells. This study also provided evidence on the pharmaceutical potential of *Ficus pseudopalma* Blanco leaves as a chemotherapeutic agent against prostate cancer.

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