

ANTIPROLIFERATIVE AND APOPTOTIC EFFECTS OF FICUS PSEUDOPALMA BLANCO (MORACEAE) AGAINST HEPATOCARCINOMA (HEPG2)LIBRADO A SANTIAGO^{1,2,3*}, ANNA BEATRIZ R MAYOR^{1,3}

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ABSTRACT

Objective: Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer-related death in the Philippines. The prevalence of this disease continuously poses a great challenge to the medical and health sectors of the country, especially in discovering safe, efficacious and cheap alternative medicines with less or no adverse side-effects. Among our endemic medicinal plant species that belong to Moraceae family, *Ficus pseudopalma* (FP) remains understudied. To the best of our knowledge, there are no reports into the molecular mechanisms underlying the growth inhibitory effects of FP against HCC. We provided evidence that FP enhances apoptotic cell death of HepG2, specifically targeting DNA damage.

Methods: To assess the apoptotic and genotoxic abilities of the plant extract, trypan blue exclusion test, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, comet, terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling and caspase-3 activations assays were performed.

Results: Results showed that the crude extract of FP dose-dependently inhibited cell growth of HepG2 recording an inhibitory concentration of 353.342 µg/ml. Interestingly, all assays indicated apoptotic DNA fragmentation of HepG2 cells at concentrations of 300 µg/ml and 1000 µg/ml. In particular, changes in morphological features such as aggregation of chromatin, cell shrinkage, and the formation of apoptotic bodies were also observed in treated HepG2 cells.

Conclusion: The data strongly suggest that FP may be used as an alternative, natural, cheap, readily available, and potent cancer chemoprotective agent by inhibiting cell growth and promoting cell death. The study provides the first molecular evidence on the plant that induces apoptosis for the development of phytotherapeutic regimen for cancer prevention or treatment.

Keywords: *Ficus pseudopalma*, Cytotoxicity, Apoptosis, Comet assay, Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling assay, Caspase assay.

INTRODUCTION

The prevalence of cancer had been continuously increasing all throughout the world. In the Philippines, cancer ranks the third leading cause of mortality [1]. Among the most common types of cancer, hepatocellular carcinoma (HCC), which is usually associated with liver cirrhosis, hepatitis B and hepatitis C, is prevalent in the country.

To date, the current conventional available treatment on HCC had shown adverse side effects that pose a threat to the health and general well-being of patients with liver cancer. Phytotherapy is one of the current medical therapies available in developing countries in treating cancer concomitant with the orthodox practice allowing use of safe and effective botanicals instead [2].

Preliminary results on the crude extract of *Ficus pseudopalma* (FP), one of the 150 endemic ethnomedicinal *Ficus* species in the Philippines that belong to the Moraceae family, revealed antioxidant [3,4] and anti-urolithiatic activities [5] as well as cytotoxic against human hepatocarcinoma (HepG2) [6] and anti-proliferative and apoptotic activities to prostate cancer cells (PRST2) [7]. Its pharmacologic effects have been attributed to its contents such as phytosterols, pentacyclic triterpenes, phenolics and flavonoids [4,8]. To date, however, the putative molecular bases are sorely lacking using HepG2. Therefore, it was the objective of this study to further explore the mechanisms of action of FP, specifically its anti-proliferative and apoptotic effects on HepG2.

METHODS**Plant preparation and extraction**

Fresh leaves of FP were harvested in Bicol, Philippines. The plants were sent to the National Museum of the Philippines for proper identification. Extraction procedure was based on the protocol discussed [3]. Briefly, dried leaves of FP were ground and soaked with 95% ethanol (RCI Labscan) for 1 week, with a 24 hrs collection interval. The resulting ethanolic extract was concentrated through rotary evaporator (Eyela, USA). The collected crude extract was air-dried and kept in an amber bottle at 4°C.

Cell culture and maintenance

Liver cancer cell lines, HepG2 (85011430), was purchased from the European Collection of Cell Culture, Health Protection Agency, Porton Down, Salisbury, Wiltshire, United Kingdom through Sigma-Aldrich. The cell line was authenticated using the Amp FISTER® SGM Plus® polymerase chain reaction (PCR) amplification kit and the ABI Prism 3730 genetic analyzer. Further analysis was performed such as DNA barcoding of the mitochondrial cytochrome C oxidase sub-unit 1 gene, mycoplasma detection using Vero indicator cell line and Hoechst 33258 fluorescent detection system as well as PCR analysis. Cell count, viability and confluency tests of cells on resuscitation from frozen condition was also performed.

HepG2 was cultured in a T-75 flask using Dulbecco's modified Eagle medium (DMEM) nutrient mixture F-12 HAM supplemented with 15 mM HEPES, NaHCO₃, pyridoxine, L-glutamine (Sigma-Aldrich) and 10%

fetal bovine serum (FBS) (Sigma-Aldrich) as the culture media. The cells were maintained at 37°C in 5% CO₂ incubator, wherein the culture medium was changed every 2-3 days. The study was performed in the tissue culture laboratory of the University of Santo Tomas Research Center for Natural and Applied Sciences.

Trypan blue exclusion test

Confluent HepG2 cells were seeded in a 6-well microplate at a density of 1.0×10^6 cells/mL and were incubated for 24 hrs at 37°C. The cells were then treated with different concentration of the crude ethanolic leaf extract of FP for 24 hrs and incubated at 37°C. After which, the cells were detached using 0.05% trypsin - ethylenediaminetetraacetic acid (EDTA) (Gibco, Life Technologies) and were centrifuged at 3000 rpm for 5 minutes. Cell supernatant was discarded, and the cells were re-suspended in DMEM. An aliquot of 10 μ L of the cells suspension was added with similar volume of 0.4% trypan blue solution (Sigma-Aldrich). Cell viability was assessed using TC-10 automated cell counter (Bio-Rad).

Cytotoxicity test: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Confluent (80%) HepG2 cells were seeded in 96-well microplates at a density of 3.0×10^4 cells/mL and were incubated for 24 hrs at 37°C. The culture media of each cell were replaced with different concentrations of the crude ethanolic leaf extract of FP, doxorubicin (Dx) and paclitaxel (Px). About 150 μ L of the test sample and standard drugs were added to each well, and the cells were incubated for 24 hrs at 37°C. After incubation, the test sample and standard drugs were removed, and the plate was washed with phosphate buffered saline (PBS), pH 7.4 (Gibco, Life Technologies). Then, 100 μ L of PBS was added to each well together with 10 μ L of 5 mg/mL MTT reagent (Invitrogen). The plate was incubated for 4 hrs at 37°C. Then, 50 μ L of the current solution was removed and replaced with 50 μ L of dimethyl sulfoxide (DMSO) Hybri-Max® (Sigma-Aldrich) to dissolve the purple formazan crystals that were formed [9]. The plates were then read at 570 nm using corona microplate reader SH-1000 (Hitachi, Japan) [6,7].

Assessment of the genotoxic activity against HepG2 cells by comet and terminal deoxynucleotidyl transferase (TdT) deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assays

Comet assay was used in determining the extent of DNA damage that has been inflicted by a cytotoxic agent to a certain cell type. As patterned on the previously discussed protocol [10,11], 5.0×10^5 cells/mL HepG2 cells were used for every trial. Materials consist of cell culture grade phosphate buffered saline (pH 7.5), a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM trizma base, 1% triton X-100 and 10% DMSO), electrophoresis buffer (300 mM NaOH and 1 mM EDTA), neutralization buffer (0.4 M Tris, pH 7.5) and a staining solution (2 μ g/ml ethidium bromide, Sigma-Aldrich).

The HepG2 cells were treated with trypsin to detach it from the cell culture flask. Then, they were washed with PBS and were centrifuged for 5 minutes at 3000 rpm. The cells were then suspended back to DMEM supplemented with 10% FBS to wash off the effect of trypsin. The cell viability of HepG2 cells were determined by trypan blue exclusion test and the cell density was adjusted and maintained at 1.0×10^5 cells/mL. 10 μ L of cells were then transferred to a tube containing 75 μ L of 0.5% low melting agar (Sigma-Aldrich). The resulting solution was transferred to a covered glass slide (1% normal melting agar, Sigma-Aldrich) and was chilled (4°C) for 10 minutes. After which, the slide was placed on a container with the lysing solution and was chilled for 2 hrs to allow the lysis of the cells. After 2 hrs, the slide was then washed off with the lysing solution and was transferred to the electrophoresis set up containing the alkaline electrophoresis buffer (pH>13). The slide was allowed to stand for 20 minutes on the electrophoresis buffer before the run was started. After that, the machine was set to 30 V, 3000 mA for 30 minutes. Then, the slide was washed with the neutralization buffer thrice and was stained with ethidium bromide. The slide was then viewed under a fluorescent microscope (Optika, Italy).

TUNEL assay was performed using Click-iT® TUNEL Alexa Fluor® imaging assay (Invitrogen). The kit utilize a dUTP modified with an alkyne, a

small, bio-orthogonal functional group that enables the nucleotide to be more readily incorporated by TdT than other modified nucleotides.

The assay was done based on the manufacturer's specifications. Briefly, HepG2 cells, cultured in cover slips, were treated with 300 μ g/ml and 1000 μ g/ml crude ethanolic leaf extract of FP, 10 μ g/ml Px and 20 μ g/ml Dx for 24 hrs. After which, the cells were washed with PBS prior to ethanol fixation (70%). Then, they were permeabilized using 0.25% Triton X-100. After an incubation for 20 minutes, the cells were washed ($\times 2$) with DNase free ultrapure water (Invitrogen). After which, TdT reaction buffer was added to each cover slips prior to 60 minutes incubation with tetrathionate reaction cocktail (TdT reaction buffer, EdUTP, TdT enzyme). Then, the cover slips were washed with 3% bovine serum albumin (BSA) (Sigma-Aldrich) and were added with click-iT reaction cocktail (click-iT reaction buffer and reaction buffer additive). After incubating for 30 minutes, the cover slips were washed with 3% BSA and were added with 100 μ L $\times 1$ Hoechst 33342 dye. The cover slips were then washed with PBS ($\times 2$) and were viewed under a fluorescent microscope (Optika, Italy).

Caspase-3/CPP32 colorimetric protease assay

The assay was performed to determine the proteolytic activity of caspase-3 in mammalian cell lysates using caspase-3/CPP32 colorimetric protease assay (Invitrogen). Briefly, apoptosis was induced by treating HepG2 cells (1.0×10^6 cells/mL) with the crude ethanolic leaf extract of FP (300 μ g/ml and 1000 μ g/ml), Px (10 μ g/ml) and Dx (20 μ g/ml). Treated cells were washed with PBS and lysed using the lysis buffer provided in the kit. The lysed cells were centrifuged for 1 minute at $\times 10,000$ g and the supernatants were collected on another tubes. Protein concentration of each supernatants was determined using Bradford assay. About 150 μ g of protein from each supernatant was transferred to a 96-well microplate and was added to the reaction buffer containing 1.0 M dichlorodiphenyltrichloroethane. Then, 4 mM DEVD-pNA (Asp-Glu-Val-Asp p-nitroanilide) was added to each well, and the plate was incubated for 2 hrs at 37°C. Lastly, the absorbance was read at 450 nm using corona microplate reader SH-1000 (Hitachi, Japan).

RESULTS AND DISCUSSION

Cytotoxicity of the crude ethanolic leaf extract of FP

Trypan blue exclusion test is used to rapidly evaluate the viability response of the cells against an external factor. Uptake of the dye will be clearly observed in dead cells wherein the permeability of the cellular membrane is lesser compared to live cells.

Results showed that the crude ethanolic leaf extract of FP was able to inhibit the growth of HepG2 cells by decreasing its viability as the extract's concentration increases (Fig. 1).

In order to support the findings that were initially obtained, another in-vitro cytotoxicity test for the crude ethanolic leaf extract of FP was

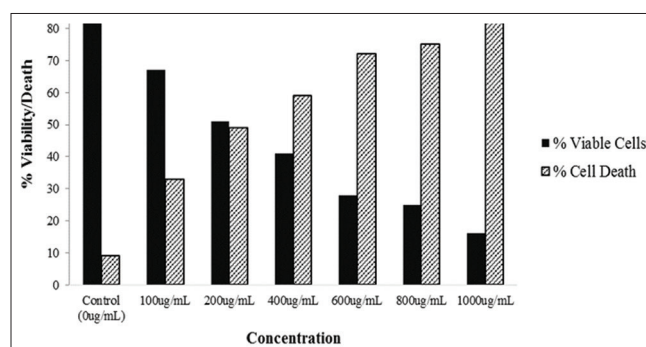


Fig. 1. Trypan blue exclusion test. Concentration-dependent inhibition of HepG2 cell proliferation by the crude ethanolic leaf extract of *Ficus pseudopalma*. Results are expressed as mean \pm standard error mean (n=3; p<0.05)

assessed against HepG2 cell lines by MTT assay. The ability to inhibit cell proliferation and induce death to cancer cells were measured through the direct reduction of the MTT salt to purple formazan. Initially, the plant extract was dissolved in DMSO and was further diluted to the desired concentration using DMEM. The final concentration of DMSO was maintained below 0.5%, which was found to be non-toxic to cells [12]. Results showed that the crude ethanolic leaf extract had a concentration-dependent inhibition of HepG2 cell proliferation, with an inhibitory concentration 50 (IC_{50}) of 353.342 $\mu\text{g}/\text{ml}$. The observed activity was then compared with that of the activities of Px (IC_{50} <10 $\mu\text{g}/\text{ml}$) and Dx (IC_{50} =18.5 $\mu\text{g}/\text{ml}$) (Fig. 2).

The obtained results corroborated with the previous study that was conducted using defatted methanolic leaf extract of FP leaves, wherein similar concentration-dependent inhibition of HepG2 cells (IC_{50} =300 $\mu\text{g}/\text{ml}$) was observed [6]. Furthermore, ethanolic leaf extract of FP, and its two fractions (ethyl acetate and chloroform fractions), demonstrated a concentration-dependent inhibition of prostate cancer cell lines (PRST2) as previously discussed [7].

Structural analysis of the bioactive compounds present in FP has been discussed previously [8] and it was found to contain ursenone, β -amyrin fatty acid ester, α -amyrin acetate, β -amyrin acetate, oleanone, lupeol fatty acid ester, lupenone, squalene, and polyprenol. Separate studies have demonstrated the anticancer potential of these compounds though in a different chemical form. Oleanolic and ursolic acid were shown to have a good cytotoxic activity toward various cancer cell lines. The 2 α -hydroxyloleanolic acid inhibited the growth of HepG2, MCF-7 and Caco-2 cell lines [13]. In a different study, combination of oleanolic acid and 5-fluorouracil had synergistically potentiated cell death of Panc-28 human pancreatic cells [14]. On the other hand, ursolic acid induced apoptosis in HT-29 and HCT116 colon tumor models [15] and some of its derivatives were cytotoxic against HepG2, BGC-823, SH-SY5Y, HeLa and HELF cell lines [16]. In addition, a study showed that the ester derivatives of ursolic acid inhibited the growth of several lung, colon, breast and renal cancers, melanoma and leukemia cell lines [17]. Considering these findings, the cytotoxic activity of the crude ethanolic leaf extract of FP can be attributed to its bioactive constituents.

Genotoxicity of the crude ethanolic leaf extract of FP

To further elaborate the observed cytotoxicity of FP leaf extract, its ability to induce DNA fragmentation was assessed by comet and TUNEL assay.

Assessment of cellular apoptosis can be based on both morphological and biochemical parameters. The morphology of apoptotic cells usually demonstrate the fragmentation of nuclei into sharply defined chromatin clumps, early preservation of cell membranes and absence of inflammatory reaction [18]. Changes in chromatin structures are

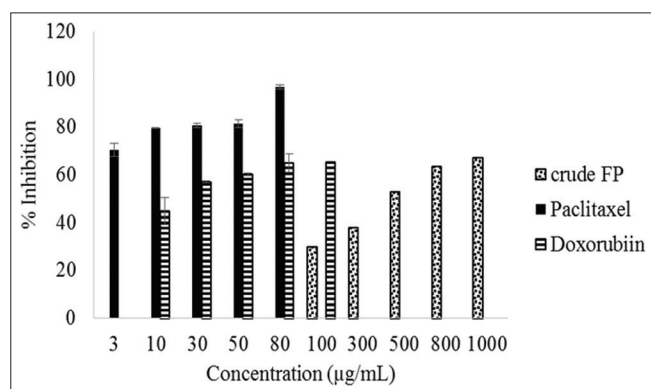


Fig. 2: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Concentration-dependent inhibition of HepG2 cell proliferation by the crude ethanolic leaf extract of *Ficus pseudopalma*, paclitaxel and doxorubicin. Results are expressed as mean \pm standard error mean (n=3; p<0.05)

essential in apoptosis and are assumed to mediate DNA fragmentation by endonucleases resulting to a "ladder" of oligonucleosomal fragments that are observable on agarose gel electrophoresis [19,20].

In this study, alkaline comet assay was used due to its high sensitivity and its ability to detect a broad spectrum of DNA lesions [21,22] and is useful in measuring DNA strand breaks in eukaryotic cells [23]. The results showed that the crude ethanolic leaf extract of FP was able to fragmentize the DNA of HepG2 cells at 300 $\mu\text{g}/\text{ml}$ and 1000 $\mu\text{g}/\text{ml}$ (Fig. 3). Fragmentation is shown as the tail of the comet, which consists of denatured DNA strands that migrated out of the cellular membrane due to the influence of electric potential [23]. Studies pertaining to other *Ficus* species showed that the comet assay is effective in demonstrating the genotoxicity toward cancer cells of the said plant species. In a particular study, two phenolic compounds isolated from *Ficus rumphii* displayed a significant activity against HL-60 with IC_{50} values of 3.3 and 2.3 μM , respectively [24].

In addition to that, TUNEL assay was performed in order to further confirm the DNA degrading activity of the crude ethanolic leaf extract of FP towards HepG2 cells. The assay is a good combination of molecular biology and morphological observation that are both useful in the quantification of apoptotic cells [25]. Moreover, it identifies apoptotic cells, wherein genomic DNA is cleaved to a large number of DNA double-strand breaks, which resulted from endonucleases activation [18,22] which is why it is often used for cytochemical quantification of apoptosis induced by various plant extracts toward human cancer cell lines [2,26,27] and other in-vivo using rat models [28].

As shown in Fig. 4, the crude ethanolic leaf extract of *F. pseudopalma* had induced apoptosis to HepG2 cells both at 300 $\mu\text{g}/\text{ml}$ and 1000 $\mu\text{g}/\text{ml}$, though there were more observable condensation and small vesicle formation on the cells treated with higher concentration of the crude extract. This study corroborates with the results that were previously obtained [7] regarding the apoptosis inducing activity of FP and its

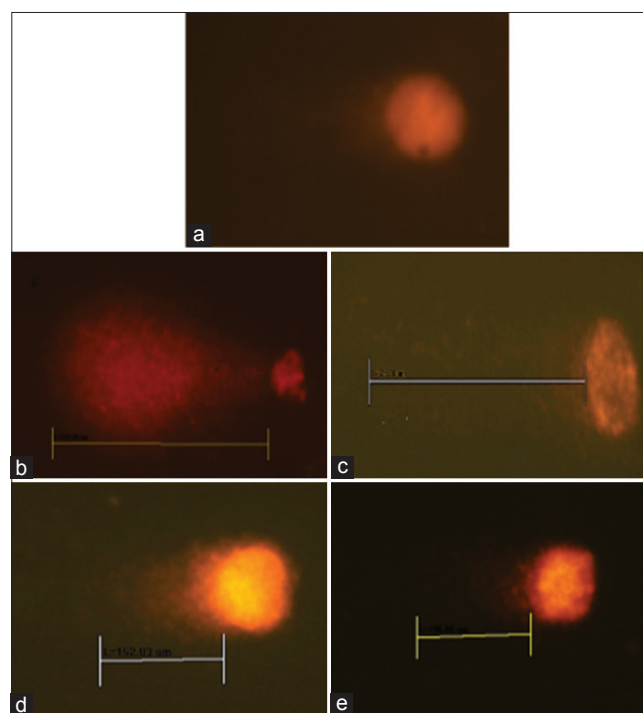


Fig. 3: Comet assay. Fluorescence micrographs ($\times 400$) of untreated HepG2 (negative control) (a), HepG2 treated with 10 $\mu\text{g}/\text{ml}$ paclitaxel (b), HepG2 treated with 20 $\mu\text{g}/\text{ml}$ doxorubicin (c), HepG2 treated with 300 $\mu\text{g}/\text{ml}$ of *Ficus pseudopalma* (FP) extract (d), HepG2 treated with 1000 $\mu\text{g}/\text{ml}$ of FP extract (e)

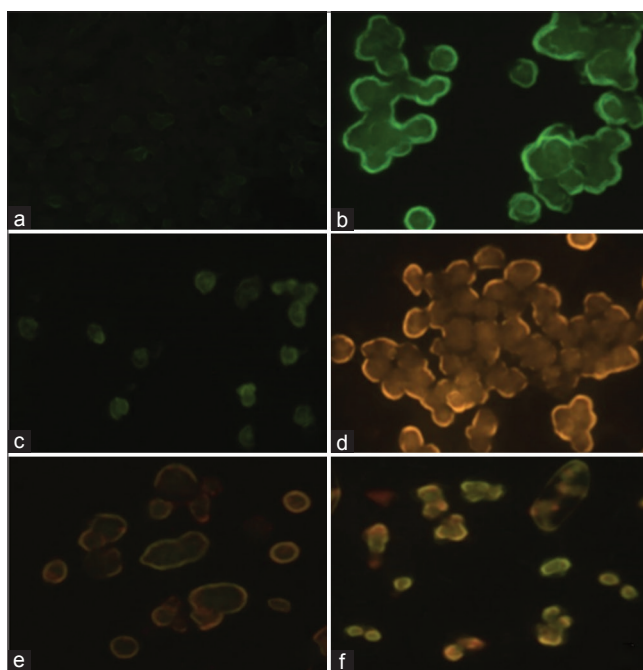


Fig. 4. Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling assay. Fluorescent micrographs ($\times 400$) of DNase-treated control HepG2 cells (positive-control) (a), untreated HepG2 (negative-control) (b), HepG2 treated with 10 $\mu\text{g/ml}$ paclitaxel (c), HepG2 treated with 20 $\mu\text{g/ml}$ doxorubicin (d), HepG2 treated with 300 $\mu\text{g/ml}$ *Ficus pseudopalma* (FP) extract (e) and HepG2 treated with 1000 $\mu\text{g/ml}$ of FP extract (f)

fractions, wherein a concentration-dependent increase in the number of apoptotic-labeled human PRST2 was observed. Likewise, other *Ficus* species such as *Ficus benghalensis*, *Ficus deltoidea* and *Ficus indica* were shown to have a good genoprotective and anti-mutagenic activities [29-31]. These attributes can also be related to the ability of *Ficus* species to prevent the occurrence of cancer and other related diseases.

Induction of apoptosis by caspase-3 activation

Caspase-3 belongs to the effector caspases that are responsible for most of the cleavages that disassemble the cell leading to apoptosis [32,33]. In connection to this, discovery of caspase activators had been given much attention, especially in the field of anticancer drug discovery [34,35].

Measurement of caspase activities usually involves peptide substrates containing fluorogenic residue at the carboxyl terminal side of the cleavage site [32]. In this study, DEVD tetrapeptide backbone was used since it was shown to be the consensus sequence of most substrates for caspase-3 [36].

Results shown in Fig. 5 demonstrated that the crude ethanolic leaf extract of FP was able to stimulate the release of caspase-3 protease as reflected on the increased optical density (OD) that were recorded for both 300 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ concentrations. It is, however, noticeable that Px treated cells had a lower OD measurement as compared to that of the Dx - treated cells that contradicts the results presented above (Fig. 2). This can be explained by the fact that Px can induce integrin-mediated cell death resulting from inappropriate cell adhesion or loss of cell adhesion to the extracellular matrix [37]. Since lower number of intact Px - treated cells were obtained, lower amount of protein was obtained and was assayed.

There are no data yet on the ability of other *Ficus* species in activating apoptosis via caspase-3 pathway though there are some studies that provide insights on the capability of various phytochemicals in inducing the release of caspases from cells.

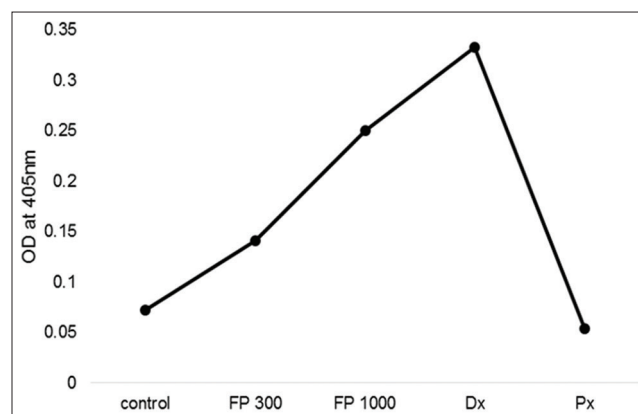


Fig. 5: Caspase-3 assay. Induction of caspase-3 proteases by the crude ethanolic leaf extract of *Ficus pseudopalma*, doxorubicin and paclitaxel

CONCLUSION

The study showed that the crude ethanolic leaf extract of FP was able to induce apoptosis to HepG2 cells and induce DNA fragmentation in HepG2. Therefore FP has a promising potential as a chemopreventive agent against HCC.

Extensive studies on the endemic ethnomedicinal plants of the country, such as FP, had shown to be useful in unraveling more medical potentials and provide deeper understanding, through molecular study, on the biological and pharmacological actions of these plants.

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