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Cytotoxic and genotoxic activities of the crude ethanolic leaf extract of *Ficus odorata* (Blco). Merr. against human hepatocellular carcinoma

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Abstract
Due to the prevalence of liver cancer in the Philippines, the study focused on the use of endemic medicinal plant, *Ficus odorata*, as a possible chemopreventive agent. In this study, it was demonstrated that the crude ethanolic leaf extract of *F. odorata* has a potent cytotoxicity towards HepG2 cells and was further supported by its genotoxic activity as assessed by both Comet and TUNEL assays. The crude extract was also able to induce the release of caspase 3 protease that activates cell apoptosis. Considering these, *F. odorata* is a potential anticancer agent.

Keywords: *Ficus odorata,* endemic, cancer, HepG2, cytotoxic, genotoxic

1. Introduction
The prognosis of liver cancer is often associated with several risk factors such as hepatitis B and C infections, cirrhosis, alcohol consumption, obesity and diet contamination [1]. Due to this combined occurrence of diseases, it has become a problem to suppress the severity of liver cancer, making it the 3rd most common type of cancer that contributes to the mortality rate in the Philippines [2].
In developing countries such as the Philippines, the use of medicinal plants for the treatment of a wide range of diseases, including antimicrobial, antidiabetes, antihypertension, antioxidants and anticancer are still popular. It is noteworthy that there are endemic plants to the country that are comparably effective to that of the medicines available in the market.
Among which is *Ficus odorata* (Blanco) Merr. that is frequently used for the treatment of diabetes, allergy, asthma and cancer [3]. The dichloromethane (DCM) extract of *F. odorata* exhibited cytotoxicity towards certain cancer cell lines such as lung adenocarcinoma epithelial cells (A549), human stomach adenocarcinoma cells (AGS), human colon adenocarcinoma (HT29) and human prostate cancer cell line (PC3). Furthermore, same study on the extract revealed the presence of 1-sitosteryl-3-β-glucopyranoside-6'-O-palmitate, squalene, lutein, α-amyrin acetate, lupeol acetate, and β-carotene [4].
Related study demonstrated that the crude extract of *F. odorata* has prooxidant activity towards DPPH, superoxide anions, hydrogen peroxide, hydroxyl and nitric oxide radicals [5]. Although antioxidants have been often link to anticancer or antitumor activities, current studies have revealed that prooxidants can also be beneficial in fighting cancer.
The present study aimed to assess the cytotoxic and genotoxic effect of *F. odorata* against HepG2, a cell line of hepatocellular carcinoma, to assess its potential cancer chemopreventive activity. This is also the first study to screen the phytochemicals in the plant and its phenolic and flavonoid content.

2. Materials and Methods
2.1. Plant Collection, Preparation and Extraction
Ficus odorata leaves were collected from Barangay Igbac, Buhi, Camarines Sur in Bicol, Philippines and was authenticated by the Botany Division of the Philippine National Museum. Air-dried leaves were processed and extracted according to the methods of Santiago and Valerio [6]. Briefly, 1 kg of ground leaves were soaked in 2.5L ethanol (p5%) for five days with extract collection every after 24 hrs.

Collected leaf extracts were concentrated using rotary evaporator (Eyela, USA) at 40 ºC.

2.2. Phytochemical Screening and Thin Layer Chromatography

Qualitative determination of the phytochemical constituents of the crude ethanolic leaf extract of *F. odorata* was performed based on the established protocol [7]. The crude leaf extract was tested for the presence of carbohydrates, tannins, saponins, flavonoids, alkaloids, anthocyanins, β-cyanin, quinones, glycosides, terpenoids, steroids, phenols, coumarins and other acidic compounds.

The total phenolic and flavonoid content were both quantified [8, 9, 10] with some modifications. The crude leaf extract was prepared at 1mg/mL and standards, gallic acid and quercetin, were used as reference. For total phenols, 1 mL of Na_2CO_3 (0.2 M), 2 mL of distilled water and 0.20 mL of Folin-Ciocalteu reagent were added to 1ml of the crude leaf extract and was diluted to 5 mL prior to heating (100 ºC) for 15 min. An aliquot of 150 µL was transferred to a microplate and the absorbance was read at 710nm. The result was compared to that of standard gallic acid. On the other hand, total flavonoid content was determined using quercetin as the standard. The crude leaf extract was prepared at 10mg/mL and was treated with 2 mL concentrated hydrochloric acid. It was then incubated for 20 min at 25 ºC and 150 µL aliquot was transferred to a microplate and absorbance was read at 450 nm. All absorbance readings were prepared in triplicates and was read using Corona Microplate Reader SH-1000 (Hitachi, Japan).

Thin layer chromatography (TLC) was performed following the protocol discussed [5]. In brief, the crude leaf extract, together with the standards, was spotted onto silica gel G 250 analytical plates (60 mm x 80 mm x 0.2 m). The plate was then allowed to develop in toluene: ethyl acetate: methanol (6:3:1) and was observed under visible light, ultraviolet light (254 nm and 366 nm) and visualized using iodine crystals.

2.3. Cell Culture and Maintenance

Hepatocellular carcinoma cell line (HepG2; Acc No. 85011430) was obtained from the European Collection of Cancer Cell Lines. The cells were cultured in a cell culture flask using Dulbecco's Modified Eagles Medium (DMEM) Nutrient Mixture F-12 HAM supplemented with 10% fetal bovine serum (FBS), 15mM HEPES, $NaHCO_3$, pyridoxine and L-glutamine. The cells were maintained in a 5% CO_2 incubator with 97% humidity at 37 ºC.

2.4. Cytotoxicity Test (MTT Assay)

The cells were ascertained to at least 80% confluent before use. Three milliliters of trypsin was then added to each culture flasks of cells and were incubated at 37 ºC for 5 min. After incubation, the cells were collected in a falcon tube, were centrifuged at 3000 rpm for 5 min and the supernatant was decanted. The cells were the homogenized and 20 µL of cell suspension was transferred to a small tube and was added with 20 µL of tryphan blue. Viable cells were counted using hemacytometer, seeded in 96 well at 3.0 x 10^4 cell/mL and were incubated at 37 ºC for 24 hours.

MTT assay was done according to the previously described method [11]. The cells were treated with different concentrations of crude ethanolic leaf extract of *F. odorata* (FO), doxorubicin (Dx) and paclitaxel (Px). The cells were then incubated at 37 ºC for 24 hours. After incubation, 20 µL of 5mg/mL MTT dissolved in phosphate buffered saline (PBS, pH 7.4) was added to each well of cells and was incubated at 37 ºC for another 4 hours. The medium was then carefully aspirated and 100µL of dimethyl sulfoxide (DMSO) was added to each well. The absorbance was then read at 595 nm using Corona microplate reader SH-1000 (Hitachi, Japan).

2.5. Assessment of DNA Fragmentation Activity

2.5.1. Comet Assay

Modified protocol was followed for the assay [12, 13]. Ten microliters of treated Hepg2 cells were mixed with 75µL of 0.5% low melting point (LMP) agarose. This were then, spread to frosted pre-coated microscope slide (1% normal melting point (NMP) agarose. The cells were covered with cover slips and were allowed to solidify for 5 min at 4 ºC. After which, the cover slips were removed and the slides were immersed in a fresh cold lysing solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 10% DMSO and 1% Triton X-100) for 2 h. The slides were washed with deionized water and then immersed in an alkaline solution containing 300 mM Na OH, 1mM Na_2EDTA (pH>1)3 for 30 minutes to unwind the DNA. The samples undergone electrophoresis at a constant voltage of 30V and the conditions were controlled beginning with 300mA for 30 minutes. Identical alkaline solution levels were achieved by filling the spaces with blank slides. The comet assay samples were stained with 20µL diluted ethidium bromide before viewing. Fluorescent microscope (Optika, Italy) was used to view the result of the samples of comet assay. The comets that were scored are those within the circular area that was half the diameter of the gel loading circle.

2.5.2. dUTP Nick End Labeling (TUNEL) Assay

TUNEL assay was performed using Click-iT® TUNEL Alexa Fluor® Imaging Assay (Invitrogen). The kit utilize a deoxyuridine triphosphate (dUTP) modified with an alkyne, a small, bio-orthogonal functional group that enables the nucleotide to be more readily incorporated by terminal deoxynucleotidyl transferase (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 20 µg/mL and 1000 µg/mL crude ethanolic leaf extract of *F. odorata,* 10 µg/mL Paclitaxel and 20 µg/mL Doxorubicin for 24 hours. After which, the cells were washed with PBS prior to ethanol fixation (70%). Then they were permeabilized using 0.25% Triton X-100. After incubation for 20 min, the cells were washed (2x) with DNase free ultrapure water (Invitrogen). After which, TdT reaction buffer was added to each cover slips prior to 60-minute incubation with TT reaction cocktail (TdT reaction buffer, EdUTP, TdT enzyme). Then, the cover slips were washed with 3% bovine serum albumin (BSA) and were added with Click-iT reaction cocktail (Click-iT reaction buffer and reaction buffer additive). After incubating for 30 mins, the cover slips were washed with 3% BSA and were added with 100 µL 1x Hoechst 33342 dye. The cover slips were then washed with PBS

(2x) and were viewed under a fluorescent microscope (Optika, Italy).

2.6. Caspase-3/CPP32 Colorimetric Protease Assay

The assay was performed to determine the proteolytic activity of Caspase-3 in mammalian cell lysates. Briefly, apoptosis was induced by treating HepG2 cells ($1.0x10^6$ cells/mL) with the crude ethanolic leaf extract of *F. odorata* (200 µg/mL and 1000 µg/mL), paclitaxel (10 µg/mL) and doxorubicin (20 µg/mL). Treated cells were washed with PBS and lysed using the lysis buffer provided by the kit. The lysed cells were centrifuged for 1 min at 10000 x g and the supernatants were collected on another tubes. Protein concentration of each supernatants were determined using Bradford Assay. About 150 µg of protein from each supernatant was transferred to a 96-well microplate and was added with the reaction buffer containing 1.0M dichlorodiphenyltrichloroethane (DDT). Then, 4mM DEVD-*p*NA (Asp-Glu-Val-Asp *p*-nitroanilide) was added to each well and the plate was incubated for 2 hrs at 37 ºC. Lastly, the absorbance was read at 450 nm using Corona Microplate Reader SH-1000.

3. Results and Discussions

3.1 Extraction Yield

The dried ethanolic extract of *F. odorata* was physically examined for general appearance such as color, odor, and texture. The extract of *F. odorata* is dark green in color with aromatic tea-like odor and sticky-oily texture. These findings were verified through sensory panel of four individuals. The percentage yield obtained was 6.69%.

3.2 Qualitative and Quantitative Chemical Profiling

3.2.1 Qualitative Phytochemical Screening

Phytochemicals present in a plant can determine the number of possible biological activity of a plant that can either be beneficial or harmful towards human. Table I summarizes the result of the phytochemical screening performed in the crude ethanolic extract of *F. odorata.*

Seven out of the ten phytochemical present in the crude extracts exhibit anticancer or antitumor activity namely phenols, flavonoids, terpenoids, alkaloids, tannins, β-cyanins and coumarins. While three out of ten exhibits antioxidant activity: phenols, flavonoids, and β-cyanins. Steroids and glycosides are known to be beneficial in lowering blood cholesterol and increasing cardiac output, respectively.

Table 1: Preliminary phytochemical screening of the crude ethanolic extract of *F. odorata.*

Phytochemical Test	Result	Phytochemical Test	Result
Phenols	(+)	Alkaloids	(+)
Flavonoids	(+)	Betacyanin	(+)
Tannins	(+)	Quinones	(–)
Carbohydrates	(–)	Glycosides	(+)
Saponin	(–)	Cardiac glycosides	(+)
Acids	(–)	Terpenoids	(+)
Sterols	(+)	Coumarins	(+)

*Each phytochemical screening were performed in triplicates.

3.2.2 Thin layer Chromatography

The chromatograph showed 19 spots under visible light and UV light (254 and 366 nm). In addition, the chromatograph with the triterpenoid standards showed that spot 12 had the same Rf value as lupeol and α-amyrin (Rf=0.797) while spot 14 had the same Rf value as oleanolic acid and ursolic acid (Rf= 0.672) viewed using iodine crystals (Figure 1).

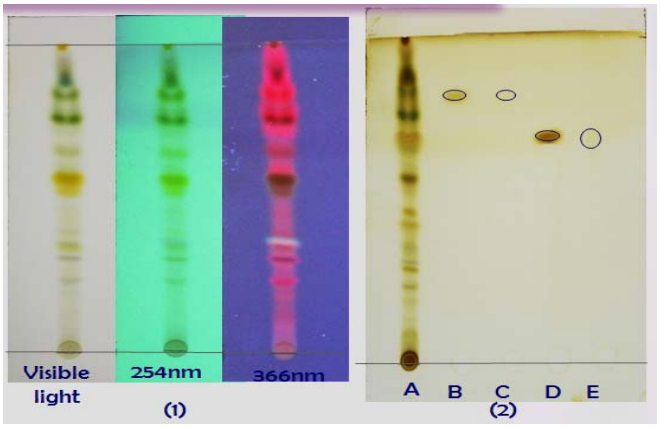

Fig 1: Thin Layer Chromatography. Chromatogram of the crude extract of *F. odorata* viewed under visible light and ultraviolet light (254nm and 366nm) (**1**). Comparison of the spots of *F. odorata* leaf extract (**A**) and standards lupeol (**B**) α-amyrin (**C**), oleanolic acid (**D**) and ursolic acid (**E**) visualized using iodine crystals (**2**).

The four triterpenoids were used as standards due to a large range of studies that indicated that they are good anticancer agents and also because of their hepatoprotective activity. Recent studies have shown that ursolic acid, lupeol and oleanolic acid have anticancer properties. Their action induces apoptosis in tumor cell [14], modulate the host system which potentially enables more robust antitumor responses [15] and induces apoptosis specific for HepG2 [16], respectively. α- amyrin, was indicated to be a potential hepatoprotective agent against toxic liver injury [17]. Hence, the presence of these triterpenenoids in the crude extract of *F. odorata* may explain the cytotoxicity exhibited towards HepG2.

3.2.3 Quantitative Phytochemical Screening

Results of TPC and TFC assays showed that the crude extract of *F. odorata* has 94.26±0.0005 µgGAE/mg extract and 88.55±0.04 µgQE/mg extract, respectively. Different plant materials rich in phenolic compounds are now popular in the food industry because of their antioxidant and anticancer properties. Flavonoids have shown a wide range of antibacterial, antiviral, anti-inflammatory, anticancer and anti-allergic activities. The high total phenolics and flavonoids content of *F. odorata* strongly suggest that it can be cytotoxic and genotoxic towards cancer cells.

3.3. Cytotoxic Activity of *Ficus odorata*

Cytotoxicity assay revealed that the extract of *F. odorata* inhibited the proliferation of HepG2 cells (IC_{50}=25 µg/mL). This median inhibitory concentration (IC_{50}) was significantly comparable to that of two chemotherapeutic drugs Dx (18.49 µg/mL) and Px (IC_{50}<10 µg/mL).

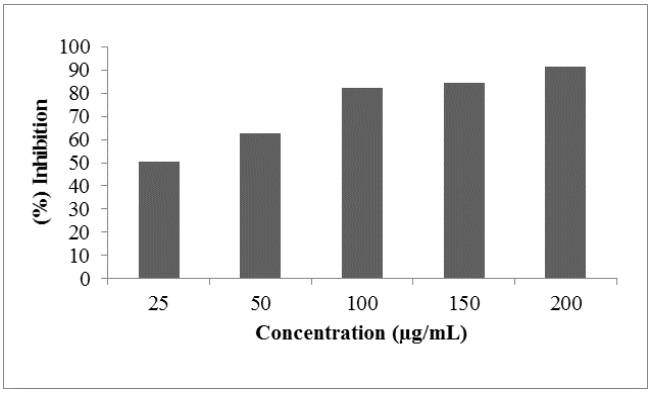

Fig 2: MTT assay. Concentration-dependent HepG2 cell inhibition of *Ficus odorata* on HepG2 cells. Values are presented as mean±SEM and are significant at $p<0.05$.

There is an increasing trend in the inhibitory activity of the extract in relation to its increasing concentration. In addition, the extract showed its highest inhibitory activity at 200 µg/mL wherein it exhibited 91.5% inhibition against HepG2 (Figure 2). The cytotoxic activity of the extract can be attributed to the different secondary metabolites present in its crude extract. The ability of the extract to inhibit the proliferation of HepG2 cells at a low concentration makes it a possible potent chemopreventive agent. In addition, the median inhibitory concentration exhibited by the extract was significantly comparable to Dx and Px which are chemotherapeutic drugs use in the market today.

3.4. Genotoxicity of the Crude Ethanolic Leaf Extract of F. odorata

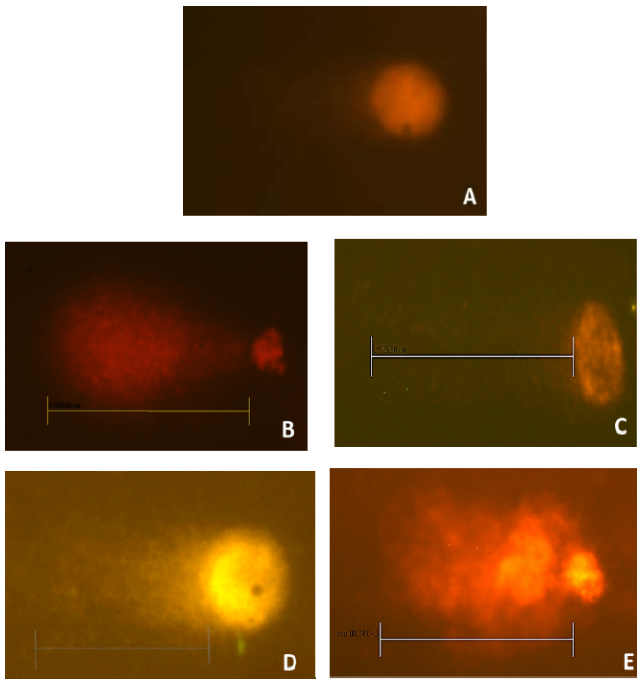

Fig 3: Comet Assay. Fluorescent micrographs (400x) of untreated HepG2 (negative control) (A), HepG2 treated with 10µg/mL Paclitaxel (B), HepG2 treated with 20µg/mL Doxorubicin **(**C**),** HepG2 treated with 20µg/mL of *F. odorata* extract (D), HepG2 treated with 1000µg/mL of *F. odorata* extract (E)

Most cytotoxic assays only measure cell death by determining the number of cells that undergo lysis. Thus, as for the genotoxic activity, Comet assay was performed to better examine the extent of the extract's chemopreventive activity against HepG2 cells by determining its capability to damage the harmful DNA of cancer cells.

The genotoxic activity of *F. odorata* was assessed by Comet and TUNEL assays. In Comet assay, cells with increased DNA damage display increased migration of chromosomal DNA from the nucleus which resembles the shape of a comet. This is due to the ability of denatured and cleaved DNA fragments to migrate out of the cell under the influence of an electric potential whereas intact DNA strand remain within the cell membrane. The intensity of the comet tail relative to the head reflects the number of DNA breaks. In Figure 3, the extract exhibited genotoxic activity at 20 µg/mL and 100 µg/mL, Dx (20 µg/mL) and Px (10 µg/mL) as illustrated by the comet-like appearances of the cells.

Another defining characteristics of cancers is its ability to resist cell death or to by-passed apoptosis [18]. TUNEL assay is one of the most established apoptosis detection methods. Extensive DNA fragmentation that generates a multitude of DNA double-strand breaks with accessible 3'-hydroxyl groups is one of the hallmarks of late apoptosis [19]. Using TUNEL assay, the extent of the apoptotic capability of *F. odorata*, doxorubicin and paclitaxel can be estimated by identifying apoptotic cells using the TdT-mediated addition of labelled dUTPs to the 3'-OH end of the cells with DNA fragmentation.

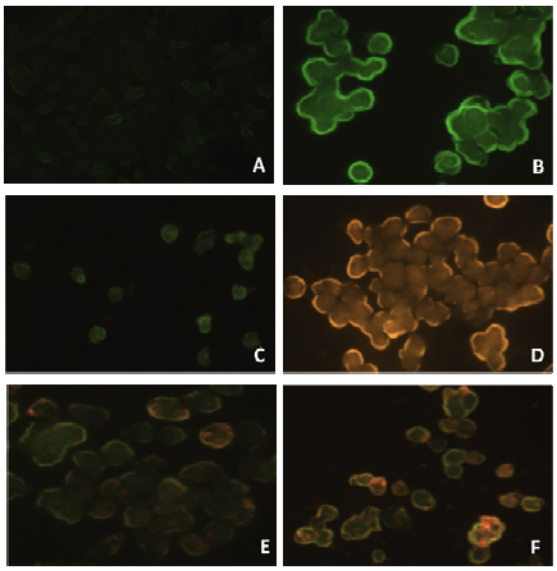

Fig 4: TUNEL Assay. Fluorescent micrographs (400x) of DNase-treated control HepG2 cells (positive control) (A), untreated HepG2 (negative control) (B), HepG2 treated with 10µg/mL Paclitaxel (C), HepG2 treated with 20µg/mL Doxorubicin (D), HepG2 treated with 20 µg/mL of *F. odorata* extract (E) and HepG2 treated with 1000 µg/mL of *F. odorata* extract (F)

In Figure 4, the DNase treated control did not fluoresce due to extensive cell damage causing the assay to not detect any free 3'-OH for binding with the dUTPS. DNase treatment of cells can fragment the DNA either by simultaneous

cleavage of both strands or by generating nicks on each dsDNA strands to produce dsDNA breaks. This would explain the lesser green fluorescent intensity in the positive control treatment where most of the DNA of the cells were expected to be fragmentized. On the contrary, untreated cells exhibited greater green fluorescence showing that it contains cleaved PARP where the dye could bind and would indicate no DNA damage. As for the extract, it exhibited a yellow fluorescent that indicates its ability to both cleaved PARP and DNA-strand. In addition, it can be observed that as the concentration of *F. odorata* increases, its ability to induce apoptosis in HepG2 also increases. Lastly for the standards, only Dx (20 µg/mL) exhibited efficiency in inducing apoptosis which also exhibited a yellow fluorescent while Px (10 µg/mL) only exhibited deem green fluorescent. The TUNEL assay for paclitaxel can be explained by the ability of the standard drug to totally degrade the cellular DNA.

Combined results of the Comet and TUNEL assays showed that the crude ethanolic leaf extract of *F. odorata* indeed caused DNA fragmentation in HepG2 cells leading to apoptosis. Salient morphological features of apoptotic cells were also observed on the treated HepG2 cells such as membrane blebbing and cellular condensation with intact cellular membrane. Other characteristics of apoptotic cells that can be useful in the evaluation of apoptotic induction are the presence of chromatin aggregates and formation of apoptotic bodies (small vesicles).

3.5. Caspase-3/CPP32 Activation

The 20 µg/ml *F. odorata* revealed a slightly elevated caspase-3 activity due to the low concentration of the plant extract whereas 1000 µg/ml of *F. odorata* is comparable to that of the caspase-3/ CPP32 measured in the cells that were treated with Dx (Figure 5). This means that the extent of apoptosis is significantly high in the concentration of *F. odorata* and Dx as stated above. On the contrary, low signals were recorded for Px, which may be due to the ability of this drug to lessen the cellular adhesion upon induction of apoptosis, resulting to smaller number of cells were protein can be isolated. This ability may be attributed to possible induction of intergrin-mediated cell death or "anoikis", which is a type of apoptosis that results from the loss of cell adhesion to the extracellular matrix or inappropriate cell adhesion [20].

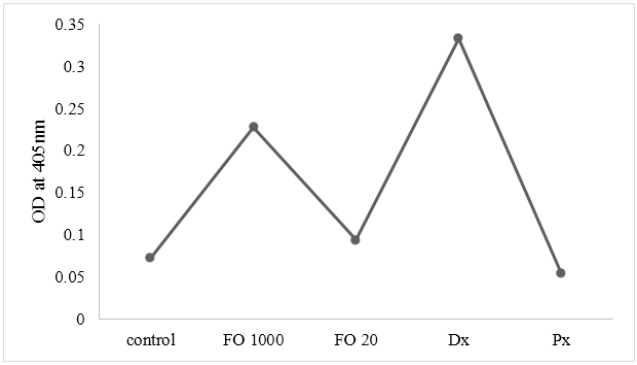

Fig 5: Caspase-3 Assay. Optical density (OD) obtained from the induction of caspase of *F. odorata* (FO) at 20 µg/mL and 1000µg/mL in contrast with standard doxorubicin (Dx) and paclitaxel (Px).

The presence of caspase-3/ CPP32 is found to be indicative of the cell undergoing apoptosis [21]. This was detected by the substrate DEVD-pNA. The sequence DEVD is recognizable by the cleavage site of caspase-3 in poly (ADP ribose) polymerase (PARP), the DEVD-pNA would then undergo cleavage and the free pNA provides the colorimetric measurement that is directly proportional to the caspase-3 present in the cells [22]. Furthermore, the low caspase activity detected in the drug, Paclitaxel, can be explained by its ability to extensively destroy the cell thus, making the substrate DEVD-pNA unable to attach, and free the pNA, with the cleaved PARP.

Caspase 3 belongs to the group of effector caspases that are activated by the action of initiator caspases (caspase-2, -8, -9 and -10). Effector caspases such as caspase-3 activates intrinsic DNase that fragments DNA and activates some enzymes that remodel the cytoskeleton, which in turn cause cellular condensation or cell shrinkage. Proteins that are cleaved by caspases during apoptosis include PARP-1 (cleavage inactivates it), DNA repair and cell- cycle regulatory proteins (including p53), and some proteins involved in signal transduction.

4. Conclusion

The presence of several phytochemicals in *F. odorata* may have contributed to its potency towards HepG2 cells. This has been confirmed by the low median inhibitory concentration of the crude extract as well as its ability to induce DNA damage at lower concentrations. Furthermore, it ability to activate apoptotic caspases may also support its cytotoxicity towards HepG2 cells. Hence, the crude ethanolic leaf extract of *F. odorata* can be used as an alternative or complementary medicine against hepatocellular carcinoma.

5. Acknowledgement

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6. Conflict of Interest

The authors declare no conflict of interests.

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