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EVALUATION OF THE ANTI - PROLIFERATIVE EFFECT, ANTIOXIDANT AND PHYTOCHEMICAL CONSTITUENTS OF *FICUS PUMILA* LINN.

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Keywords:

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ABSTRACT: This study sought to evaluate the heavy metal content of the raw powder and extract of the plant, the phytochemical constituents, antioxidant effect by the use of DPPH assay, the total phenolic content using Folin Ciocalteu assay and the cytotoxic effect using the MTT Assay of *Ficus pumila* ethanolic extract, methanolic and hydro fractions on liver cancer cells (HepG2), Leukemic cells (Jurkat) and normal liver cells (Chang). FTIR and Gas Chromatography-Mass Spectrometry (GC-MS) was used to identify the functional groups and major constituents of the most active fraction of *F. pumila*. Alkaloids, terpenoids, flavonoids, cardiac glycoside, saponins, and tannins were present in the ethanolic extract of *F. pumila*. The heavy metal analysis revealed the presence of Iron in both the raw powder (1.97 ± 0.11 mg/l) and extract (0.92 ± 0.02 mg/L). Zinc was also detected in both the raw powder (1.19 ± 0.00 mg/l) and extract (0.6595 ± 0.02 mg/l). The results from the FTIR revealed the presence of alkynes, alkyl halides, aromatics and aliphatic amines common to all fractions and compounds such as Phenol, 2,4-bis (1,1-Dimethylethyl) and Dodecane, 2,6, 10-trimethyl were detected in the samples by GC-MS. The DPPH assay also showed that all the fractions scavenged DPPH free radical in a dose-dependent manner as compared to the positive control (Ascorbic acid) and positively correlated to the phenolic contents. The MTT assay revealed that methanolic fraction was selective towards the Jurkat cell lines (Selectivity Index = 2.822). This increases the prospects that this plant contains compound(s) which could serve as leads for novel anticancer drugs.

INTRODUCTION: The burden of cancer in Africa promises to increase over the coming years due to increases in life expectancy, a lower burden of communicable diseases and changes in diet and lifestyle.

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The International Agency for Research on Cancer (IARC), [1] reported that about 715,000 new cancer cases and 542,000 cancer deaths occurred in 2008 in Africa and these numbers are projected to nearly double (1.28 million new cancer cases and 970,000 cancer deaths) by 2030. This could be as a result of aging and growth of the population and could even be higher due to the adoption of certain behaviours and lifestyles such as physical inactivity, unhealthy diet, and smoking.

In Africa, cancer continues to receive low public priority, despite this growing burden. This could be

attributed to limited resources and other urgent health problems which include some communicable diseases like Ebola, HIV/AIDS and malaria [2]. It is obvious that the best way to outwit cancer is to prevent it altogether. A lot of researches have been made into this effect, with most of them still ongoing. The fact, however, remains that lots of people are suffering from cancer. Curative surgery is the first option for patients with early-stage cancer while radiotherapy and chemotherapy have proven to be effective treatments for patients in the advanced stages. However, the curative effect of traditional chemotherapeutic drugs is limited, expensive and their side effects such as neurological and renal and cardiac toxicity are serious [3]. Traditional plant remedies have been used for the treatment of diseases like cancer for centuries, but only a few have been scientifically evaluated. Ornamentals which are usually cultivated for aesthetic purposes are found to contain chemical agents that could offer therapeutic potential in cancer treatment. One of such plants is *Ficus pumila.* This plant has been reported safe for use [4].

This study sought to evaluate the heavy metal content of the raw powder and extract of the plant, antioxidant effect, the total phenolic content and the cytotoxicity effect using the MTT assay, of *Ficus pumila* crude extract, methanolic and hydroethanolic fractions on liver cancer cells (HepG2), Leukemic cell (Jurkat) and normal liver cells (Chang). FTIR analysis was also done to determine the functional groups of the active components of the *F. pumila* fractions. The Gas Chromatography-Mass Spectrometry (GC-MS) was used to determine major compounds present in the most active fraction of *F. pumila*.

MATERIALS AND METHODS:

Cell Lines and Reagents: The cell lines for the study included Jurkat, HepG2 and Chang cells and were obtained from the Cell Bank of the Noguchi Memorial Institute for Medical Research (NMIMR), Legon. Culture media used included Dulbecco's Minimum Essential Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI), foetal bovine serum (FBS), supplemented with antibiotics (Penicillin, Streptomycin and Glutamine), phosphate buffered saline, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, acidified isopropanol, trypan blue solution, absolute ethanol, ethyl acetate, methanol, 2, 2- diphenyl-1-picrylhydrazyl (DPPH), Folin Ciocalteu, and Sodium Carbonate were of analytical grade and were purchased from Sigma Aldrich (USA). All experiments were conducted at the Department of Biochemistry and Biotechnology, Central Laboratory, KNUST-Kumasi and the Clinical Pathology Laboratory of NMIMR, Legon.

Plant Material: *Ficus pumila* plant (with voucher number KNUST/HM1/2014/L093) was collected near the Bomso Clinic, Kumasi and the leaves were separated from the stems. The leaves were washed, air-dried, pulverized and packaged in zip-locks and stored at room temperature.

Preparation of Plant Extracts and Fractionation: The 50% ethanolic extraction of the leaves was carried out by suspending 6000 grams of the powder of the leaves in 6000 ml of 50% ethanol in distilled water (50:50 v/v). The extraction was done by cold maceration for 24 h at room temperature on a shaker. The extract was then filtered through cotton wool, concentrated using a rotary evaporator and freeze-dried to obtain the *F. pumila* ethanolic leaf (FPL) extract.

FPL extract was sequentially extracted with solvents of increasing polarity starting with petroleum ether and followed by methanol. This was done by suspending 60 g of the ethanolic crude extract in 400 ml of the petroleum ether in a separating funnel. The mixture was shaken and left for 48 hours at room temperature after which the liquid portion was separated from the solid residue. This step was repeated for methanol. The remaining residue was designated hydro fraction.

For each fractionation step, extraction was performed twice with 400 ml of solvent. The Petroleum ether, methanol, and hydro fractions were then concentrated by air drying and freeze-drying for an aqueous fraction.

Determination of Extract Yield: The percentage yield was obtained using the formula; Yield = $(W_2-W_1/W_0) \times 100\%$; where W_2 is the weight of the extract and the container, W_1 the weight of the container alone and W_0 the weight of the initial dried sample.

Heavy Metal Analysis: About 1 g of each powdered sample (raw powder and extract) was weighed into 50 ml digestion tube, 1 ml H_2O, 2 mL HCl, 5 mL of 1:1 HNO_3: $HClO_4$ and 2 ml H_2SO_4 were added. Samples were allowed to stand for about 20 min at room temperature to enable the foam that formed to settle. They were then heated in a digestion block on a hot plate for about 2 h at a temperature of 150 ºC. The digested samples were allowed to cool and diluted to 50 ml. Blanks were prepared alongside. The samples were then stored for analysis by the Atomic Absorption Spectrophotometer. Metals analyzed included lead, copper, cadmium, nickel, zinc, and iron.

Total Phenolic Content Determination:

Principle: The total phenolic content assay is on the basis that all phenolic compounds contained in a mixture of antioxidant compounds or extract are oxidized by Folin-Ciocalteu reagent. This reagent is formed from a mixture of phosphotungstic acid, $H_3PW_{12}O_{40}$, and phosphomolybdic acid, $H_3PMo_{12}O_{40}$, which, after oxidation of the phenols, is reduced to a mixture of blue oxides of tungstate (W_8O_{23}), and molybdate (Mo_8O_{23}). The blue coloration produced has maximum absorption in the region of 750 nm, and is proportional to the total quantity of phenolic compounds originally present.

Procedure: Stock solutions of the ethanolic crude extract, methanolic and hydro fractions were prepared by dissolving 10 mg of each of the dried samples in 1 ml of the respective solvent and filtering. A stock solution of 5 mg/ml of standard (gallic acid) was prepared by dissolving 50 mg of it in 1 ml absolute ethanol. This was then diluted in 9 mL distilled water to obtain the 5 mg/ml stock solution. Two-fold serial dilutions were carried out on the gallic acid standard to obtain six different concentrations 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 mg/ml. An ethanol blank was also prepared. A two-fold serial dilution was also carried out on the samples to obtain three different concentrations of extract and fractions (10, 5, 2.5 mg/ml). Distilled water without extracts, were also prepared as blanks.

A volume of 10 µl of each sample dilutions and gallic acid dilutions were aliquoted into a 2.0 mL Eppendorf tube. Aliquots of 790 µl of distilled water were then added, and this was followed by the addition of 50 µl of Folin-Ciocalteu reagent. The mixture was mixed thoroughly by vortexing for five seconds. This was followed by incubation of the tubes in darkness at room temperature for eight minutes. Afterward, a volume of 150 µl of 7% sodium carbonate solution was added to each tube, mixed thoroughly by vortexing for five seconds and further incubation of the tubes in darkness at room temperature was done for two hours. After the two-hour incubation, a volume of 200 µL of each extract and gallic acid were aliquoted into wells on a 96-well plate in triplicate, and absorbance read at 750 nm using microplate spectrophotometer (Synergy H1, USA). A graph of absorbance against concentration was plotted for the gallic acid standard. The concentration of phenolics in each of the samples was determined using the gallic acid standard plot and the gallic acid equivalence for each fraction also calculated.

***In-vitro* Antioxidant Assay:**

Pricinple: When an antioxidant compound or extract that can donate hydrogen reacts to DPPH (a stable N centered radical purple in color), it reduces the DPPH to yellow color. This color change can be measured at 517 nm using a UV/VIS light spectrophotometer. The antioxidant effect was evaluated by calculating the EC_{50} (Effective concentration at 50%) value, which is the concentration of the extract / fraction that can scavenge 50% of the free radicals. The smaller, or the closer this value is to zero, the better the antioxidant effect of the fraction or compound [5].

Procedure: The stock solutions of the ethanolic extract, methanolic and hydro fractions were used for the determination of antioxidant activity as described by Brand-Williams *et al.* [6] Also, stock solutions of 10 mM of standard (Ascorbic acid) and 0.5 mM of DPPH were prepared by dissolving 0.176 mg of Ascorbic acid and 3 mg of DPPH in 1mL of distilled water and 15 mL absolute methanol respectively. The solutions were then vortexed until complete dissolution was achieved. The DPPH solution was immediately kept in the dark as it photo-bleaches in light.

In 1.5 mL Eppendorf tubes, the samples were serially diluted in distilled water to obtain a concentration range of 0.156-10 mg/ml. A hundred microliters of each concentration of the test sample

were transferred into a 96 well plate. This was followed by the addition of 100 μL of 0.5 mM DPPH. For positive control or standard, Ascorbic acid was used at a concentration range of 0.156-10 mM in distilled water. Distilled water was used as blank. Triplicate experiments were performed. The plates were covered with aluminium foil, shaken gently and kept in the dark for 20 min after which an absorbance was read on the plate reader at the absorbance wavelength of 517 nm. Percentage scavenging activity was determined by;

% Scavenging = [Absorbance of blank (OD0) - Absorbance of test (OD1)] × 100 / Absorbance of blank (OD0)

The mean percentage antioxidant activity for the triplicate experiment was plotted for the standard and fractions and their effective concentration at 50% (EC_{50}) values, which is the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, were determined by nonlinear regression analysis.

FTIR Spectroscopic Analysis: FTIR analysis was performed on each fraction using a PerkinElmer Spectrophotometer system from the USA, which was used to detect the characteristic peaks and their functional groups. The peak values of the FTIR were recorded and compared to standard values.

Gas Chromatography-Mass Spectrometry Analysis: GC-MS analysis of the methanolic and hydro fractions, were performed using a PerkinElmer GC Clarus 580 and a Gas Chromatograph interfaced to a Mass Spectrometer PerkinElmer (Clarus SQ 8 S) equipped with Elite-5MS (5% diphenyl / 95% dimethyl polysiloxane) fused with a capillary column (30 × 0.25 µm ID × 0.25 µm DF). For GC-MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1ml/min, and an injection volume of 2 µl was employed (split ratio of 10:1); Injector temperature 80 °C; Ion-source temperature 250 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min.), with an increase of 10 °C/min, to 200 °C, then 5 °C/min to 250°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scanning interval of 0.5 sec and fragments from 45 to 450 Da. Total GC running time was noted (29 min).

The mass-detector used in this analysis was Turbo-Mass, and the software adapted to handle mass spectra and chromatograms was a Turbo-Mass ver-6.1.0. The components were identified based on comparison of their relative retention time and mass spectra with those of NIST Library data. The results were confirmed by the comparison of the compounds elution and order with their relative retention indices on non-polar phases reported in the literature. The name, structure, molecular weight and chemical formula of the components of the test material were ascertained.

Phytochemical Analysis: The crude ethanolic extract was screened for the presence of alkaloids, tannins, terpenoids, saponins, and flavonoids according to the methods described by Ayoola *et al*., [7] with slight modifications.

Terpenoids: One milliliter of absolute chloroform was added to 10 mg of each extract and standard, ursolic acid, and 1 ml of 0.1M sulphuric acid was subsequently added. A reddish-brown color at the interface was indicative of the presence of terpenoids.

Saponins: One milliliter of distilled water was added to 10 mg of each plant extract and shaken vigorously for 1 min. A stable, persistent froth indicates the presence of saponins.

Tannins: Ten milligrams of each extract and standard, gallic acid, was boiled with 2 ml of distilled water. The boiled extract was centrifuged to obtain supernatant to which three drops of 0.1% $FeCl_3$ was added to each supernatant. A blue-black coloration indicates the presence of tannins.

Alkaloids: Ten milligrams of standard, quinidine, and crude ethanolic plant extracts were dissolved in 2 ml of acid alcohol (Concentrated HCl in 70% Ethanol) (v/v). The solution was boiled for three minutes and centrifuged to obtain a supernatant. One milliliter of dilute ammonia was added to the supernatant. Subsequently, 2 ml of absolute chloroform was added and shaken gently to extract the alkaloidal base. The chloroform fraction was then extracted with 2 ml of acetic acid. After adding four drops of Draggendorf's reagent to each extract and standard, a reddish-brown precipitate indicated the presence of alkaloids.

Flavonoids: Two milliliters of dilute ammonia was added to 2 ml portions of aqueous supernatant of each plant extracts and standard, quercetin. Subsequently, 1ml of 0.1 M sulphuric acid was added to the mixture. A yellow coloration that disappears on standing for 5 min indicated the presence of flavonoids.

Cardiac Glycoside: One milliliter aqueous supernatant of the crude extract and its fractions was added to 1 ml of diluted hydrochloric acid and was boiled for 10 min in a water bath. Filtrate after boiling was obtained using benzene, and 1 ml of ammonia solution added subsequently. Glycoside was indicated through the appearance of the red colour in the ammonia layer.

Sterols: A mass of 0.5 g each of extracts was added to 2 ml of acetic anhydride; afterwards 2 ml of sulphuric acid was also added. The presence of sterol was indicated by a blue coloration.

***In-vitro* Cytotoxicity (MTT) Assay:**

Principle: The MTT assay was used to determine the cytotoxicity of the extract and fractions on the principle that, the mitochondria of metabolically active living cells produce the mitochondrial enzyme reductase which can reduce the yellow water-soluble substrate, MTT, into a purple formazan crystal. This crystal is soluble in acidified isopropanol or dimethyl sulfoxide (DMSO). The purple color change can be measured at 570 nm with the use of a spectrophotometer, which gives a measure of cell viability.

Procedure: The *in-vitro* cytotoxicity of the fractions was performed on cancer (liver - HepG2 and leukemic - Jurkat) and normal (liver - Chang) cell lines. Cells in exponential growth were seeded into 96-well plates at a concentration of 10^4 cells/well. The cells were then treated with various concentrations of the fractions of *F. pumila* at a concentration range of 0-1000 µg/ml (in 1% DMSO). Negative control (untreated) experiment was included. Culture medium was used as blank.

A color control plate was also set up for each test extract. After 72 h of incubation at 37 ºC, under 5% CO_2, in the humidified atmosphere, 20 µL of 2.5 mg/mL of MTT was added to each well, and the plates were kept in the dark for 4 h. Subsequently, 150 µL of acidified isopropanol was added to stop the reaction and solubilize the formazan crystals formed. Absorbance readings were taken at 570 nm on a microplate reader after overnight incubation of the plates in the dark. Triplicate experiments were performed. Dose-response curves were plotted as percentages of cell viability against concentration.

$$\% \text{ Cell Viability} = [(ODT_0\text{-}ODT_1)/(ODU_0\text{-}ODU_1)] \times 100$$

Where ODT_0 is the average absorbance of wells treated with test extracts for all cell lines; ODT_1 is the average absorbance of wells with curcumin or test extract control; ODU_0 is the average absorbance of wells with untreated cells (negative control) for all cell lines; ODU_1 is the average absorbance of wells containing blank (culture media only). The inhibition concentration at 50% (IC_{50}) values, that is, the concentration of test substance that caused 50% inhibition of various cell lines were determined from the dose response curves by nonlinear regression analysis. The selectivity index (SI), a measure of cytotoxic selectivity, was calculated for the samples. This is the ratio of the IC_{50} values of each crude extract or fraction in the normal cell line (Chang liver) to IC_{50} values in the cancer cell lines (HepG2 and Jurkat). Samples with SI greater than 2 were considered to have a good selectivity towards cancer cells.

Data Analysis: Microsoft Excel Version 2010 was used for the calculation and plotting of mean and S.D estimates in the graph. Mean EC50 and IC50 values were compared by one way ANOVA using SPSS Version 16.0 and values with $p<0.05$ were considered statistically significant.

RESULTS:

Extract Yield: Six thousand grams of *Ficus pumila* dry powder yielded 89 g of crude ethanolic extract which is 1.483% yield. Sequential fractionation was done using 60 g of the crude ethanolic extract. In the fractions, the methanolic fraction gave a yield of 6.43% (3.809 g). However, petroleum ether gave an insignificant yield. The remaining residue after the entire fractionation step - the hydro fraction, was 56.191 g (93.66% yield).

Heavy Metal Analysis: Heavy metal analysis by Atomic Absorption Spectrophotometry (AAS) revealed that *Ficus pumila* raw powder contained 1.97 mg/l of Iron and 0.19 mg/l of Zinc as shown in **Table 1**. The extract also contained 0.92 mg/l of

Iron and 0.66 mg/l of zinc. Out of the six heavy metals analyzed in both the raw and the extracts of *F. pumila*, the levels of the rest of the metals; Copper, Cadmium, Nickel, and Lead, were below the detection limit (0.00001 mg/l). The content of iron and zinc were within acceptable limits as accepted by the WHO.

TABLE 1: HEAVY METAL CONTENT IN *F. PUMILA* RAW POWDER AND EXTRACT

Heavy Metal	Concentration in mg/l		
	Blank	*F. pumila* Raw	*F. pumila* Ethanolic Extract
Iron (Fe)	BDL	1.97 ± 0.11	0.92 ± 0.02
Copper (Cu)	BDL	BDL	BDL
Zinc (Zn)	BDL	1.19 ± 0.00	0.66 ± 0.02
Cadmium (Cd)	BDL	BDL	BDL
Nickel (Ni)	BDL	BDL	BDL
Lead (Pb)	BDL	BDL	BDL

BDL means below detection limit; Detection Limit – 0.00001mg/l

Total Phenolic Content: A linear plot of Gallic acid standard produced a straight line (y = 0.2435x + 0.0413, R^2=0.9967) as shown in **Fig. 1.** The total phenolic content of the three fractions was extrapolated from the gallic acid standard curve and was estimated as gallic acid equivalent (GAE); [Phenolic] mg GAE/100g as shown in **Fig. 2**.

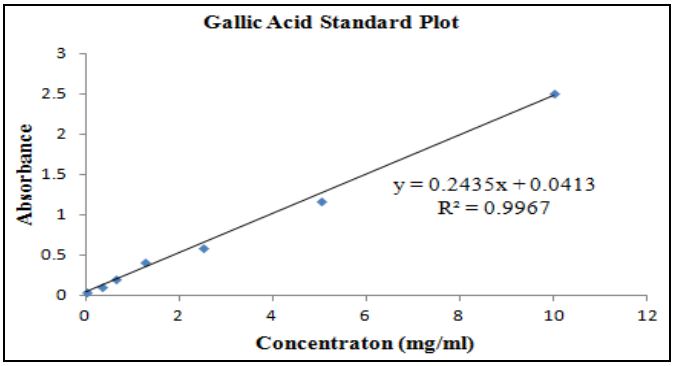

FIG. 1: STANDARD CONCENTRATION CURVE OF ABSORBANCE AGAINST CONCENTRATION OF STANDARD GALLIC ACID

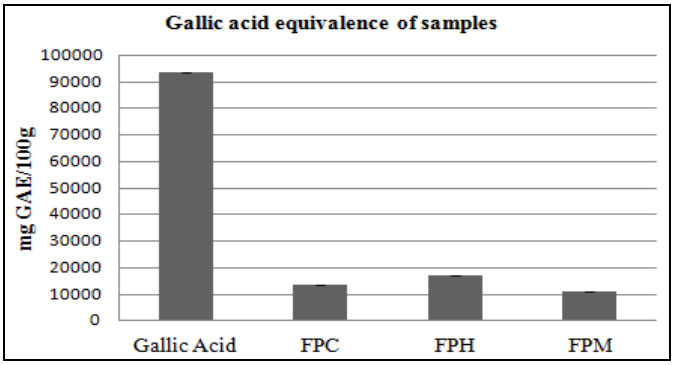

FIG. 2: TOTAL PHENOLIC CONTENTS (mg/ml) IN THE VARIOUS *F. PUMILA* FRACTIONS AGAINST GALLIC ACID STANDARD. FPC- *Ficus pumila* ethanolic crude extract, FPH - *Ficus pumila* hydro fraction, FPM – *Ficus pumila* methanolic fraction. Each bar represents a mean ± standard deviation, n=3, p<0.0001 between extracts and Gallic acid.

The hydro fraction recorded the highest total phenolic content (0.85 ± 0.02 mg/ml). This was followed by the crude ethanolic extract (0.69 ± 0.00 mg/ml) and finally, methanolic fraction (0.57 ± 0.04 mg/ml). In terms of Gallic acid equivalence, the total phenolic content in the hydro fraction was found to be 16950 ± 331.95 mg GAE/100 g. The crude ethanolic extract and methanolic fraction recorded Gallic acid equivalence of 13883.6 ± 47.42 and 11310.1± 716.041 mg GAE/100 g, respectively (p<0.001).

***In-vitro* Antioxidant Assay: Table 2** shows the comparison of the EC_{50} values of DPPH scavenging activities of the extract and fractions of *Ficus pumila*.

TABLE 2: ANTIOXIDANT ACTIVITIES OF ASCORBIC ACID AND EXTRACTS OF *F. PUMILA*

Sample / Standard	EC_{50} Value (mg/ml) n=3	P value
Ascorbic Acid (Standard)	0.0852 ± 0.0037	
Crude ethanolic extract	0.1722 ± 0.0319	0.016
Hydro fraction	0.0900 ± 0.0006	0.996
Methanolic fraction	0.4170 ± 0.0420	0.000

All fractions reduced DPPH to diphenyl-picrylhydrazine and diminished the absorbance at 517 nm. The results showed that the hydro fraction scavenge DPPH radical more strongly (EC_{50} = 0.09 ± 0.0006 mg/ml) compared to the other fractions, with the methanolic fraction having the least antioxidant activity (EC_{50} = 0.417 ± 0.042 mg/ml), and all followed a concentration-dependent pattern compared to the positive control (Ascorbic acid). The EC_{50} value is the effective concentration at which 50% of free radicals are scavenged. The smaller the EC_{50} value, the better the antioxidant

effect of the fraction. Generally, the difference between the various EC_{50} values for the different extract was statistically different (p=0.002, n=3).

FTIR Spectroscopic Analysis: The Fourier Transform Infrared Spectroscopy (FTIR) analysis was done to determine the functional groups of the active components of *F. pumila* fractions based on peak value in the region of infrared radiation. The results of FTIR analysis of *F. pumila* is shown below.

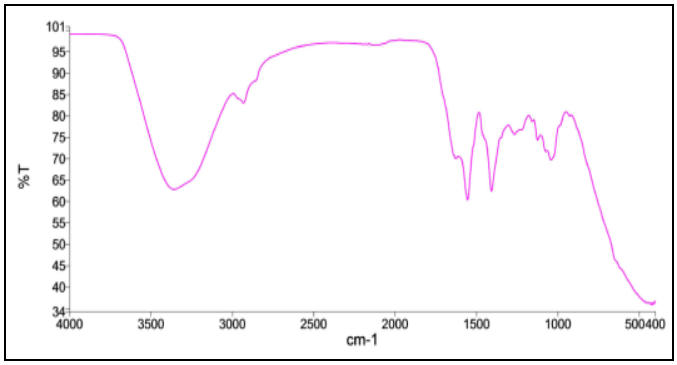

FIG. 3: FT-IR SPECTRA OF METHANOLIC FRACTION OF *F. PUMILA*

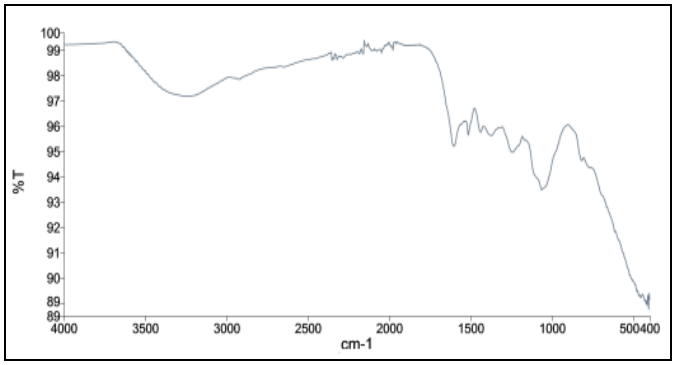

FIG. 4: FT-IR SPECTRA OF HYDRO FRACTION OF *F. PUMILA*

TABLE 3: FTIR PEAK VALUES OF METHANOLIC FRACTION OF *F. PUMILA*

Peak	X(cm^{-1})	Y (%T)	Bond	Functional Group
1	3367.89	62.9	N–H stretch	1°, 2° amines, amides
2	2934.06	83.11	C–H stretch	Alkanes
3	2171.8	96.87	–C≡C– stretch	Alkynes
4	2122.98	96.68	–C≡C– stretch	Alkynes
5	1626.79	70.08	N–H bend	1° amine
6	1554.33	60.4	Unknown	Unknown
7	1407	62.4	C–C stretch (in–ring)	Aromatics
8	1266.73	75.82	C–H wag (–CH_2X)	Alkyl halides
9	1224.26	76.78	C–N stretch	Aliphatic Amines
10	1156.46	78.71	C–H wag (–CH_2X)	Alkyl halides
11	1123.09	74.46	C–N stretch	Aliphatic Amines
12	1072.14	71.82	C–N stretch	Aliphatic Amines
13	1041.86	69.8	C–N stretch	Aliphatic Amines
14	925.99	80.07	O–H bend	Carboxylic acids
15	439.13	36.14	Unknown	Unknown
16	423.39	35.87	Unknown	Unknown
17	415.96	35.84	Unknown	Unknown
18	403.88	36.03	Unknown	Unknown

TABLE 4: FTIR PEAK VALUES OF HYDRO FRACTION OF *F. PUMILA*

Peak	X(cm^{-1})	Y (%T)	Bond	Functional Group
1	3241.04	97.21	O–H stretch, H–bonded	Alcohols, phenols
2	2355.9	98.63	Unknown	Unknown
3	2323.94	98.66	Unknown	Unknown
4	2162.57	98.89	–C≡C– stretch	Alkynes
5	2143.91	99.18	–C≡C– stretch	Alkynes
6	2050.34	98.93	Unknown	Unknown
7	1980.25	99.04	Unknown	Unknown
8	1603.29	95.23	Unknown	Unknown
9	1516.13	95.68	N–O asymmetric stretch	Nitro compounds
10	1440.08	95.79	C–C stretch (in–ring)	Aromatics
11	1374.09	95.65	Unknown	Unknown
12	1243.56	94.99	C–N stretch	Aliphatic Amines
13	1063.96	93.5	C–N stretch	Aliphatic Amines
14	818.98	94.65	C–Cl stretch	Alkyl halides and Alkenes
15	454.99	89.22	Unknown	Unknown
16	416.33	88.93	Unknown	Unknown
17	406.93	88.77	Unknown	Unknown

Table 3 and **4** show the functional groups present in the *F. pumila* methanolic and hydro fractions, respectively, as well as the nature of their bonds. The frequencies and nature of the various peaks were used to identify the various functional groups by comparing the values with standard values.

***In-vitro* Cytotoxicity Assay:**

MTT Assay: The anti-proliferative effect of the ethanolic extract (FPC) and fractions (FPM and FPH) were evaluated using the MTT assay against two cancer cell lines (Jurkat and HepG2) and a normal cell line (Chang's liver). The samples exhibited a cytotoxic effect against the cancer cell lines as shown in **Fig. 5**, **6** and **7**.

Their cytotoxic effects were evaluated by calculating the IC_{50} values and compared to the standard curcumin. The smaller this value, the stronger the cytotoxic effect.

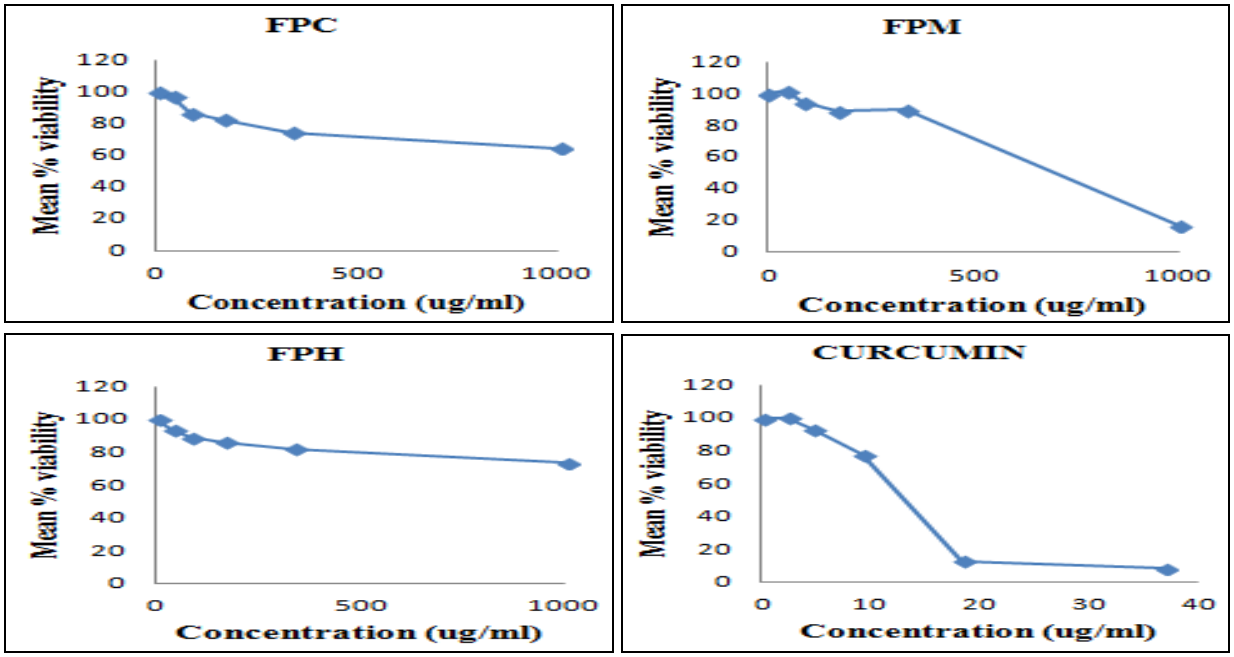

FIG. 5: CELL VIABILITY CURVES SHOWING CYTOTOXICITY EFFECT OF VARIOUS SAMPLES ON CHANG LIVER CELL LINES

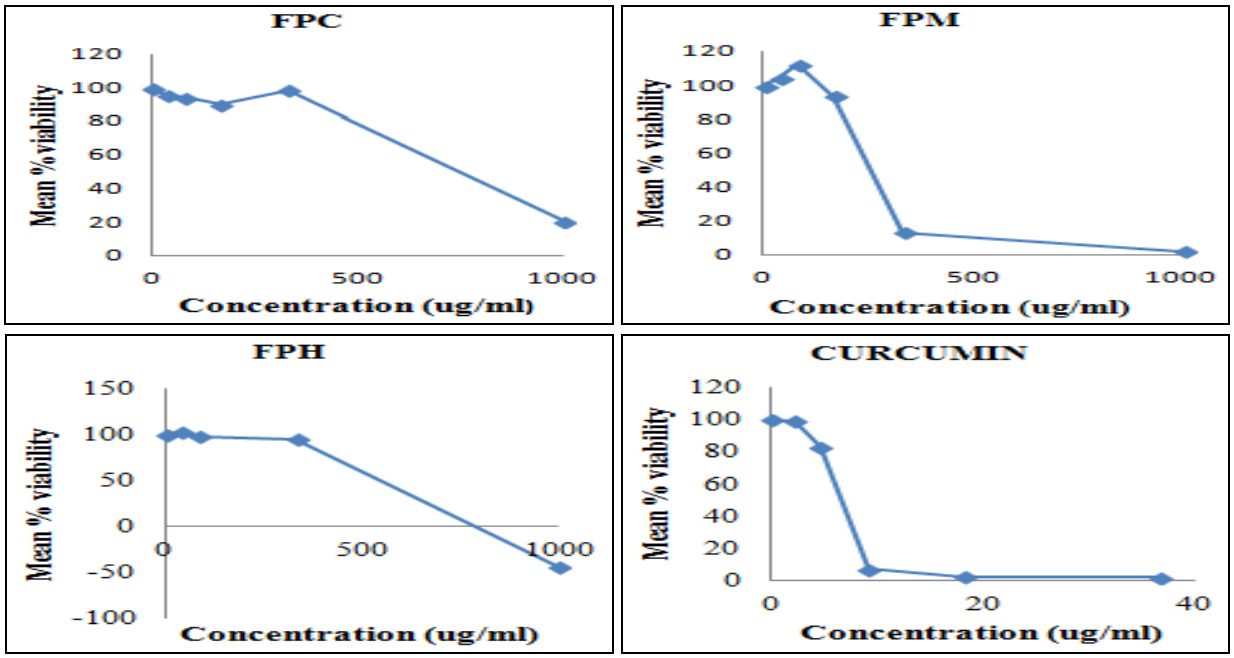

FIG. 6: CELL VIABILITY CURVES SHOWING CYTOTOXICITY EFFECT OF VARIOUS SAMPLES ON JURKAT CELL LINES

The ethanolic crude extract (FPC) and a hydro fraction (FPH) were seen to decrease the cell viability to a lesser extent as concentration increased compared to standard curcumin as seen in **Fig. 5**. Methanolic fraction (FPM), however, decreased the viability of the cells, hence killing 50% of the cells at a concentration of 700.19 µg/ml. The standard curcumin was also cytotoxic against the cells with an IC_{50} value of 13.18 µg/ml. The difference between the IC_{50} values of the various samples was statistically significant ($p<0.05$).

All the samples were cytotoxic against the Jurkat cell lines **Fig. 6**. The methanolic fraction showed the highest cytotoxicity, with IC_{50} of 248.10 ± 0.79 µg/ml. This was followed by the hydro fraction with IC_{50} of 546.15 ± 6.98 µg/ml. The crude ethanolic extract gave an IC_{50} value of 749.07 ± 27.2 µg/ml. The standard curcumin was also cytotoxic against the cells with an IC_{50} value of 6.61 ± 0.44 µg/ml. The difference between the IC_{50} values of the various samples was statistically significant ($p<0.01$).

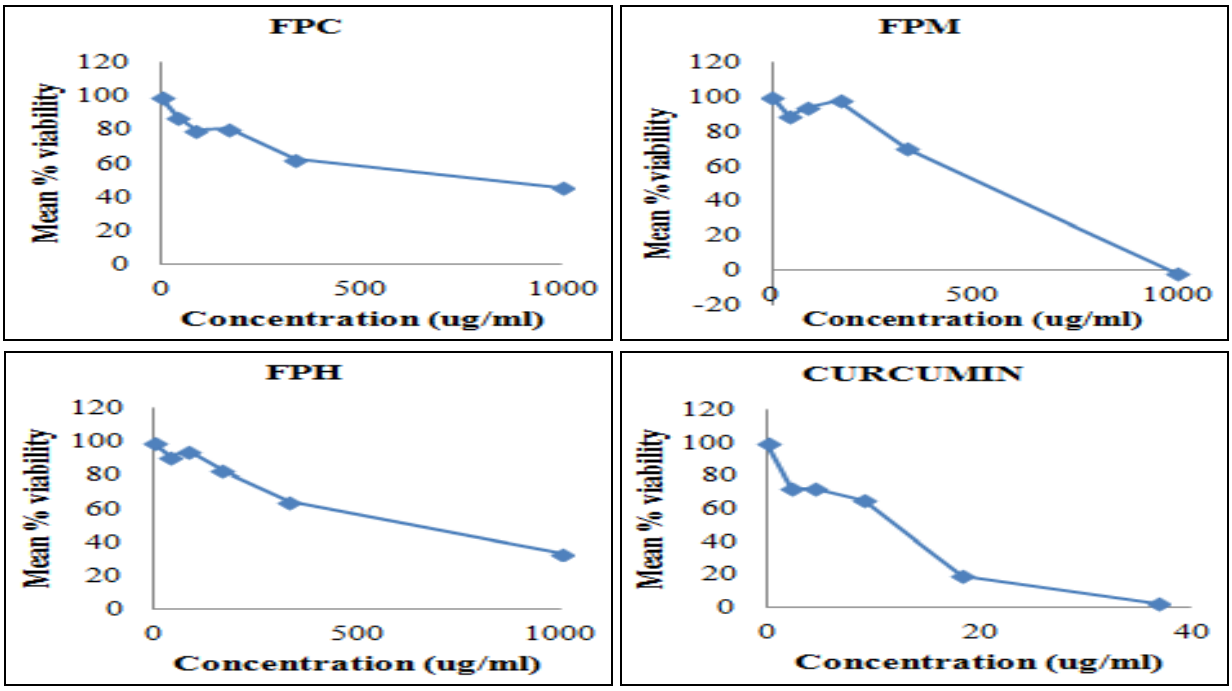

FIG. 7: CELL VIABILITY CURVES SHOWING CYTOTOXICITY EFFECT OF VARIOUS SAMPLES AGAINST HEPG2 CELL LINES

All the samples were cytotoxic against the HepG2 cell lines **Fig. 7**. The methanolic fraction showed the highest cytotoxicity, with IC_{50} of 515.86 ± 20.81 µg/ml. This was followed by the hydro fraction with IC_{50} of 621.77 ± 45.92 µg/ml. The crude ethanolic extract gave an IC_{50} value of 705.53 ± 65.47 µg/ml. The standard curcumin was also cytotoxic against the cells with an IC_{50} value of 12.28 ± 0.70 µg/ml. The difference between the IC_{50} values of the various samples was statistically significant ($p<0.01$).

TABLE 5: COMPARISON OF IC_{50} VALUES OF SAMPLES TESTED ON THE VARIOUS CELL LINES

	IC_{50} (µg/ml)		
Sample	**Chang**	**Jurkat**	**HepG2**
Crude ethanolic Extract	>1000	749.07 ± 27.2	705.53 ± 65.47
Hydro fraction	>1000	546.15 ±6.98	621.77 ± 45.92
Methanolic Fraction	700.19 ± 20.28	248.10 ± 0.79	515.86 ± 20.81
Curcumin	13.18 ± 0.35	6.61 ± 0.44	12.28 ± 0.70
P value	0.00	0.00	0.00

The p-value represents the comparison between the IC_{50} values of one sample among the three cell lines.

Selectivity Indices: SI of the various samples against the cancer cell lines were calculated by dividing the IC_{50} value of the normal cells by that of the cancerous cells. The SI values of the various samples are shown in **Table 6**.

TABLE 6: SELECTIVITY INDICES OF THE CRUDE ETHANOLIC EXTRACT, FRACTIONS, AND STANDARD

	Selectivity Index	
	JURKAT	HEPG2
Ethanolic Extract	1.335	1.417
Hydro Fraction	1.832	1.608
Methanolic Fraction	2.822	1.357
Curcumin	1.994	1.074

Qualitative Phytochemical Analysis: The phytochemicals present in the crude ethanolic extract of *F. pumila* are presented in **Table 7**.

GC-MS Analysis of Fractions: The Gas chromatography-mass spectrophotometric analysis was carried out on the *Ficus pumila* hydro fraction and methanolic fraction. **Fig. 8** and **9** show the total ion chromatogram for the fraction which sums up intensities of all mass spectral peaks.

TABLE 7 PHYTOCHEMICAL CONSTITUENTS OF *F. PUMILA* CRUDE ETHANOLIC EXTRACT

Phytochemical	Crude ethanolic extract
Alkaloid	+
Terpenoids	+
Flavonoid	+
Cardiac glycoside	+
Sterols	-
Saponins	+
Tannins	+

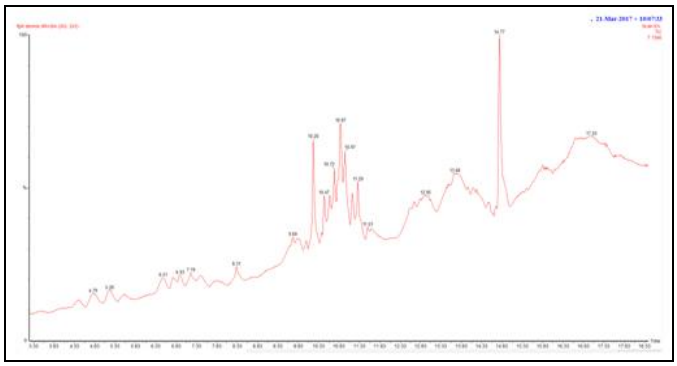

FIG. 8: TOTAL ION CHROMATOGRAM (TIC) OF *FICUS PUMILA* HYDRO FRACTION

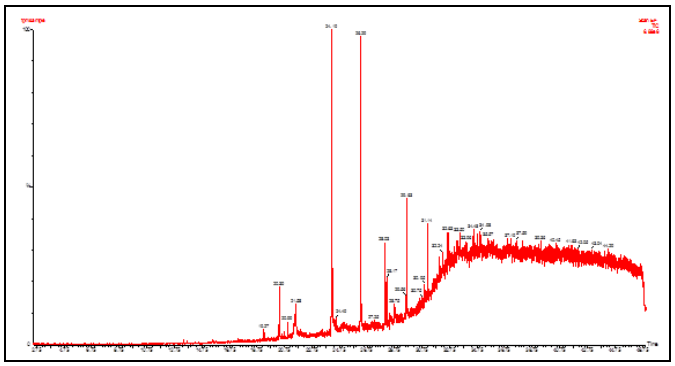

FIG. 9: TIC OF *FICUS PUMILA* METHANOLIC FRACTION

The active compounds with their retention time (RT), molecular formula and molecular weight (MW) in the hydro fraction of *F. pumila* are presented in **Table 8**. Six compounds were identified.

TABLE 8: COMPOUNDS PRESENT IN THE HYDRO FRACTION OF *FICUS PUMILA*

Peak	RT	Compound name	Molecular formula	MW
1	8.31	Phenol, 2,4 -bis(1,1-Dimethylethyl)-	$C_{14}H_{22}O$	206
2	10.20	2,4,4,6,6,8,8-Heptamethyl-1-nonene	$C_{16}H_{32}$	224
3	10.47	1-Hexadecanol, 3,7,11,15-tetramethyl-	$C_{20}H_{42}O$	298
4	10.72	1-Decanol, 2-hexyl-	$C_{16}H_{34}O$	242
5	10.72	Nonadecylheptafluorobutyrate	$C_{23}H_{39}O_2F_7$	480
6	10.87	Heptacosylheptafluorobutyrate	$C_{31}H_{55}O_2F_7$	592
7	14.77	2,4,4,6,6,8,8-Heptamethyl-1-nonene	$C_{16}H_{32}$	224

TABLE 9: COMPOUNDS PRESENT IN THE METHANOLIC FRACTION OF *FICUS PUMILA*

Peak	RT	Compound name	Molecular formula	MW
1	19.27	Dodecane, 2, 6, 10- trimethyl-	$C_{15}H_{32}$	212
2	20.39	Benzene, 1,3-bis (1,1-dimethylethyl)-	$C_{14}H_{22}$	190
3	20.99	Sulfurous acid, pentyl undecyl ester	$C_{16}H_{34}O_3S$	306

DISCUSSION: The presence of heavy metals such as Iron (Fe), Copper (Cu), Zinc (Zn), Cadmium (Cd), Nickel (Ni) and Lead (Pb) in medicinal plants can pose some health risks on individuals, although some of these metals serve as micronutrients which are important for the proper functioning of some vital organs in the human body. Iron, for instance, is a component of the hemoglobin and other compounds that are used in respiration [8, 9]. Copper and zinc are also very essential in the body. They play important roles biochemically and physiologically in maintaining good health throughout life [10]. Excessive concentrations of these metals in foods and medicines, however, is associated with several diseases, most especially diseases of the cardiovascular, nervous, renal and

skeletal systems [11]. It is therefore vital to have good quality medicinal plants to protect consumers from contamination.

Even though a lot of phytochemical and bioactivity studies have been carried out on many medicinal plants in Ghana, not much has been reported on the heavy metal contents of these plants [10]. This study, therefore, sought to establish the presence and quantity of six heavy metals (Iron, copper, zinc, nickel, cadmium, and lead) in *F. pumila* raw powder and crude ethanolic extract. The results revealed the highest concentration of Iron in the raw powdered leaf sample (1.97 ± 0.11 mg/l) and the ethanolic extract (0.92 ± 0.02 mg/l) but at a lower concentration. Zinc was also detected in both the raw powder (0.19 ± 0.00 mg/l) and extract (0.66 ± 0.02) of *F. pumila* as indicated in **Table 1**. The permissible limit of iron set by FAO/WHO [12] in edible plants is 20 ppm (20 mg/l) whiles that of zinc 27.4 mg/l, thus these levels in plant materials were below maximum permissible limits. This means that, concerning iron and zinc levels, the plant extract and raw powder will not cause any negative effect resulting from excess iron and zinc, when taken in as medicine.

The remaining heavy metals (copper, nickel, cadmium, and lead) were all below the detection limit of the atomic absorption spectrophotometer. These values indicate that the *Ficus pumila* powder and ethanolic extract from the Bomso clinic can be consumed without any effect resulting from these heavy metals that were screened. *Ficus pumila* has also been reported safe for use according to the toxicity study conducted by Larbie *et al.* [4] In this study, the median acute toxicity (LD_{50}) values of ethanolic extract were determined to be <5 g/kg body weight in mice. Observations after sub-chronic toxicity included hypolipidaemia, increased ALP at higher doses and normal creatine and urea levels. The extract did not produce any toxic effect on vital organs except decreases in uterus weight in female rats and hence, considered safe at moderate doses [4].

At low or moderate concentrations, reactive oxygen species (ROS) and reactive nitrogen species (RNS) play vital roles in the physiological functions of the human body, despite their harmful effects. Some of these roles include signal transduction pathway, defense against infectious agents, smooth muscle relaxation and cell growth [13]. When these free radicals accumulate in the body, however, they lead to a phenomenon known as oxidative stress. Oxidative stress refers to an imbalance between antioxidants and oxidants in favor of the oxidants, which potentially leads to the damage of cells [14]. The occurrence of oxidative stress eventually leads to numerous deteriorating effects to the cellular biomolecules which include lipid peroxidation, DNA damage, protein degradation, tissue injury, among others. Free radicals are therefore known for their contribution to diverse forms of diseases such as arthritis, atherosclerosis, neurodegenerative disorders and aging [15].

Additionally, *Ficus* species have also been reported as rich sources of naturally occurring antioxidants and play very vital roles in preventing several diseases that are related to oxidative stress such as cardiovascular diseases and cancer. Due to their strong antioxidant properties, *Ficus species* are also known to diffuse the toxic free radicals such as Hydroxyl radicals and hence, can be used in nutraceutical and biopharmaceutical industries and can also be used as a possible food additive [14]. Preliminary work done on the antioxidant effect of *Ficus pumila* ethanolic crude extract revealed a strong antioxidant effect (EC_{50} = 0.07 mg/ml; [5]). This current study sought to evaluate the antioxidant effect of *Ficus pumila* fractions compared to standard, L-Ascorbic acid.

The results of the DPPH assay showed that *F. pumila* hydro fraction had the highest antioxidant activity with an EC_{50} value of 0.09 ± 0.00 mg/ml. There was a difference between the EC_{50} values of the hydro fraction and the standard Ascorbic acid (EC_{50} = 0.0852 ± 0.0037 mg/ml). *F. pumila* methanolic fraction had the lowest antioxidant effect (EC_{50} = 0.42 ± 0.04 mg/ml) among the three fractions. Even though methanolic fraction had the least EC_{50} value, it still possessed good antioxidant activity.

Results from the antioxidant activity suggest that the extract and fractions could play a very vital role as health protecting factors. They could do so by scavenging free radicals from the body. There is scientific evidence that suggests that antioxidants reduce the risk for chronic diseases which includes

heart diseases and cancer [16] and hence *Ficus pumila* could help in the prevention of these diseases.

The total phenolic content was carried out based on the absorbance values of the various solutions of the fractions which reacted with Folin-Ciocalteu reagent and compared with the standard solutions of gallic acid. The data from the total phenolic content support the key role of phenolic compounds in the antioxidant activity of the plant. Phenolic compounds were present in all the fractions. Hydro fraction recorded the highest total phenolic contents, and methanolic fraction recorded the least. There are lots of studies that have reported a positive correlation between total phenolic contents of various plants extracts and their antioxidant activities. For instance, Hossain *et al*., [17] reported a positive relationship between antioxidant potential and the amount of phenolic compounds. Another study conducted by Larbie *et al*., [5] also confirms this correlation. This current study also confirms the fact that hydro fraction, which had the highest total phenolic content, had the highest antioxidant activity with the least EC_{50} value. This was followed by the crude ethanolic extract and then methanolic fraction concerning the EC_{50} values calculated.

Fruits and vegetables are the predominant sources of antioxidant vitamins (vitamin A, C, and E), which act as free radical scavengers, making these foods essential to human health. However, more than 80% of the total antioxidant activity in fruits and vegetables come from the ingredients other than antioxidant vitamins, indicating the presence of other potentially important antioxidants in these foods. Examples of these ingredients are total phenols, flavonoids, alkaloids, glutathione, among others. Therefore, the presence of some of these ingredients could give rise to the antioxidant effect observed in the fractions and not only due to the presence of the phenolic compounds in the fractions.

Fourier Transform Infrared (FTIR) Spectroscopy is a high-resolution analytical technique which is used in the identification of bioactive chemical constituents of compounds. It is also used to reveal the structure of compounds. In this technique, molecules such as organic compounds show absorption in a characteristic range of frequency mainly in the range of 4000-400 cm^{-1} which play a key role in the study of these compounds [18]. FTIR is perhaps the most powerful tool used for the identification of the functional groups and the types of chemical bonds present in compounds. The wavelength of light that is absorbed is characteristic of the chemical bond which is observed in the annotated spectrum. The chemical bond in a molecule can be determined by the interpretation of the infrared absorption spectrum [19, 20].

The **Fig. 7** and **8** show the FTIR spectrum of the various fractions and their interpretations are shown in **Table 3** and **4**. The FTIR spectroscopic studies of *F. pumila* methanolic fraction revealed 18 characteristic peak values with various functional compounds in the fractions. This analysis revealed the presence of primary and secondary amides, alkanes, alkynes, alkyl halides, aromatics, aliphatic amines, and carboxylic acid. Alcohols, phenols, alkynes, alkenes, aromatics, nitro compounds, aliphatic amines, and alkyl halides were also shown in the hydro fraction of *F. pumila*, which revealed 17 peaks. Alcohols and phenols were only observed in the hydro fraction, with a very high transmittance value, and this shows the relatively higher total phenolic contents observed in this fraction by the Folin-Ciocalteu assay. However, aromatic compounds were observed in the methanolic fraction, but with a relatively lower transmittance value. These aromatic compounds could include phenols, and this could account for the presence of the total phenols observed in the Folin-Ciocalteu assay of these fractions.

This current work sought to determine the cytotoxicity effect of the crude ethanolic extract of *Ficus pumila* as well as its fractions - methanolic and hydro, with curcumin as standard on cancer and normal cells. The results revealed that the crude extract and hydro fraction had no cytotoxic effect on the normal cells - Chang cell. However, the methanolic fraction and standard curcumin decreased the viability of the normal cells with IC_{50} values 700.19 ± 20.28 and 13.18 ± 0.35 µg/ml, respectively. Comparing the methanolic fraction to the curcumin, it was observed that the curcumin was highly toxic to the normal cells than the methanolic fraction.

All the samples showed some level of cytotoxicity on the cancerous cell lines. The methanolic fraction gave an IC_{50} value of 248.10 ± 0.79 µg/ml while the hydro fraction also gave an IC_{50} value of 546.15 ± 6.98 µg/ml. This shows that though the two fractions are moderately cytotoxic against the Jurkat cells, the methanolic fraction had a better activity. Comparing the cytotoxicity effect of the crude ethanolic extract and fractions against the standard curcumin, it was observed that the differences were statistically significant. A similar trend was observed with the HepG2 cells. All the samples were cytotoxic against the HepG2 cell lines. The methanolic fraction recorded the highest cytotoxicity against the HepG2 cells (IC_{50} = 515.86 ± 20.81 µg/ml); hydro (621.77 ± 45.92 µg/ml) and crude ethanolic extract (705.53 ± 65.47 µg/ml). All the samples had statistically significant cytotoxicity effect on the three cell lines as shown in **Table 5**.

Overall, the samples (crude ethanolic extract and fractions) had varying cytotoxic effects on the cancer cell lines. However, to be named as an anticancer agent, the sample should show good selectivity (SI > 2) for cancer cells only [21]. This SI value means that the sample is twice more cytotoxic to the cancer cell line as compared with the normal cell line. From the results, it was observed that only the methanolic fraction was selective towards the Jurkat cell line (SI = 2.822). The remaining fraction had an SI value less than 2 and hence can be said to be non-selective towards the cancer cell lines, even though they all exhibited a cytotoxic effect on the cancerous cell lines.

From the result, the Jurkat cell line showed the highest sensitivity toward all the samples. Ethanolic extract of *F. pumila* was shown to possess anti-proliferative effect against Jurkat, CEM, and HL-60 leukemic cell lines in a preliminary work done by Larbie *et al*. [5] This study has confirmed the anti-leukemic effect of the crude ethanolic extract and has also revealed that this property is inherent in the methanolic fraction. Phytochemicals present in *F. pumila* could be the contributing factor for the observed cytotoxic activity. According to Bhandari *et al*., [22] phytochemicals such as flavonoids, steroids, and terpenoids have been shown to have anti-cancer potential, and these phytochemicals could be abundant in the methanolic fraction.

The GC-MS analysis revealed a variety of compounds with some medicinal values. For instance, Phenol-2,4-bis (1,1-dimethylethyl) that was found in the hydro fraction of *F. pumila*, possesses some therapeutic effect like anti-arthritic, anti-inflammatory, and anti-hyperlipidemic activities as reported by Vinjamuri and Sharad [23]. 1-Hexadecanol-3, 7, 11, 15-tetramethyl- which was present in *F. pumila* hydro fraction also possesses anti-mycobacterial activity [24]. These activities could be as a result of their antioxidant activities; hence the higher antioxidant activity observed in the hydro fraction in the DPPH assay. The methanolic fraction contained Dodecane, 2, 6, 10-trimethyl-, Benzene, 1,3-bis (1,1-dimethylethyl)- and Sulfurous acid, which could be responsible for the cytotoxic activity observed.

CONCLUSION: *Ficus pumila* crude ethanolic extract and raw powder contain iron and zinc whose concentrations were below the permissible level proposed by FAO/WHO for medicinal plants and hence, with respect to iron and zinc levels, the plant extract and raw powder will not cause any negative effect resulting from excess iron and zinc, when taken in as medicine. *Ficus pumila* crude ethanolic extract also contains phytochemicals like tannins, flavonoids, terpenoids, alkaloids, and saponins which provides it with medicinal properties. The hydro fraction of *F. pumila* had the highest total phenolic content and antioxidant effect, and hence, can be used to prevent the occurrence of diseases associated with oxidative stress like cardiovascular diseases and cancers. The methanolic fraction had the highest cytotoxicity effect and was highly selective for Jurkat cell lines. This cytotoxic selectivity between the Jurkat cells and normal (Chang's liver) cell lines increases the prospect that this plant contains a compound(s) which could serve as leads for novel anticancer drugs. The most active fraction in terms of the antioxidant assay was the hydro fraction and with respect to the cytotoxicity assay was the methanolic fraction.

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