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Phytochemical profiling (GC-MS and HPLC) and cytotoxicity evaluation of methanol leaf extract of *Luffa cylindrica* on human colon cell lines (HT-29 and HCT 116)

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Abstract
Phytochemicals present in *Luffa cylindrica* (L.) M. Roem plant may be responsible for its cytotoxicity activity against cancer cell lines. The present study investigated the phytoconstituents present in *Luffa cylindrica* methanolic leaf extract and evaluates its cytotoxicity activity on colorectal cell lines using MTT assay method. Gas chromatography- mass spectrometry (GC-MS) and High performance liquid chromatography (HPLC) methods were used to identify the phytochemical constituents of *Luffa cylindrica* methanol leaf extract. GC-MS analysis result revealed the presence of various volatile organic compounds ranging from alcohols, triterpenes, phenols, fatty acids, esters, and steroids. A total number of 18 compounds were identified with GC-MS while HPLC analysis identified 12 different phytochemicals, some of which are known to possess cytotoxicity activities both *in vitro* and *in vivo*. The plant extract showed a dose dependent cytotoxicity activity against the tested cell lines. The result from this finding shows that methanol leaf extract of *Luffa cylindrica* possess anticancer properties which could be further investigated for the development of cancer therapy.

Keywords: *Luffa cylindrica*, GC-MS analysis, HPLC analysis, MTT assay, cytotoxicity, colon cancer

1. Introduction
Cancer is a broad term used to encompass several malignant diseases ravaging different parts of the body. The hallmark of cancer is uncontrolled cellular growth with subsequent cell invasion and organ metastasis which is the major causes of most cancer related mortality.
Colorectal cancer, is the third most commonly diagnosed cancer worldwide with nearly 1.4 million new cases and about 693,900 deaths reported in 2012 [1]. In Nigeria, the common types of cancer affecting people are the cancer of the breast, prostate, cervix, colon and liver [2]. Till date, the conventional treatments available for cancer are chemotherapy, radiotherapy and surgery which are quite expensive with array of undesirable side effects. This has made the search for new natural agents with no or relatively low side effects imperative.
Over time plants have proven to be highly effective in treatment and management of several human diseases including cancer. In recent time, much attention has been given to natural products with diverse pharmacological properties that could be employed in the prevention and treatment of various diseases including cancer. Many plants-derived compounds today have been identified to possess anti-tumor properties, for example, induction of apoptosis and inhibition of cell proliferation which ultimately reduce the risk of cancer [3, 4].
Luffa cylindrica, otherwise known as sponge gourd is a cucurbit fibrous plant which grows as a flowering annual vine with pollinated flowers developing into cylindrical green fruits. Other members of the family include cucumis sativus (cucumber), pumpkins, snake gourd, ridge gourd, bitter apple and watermelon. Cucurbits are edible crops, belonging to the family Cucurbitaceae. They are climbers with reserves in roots and are distributed mainly in tropical zones. *Luffa cylindrica* is grown in Africa, Asia and other parts of the globe. In Nigeria, it is used for medicinal purposes; nearly all parts of the plant are used traditionally in the treatment and prevention of array of diseases and disorders including asthma, diabetics, convulsions, intestinal disorders, emetics, snake bite, sinusitis among others. In the present study, *Luffa cylindrica* methanol leaf extract (LCMLE) was investigated for its phytoconstituents and cytotoxicity effect on colon cancer cell lines.

2. Materials and Methods
2.1 Plant material collection and extract preparation
The fresh leaves of *Luffa cylindrica* were collected from farm land in Oba-Ile Akure, Ondo state, Nigeria. The plant was identified and authenticated by a taxonomist, Mr. Omomoh B.E

of the Department of crop Soil and Pest, Federal University of Technology Akure, Ondo State, Nigeria. A voucher specimen (0252) of the identified and authenticated *Luffa cylindrica* leaves and fruits were deposited at the herbarium of the same department.

The collected fresh leaves of *Luffa cylindrica* were thoroughly washed, air dried and powdered using electric grinder and soaked in methanol for 72 h at room temperature after which was filtered, and the filtrate was concentrated to dryness using rotary evaporator (Büchi Rotavapor R-200). The resulting crude extract was weighed and stored in an air tight container for phytochemical profiling and cytotoxicity studies in cell model.

2.2 GS-MS analysis

Analysis was done using a Varian 3800 gas chromatograph equipped with a Agilent MS capillary column (30 m × 0.25 mm i.d) connected to a Varian 4000 mass spectrometer operating in the EI mode (70 eV; m/z 1 – 1000; source temperature 230°C and a quadruple temperature 150°C). The column temperature was initially maintained at 200°C for 2 min, increased to 300°C at 4°C/min, and maintained for 20 min at 300°C. The carrier gas was Nitrogen at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 300°C with a split ratio of 50:1. A sample volume of 1µL in chloroform was injected using a split mode, with the split ratio of 50:1. The mass spectrometer was set to scan in the range of m/z 1-1000 with electron impact (EI) mode of ionization.

Analysis of the sample was carried out with a runtime of 34.00 minutes. Using computer searches on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC – MS compounds present in the sample were identified.

2.3 HPLC analysis

The extract (10 mg) was dissolved in 10 ml (v/v) aqueous acetonitrile and was mixed vigorously for 30 min. After mixing, the aqueous end was run off while the organic solvent end was collected into a 25 ml standard flask. The analysis was performed on Shimadzu (Nexera MX) HPLC system fitted with u BONDAPAK C18 column (length 100 mm, diameter 4.6 mm, and thickness 7 µm). The mobile phase consisted of a mixture of an aqueous acetonitrile (Acetonitrile/Water, 70:30). Sample was extracted with Acetonitrile, the extract stabilized with Ethyl Acetate, put in 25ml Standard flask, and made up to the mark. 5ul injected @ 2ml/min flow rate. Compounds were detected by a UV detector (Diode Array Detector, DAD) at 254 nm. The retention times of the identified compounds were measured by a single standard solution at a concentration of 15.69 mg/g.

2.4 Cell lines and cell culture

Human colorectal adenocarcinoma cell line HT-29, and human colon carcinoma cell line HCT116 were used in this study. The cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured at 37^{oc} in a humidified incubator with CO_2 (5%) in Dulbecco Modified Eagle's Medium, supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin.

2.5 MTT assay

MTT assay is a quantitative rapid colorimetric assay for measuring cell growth, survival and cell proliferation ability of living cells. The principle of the assay is based on the reduction of MTT (yellow colored) and other tetrazolium dyes depends upon cellular metabolic activities due to NAD(P)H-dependent cellular oxidoreductase enzymes [5, 6]. MTT is cleaved by mitochondrial enzyme dehydrogenase of viable cells, yielding a measurable purple product formazan. The healthy and rapidly growing cells exhibit high rates of MTT reduction to formazan ((E, Z)-5-(4, 5-dimethylthiazol-2-yl)-1,3-diphenylformazan) while the dead or inactive cells do not. The final product of MTT reduction is a purple color formazan that can be easily dissolved in DMSO. Viability in the MTT assay is connected with the quantification of formazan at visible wavelength on a spectrophotometer, which is linearly associated with the enzyme activity and indirectly with the number of viable cells. High purple color intensity denotes higher cell viability while the decrease in purple color intensity signifies the reduced cell number and thus, cytotoxicity of the given substance. Formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity.

2.5.1 Assay procedure

Cell culture with the concentration of 2 $x10^3$ cells/ml was plated (100 µl/well) onto 96-well plates. The diluted ranges of plant extract were added to each well with concentrations; 1, 5, 10, 20, 50, 100, and 500 µg/ml and incubated for 72 hr. The negative control was performed using the complete growth media while 5-fluorouracil (5-FU), a conventional chemotherapeutic agent was used as positive control. MTT solution was added by the end of incubation period and continued incubation for 3 hours after which the supernatant was removed. After solubilization of the purple formazan crystals using DMSO were completed, the Optical Density (OD) of the plant extract was measured using an ELISA reader at a wavelength of 570 nm. The percentage cell viability was calculated using the formula given below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance sample (mean)}}{\text{Absorbance control (mean)}} \text{ X } 100$$

After the determination of the percentage of cell viability, graphs were plotted with the percentage of cell viability against their respective concentrations and the IC_{50} value (drug and extract concentration causing 50% growth inhibition of the tumor cells) was deduced.

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation

3. Results and discussion

This present study sought to determine the phytoconstituents of *Luffa cylindrica* methanolic leaf extract and evaluates its *in vitro* cytotoxicity activity on HT-29 and HCT116 cells.

In the present study, 12 distinct peaks representing different chemical compounds were identified in methanolic leave extract of *Luffa cylindrica* using HPLC analysis as shown in Table 1. As represented in Fig 1, peaks representing P-coumaric acid, diosmetin, sapogenin 1, sapogenin 2 and aegyptinin A were the prominent peaks with sapogenin 1 being the highest peak. The identification of the compounds with GC MS as shown in Table 2 revealed that LCMLE consists of various volatile phytoconstituents ranging from alcohols, terpenes, phenols, fatty acids, esters, and steroids. The chromatogram was presented in Fig. 2. Most of the compounds (Squalene, Dodecanoic acid, 4(1H)-Pyrimidinone,

2, 6,-diamino, Tetradecanoic acid, Hexadecanoic acid, methyl ester, Phenol, 2,4-bis(1,1-dimethylethyl), Furaneol) identified with GC-MS analysis belong to the group of compounds that exhibit antioxidant properties [7]

Some of the identified compounds are unarguably known to possess antiproliferative and cytotoxic activities against various cancer cell lines. For examples, β- Sitosterol, a phytosterol with a chemical structure similar to cholesterol, echinocystic acid and squalene have been reported by several studies for their antiproliferative activities against different cell lines. β- Sitosterol is an important meal in the diet as its present in many oils from plants and vegetables. It is a well-known lipid-soluble cellular antioxidant and free radical scavenger which protects cellular integrity from various toxic moieties. Importance of β-sitosterol for proper cellular and biological functioning makes it an essential nutrient [8]. A study has demonstrated that β- sitosterol modulate the growth of estrogen- responsive breast cancer cells *in vitro* as well as in ovariectomized athymic mice and also down regulate the expression of antiapoptotic marker Bcl-2 while echinocystic acid has been reported for its anticancer properties against human breast cancer cell lines [9, 10]. Squalene, a hydrocarbon and a triterpene is a precursor for synthesis of all plant and animal sterols. It has been investigated to enhance the expression of anti-inflammatory enzymes by targeting pro and anti-inflammatory mediators and pathways to modulate over-activation of neutrophils, monocytes and macrophages [11]. Also diosmetin, an identified phytoconstituent of LCMLE is the aglycone of the flavonoid glycoside diosmin which occurs naturally in citrus fruits and certain legumes. Intestinal microflora enzymes usually hydrolyze diosmin to diosmetin which is ultimately absorbed into the body. Pharmacologically, diosmetin is reported to exhibit anticancer, antimicrobial, antioxidant, oestrogenic and anti-inflammatory activities. [12, 13, 14].

Various pharmacological activities of another major phytoconstituents of LCMLE, saponin and its aglycone sapogenin, have been reported ranging from immunostimulant, antiproliferative, antiangiogenesis and apoptotic effects [15, 16]. Saponins have also been shown to be effective in the treatment of various diseases including diabetes, obesity and osteoporosis [17, 18]. Ability to swell and ruptures red blood cells to release hemoglobin and causing frothing when mixed with warm water in test tube have been used to identify saponin. Also Phytol, an acyclic diterpene alcohol usually found in the essential oils of some plants most especially aromatic plants [19] has been reported in past studies for its antibacterial, antispasmodic, anticonvulsant, antioxidant and anticancer activities [20, 21, 22, 23].

Active principles extracted from medicinal plants is believed to produce better, specific, safe drugs and may reduce the risk of toxicity as well as maintain their therapeutic efficacy when put to use in clinical application [24].

Past studies have indicated that plants used in traditional medicine possess cytotoxic effect on different cancer cell lines as well as antiproliferative effect mainly because of various classes of phytoconstituents present in them. *Luffa cylindrica*, given its richness in phytoconstituents such as polyphenols and flavonoids, it has also been reported to possess anti-inflammatory, immunodulatory and antioxidant activities, which are all relevant in the pathogenesis of cancer. [25, 26] It is therefore pertinent to explore its cytotoxic effect to substantiate its folkloric use in the treatment of cancer, intestinal disorders and other diseases.

In this study, the crude extract showed a dose dependent cytotoxicity activity on the tested cell lines as shown in Tables 3 and 4. Cytotoxicity of the plant extract increased as the concentration increases. This trend was also observed for the synthetic drug 5-FU used as positive control.

The crude extract showed stronger activity on HCT 116 cells than HT-29; IC_{50} = 88.2, 94.2 (µg/ml) on HCT116 and HT-29 cell respectively while standard 5-FU showed the IC_{50} values of 4.2 and 3.9 on the respective cells. The summary of the IC_{50} results were presented in Table 5.

Luffa cylindrica methanol leaf extract inhibits the proliferation of HCT 116 and HT-29 human colon cancer cell lines in a dose-dependent manner, with greater effect on HCT116 cancer cells.

Antiproliferative/cytotoxicity activity of different fractions and different parts of *Luffa cylindrica* plant using various cell lines has been reported. The cytotoxic effect of the n-hexane, chloroform and ethyl acetate extracts of leaves of *Luffa cylindrica* was studied using brine shrimp lethality assay. All extracts showed considerable general toxicity towards brine shrimps. The LC_{50} values of the extracts were of 15.92 to 33.69 µg/ml compared to standard drug vincristine sulphate (LC50 = 0.91 µg/ml) [27].

The anticancer activity of the hot water extract of the whole *Luffa cylindrica* was studied using circulating tumor cells and cancer stem cells isolated from the peripheral blood of hepatocellular carcinoma patients *in vitro*. *Luffa cylindrica* hot water extract showed cytotoxic activity against circulating tumor cells of hepatocellular carcinoma especially the cells sub-population CD133+/CD44+ with little effect among CD133+/CD44- sub-population. It was therefore postulated that *Luffa cylindrica* hot water extract whole plant could possibly decrease the ratio of cancer stem cells in blood of hepatocellular carcinoma (HCC) patients as well as being used to minimize recurrence and metastasis [28].

The anticancer effects of the aqueous ethanol extract of *Luffa cylindrica* leaves were studied in different types of breast cancer cell lines representing different molecular subtypes of the disease. Cell cycle analysis and molecular analysis of apoptotic and proliferative markers showed that the ethanol extract of *Luffa cylindrica* leaves possessed anticancer effects. The major active constituents of the extract were identified as phenolic compound derivatives and saponin which may be responsible in part for the anticancer activity of the extract.[29]

Other pharmacological activities of *Luffa cylindrica* reported include hepatoprotective activity [30, 31], antimicrobial activity, [32, 33], antioxidant activity [34, 35] antiviral activity [36] and hypoglycemic activity [37] amongst others.

Table 1: HPLC analysis of *Luffa cylindrica* methanol leaf extract (LCMLE)

Component	Rt time	Area	Concentration
P-coumaric acid	1.266	1292.227	2.326034
Diosmetin	2.75	2858.627	5.145583
Luteolin	4.45	962.2385	1.732048
Apigenin	5.466	485.425	0.873774
Echinocystic acid	6.483	363.6485	0.654574
Gypsogenin lacto	7.33	137.003	0.246608
Lucyoside 1	7.95	167.036	0.300668
β-Sitosterol	9.35	140.297	0.252537
Sapogenin 1	11.05	9744.667	17.54059
Sapogenin 2	12.166	3876.375	6.97755
Aegyptinin A	13.7	3496.598	6.293944
Ginsenoside	17.616	525.702	0.946274

Table 2: GC-MS analysis of *Luffa cylindrica* methanol leaf extract (LCMLE)

	Compound	RT (min)	Peak area (%)	Formula	Mol. weight	% Composition
1	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	4.02	11.38	$C_6H_6O_4$	142	2.65
2	4H-Pyranone-2,3-dihydro-3,5-dihydroxy-dihydroxy-6-ethyl	4.08	4.92	$C_6H_8O_4$	144	1.42
3	4(1H)-Pyrimidinone,2,6,-diamino	5.17	3.69	$C_4H_6N_4O$	126	2.07
4	1,2,3-Propanetriol, mono acetate	5.63	4.74	$C_5H_{10}O_4$	134	2.12
5	Benzoic acid, 4-hydroxy-3,5-dimethoxy	6.00	2.15	$C_9H_{10}O_5$	198	0.58
6	Furaneol	7.01	1.23	$C_6H_8O_3$	128	2.25
7	Tetradecanoic acid	8.42	3.51	$C_{14}H_{28}O_2$	228	2.18
8	Phenol 2,4-bis(1,1-dimethylethyl)	10.00	0.80	$C_{14}H_{22}O$	206	3.12
9	Pentadecanoic acid	10.50	16.31	$C_{15}H_{30}O_2$	242	0.67
10	Hexadecenoic acid, Z-11	24.36	27.08	$C_{16}H_{30}O_2$	254	17.04
11	n-Heneicosane	25.00	0.30	$C_{21}H_{44}$	296	1.63
12	Octadecanoic acid	25.04	16.31	$C_{18}H_{36}O_2$	284	20.42
13	Phytol	25.15	2.46	$C_{20}H_{40}O$	296	
14	Hexadecanoic acid, methyl ester	27.83	12.00	$C_{17}H_{34}O_2$	270	21.21
15	9, 12,15-Octadecadienioc acid, methyl ester (Z,Z,Z)	28.09	3.08	$C_{19}H_{34}O_2$	292	3.38
16	Hexadecanoic acid, 2,3-dihydroxypropyl ester	28.50	2.15	$C_{19}H_{38}O_4$	330	
17	Squalene	32.00	0.92	$C_{30}H_{50}$	410	6.83
18	1-Eicosanol	32.49	2.15	$C_{20}H_{42}O$	298	4.21

Table 3: Optical Density value and percentage of cell viability of LCMLE and 5-FU exposed to HT-29 cells

	LCMLE		5-FU	
Concentration (µg/ml)	Absorbance (Mean ± SD)	% Viability	Absorbance (Mean ± SD)	% Viability
500	0.142 ± 0.012	12.55	0.241 ± 0.016	20.50
100	0.551 ± 0.030	48.68	0.244 ± 0.016	20.73
50	0.679 ± 0.080	59.92	0.269 ± 0.029	22.88
20	0.823 ± 0.024	72.67	0.311 ± 0.053	26.47
10	0.892 ± 0.033	78.74	0.339 ± 0.041	28.80
5	1.024 ± 0.033	90.43	0.408 ± 0.039	34.69
1	1.077 ± 0.156	95.10	0.655 ± 0.257	55.71
Control	1.133 ± 0.285	100	1.176 ± 0.138	100

Table 4: Optical Density value and percentage of cell viability of LCMLE and 5-FU exposed to HCT116 cells

	LCMLE		5-FU	
Concentration (µg/ml)	Absorbance (Mean ± SD)	% Viability	Absorbance (Mean ± SD)	% Viability
500	0.207 ± 0.018	12.25	0.158 ± 0.016	10.78
100	0.828 ± 0.057	48.90	0.167 ± 0.005	11.37
50	0.906 ± 0.022	53.58	0.170 ± 0.007	11.58
20	1.044 ± 0.026	61.75	0.172 ± 0.015	11.74
10	1.174 ± 0.074	69.39	0.194 ± 0.008	13.19
5	1.449 ± 0.089	85.66	0.411 ± 0.018	28.01
1	1.486 ± 0.120	87.86	0.819 ± 0.042	55.83
Control	1.691 ± 0.125	100	1.468 ± 0.226	100

Table 5: Summary of the results (IC_{50} in µg/ml)

No	Sample	Type of cell	Result (IC50 in µg/ml)
1	LCMLE	HT-29	94.2
2	5-FU	HT-29	3.9
3	LCMLE	HCT116	88.2
4	5-FU	HCT116	4.2

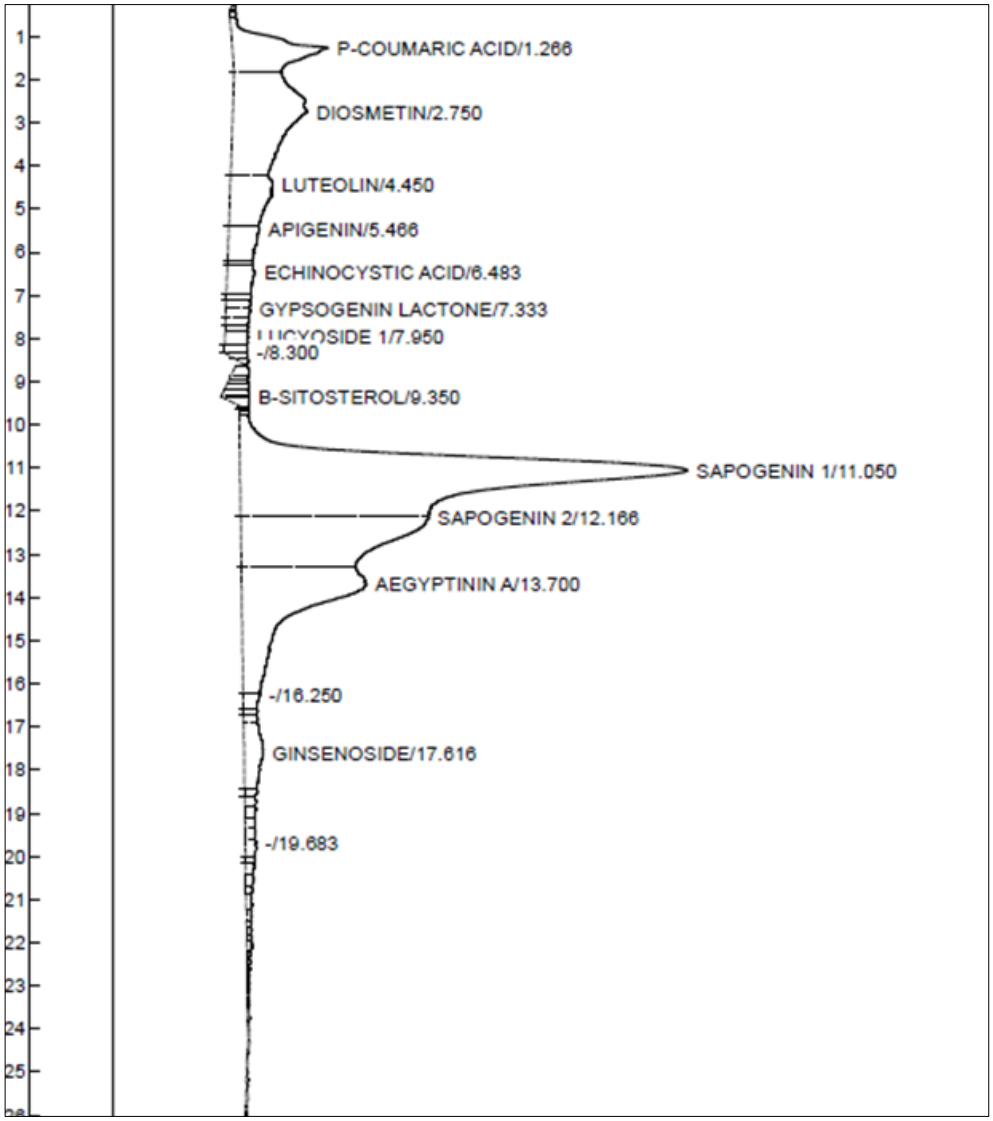

Fig 1: HPLC Chromatogram of Luffa cylindrica methanolic leaf extract

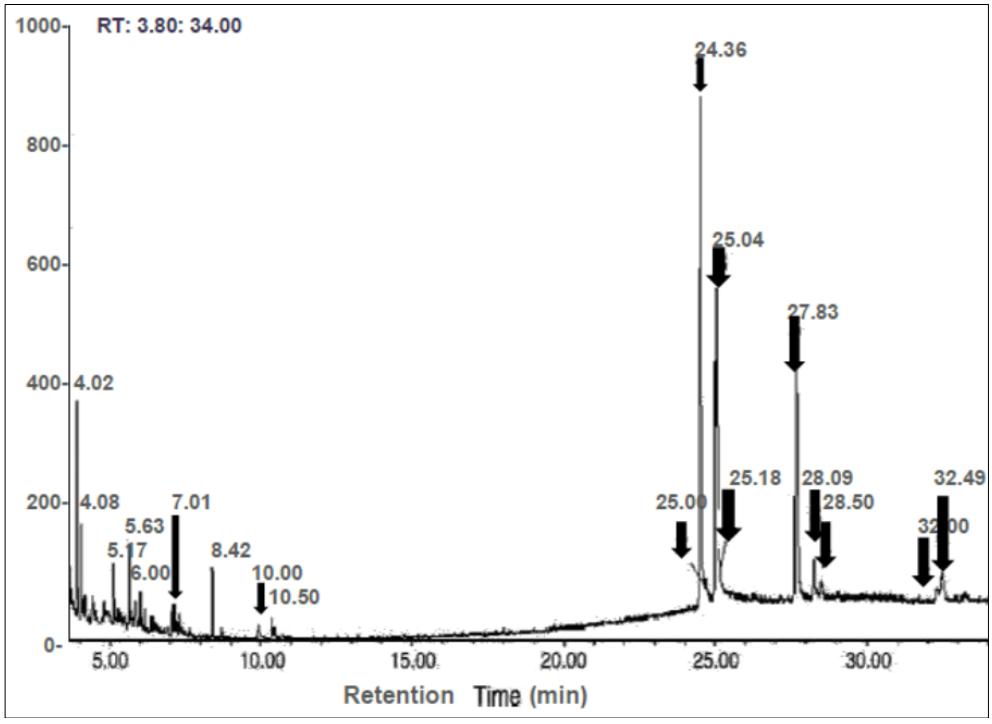

Fig 2: GC-MS Chromatogram of Luffa cylindrica methanolic leaf extract

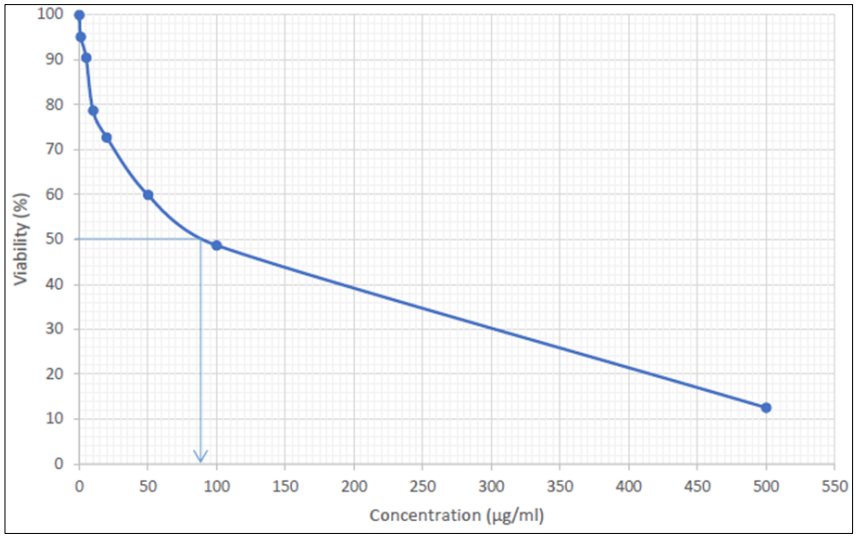

Fig 3: Effect of different concentrations of LCME on HT 29 Cell line after 72hr exposure

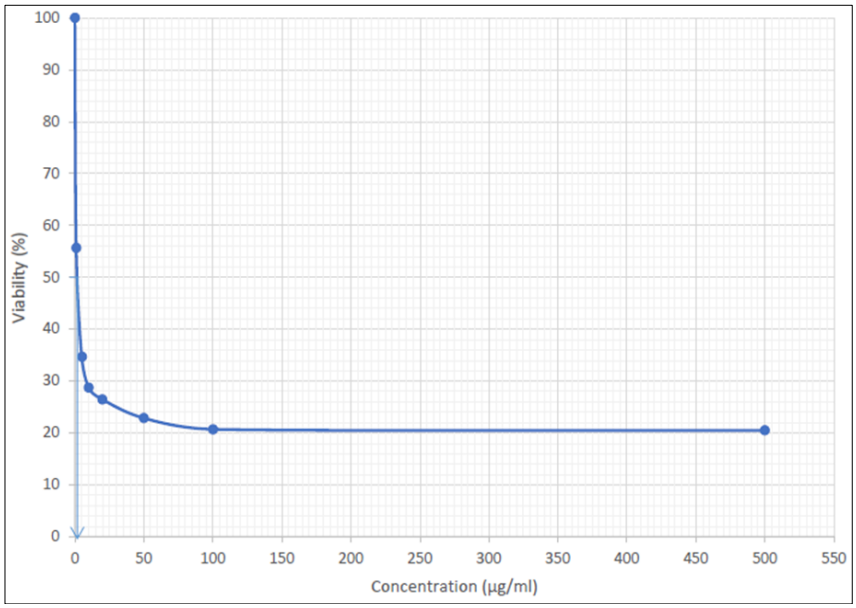

Fig 4: Effect of different concentrations of 5-FU on HT 29 Cell line after 72hr exposure

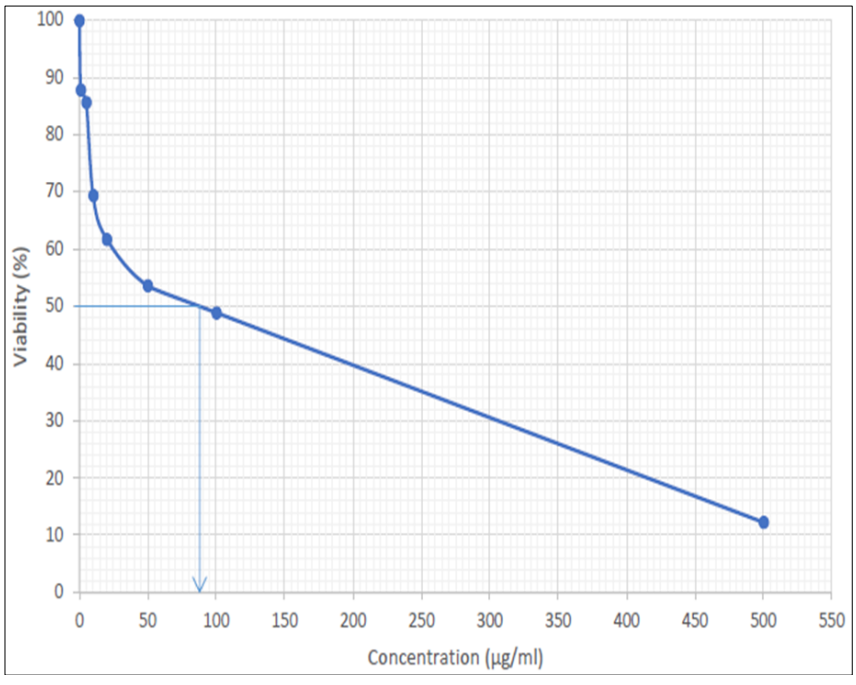

Fig 5: Effect of different concentrations of LCME on HCT 116 Cell line after 72hr exposure

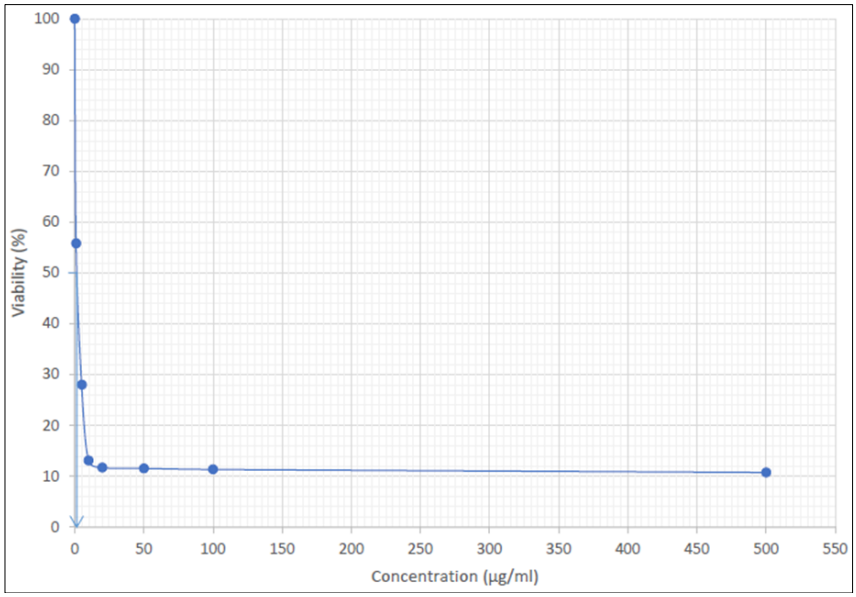

Fig 6: Effect of different concentrations of 5-FU on HCT 116 Cell line after 72hr exposure

4. Conclusion

The result of this investigation showed that methanol leaf extract of *Luffa cylindrica* contain bioactive compounds which are essential in the treatment of various diseases including cancer as it exhibited cytotoxicity activities against cancer cell lines and therefore could be new sources of development of plant based cancer therapy. The result of this study gives credence to the use of *Luffa cylindrica* plant in traditional medicine for therapeutic purposes. However further studies needed to be carried out to isolate and characterize the crude extract to get the actual biomolecule(s) responsible for the observed cytotoxic activity against the cell lines used in this study. Isolated pure compound tends to have higher anticancer activity.

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