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Extracts of *Codiaeum variegatum* (L.) A. Juss is Cytotoxic on Human Leukemic, Breast and Prostate Cancer Cell Lines

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ABSTRACT

The high cost of treatment of cancer coupled with the emergence of drug resistance makes it imperative for new drug interventions to curb its occurrence. Hence, the objective of this research was to determine the phytochemical, total phenolic content, antioxidant and antiproliferative effect of *Codiaeum variegatum* crude extracts and fractions. The MTT cell viability and DPPH assays among others were used to determine the selected properties of the plants. The presence of general glycosides, tannins, alkaloids, flavonoids and sterols was observed in its stem bark and leaf. Triterpenoids were present in the leaf only while saponins were observed in the stem bark only. Strong antioxidant activities were observed in both stem bark and leaf with EC_{50} values of 0.053±0.004 mg/mL and 1.396±0.073 mg/mL respectively. Both crude extracts showed antiproliferative activity towards all cancer cell lines with the stem bark exhibiting the strongest cytotoxicity. However, both showed strong cytotoxicity towards normal cells as well. The mechanism of cell death was determined to be apoptosis. Further testing of fractions from the stem bark crude extract revealed an increase in cytotoxicity of its chloroform fraction against Jurkat cells with an IC50 of 44.71±0.44 µg/mL. These results establish the antiproliferative nature of this plant.

INTRODUCTION

Cancer is a term used to describe a large group of diseases characterized by the uncontrolled proliferation and spread of abnormal cells (Hayflick, 1997). Its incidence and mortality has risen tremendously over the past decade causing the need for effective control measures (Ferlay *et al*., 2013). It is estimated that the number of new cases is expected to rise by about 70% over the next two decades (World Cancer Report, 2014). Recent statistics by the International Agency for Research on Cancer (IARC) indicates a changing trend in recorded incidence and mortality of which less developed countries now record the highest number of cases (Ferlay *et al*., 2013). More than 60% of the world's total annual new cases occur in Africa, Asia and Central and South America, with these regions accounting for 70% of the world's cancer deaths (World Cancer Report, 2014). Though there are several treatment options available, each comes with a high price tag often coupled with adverse side effects. The recently evolving paradigm of drug resistance to chemotherapeutic agents is also posing a great barrier to reducing the incidence and mortality of cancer (Shervington and Lu, 2008). Hence there is the need to exploit other remedies with possibly less known adverse effects and from readily accessible sources like plants. Plants could serve as a major source of bioactive compounds with potential efficacy against cancers (Talalay and Fahey, 2001). *Codiaeum variegatum* L. belongs to the family Euphorbiaceae and it's native to India, Philippines, Sri Lanka, Thailand, Indonesia, Malaysia and some other Pacific Islands (Stamps and Osborne, 2003). It is a common perennial plant with over 200 varieties worldwide; each different from the other with respect to the pattern and shades of colour as well as the size and shape of it leaf (Figure 1).

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Aside from its main purpose in serving as an ornamental, the root decoction of *C. variegatum* is used to treat gastric ulcers. The leaves have antibacterial and antiamoebic properties and can be crushed and drunk to cure diarrhoea (Moundipa *et al*., 2005). In indigenous Malaysian medicine, the plant is used as an anti-infective and an anti-cancer agent (Ali *et al*., 1996). Research conducted by Hassan *et al*. (2013) exposed the cytotoxicity effect of *C. variegatum* cv. *petra* leaves on human caucasian breast adenocarcinoma (MCF7), hepatocellular carcinoma (HepG2), colon cell line (HCT116) and lung carcinoma cell line (A549) with activities ranging from 17.3% to 98%.

Fig. 1: *Codiaeum variegatum* cv Gold Dust.

The purpose of this research was to evaluate the cytotoxicity of 50% hydroethanolic extracts of *C. variegatum* CV gold dust on leukemia (Jurkat), breast (MCF 7) and prostate (PC 3) cancer cells. *C. variegatum* crude stem bark and leaf extracts were also analyzed for their phytochemical constituents, antioxidant activity, total phenolic content (TPC) and mode of cytotoxicity induction.

MATERIAL AND METHODS

Cell lines and reagents

The cell lines used (Jurkat, MCF 7, PC 3, WRL 68, HepG2) were obtained from RIKEN BioResource Centre Cell Bank (Japan). Culture media (RPMI and α-MEM), 96 well plates, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, isopropanol, HCl, trypan blue solution, absolute ethanol, foetal bovine serum (FBS), antibiotics (penicillin and streptomycin), 2, 2-diphenyl-1-picryl hydrazyl (DPPH), and phosphate buffer saline were obtained from Sigma-Aldrich Company (St. Louis, MO, USA).

Plant material and extraction process

Codiaeum variegatum cv. *gold dust* samples were handpicked from the environs of the New Times Corporation (5°34'10.5"N 0°13'20.5"W), North Industrial Area, Accra in April, 2014 before 9.00 am. Specimen of the plant was sent to the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi for authentication by a taxonomist and a voucher specimen was deposited at the Herbarium for reference purpose (KNUST/HMI/2014/L094). The plant was sorted into stem bark and leaves. The stem bark component was chopped into pieces and both parts were washed separately with water three times and air dried at room temperature for three weeks. The dried samples were separately pulverized and packaged in zip-locks for further use. Preparation of 50% hydroethanolic extraction of the plant leaves and stems were carried out separately, by suspending 50 g of the powder of each part in 500 mL of 50% ethanol (50:50 v/v). The extraction was done by cold maceration for 24 hrs at room temperature on a shaker. The extracts were filtered through cotton wool, concentrated using a rotary evaporator and freeze-dried to obtain the *C. variegatum* hydroethanolic leaf and stem bark crude extracts.

Fractionation of *C. variegatum* stem bark hydroethanolic extract

Fractionation of the hydroethanolic stem bark extract of *C. variegatum* was carried out in a separating funnel using solvents of increasing polarity, petroleum ether, chloroform and ethyl acetate. A mass of 1.5 g was dissolved in 15 mL of 50% ethanolic solution and was successively partitioned with petroleum ether, then chloroform and finally with ethyl acetate, each having a volume of 30 mL, to obtain petroleum ether, chloroform and ethyl acetate fractions. This was done for two to three times as polarity increased. The remaining portion was designated as hydroethanolic fraction.

Phytochemical screening

The phytochemicals tested for were general glycosides, anthracene glycosides, saponins, tannins, alkaloids, flavonoids, sterols and triterpenoids. The presence of these phytochemicals in the crude extracts was analyzed using standard methods (Trease and Evans, 1989; Sofowora, 1993; Harborne, 1998).

Determination of total phenols

Total phenolic content (TPC) of leaf and stem bark was determined using the Folin–Ciocalteau assay with slight modification (Marinova *et al*., 2005). To a volume of 10 µL of sample, 790 µL of distilled water was added. The concentration of the leaf and stem bark extracts tested was 5 mg/mL. A volume of 50 µL of Folin–Ciocalteau reagent was added to the diluted samples and thoroughly mixed. The mixtures were incubated in the dark for 8 mins. Subsequently, 150 µL of 7% Na_2CO_3 was added before incubation of the mixture for 2 hrs in the dark at room temperature. Triplicate experiments were performed. The absorbance was read at a wavelength of 750 nm using a microplate reader (Tecan Infinite M200, Austria). Gallic acid (GA) was used as the standard phenolic compound. A GA calibration curve was plotted and used to determine the total phenolic content. The results were expressed in milligrams of GA equivalents per gram dry mass (mg GAE/g DM).

Determination of antioxidant activity

The antioxidant activity of *C. variegatum* leaf and stem bark extracts was determined using the free radical scavenging activity by DPPH method with some modification (Blois, 1958). Methanolic solution of DPPH (0.5 mM) was added to equal volumes of various concentrations of each extract (concentration range 0-5 mg/mL). After 20 mins incubation at room temperature, the absorbance was read at a wavelength of 517 nm (Tecan Infinite M200 Pro plate reader, Austria). The inhibition concentration at 50% (IC_{50}) value of each extract was calculated from the following formula:

$$\% \text{ Antioxidant activity} = [(A_0 - A_1)/A_0 \times 100]$$

Where A_0 is the absorbance of negative control (methanol), and A_1 is the absorbance of test sample with DPPH. Butylated hydroxytoluene (BHT) was used as standard control. Triplicate experiments were performed. The half maximal effective concentration (EC_{50}) value, which is the concentration of the extracts that can cause 50% free radical scavenging activity, was determined.

MTT assay

L-RPMI and α-MEM culture media, respectively, supplemented with 10% foetal bovine serum (FBS), containing penicillin, streptomycin, and L-glutamine were maintained in culture at 37°C in a humidified 5% CO_2 atmosphere. The tetrazolium-based colorimetric assay (MTT) was used to determine the cytotoxicity of *C. variegatum* on the cancer and normal cell lines (Ayisi *et al*., 2011). Triplicate experiments were performed. Cells were seeded into the 96-well plates at the concentration of 1×10^4 cells/well, treated with varying concentrations of the plant extracts (0-1000 µg/mL) and incubated as indicated above for 72 hrs. A color control plate was also setup for each extract including the positive control, curcumin. MTT solution (0.5 mg/mL) was added to each well on the plate, and incubation continued for further 4 hrs. The reaction was stopped with acidified isopropanol solution, and the plate incubated in the darkness overnight at room temperature before reading the absorbance at 570 nm using a microplate reader (Tecan Infinite M200 Pro, Austria). The percentage cell viability was determined as follows:

$$\% \text{ Cell Viability} = ((\text{Absorbance of treated cells} - \text{Absorbance of blank})/(\text{Absorbance of untreated cells} - \text{Absorbance of blank}))\text{x}100$$

The IC_{50} values were determined from the plot of percent cell viability on the y-axis against extract concentrations on the x-axis.

Nuclear morphology examination (Hoechst staining)

MCF-7 cells were seeded at 1×10^6 cells /mL in a total volume of 6 mL in sterile petri dishes and incubated for 24 hrs at 37 °C in 5% CO_2 to allow the cells to adhere to the dishes. The cells were then treated with two different concentrations (20 and 40 µg/mL) of the most active crude extract (*C. variegatum* stem bark) and standard urosolic acid (5.7 µg/mL) and then re-incubated for 24 hrs at 37 °C in 5% CO_2. The cells were scraped from the petri dishes with a cell lifter and transferred into 15 mL centrifuge tubes. Centrifugation was done at 1000 rpm for 5 min and the supernatant was discarded. The remaining cell pellets were re-suspended in 1 mL of phosphate buffered saline (PBS). The cells were then transferred into 1.5 mL eppendorf tubes and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cell pellets were treated with 200 µL of 1% glutaraldehyde and then incubated at room temperature for 30 min. The centrifugation was subsequently repeated (as above) and the supernatant was removed. A volume of 50 µL of PBS and 8 µL of Hoechst solutions was finally added and mixed gently but uniformly. The samples were applied on microscope slides, covered with cover slips, mounted and examined on a fluorescent microscope (Olympus, U.S.A).

Flow-cytometry analysis

MCF-7 cells were seeded at 2×10^5 cells /mL in a total volume of 3 mL in sterile petri dishes and incubated for 24 hrs at 37 °C in 5% CO_2. The cells were then treated with different concentrations (20 and 40 µg/mL) of the most active crude extract (*C. variegatum* stem bark) and standard curcumin and then re-incubated for 24 hrs at 37 °C in 5% CO_2. The cells were scraped from the petri dishes with a cell lifter and stirred gently and uniformly. A volume of 100 µL was aliquoted into wells in a 96 well plate. An equal volume of the Guava Nexin Reagent was aliquoted into each well, mixed thoroughly and incubated for 20 mins. The plates were read in a flow cytometer (Guava Easycyte, Germany) (Vermes *et al*., 1995).

Statistical analysis

Data were analyzed by one-way analysis of variance and the means assessed by Tukey's test at 5% level of significance ($p < 0.05$) using Graph pad Prism version 5.0. The results were expressed as mean ± SD.

RESULTS AND DISCUSSION

Phytochemical screening

Medicinal plants are of great importance to the general health of individuals and communities. It is also an immense source of medicines in pharmacognosy. Research by Ogunwenmo *et al*. (2007) proved a difference in the phytochemical constituents among various *C. variegatum* varieties. The presence of alkaloids, saponins, and tannins were reported by Ogunwenmo *et al*. (2007) to be varying in levels among varieties of this plant. With respect to the *C. variegatum* cv gold dust tested in this research, both its stem bark and leaf tested positive for general glycosides, tannins, alkaloids, flavonoids and sterols as shown in table 1. However, saponins were absent from the crude extract of *C. variegatum* leaves, (probably due to the difference in variety as compared to that used by Ogunwenmo *et al.,* 2007) while its stem bark showed no traces of triterpenoids. The presence of alkaloids, saponins, tannins in such high concentrations could render this plant,

antibacterial and antiamoebic and this could be related to its use in the treatment of diarrhoea (Moundipa *et al*., 2005).

Table 1: The phytochemical constituents present in hydroethanolic extracts of *C. variegatum*.

Phytochemical	Stem bark	Leaf
General glycoside	++	++
Anthracene glycoside	-	-
Saponins	+++	-
Tannins	+++	+++
Alkaloids	++	+++
Flavonoids	+++	+++
Sterols	+++	+++
Triterpenoids	-	+++

The data show the intensities of observed colours or froths as compared to standards.
+++ present at high concentration; ++ present in moderate concentration; + present in low concentration; - absent

Total phenol content

The total phenolic content was extrapolated from the standard calibration curve ($y = 0.392x + 0.036, R^2 = 0.991$) obtained from GA. Figure 2 shows the levels of TPC in the leaf and stem bark extracts. The TPC of the stem bark extract was significantly higher ($p= 0.0005$) as compared to the leaf extract as shown in Figure 2.

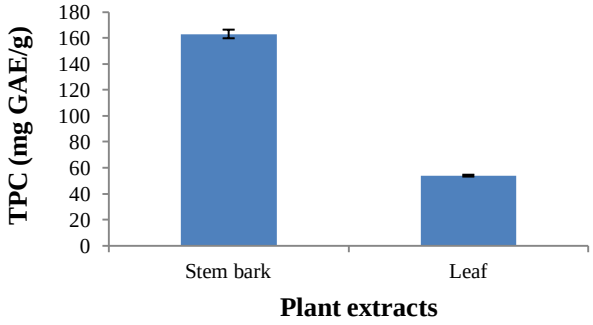

Fig. 2: Total phenolic content of hydroethanolic extracts of *C. variegatum* stem bark and leaf expressed as mean±standard deviation ($p = 0.0005$).

Antioxidant activity

Figure 3 shows the antioxidant activity of *C. variegatum* stem bark and leaf extracts. Both extracts and standard BHT exhibited strong antioxidant activity in a dose dependent manner. All the samples analysed showed an increase in antioxidant activity with increasing concentrations, thus exhibiting a concentration dependent pattern of free radical scavenging ability. The stem bark extract of *C. variegatum* recorded the strongest antioxidant activity. The stem bark of the *C. variegatum* appeared to have stronger antioxidant activity as compared with the standard (BHT) ($p<0.0001$). Members of the Euphorbiaceae plant family possess strong antioxidant activities which are greatly associated with the presence of phenolic compounds (Shahwar *et al*., 2010). For instance, analysis of the leaf extract of *C. variegatum* cv spiral and royal-like by HPLC-DAD showed that ellagic acid, a phenolic compound may be responsible for its antioxidant activity (Saffoon *et al*., 2014). Findings from this research proved that the stem bark of the *C. variegatum* recorded the highest concentration of total phenolics for this plant, suggesting that the observed antioxidant activity could be partly attributed to the high levels of total phenolics present in the sample. (Figure 3)

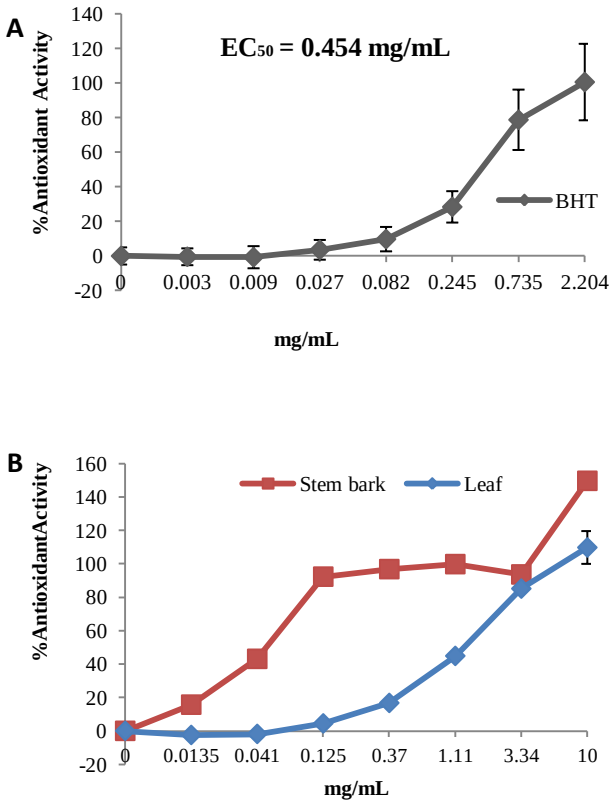

Fig. 3: Antioxidant activity of (A) BHT, (B) *C. variegatum* stem bark and *C. variegatum* leaf. Each point represents three determinations.

Antiproliferative activity of extracts and curcumin

Table 2 shows the antiproliferative effect of the extracts and standard (curcumin) on Jurkat cells, MCF 7, PC 3 and WRL 68 cell lines. The stem bark extract showed stronger inhibitory effect on all the cell lines compared to the leaf extract. All the extracts and curcumin inhibited the growth of the normal cell WRL 68, indicating a poor selectivity (Table 2).

A previous study on *C. variegatum* cv petra established the cytotoxicity of the leaves of this plant on human caucasian breast adenocarcinoma (MCF7), hepatocellular carcinoma (HepG2), colon cell line (HCT116) and lung carcinoma cell line (A549) with activities ranging from 17.3% to 98% (Hassan *et al*., 2013). This was confirmed in this research since both parts of the plant showed various levels of cytotoxicity against MCF 7 cells, with the stem bark of *C. variegatum* being the most cytotoxic part with an IC_{50} of 35.55 ± 1.50 µg/mL. A similar trend was observed with respect to leukaemia (Jurkat) and prostate (PC 3) cancer cell lines where the stem bark recorded an IC_{50} of 59.71 ± 12.20

µg/mL and 52.54 ± 1.88 µg/mL as compared to 62.03 ± 8.49 µg/mL and 211.20 ± 77.09 µg/mL recorded by its leaf. This suggests that the stem bark of *C. variegatum* could possibly be a better source of bioactive compounds for chemotherapy than its leaf. In elucidating the cytotoxic effect on the normal human liver cell line (WRL 68), it was observed that the stem bark of *C. variegatum* was the most toxic part. None of the extracts showed good selectivity against the cancer cell lines with respect to the normal human liver cells. This suggests that even the most active part of the plant could in one way or the other cause damage to the liver and probably other organs of the body when this medicine is administered. However, further studies will be needed to eliminate the toxic components of the extracts and isolate the active principle.

Table 2: Cytotoxic activities of *C. variegatum* crude extracts.

	IC_{50} values (µg/mL)			
Cell Line	**Stem bark**	**Leaf**	**Curcumin**	***p*-value**
Jurkat	59.71 ±12.20	62.03 ± 8.49	1.84 ± 0.16	0.0002
MCF 7	35.55 ± 1.50	84.44 ± 1.53	3.65 ± 0.08	< 0.0001
PC 3	52.54 ± 1.88	211.20 ± 77.09	8.10 ± 0.82	0.0032
WRL 68	49.37 ± 2.7	74.55 ± 4.8	8.35 ± 0.40	< 0.0001

Tabulated values represent mean ± standard deviation of three replicates. *p*-values compare the statistical difference between the calculated means using Tukey's test. For each plant extract tested n=3

Nuclear morphology examination (Hoechst staining)

Apoptosis is often characterised by nuclear condensation, chromosomal DNA fragmentation and the formation of cell fragments called apoptotic bodies (Bruce *et al*., 2008). Cells that are not undergoing cell death often possess a nucleus with the normal, roughly spherical morphology. The most active crude extract among all the extracts tested, *C. variegatum* stem bark, was analysed to elucidate its molecular mechanism of action. The effect of this selected crude extract on the nuclear morphology of breast cancer cells (MCF 7) and its specific mode of action was analysed using the Hoechst staining. The presence of fragmented or shrunk nuclei was observed in *C. variegatum* stem bark treated cells as well as standard urosolic acid. Figure 4 shows the effect of *C. variegatum* stem bark extract on MCF 7 cells.

Hoechst staining showed that there were significant morphological changes in nuclear chromatin similar to the changes observed in the apoptotic mechanism of action by other members of the Euphorbiaceae family on MCF 7 cells (Aslanturk and Celik, 2013).

Flow cytometry analysis

Figure 5 shows the mechanism of action of *C. variegatum* stem bark on MCF 7 cells. The extract induced cytotoxicity in a dose dependent manner and the mode of cytotoxicity was confirmed to be apoptosis. (Figure 5)

Data generated from the flow cytometry assay indicated and confirmed a dose-dependent and apoptotic mode of cytotoxicity for *C. variegatum* stem bark at 20 and 40 µg/mL. Emerging evidence has demonstrated that, the anticancer activities of certain chemotherapeutic agents involved in the induction of apoptosis have no side effects on normal tissues, and are thus regarded as the preferred method of treating cancer (Xiao, 2007). Hence, the apoptotic nature of *C. variegatum* renders it a good candidate for chemoptherapy.

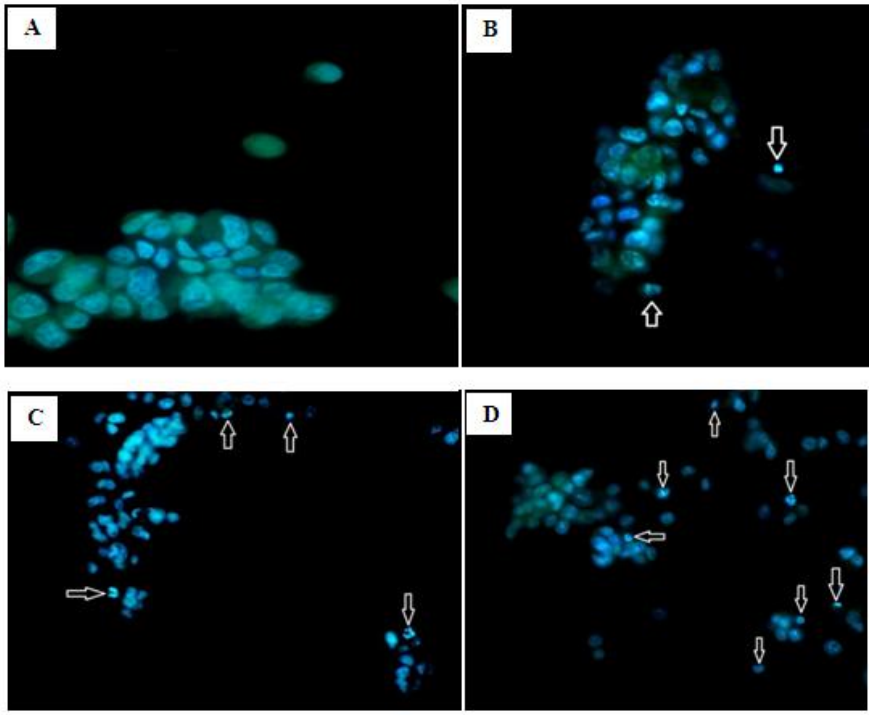

Fig. 4: Nuclear morphology of MCF 7 cells after 24 hrs of incubation without any treatment, control (A), treatment with 20 µg/mL (B), 40 µg/mL of *C. variegatum* stem bark extract (C) and urosolic acid (standard) (D). Arrows point to nuclei that are fragmented/shrunk

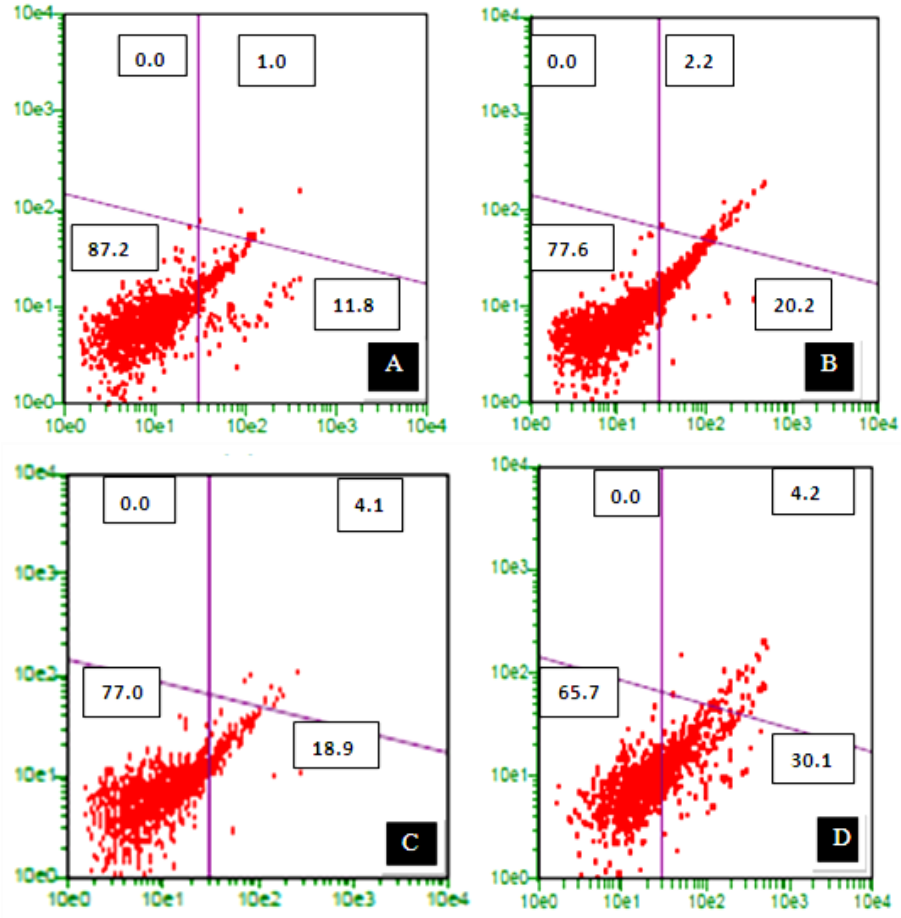

Fig. 5: Flow cytometry evaluation of apoptotic effects on MCF 7 cells after 24 hrs of incubation without any treatment, control (A) and treatment with 20 µg/mL (B), 40 µg/mL (C) of *C. variegatum* stem bark extract and curcumin (standard) (D). Inserted values represent percentage of cells of two replicates., Upper left quadrant represents nuclear debris, upper right quadrant represents cells in late apoptotic stage, lower left quadrant represents non apoptotic cells and lower right quadrant represents cells in early stage apoptosis.

Table 3: Cytotoxic activities of fractions of *C. variegatum* stem bark .

Cell Line	IC_{50} values (µg/mL)					
	Pet-ether fr.	Chloroform fr.	Ethyl acetate fr.	Hydroethanol fr.	Curcumin	*p*-value
Jurkat	80.87 ±13.90	44.71 ±0.44	560.27 ± 22.16	498.17±4.74	1.90±0.16	< 0.0001
MCF 7	736.56±183.7	675.80±33.06	>1000	>1000	2.93±0.62	0.003
HepG2	>1000	>1000	>1000	>1000	24.08±1.62	--

Tabulated values represent mean ± standard deviation of three replicates *p*-values compare the statistical difference between the calculated means using Tukey's test. For each fraction n=3

Antiproliferative activity of stem bark fractions and curcumin

The antiproliferative activity of petroleum ether, chloroform, ethyl acetate and hydroethanolic fractions against Jurkat, MCF 7 and HepG2 was assessed. (Table 3).

Fractionation of the stem bark extract was performed to verify if the fractions could elicit an increase in cytotoxic activity. The fractions were tested against MCF 7, Jurkat and HepG2 cell lines. With the exception of the chloroform extract of *C. variegatum* stem bark which recorded an increase in cytotoxicity towards Jurkat cells, with an IC_{50} of 44.71 ±0.44 µg/mL compared to the extract; all the other fractions were relatively less toxic against the cancer cells as compared to the crude extract. This could be attributed to the fact that the active molecules in the extract worked in a synergistic manner (or the activity of the active compound was complemented by another compound) and individually was not that effective. On the other hand, it is possible that the solvents used for fractionation were unsuitable for the purpose.

CONCLUSION

This study provides information on the phytochemical constituents, antioxidant and antiproliferative effect of *C. variegatum*. From the study the stem bark of *C. variegatum* exhibited a stronger free radical scavenging activity than its leaf, though both had good antioxidant properties. However, further

study on the stem bark of *C. variegatum* is needed to reveal its active principle.

Findings from this study indicates that *C. variegatum* has antiproliferative effect on leukaemia (Jurkat), breast (MCF 7) and prostate (PC 3) cancer cell lines and possibly contain bioactive compounds with anticancer properties.

However, though the stem bark and leaf of *C. variegatum* showed interesting anticancer activities both were very toxic to the normal human liver cells (WRL 68), suggesting a possible health risk to individuals who take the preparations of this extract for the purpose of alleviating illnesses especially on chronic users. However, it is imperative that this research is repeated in animal models such as mice. This will help serve as an index of its potential toxicity in humans.

Also, further fractionation of the stem bark revealed an increase in cytotoxicity of its chloroform fraction towards Jurkat relative to the crude extract, indicating the presence of some bioactive compound(s) in this fraction.

In addition to the anticancer evaluation, the molecular studies conducted on the *C. variegatum* stem bark crude extract using the Hoechst staining and flow cytometry revealed an apoptotic mechanism of action. This renders it a great candidate with promising leads to medicines against cancer.

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Conflict of Interests: There are no conflicts of interest.

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