COMPARATIVE *IN VITRO* ASSESSMENT OF THE METHANOL EXTRACTS OF THE LEAF, STEM AND ROOT BARKS OF *CNIDOSCOLUS ACONITIFOLIUS* ON LUNG AND BREAST CANCER CELL LINES

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ABSTRACT

Cnidoscolus aconitifolius (Mill.) I.M.Johnstone is a medicinal plant widely used in ethnomedicine for the treatment of cancer, and other diseases. The effects of the methanol extracts of the leaf, stem, and root barks were evaluated on the breast (MCF-7), and lung (NCI-H460) cancer cells at 1-250 µg/mL using SRB assay and the extracts were screened for phytochemicals using standard method. The stem and root extracts showed no activity at the maximum concentration, while the leaf extract at 100 µg/mL showed a remarkable cell growth inhibition against breast (-14.50 ± 0.58), and lung cancer (+53.29 ± 4.57 %) *in vitro*. The extracts showed the presence of saponins, terpenes, cardiac glycosides, and phenolic compounds. Partitioning of the active leaf extract further enhanced its activity as the chloroform fraction exhibited GI_{50}, LC_{50} and total growth inhibition (TGI) of ~22.5, ~68.75, and ~43.75 µg/mL against breast cancer, respectively, where as GI_{50}, and TGI of ~35.4 and ~55.8 µg/mL against lung cancer cells, respectively. However, the aqueous fraction showed no cytotoxicity against both cell lines. These results have justified the ethnomedicinal uses of the plant against tumor-related ailments. Isolation of the constituents responsible for the observed activity needs to be carried out to further support this claim.

Keywords: Cytotoxicity, *Cnidoscolus aconitifolius,* growth inhibition, MCF-7, NCI-H460, cancer cells

INTRODUCTION

Traditionally, medicinal plants have found applications in the formulation and production of modern drugs. These are used in the treatment of severe life threatening ailments including cancer especially in developing countries.[1] Over the years, cancer patients have relied on surgery, radiotherapy and chemical derived drugs for their treatment which further damage patient health and increase their sufferings. Hence, there is a need to search and focus on medicinal plants that are used traditionally in treating tumor related ailments. One of such plant, *Cnidoscolus aconitifolius* (Mill.) I.M.Johnstone has been used as a constituent of herbal preparations for the treatment of cancer patients by Nigerian herbalists.

Cnidoscolus aconitifolius (Euphorbiaceae) originates from South East Mexico and Guatamela and in South Western part of Nigeria.[2] It is locally known as Iyana Ipaja.[3] In Nigerian traditional health practices, it is also referred as "Hospital too far" due to its rapid healing properties against certain health conditions.[4] In ethnomedicine, it has been reported to have blood boosting effect in both pregnant and young anaemic children [5], and as an antidote for alcoholism, insomnia, and scorpion bite. Previously, the cytotoxic and anti-proliferative activity of the methanol extracts of *C. aconitifolius* leaves, stem, and root barks against tadpoles of *Ranicep ranninus* and radicle length of *Sorghum bicolor* have been reported.[6] The cytotoxicity of ethanol extract of its leaf against Brine Shrimp has also been noted.[7] However, there is presently no report for the anticancer activity of this plant. Therefore, present study was aimed at validating the antitumor ethnomedicinal uses of *Cnidoscolus aconitifolius* by using human breast, and lung cancer cell lines.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), gentamycin sulphate, L-glutamine penicillin streptomycin solution (GPSS), Roswell Park Memorial Institute-1640 medium (RPMI-1640), sulforhodamine B (SRB), trichloroacetic acid (TCA), tris base, trypan blue and trypsin-EDTA were purchased from Sigma Co (St. Louis, Mo, USA). Acetic acid (Lab Scan, Ireland), amphotericin B (Formepharma, Pak), and doxorubicin (ICN, USA) were also obtained.

Consumables

The cell culture boats, flasks (25 and 75 cm^2), centrifuge tubes (15 and 50 mL), culture plates (96-well, transparent with flat-bottom), and serological pipettes (1, 5, and 10 mL) were purchased from Falcon BD (USA). Micro centrifuge tubes (2 mL) were purchased from Kartel (Italy).

Equipments

The equipments include analytical balance, milligram balance and pH meter (Precisia, Switzerland), centrifuge, and CO_2 incubator (Kendro Lab Products, Germany). Safety cabinet class 2 (Heraeus Germany), Microscope: inverted TS-100 (Nikon, Japan), multiwell micro plate reader (Stat fax 2100, Awareness technology, USA), multiwell plate shaker (PMS 1000, Grant instruments, England), Ultrasonic bath (MXB6, Grant, England), and Neubauer's chamber (0.1 mm and 0.0025 mm^2, HBG, Germany).

Collection and processing of the plant.

The leaves of *Cnidoscolus acontifolius* **(Mill.) I.M.Johnstone** were collected in February 2014 at Sabongida-Ora in Edo State, Nigeria, and the identity of the plant was confirmed by Dr.

Shasanya Olufemi, a Plant Taxonomist. It was preserved at the Forest Research Institute of Nigeria (FRIN), Ibadan, with a herbarium specimen number FHI 109574. The plant material was air dried in the laboratory for 5 days at room temperature, followed by oven drying at 40°C and subsequently grinded to a powder form and stored in an air tight container.

Extraction of plant materials

About 2.5 kg of each plant part was exhaustively extracted in methanol (95%) using a Soxhlet apparatus, and dried using a rotary evaporator maintained at 40°C.

Preliminary phytochemical screening

Phytochemical screenings of the extracts (leaves stem and root barks) were carried out using standard methods previously described.[8]

Sulforhodamine-B assay

The growth inhibitory activities of methanol extract of *Cnidoscolus acontifolius* and its fractions were performed using human cancer cell lines [breast (MCF-7) and lung cancer (NCI-H460)]. [9]

The stock solutions of plant extracts and fractions were prepared as 40 mg/mL in DMSO. However, doxorubicin (1 mM) was prepared in distilled water. On the experimental day, respective dilutions were prepared in RPMI-1640 containing gentamicin (50 µg/mL).

Monolayer trypsinization, cell viability determination and cells counting from confluent flask (75 cm^2) were carried out. Cells (10000 cells/well/100 µL for MCF-7 and NCI-H460) were seeded for monolayer formation, and incubated in CO_2 incubator at 37 ºC for 24 h. Various concentrations of methanol extracts of *C. acontifolius* (1, 10, 50, 100, 200 and 250 µg/mL) and fractions (1, 25, 50, 75, 100) were added (100 µL/well) in appropriate wells, and incubated for 48 h. Appropriate controls and blanks (drug and extract) were also prepared. At the end of 48 h,

time zero-1 (T_{z1} plate) and time zero-2 (T_{z2} plate) plates were fixed with gentle addition of 50 % w/v cold TCA (50 µL/well) before and after the addition of extract and fractions in experimental plates. These were left at room temperature for 30 min, washed (3x) and dried overnight. After 48 h, experimental plates were also fixed in similar manner.

The dried fixed plates were stained with 100 µL of sulforhodamine solution (0.4 % wt/vol prepared in 1 % acetic acid) for 10 min, followed by washing (5x) with 1 % acetic acid to remove excess stain and air-dried. Finally, 100 µL of tris base solution (pH 10.2, 10 mM) was added to solubilize protein bound stain and absorbance was recorded at 545 nm using a micro-plate reader. All the experiments were conducted in triplicates. The results of the extracts and fractions were presented as GI_{50}, TGI and LC_{50} (µg/mL) values.

Fractionation of methanol extract of the leaf of *C. aconitifolius*

About 25.0 g of the crude methanol extract were re-dissolved in methanol-water (1:1) and partitioned exhaustively with chloroform (400 mL ×4) volumes in a separating funnel. The chloroform layer (lower) was collected first, followed by the aqueous fraction. This was repeated until a clear lower layer was obtained. The aqueous and the chloroform fractions were concentrated to dryness on a rotary evaporator and their respective yields noted.

Results

The 2.5 kg of the powdered leaves, stem and roots of *C. aconitifolius* yielded 87.65, 71.10, and 68.43 g of the methanol extracts which corresponds to 3.5, 2.8, and 2.7 % respectively.

Phytochemical screening of extract of the leaves, stem and roots of *C. aconitifolius* showed the presence of saponins, tannins, terpenes, flavonoids in varying intensities. However, alkaloids and anthraquinones were absent (Table 1)

Table 1: Results of the phytochemical screening of methanol extracts of the leaves stem and roots of *C. aconitifolius*

Phytochemical groups	leaves	Stem bark	Root bark
Alkaloids	-	-	-
Anthraquinones	-	-	-
Tannins/Phenolic compounds	+++	+	+
Flavonoids	+	-	-
Saponins	++	+	+
Cardiac glycosides	+	+	++
Terpenes	+++	+	++

Key: +++: appreciable amount; ++: moderate amount; + : minute amounts; - : not detected

Results of the effect of the methanol extracts on MCF-7 and NCI-H460 cell lines

The methanol extract of leaves produced growth inhibitory and cytotoxic effects on MCF-7 to varying extents. At a concentration of 50 µg/mL, the extract had +63.08 ± 3.63 % growth inhibition which became more cytotoxic at 200 and 250 µg/mL as –14.70 ± 0.76, and –26.25 ± 2.18 % were recorded. The GI_{50} and TGI were recorded as 26.67 ± 3.33, and 95 µg/mL while the LC_{50} noted to be greater than 250 µg/mL (Table 2). At the maximum concentration of 250

µg/mL, stem and root barks extracts of *C. aconitifolius* showed no significant activities against breast cancer cell lines.

Extract of *C. aconitifolius* at 50-100 µg/mL exhibited cell growth inhibitory effects on the lung cells unlike the breast cancer cells. A significant growth inhibiton ranging between ~28-77% were recorded in a concentration dependent manner against human lung cancer cell line (NCI-H460). The GI_{50} value of ~59.67 µg/mL was recorded, the LC_{50} and TGI were observed to be greater than 250 µg/mL (Table 3).

Table 2: Cytotoxicity of methanol extract of *C. aconitifolius* against breast cancer cell-line (MCF-7).

Extract	(µg/mL)	% Growth inhibition/ cytotoxicity	GI_{50}	LC_{50} (µg/mL)	TGI
	1	+18.04± 0.61	+26.67±3.33	>250	95± 0
	10	+42.38 ± 2.99			
	50	+63.08 ± 3.63			
	100	–4.07 ± 0.58			
	200	–14.70± 0.76			
	250	–26.25±2.18			

Control absorbance in MCF-7 at 545 nm = 1.9 ± 0.1
Each value represents % mean ± SEM of three independent experiments as compared to control. Growth inhibition = + and cytotoxicity = –; GI_{50} and TGI = Concentration of drug causing 50% and 100 % growth inhibition of cells. LC_{50} = Lethal concentration of drug that killed 50% cells. The order of activity was significantly different from each other.

Table 3: Cytotoxicity of methanol extract of *C. aconitifolius* against lung cancer cell-line (NCI-H460).

Methanol Extract	Conc. (µg/mL)	% Growth inhibition/ Cytotoxicity	GI_{50}	LC_{50}	TGI
			(µg/mL)		
	1	0.00 ± 0.00	+59.67± 0.75	>250	>250
	10	+28.45 ± 4.26			
	50	+46.24 ± 3.24			
	100	+53.29 ± 4.57			
	200	+64.73± 2.79			
	250	+77.68±1.96			

Control absorbance in NCI-H460 at 545 nm = 2. 0 ± 0.1
Each value represents % mean ± SEM of three independent experiments as compared to control. Growth inhibition = + and cytotoxicity = –; GI_{50} and TGI = Concentration of drug causing 50 % and 100 % growth inhibition of cells. LC_{50} = Lethal concentration of drug that killed 50% cells. The order of activity was significantly different from each other.

Effects of the aqueous and chloroform fractions of *C. aconitifolius* against breast (MCF-7) and lung cancer (NCI-H460) cell lines.

The chloroform fraction at 25 µg/mL displayed a significant growth inhibiton ~48.62 % against MCF-7 cells which became cytotoxic at 50-100 µg/mL in a concentration dependent manner with GI_{50}, LC_{50}, and TGI of 22.50 , 68.75, and 43.75 µg/mL, respectively (Table 4).

Similar effects were also observed by the chloroform fraction against NCI-H460 cells, giving GI_{50} and TGI of 35.40 55.8 µg/mL with LC_{50} >100 µg/mL. The aqueous fraction did not show any activity at 100 µg/mL on both cell lines (Table 5).

Table 4: Cytotoxicity of the aqueous and chloroform fractions of *C. aconitifolius* against breast cancer (MCF-7) cell line.

Chloroform Fraction	Conc. (µg/mL)	% Growth inhibition/ Cytotoxicity	GI_{50}	LC_{50}	TGI
			(µg/mL)		
	1	+8.32± 1.60			
	25	+48.62 ± 5.30	22.50	68.75	43.75
	50	-16.10 ± 2.30			
	75	-58.30 ± 6.70			
	100	-72.00 ± 9.10			
Aqueous fraction	100	<50	>100	>100	>100

Each value represents % mean ± SEM of three independent experiments as compared to control. Growth inhibition = + and cytotoxicity = –; GI_{50} and TGI = Concentration of drug causing 50% and 100 % growth inhibition of cells. LC_{50} = Lethal concentration of drug that killed 50% cells. The order of activity was significantly different from each other.

Table 5: Cytotoxicity of the aqueous and chloroform fractions of *C. aconitifolius* against lung cancer (NCI-H460) cell line.

Chloroform Fraction	Conc. (µg/mL)	% Growth inhibition/ Cytotoxicity	GI_{50}	LC_{50}	TGI
			(µg/mL)		
	1	+3.00 ± 0.72			
	25	+21.00 ± 2.10			
	50	-2.00 ± 1.30	35.40	>100	55.8
	75	-9.60 ± 1.20			
	100	-22.00 ± 11.00			
Aqueous fraction	100	<50	>100	>100	>100

Control absorbance in NCI-H460 at 545 nm = 2. 0 ± 0.1
Each value represents % mean ± SEM of three independent experiments as compared to control. Growth inhibition = + and cytotoxicity = –; GI_{50} and TGI = Concentration of drug causing 50% and 100 % growth inhibition of cells. LC_{50} = Lethal concentration of drug that killed 50% cells. The order of activity was significantly different from each other.

DISCUSSION

Among all the human diseases, cancer remains the most deadly and life threatening pathological condition .[10] The global burden of this disease has continued to surge due to the adoption of high level of cancer inducing life styles such as smoking, eating of westernized diet, and physical inactivity .[11]

According to the global cancer statistics, breast and lung cancers are the most frequently diagnosed cancer in females and males, respectively.[12] The commonly employed treatment includes chemotherapy, radiotherapy and in some cases, surgery which also exhibit series of side effects among patients.[13] Due to this, research into ethno-medicinal plants with antitumor properties as an alternative medicine at the early stage of the disease has become necessary.

Phytochemical screening of the extracts revealed the presence of various phytochemicals including tannins and flavonoids which are known to possess free radicals scavenging activity, hence prevent the development diseases. The higher activity demonstrated by the leaf extract over the stem and root extracts could be due to the abundance of one or more secondary metabolites such as terpenes and phenolic compounds, which are known for their anti-free radical potentials and inhibition of carcinogenesis.[14,15]

Our previous work has demonstrated that the extracts from leaf, stem and root barks of *Cnidoscolus aconitifolius* on guinea corn radicle length and tadpoles displayed cytotoxic and antiproliferative effects *in vitro*. The present study further validates the previous results with as the leaf extract being most active against breast and lung cancer cells. This could happen due to variation in the chemical constituents of the different morphological parts of the plant occasioned by translocation.

The leaf extract exhibited a concentration dependent effect with higher growth inhibitory effect against breast cancer by ~2x than that of lung cancer cells. Partitioning of the extract was observed to increase the activity compared to the crude as the chloroform fraction at 100 µg/mL produces cytotoxicities of -72 ±9.1 and -22 ±11 % as well as GI_{50} of 22.5 and 35µg/mL against MCF-7 and NCI-H460 cells respectively. This variation in sensitivities of the crude extracts and fraction could be as a result of the interference of some molecular process at some stages in the cell division processes e.g. the G2/M phase and the induction of some apoptotic process such as mitochondrial trans-membrane depolarization.[14]

The results obtained have validated the traditional uses of this plant in the treatment cancer. However, further work towards the isolation of the constituents responsible for the observed activities is required.

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