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ORIGINAL ARTICLE

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

Cnidoscolus aconitifolius'un Yaprak, Gövde ve Kök Kabuklarının Metanol Ekstrelerinin Akciğer ve Meme Kanseri Hücre Hatlarında Karşılaştırmalı *İn Vitro* Değerlendirilmesi

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ABSTRACT

Objectives: *Cnidoscolus aconitifolius* Mill. I.M.Johnst. is a medicinal plant widely used in ethnomedicine for the treatment of cancer and other diseases.

Materials and Methods: The effects of methanol extracts of the leaf, stem, and root barks were evaluated on breast (MCF-7) and lung (NCI-H460) cancer cells at 1-250 µg/mL using the SRB assay and the extracts were screened for phytochemicals using the standard method.

Results: The stem and root extracts showed no activity at the maximum concentration, while the leaf extract at 100 µg/mL showed 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, and 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 either cell line.

Conclusion: These results 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.

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

ÖZ

Amaç: *Cnidoscolus aconitifolius* Mill. I.M.Johnst. kanser ve diğer hastalıkların tedavisinde yaygın olarak kullanılan tıbbi bir bitkidir.

Gereç ve Yöntemler: Yaprak, kök ve kök kabuklarının metanol ekstrelerinin etkileri, SRB yöntemi kullanılarak 1-250 µg/mL konsantrasyonda meme (MCF-7) ve akciğer (NCI-H460) kanser hücreleri üzerinde değerlendirilmiş ve ekstrelerin fitokimyasal içeriği standart yöntem kullanılarak taranmıştır.

Bulgular: Gövde ve kök ekstreleri maksimum konsantrasyonda aktivite göstermezken, yaprak ekstresi 100 µg/mL dozda meme (-14,50±0,58) ve akciğer kanserine (+53,29±4,57) karşı belirgin *in vitro* hücre büyümesi inhibisyonu göstermiştir. Ekstrelerde saponinler, terpenler, kardiyak glikozitler ve fenolik bileşiklerin varlığı saptanmıştır. Aktif yaprak ekstresinin fraksiyonlanması ile kloroform fraksiyonunda meme kanseri hücrelerine karşı sırasıyla 22,5, 68,75 ve 43,75 µg/mL GI_{50}, LC_{50} ve toplam büyüme inhibisyonu (TGI) değerleriyle; akciğer kanseri hücrelerine karşı sırasıyla 35,4 ve 55,8 µg/mL GI_{50} ve TGI değerleriyle aktivitenin arttığı tespit edilmiştir. Bununla birlikte, sulu fraksiyon her iki hücre hattına karşı sitotoksik etki göstermemiştir.

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Sonuç: Bu sonuçlar, bitkinin tümörle ilgili hastalıklara karşı etnomedikal kullanımını desteklemiştir. Bu kaydın desteklenmesi için ileri çalışmalarda aktiviteden sorumlu bileşiklerin izole edilmesi gerekmektedir.

Anahtar kelimeler: Sitotoksisite, *Cnidoscolus aitifitifolius*, büyüme inhibisyonu, MCF-7, NCI-H460, kanser hücreleri

INTRODUCTION

Traditionally, medicinal plants have found applications in the formulation and production of modern drugs. They 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 patients' health and increase their suffering. Hence, there is a need to search for and focus on medicinal plants that are used traditionally in treating tumor-related ailments. One such plant, *Cnidoscolus aconitifolius* (Mill.) I.M.Johnst., has been used as a constituent of herbal preparations for the treatment of cancer patients by Nigerian herbalists.

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

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO), fetal bovine serum, gentamicin sulfate, $_{L}$-glutamine penicillin streptomycin solution, 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 (St. Louis, MO, USA). Acetic acid (Lab Scan, Ireland), amphotericin B (Formepharma, Pakistan), 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). The microcentrifuge tubes (2 mL) were purchased from Kartel (Italy).

Equipment

The equipment included an analytical balance, milligram balance, pH meter (Precisia, Switzerland), centrifuge, CO_2 incubator (Kendro Lab Products, Germany), safety cabinet class 2 (Heraeus Germany), microscope: inverted TS-100 (Nikon, Japan), multiwell microplate reader (Stat fax 2100, Awareness Technology, USA), multiwell plate shaker (PMS 1000, Grant Instruments, UK), ultrasonic bath (MXB6, Grant Instruments), and Neubauer's chamber (0.1 mm and 0.0025 mm^2, HBG, Germany).

Collection and processing of the plant

The leaves of *C. aconitifolius* (Mill.) I.M.Johnst. 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, Ibadan, with 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 ground to 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 *C. aconitifolius* and its fractions were tested 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 cell counting from a confluent flask (75 cm^2) were carried out. Cells (10,000 cells/well/100 µL for MCF-7 and NCI-H460) were seeded for monolayer formation and incubated in a CO_2 incubator at 37°C for 24 h. Various concentrations of methanol extracts of *C. aconitifolius* (1, 10, 50, 100, 200, and 250 µg/mL) and fractions (1, 25, 50, 75, and 100) were added (100 µL/well) in appropriate wells, followed by incubation 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 (3×), and dried overnight. After 48 h, the experimental plates were also fixed in a 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 (5×) 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 microplate reader. All the experiments were conducted in triplicate. 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 was 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, corresponding 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, and flavonoids in varying intensities. However, alkaloids and anthraquinones were absent (Table 1).

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} was 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. Significant growth inhibition ranging between ~28% and 77% was recorded in a concentration-dependent manner against the human lung cancer cell line (NCI-H460). A GI_{50} value of ~59.67 µg/mL was recorded, while LC_{50} and TGI were greater than 250 µg/mL (Table 3).

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 significant growth inhibition of ~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 with the chloroform fraction against NCI-H460 cells, giving GI_{50} and TGI of 35.40 and 55.8

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	+++	+	++

+++: Appreciable amount, ++: Moderate amount, +: Minute amounts, -: Not detected

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}	TGI
			(µg/mL)		
	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 ± standard error of mean 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.
TGI: Total growth inhibition

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 ± standard error of mean 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.
TGI: Total growth inhibition

µg/mL with LC_{50}>100 µg/mL. The aqueous fraction did not show any activity at 100 µg/mL on either cell line (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	22.50	68.75	43.75
	25	+48.62±5.30			
	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 ± standard error of mean 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.
TGI: Total growth inhibition

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	35.40	>100	55.8
	25	+21.00±2.10			
	50	-2.00±1.30			
	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 ± standard error of mean 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.
TGI: Total growth inhibition

DISCUSSION

Among all 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 lifestyles such as smoking, eating of a westernized diet, and physical inactivity.[11]

According to global cancer statistics, breast and lung cancers are the most frequently diagnosed cancers 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 ethnomedicinal plants with antitumor properties as an alternative medicine at the early stage of the disease has become necessary.

Phytochemical screening of extracts revealed the presence of various phytochemicals including tannins and flavonoids, which are known to possess free radical scavenging activity, hence preventing the development of 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 antifree radical potentials and inhibition of carcinogenesis.[14,15]

Our previous work demonstrated that the extracts from the leaf, stem, and root barks of *C. aconitifolius* displayed cytotoxic and antiproliferative effects on guinea corn radicle length and tadpoles *in vitro*. The present study further validates the previous results with 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 ~2× 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 35 µg/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 transmembrane depolarization.[14]

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

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REFERENCES

1. Kolawole AO. Feasible cancer control strategies for Nigeria mini review. Am J Trop Med Pub Health. 2011;1:1-10.
2. Ross-Ibarra J, Molina-Cruz A. The Ethnobotany of Chaya (*Cnidoscolus aconitifolius* ssp. Aconitifolius Breckon): A Nutritious Maya Vegetable. Econ Bot. 2002;56:350-65.
3. Oyagbemi AA, Odetola AA, Azeez O. Ameliorative effects of *Cnidoscolus aconitifolius* on anaemia and osmotic fragility induced by protein energy malnutrition. J Biotech. 2008;7:1721-6.
4. Donkoh A, Kese AG, Atuahene CC. Chemical composition of chaya leaf meal (*Cnidoscolus aconitifolius* (Mill.) Johnston) and availability of its amino acids to chicks. Anim Feed Sci Technol. 1990;30:155-62.

5. Iwalewa EO, Adewunmi CO, Omisore NO Adebanji OA. Pro-anti oxidant effect of vegetables in south west Nigeria. J Med Food. 2005;8:531-4.
6. Ikpefan EO, Ayinde BA, Gita TD. In vitro comparative cytotoxic and growth inhibitory effects of the methanol extracts of the leaf, stem and root barks of *Cnidoscolus acontifolius* (Mill.) Johnst (Euphorbiaceae). Int J Bioassays. 2013;2:445-9.
7. Senjobi CT, Moody JO, Ettu AO. Antimicrobial and cytotoxic effects of *Cnidoscolus aconitifolius*. J Agric Biol Sci. 2011;2:21-5.
8. Khandelwal KR. Practical Pharmacognosy, Techniques and Experiments. 15th ed. New Delhi; Nirali Prakashan; 2006:15-163.
9. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst. 1990;82:1107-12.
10. Izevbigie EB, Bryant JL, Walker A. A novel natural inhibitor of extracellular signal-regulated kinases and human breast cancer cell growth. Exp Biol Med (Maywood). 2004;229:163-9.
11. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69-90.
12. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136:E359-86.
13. Ayinde BA, Ofeimun JO, Kashif M. Kashif M, Farooq AD, Choudhary MI. Growth Inhibitory Evaluations of Four Nigerian Medicinal Plants Against Cancer Cells, with Active Cytotoxic Fractions from the Leaves of *Parquetina nigrescens*. Can J Pur Appl Sci. 2014;9:3241-5.
14. Miliauskas G, Venskutonis PR, van Beek TA. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chem. 2004;85:231-7.
15. Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs. J Agric Food Chem. 2001;49:5165-70.