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Immunomodulatory, cytotoxic and antileishmanial activity of phytoconstituents of *Croton zambesicus*

Jude E. Okokon[1,*], Ashana Dar[2], Muhammad Iqbal Choudhary[2]

[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo, Uyo, Nigeria.
[2]International Centre for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan.

[*]Corresponding author: judeefiom@yahoo.com; Tel: +234-802-3453678

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Abstract

The root extract and fractions of *Croton zambesicus* were investigated for anticancer activity against HeLa cells, DNA interaction, immunomodulatory and antileishmanial activities. The GCMS analysis of the most active fraction against HeLa cell was carried. The root extract was found to exert significant anticancer activity with the hexane fraction exhibiting the most pronounced effect. The crude extract and the fractions did not interact with yeast DNA when investigated using electrophoresis. The extract demonstrated prominent antioxidant activity in whole blood, neutrophils and macrophages. The extract also exhibited moderate antileishmanial activity against promastigotes of *Leishmania major* in vitro. These results suggest that the root extract/fractions of *croton zambesicus* possesses anticancer, immunomodulatory and antileishmanial activities and these justify its use in ethnomedicine to treat cancer and microbial infections and can be exploited in primary healthcare.

Keywords: *Croton zambesicus*, anticancer, immunostimulatory, antileishmanial.

Introduction

Croton zambesicus Muell Arg. (Euphorbiaceace) (syn *C. amabilis* Muell. Arg. *C. gratissimus* Burch) is an ornamental tree grown in villages and towns in Nigeria. It is a Guineo–Congolese species widely spread in tropical Africa. Ethnobotanically, the leaf decoction is used in Benin as anti-hypertensive and anti- microbial (urinary infections) (Adjanohoun et al, 1989) and in parts of Niger Delta region of Nigeria the plant is use as antidiabetic and malarial remedy (Okokon et al., 2005a, 2006), while the Yorubas of western Nigeria use it traditionally for the treatment of Cancer (Ashidi et al.,2010). The roots are used as antimalarial, febrifuge and antidiabetic by the Ibibios of Niger Delta region of Nigeria (Okokon and Nwafor, 2009a). Boyom et al. (2002) studied the composition of essential oils from the leaves, stem and roots of *Croton zambesicus* and found the three types of oils to be similar in composition, with those from the leaves and stem rich in monoterpenes, while that of the root bark contains sesquiterpenes. The root and stem bark oils were found to be rich in oxygen-

containing compounds, with spathulenol and linalool as major components. Moha-med et al., (2009) reported the isolation of lupeol, betulinic acid, betulin, lupenone, diterpene ent kaurane-3,16,17-triol and vitexin as antioxidant principles from the seed of *Croton zambesicus.*Aderogba et al.,(2011) also isolated quercetin-3-O-*p*-600 (p-coumaroyl) glucopy-ranoside-30-methyl ether, helichrysoside-30-methyl ether, along with kaempferol-3-O-*p*-600 (p-coumaroyl) glucopyranoside, tiliroside and apigenin-6-C-glucoside, isovitexin as the antioxidant constituents from the leaf of the plant. Okokon and Nwafor (2009a) reported that the root extract contains alkaloids, saponins, terpenes, tannins, phlobatannins, anthraquinones and cardiac glycosides. Studies have reported on the antimicrobial properties of the leaf and stem (Reuben et al., 2008). The ethanolic leaf extract has been reported to possess antiplasmodial (Okokon et al., 2005a), antidiabetic (Okokon et al., 2006), anti-inflammatory, analgesic and antipyretic activities (Okokon *et al.,* 2005b), while the root extract has been reported to possess antimalarial (Okokon and Nwafor, 2009a), anticonvulsant and antiulcer (Okokon and Nwafor, 2009b), anti-inflammatory, analgesic and antipyretic (Okokon and Nwafor, 2010), antidiabetic and hypolipidemic activities(Okokon et al.,2011a). Okokon et al., (2011b) have reported on the kidney-protective activity of the root extract of the *Croton zambesicus* against gentimicin-induced kidney injury. We report in this study immunostimulatory, cytotoxicity against HeLa cell line and antileishmanial activities of the root extract and fractions.

Materials and methods

Plants collection

The plant material *Croton zambesicus* (roots) were collected in compounds in Uruan area, Akwa Ibom State, Nigeria in April, 2011. The plant was identified and authenticated by Dr. Magaret Bassey of Department of Botany and Ecological Studies, University of Uyo, Uyo, Nigeria.

Extraction

The roots were washed and shade-dried for two weeks. The dried plants' materials were further chopped into small pieces and reduced to powder. The powdered material was macerated in 70% ethanol. The liquid filtrates were concentrated and evaporated to dryness in vacuo 40°C using rotary evaporator. The crude ethanolic extract (100g) was further partitioned successively into 1L each of n-hexane, dichloromethane, ethyl acetate and butanol to give the corresponding fractions of these solvents.

Cellular antioxidant (Immunomodulatory) activity

The ethanolic crude extract was screened for cellular antioxidant activities in whole blood, neutrophils and macrophages using chemiluminescence assay. Briefly, Luminol or lucigenin-enhanced chemiluminescence assay were performed as described by Helfand *et al.* (1982) and Haklar *et al.*(2001). Briefly, 25µL diluted whole blood (1:50 dilution in sterile $HBSS^{++}$) or 25µL of PMNCs (1×10^6) or MNCs (5×10^6) cells were incubated with 25µL of serially diluted plant extract with concentration ranges between 6.25 and 100µg/mL. Control wells received $HBSS^{++}$ and cells but no extract. Tests were performed in white 96 wells plates, which were incubated at 37 ◦C for 30 min in the thermostated chamber of the lumino-

meter. Opsonized zymosan-A or PMA 25µL, followed by 25µL luminol (7×10^5M) or lucigenin (0.5mM) along with $HBSS^{++}$ was added to each well to obtain a 200µL volume/well. The luminometer results were monitored as chemiluminescence RLU with peak and total integral values set with repeated scans at 30 s intervals and 1 s points measuring time.

Anticancer activity

The growth inhibitory and cytotoxic activities of the ethanolic extracts and fractions were evaluated against HeLa cells (Cervix cancer cell) by using the sulforhodamine-B assay (Houghton et al., 2007). The cells (10000 cells/100 µL) in 96-well plate were incubated for 24 h at 37 °C in a humidified 5% CO2 incubator. The stock solutions of ethanolic extract, fractions were prepared in DMSO. Various dilutions of the ethanolic extracts and fractions (0.1, 1, 10, 100, and 250 µg/mL), were added (100 µL) in each well. After 48 h of incubation, 50 µL of cold TCA (50%) was added gently and left for 30 min at room temperature, followed by washing with distilled water and drying overnight. To each well, 100 µL of SRB solution (0.4% wt/vol in 1% acetic acid) was added and after 10 min, the unbound stain was removed by washing with acetic acid (1%), and air-dried at room temperature. The protein bound stain was solubilized with tris base (pH 10.2), and was shaken for 5 min. Absorbance was measured at 515 nm using a microplate reader. The absorbance of the appropriate blanks, including test substance blank, and control (without drug), was used to calculate the growth inhibition, and cytotoxicity of the test compounds, and represented as GI_{50}, TGI and LC_{50} (µg/mL) values.

GI_{50} = Concentration of the drug causing 50% growth inhibition of the cells
TGI = Concentration of the drug causing total growth inhibition of the cells
LC_{50} = Lethal concentration of the drug that killed 50% of the cells

DNA interaction studies using gel electrophoresis

DNA interaction assay was performed according to the protocol of Tian and Hua (2005). The reaction was carried out in an Eppendorf tube at the total volume of 15 µl containing 0.5 µg of pBR322 DNA in 3µl of 50 mM phosphate buffer (pH 7.4), and 5 µl of tested samples (DCM fraction) at concentrations 0.1, 0.5, 1.0, 10, 50 and 100 µg/ml and standard drug, paclitaxel, 20 µg/mL. Then, the mixture was incubated at 37°C for 1 h. The mixture was subjected to 1% agarose gel electrophoresis. DNA bands (open circular, supercoiled and linear) were stained with ethidium bromide and were analyzed qualitatively by scanning with Doc-IT computer program (VWR).

Antileishmanial activity

The antileishmanial activity of the extracts and fractions were evaluated against promastigotes of *Leishmania major* (DESTO) in culture using microplates. *Leishmania major* promastigotes were grown in bulk, early in modified NNN biphasic medium, using normal physiological saline. Then the promastigotes were cultured with RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS). The parasites (*Leishmania major)* were harvested at log phase and centrifuged at 3000 rpm for 10 min. They were washed three times with saline at same speed and time. Finally the parasites were counted with the

help of Neubauer chamber under the microscope and diluted with fresh culture medium to give a final density of 10^6cells/ml. In a 96-well micro titer plate, 180 ml of the culture medium was added in different wells. The extracts and fractions were dissolved in PBS (Phospate buffered saline, pH 7.4 containing 0.5% MeOH, 0,5% DMSO) to make a stock concentration of 1000 mg/ml. 20 µl of each extract/fraction concentration was added to the wells and serially diluted to get working concentrations ranging between 1.0 to 100 µg/ml. 100 ml of parasite culture (final density of 10^6cells/ml) was added in all wells. Two rows were left, one for negative and other for positive control. Negative controls received the medium while the positive controls received Pentamidine and amphotericin B as standard antileishmanial compounds. The plate was incubated between 21-22°C for 72 h. The culture was examined micrroscopically for cell viability by counting the number of motile cells on an improved Neubauer counting chamber and IC_{50} values of compounds possessing antileish-manial activity were calculated (Atta-ur-Rahman et al., 2001).

Gas chromatography-Mass spectrometry (GC-MS) analysis

Quantitative and qualitative data were determined by GC and GC-MS, respectively. The fraction was injected onto a Shimadzu GC-17A system, equipped with an AOC-20i autosampler and a split/ splitless injector. The column used was an DB-5 (Optima-5), 30 m, 0.25 mm i.d., 0.25 µm df, coated with 5 % diphenyl-95 % polydimethylsiloxane, operated with the following oven temperature programme: 50 °C, held for 1 min, rising at 3 °C/min to 250 °C, held for 5 min, rising at 2 °C/min to 280 °C, held for 3 min; injection temperature and volume, 250 °C and 1.0 µl, respectively; injection mode, split; split ratio, 30:1; carrier gas, nitrogen at 30 cm/s linear velocity and inlet pressure 99.8 KPa; detector temperature, 280 °C; hydrogen, flow rate, 50 ml/min; air flow rate, 400 ml/min; make-up (H2/air), flow rate, 50 ml/min; sampling rate, 40 ms. Data were acquired by means of GC solution software (Shimadzu). Agilent 6890N GC was interfaced with a VG Analytical 70-250s double -focusing mass spectrometer. Helium was used as the carrier gas. The MS operating conditions were: ionization voltage 70 eV, ion source 250°C. The GC was fitted with a 30 m x 0.32 mm fused capillary silica column coated with DB-5. The GC operating parameters were identical with those of GC analysis described above.

The identification of components present in the various active fractions of the plants' extracts was based on direct comparison of the retention times and mass spectral data with those for standard compounds, and by computer matching with the Wiley 229 and Nist 21 Libraries, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literatures (Adams, 2001; Setzer *et al*., 2007).

Results

Cytotoxic activity against HeLa cells

The results of cytotoxic activity of crude extract and fractions of *Croton zambesicus* shows significant activity with the hexane fraction exerting highest activity than other fractions and crude extract (Table 1). The potency order was hexane> dichloromethane> crude extract.

Table 1. Cytotoxic activity of crude extract and fractions of root of *Croton zambesicus* against HeLa cells

Extract / Fraction	GI_{50}(µg/ml)	LC_{50}(µg/ml)	TGI(µg/ml)
Crude extract	163.3±4.41	-	229.6±2.18
Hexane fraction	5.0±0.57	25.3±0.87	10.0±0.54
DCM fraction	15.0±0.08	67.3±0.32	23.0±0.54
Ethyl acetate fraction	-	-	-
Butanol	-	-	-
Aqueous fraction	-	-	-
Doxorubucin(µM)	0.61±0.03µM	7.80±0.80µM	3.60±0.30µM

Data are represented as mean ± SEM of three independent experiments. Values in the table are concentrations of extract-/fraction expressed as µg/ml. GI_{50} = Concentration of the drug causing 50% growth inhibition of the cells. TGI = Concentration of the drug causing total growth inhibition of the cells. LC_{50} = Lethal concentration of the drug that killed 50% of the cells

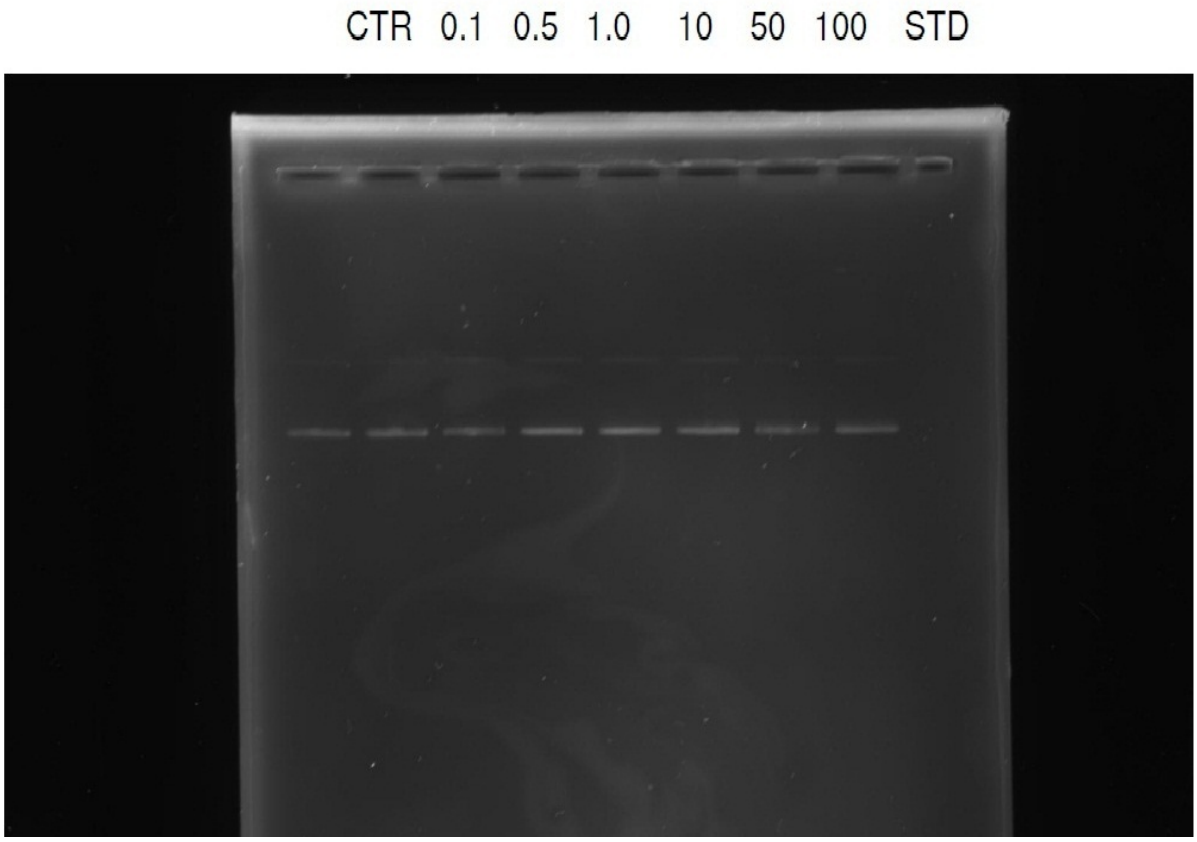

Figure 1: The effect of various concentrations of hexane fraction of *Croton zambesicus* on DNA interraction using gel electrophoresis.

Gel electrophoresis

Gel electrophoresis results shows that treatment of *E. coli* DNA with various concentrations of the hexane fraction of *Croton zambesicus* did not produce any effect on the DNA. Similar effect was also observed with the standard drug used, paclitaxel.(Figure 1).

Table 2: Cellular antioxidant activity of ethanolic root extract of *Croton zambesicus*

Cell Type	Concentration(µg/ml)	%Inhibition (RLU)
Whole Blood	1	-27.90 ± 1.29
	10	-18.20 ± 5.31
	100	66.90±1.61
Neutrophils (intracellular)	0.5	16.50± 1.74
	5	46.30 ± 0.98
	50	87.00 ±1.79
Neutrophils (extracellular)	0.5	39.30 ± 3.12
	5	59.60 ± 1.67
	50	71.70 ± 1.73
Macrophages	0.5	4.31 ± 1.02
	5	58.40 ± 3.22
	50	98.50 ± 5.20

Data are represented as mean ± SEM of three independent experiments

Table 3: Antileishmanial activity of *Croton zambesicus* (ED_{50})

Extract/ Fraction	ED_{50} (µg/ml)
Crude extract	58.18±0.14
Hexane fraction	>100
DCM fraction	>100
Ethyl acetate fraction	51.10±0.11
Butanol fraction	>100
Aqueous fraction	>100
Pentamidine	5.09±0.04
Amphotericin B	0.29±0.05

Data are represented as mean ± SEM (n=3)

Antileishmanial activity

Crude extract and fractions of ethanolic root extract of *Croton zambesicus* exerted significant antileishmanial activity when tested against promastigotes of *Leishmania major*. Ethyl acetate fraction exerted a higher activity than other fractions and crude extract though uncomparable to the standard drugs, pentamidine and amphotericin B (Table 3).

Cellular antioxidant activity

Ethanolic root extract of *Croton zambesicus* was observed to exhibit pro-oxidant effect at lower doses and antioxidant effect at higher doses especially in the whole blood, while different degrees of inhibitory effect on the oxidative burst activities in neutrophils and macrophages was also recorded and were in dose-dependent manner. The extract produced 27.90-66.90% inhibition in whole blood, 16.50 - 87% in neutrophils when activated with zymosan-A, 39.30 -71.70% in neutrophils when activated with PMA and 4.31- 98.50% in macrophages (Table 2).

GC-MS analysis

The GCMS analysis of the hexane fraction of *Croton zambesicus* revealed the presence of 24 bioactive compounds with major and minor ones as represented in Table 4.

Table 4: GC-MS analysis of hexane fraction of *Croton zambesicus*

S/No.	Name Of Compound	Mol.Wt	Chemical Formula	RI
1.	Dodecanoic acid	200	$C_{12}H_{24}O_2$	424
2.	Octadecane,6-methyl	268	$C_{19}H_{40}$	436
3.	Octadecane	254	$C_{18}H_{38}$	552
4.	Hexadecanoic acid	256	$C_{16}H_{32}O_2$	649
5.	Nonadecane	268	$C_{19}H_{40}$	606
6.	1-Hexadecanol,2-methyl	256	$C_{17}H_{36}O$	649
7.	Hexadecanoic acid , ethyl ester	284	$C_{18}H_{36}O_2$	655
8.	Linoleic acid,ethyl ester	308	$C_{20}H_{36}O_2$	737
9.	9-Octadecanoic acid(Z),2-hydroxyl-1-hydroxymethyl ethyl ester	356	$C_{21}H_{40}O_4$	740
10.	Hexadecanoic acid, 2-methyl-,methyl ester	284	$C_{18}H_{36}O_2$	745
11.	Trachylobane	272	$C_{20}H_{32}$	747
12.	Androst-4-ene-3,17-dione	286	$C_{19}H_{26}O_2$	767
13.	Androst-4-en-17-one, 3-hydroxy-,(5ά)-	286	$C_{19}H_{28}O_2$	782
14.	2,4(1H,3H)-pyrimidinedione,5-nitro	157	$C_4H_3N_3O_4$	427
15.	Retinol	286	$C_{20}H_{30}O$	876
16.	Myrcene	136	$C_{10}H_{16}$	991
17.	ϒ-Terpinene	136	$C_{10}H_{16}$	1047
18.	Linalool	154	$C_{10}H_{18}O$	1099
19.	Linalool acetate	196	$C_{12}H_{20}O_2$	1258
20.	Lupeol	426	$C_{30}H_{50}O$	1458
21.	α-Humulene	204	$C_{15}H_{24}$	1460
22.	α –Muurolene	204	$C_{15}H_{24}$	1502
23.	Stigmast-4-en-3-one	412	$C_{29}H_{48}O$	1587
24.	Lanost-7-en-3-one, 9 ά, 13 ά,14 ά,17 ά)-	426	$C_{30}H_{50}O$	1500

Discussion

Croton zambesicus has been use traditionally in the treatment of various ailments and diseases including cancer, microbial infections among other diseases. The root which has been reported to possess some pharmacological properties have been found in this study to exert pronounced cytotoxic activity against HeLa cells with the hexane fraction having the highest activity. This finding confirms and justifies the ethnomedical use of this plant to treat cancer and also corroborates the findings of Block et al.,(2002) who reported cytotoxic activity in the leaf. The cytotoxic mechanism of action was found to be unrelated to DNA interaction and is likely to involve interference with cell division processes. However, the GCMS analysis revealed the presence of some pharmacologically active compounds such as humulene and lupeol which have been implicated in the anticancer activity of plants (Ding et al., 2009; de Silva et al., 2008). Moreso, some phyto-components with compound nature of flavonoids found in this extract such as palmitic acid (hexadecanoic acid,ester and n-hexadecaonoic acid), unsaturated fatty acid and linolenic (docosatetraenoic acid and octadecatrienoic acid) are reported by Kumar et al., (2010) to have as antimicrobial, anti-inflammatory, antioxidant, hypocholesterolemic, cancer preventive, hepatoprotective, antiarthritic, antihistimic, antieczemic and anticoronary properties. These compounds and others are likely to be involved in the anticancer activity of this extract.

The root extract was also observed to exhibit strong antioxidant activity in whole blood, neutrophils (extracellular and intracellular) and macrophages. This activity may have resulted from the presence of fatty acids, lupeol and other monoterpenes and sesquiterpenes as revealed by GCMS analysis. These compounds have been reported to possess antioxidant

activity (Aderogba et al., 2011, Kumar et al., 2010). The antioxidant activity of the root extract correlate well with that of Mohammed et al., (2009) and Aderogba et al., (2011) who reported antioxidant activity in the seed and leaf respectively. lupeol, betulinic acid, betulin, lupenone, diterpene ent -kaurane-3,16,17-triol and vitexin in the seed and quercetin-3-O-p-600 (p-coumaroyl) glucopyranoside-30-methyl ether, helichrysoside-30-methyl ether, alo-ng with kaempferol-3-O-p-600(p-coumaroyl) glucopyranoside, tiliroside and apigenin-6-*C*-glucoside, isovitexin were isolated as antioxidant principles in previous studies (Mohammed et al., 2009; Aderogba et al., 2011). Although no compound was isolated in this study, these compounds present in the seed and leaf of *Croton zambesicus* are likely to be present in the root extract thereby contributing to the significant antioxidant activity observed. The significant antioxidant activity of this extract explains the strong anticancer activity of the root extract. Generation of reactive oxygen species has been implicated in the pathogenesis of cancer and other diseases (Halliwel and Gutteridge, 1999). The activities of antioxidant counteract the redox state precipitated intracellularly and hence ensure cytotoxicity. This could possibly be one of the mechanisms of cytotoxic activity of this extract.

The root extract also demonstrated antileishmanial activity. Compounds such as terpenes (mono and sesquiterpenes) as well as palmitic acid, hexadecanoic acid, hexadecanoic acid ethyl ester, and linoleic acid which have been found to be present in the root extract have been reported to possess antimicrobial activity (Habtemariam et al., 1993; Kumar et al. (2010). These compounds may have been responsible for the antileishmanial activity observed in this study.

From the results of these studies, it can be concluded that the root extract of *Croton zambesicus* has cytotoxic activity against HeLa cells, immunomodulatory and antileishmanial activities which are due to the phytochemical constituents of the extract and fractions.

Conflict of interest

There is no conflict of interest.

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