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Immunomodulatory, cytotoxic and antileishmanial activity of *Setaria megaphylla*

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Abstract

Cytotoxic, antioxidative burst and antileishmanial properties of leaf extract and fractions of *Setaria megaphylla* were investigated to ascertain the folkloric claims of its potency in inflammatory diseases and infections. The leaf extract and fractions of *Setaria megaphylla* were investigated for anticancer activity against HeLa cells using SRB method and DNA interaction activity using gel electrophoresis. Antioxidative burst activity of the extract in whole blood, neutrophils and macrophages was also investigated using luminol/lucigenin-based chemiluminescence assay. The extract and fractions were similarly screened for antileishmanial activity against promastigotes of *Leishmania major* in vitro. The GCMS analysis of the most active fraction against HeLa cells was carried out. The leaf 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 DNA when investigated using electrophoresis. The extract prominently inhibited oxidative burst activity in whole blood, isolated polymorphonuclear cells (PMNs) and mononuclear cells (MNCs) when two different phagocytosis activators (serum opsonizing zymosan-A and PMA) were used. The extract also exhibited moderate antileishmanial activity against promastigotes of *Leishmania major* in vitro. GCMS analysis of active fraction revealed pharmacologically active compounds. These results suggest that the leaf extract/fractions of *S. megaphylla* possesses cytotoxic, antioxidative burst and antileishmanial activities and these justify its use in ethnomedicine to treat inflammatory diseases and microbial infections and can be exploited in primary healthcare.

Keywords: Setaria megaphylla, Cytotoxic, antioxidative burst, antileishmanial.

Introduction

Setaria megaphylla (Steud) Dur & Schinz (Poaceae) also called broad leafed brittle grass is a tall, robust, tufted, perennial grass used mainly as pasture grass. It occurs in tropical and subtropical areas of Africa, America and India where there is high rainfall [1,2] (Van Oudtshoorn, 1999, Lowe, 1989). The plant is use traditionally by the Ibibios in Akwa Ibom State, Nigeria in the treatment of various ailments such as malaria, inflammation and diabetes. The plant has also been reported to possess antiplasmodial activity in vitro [3] and in vivo [4]. The leaf extract as reported by Okokon and Antia [5]contains flavonoid, carbohydrate, terpenes, saponins, tannins, anthraquinones and cardiac glycosides with LD_{50} of 2.4 ± 0.5g/kg. Hypoglycaemic and antidiabetic activities have been reported of the leaf and root extracts of this plant [4,6]. The antiiflammatory and antinociceptive activities of the ethanolic leaf extract of *Setaria megaphylla* have also been reported by Okokon et al.[7]. Considering the ethnomedical records of this plant in herbal medicine and the few reported biological activities, we undertook to investigate the cytotoxic, immunomodulatory and antileishmanial teffect of this leaf extract and fractions as well as GCMS analysis of hexane fraction which had not been evaluated previously to reveal the phytochemical components of leaf extract of *S. megaphylla* to provide information on medicinal potentials of this plant.

Materials and methods

Plants collection

The plant material *Setaria megaphylla* (leaf) 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 leaves 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.

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 previously [8,9]. Briefly, 25µL diluted whole blood (1:50 dilution in sterile $HBSS^{++}$) or 25µL of PMNCs ($1x10^6$) or MNCs ($5x10^6$) cells were incubated with 25µL of serially diluted plant extract with concentration ranges between 6.25 and 100 µg/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 luminometer. Opsonized zymosan-A or PMA 25µL, followed by 25µL luminol ($7x10^5$M) 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.

Cytotoxic 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 [10]. The cells (10000 cells/100 µL) in 96-well plate were incubated for 24 h at 37 °C in a humidified 5% CO_2 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 protection assay was performed according to the protocol of Tian and Hua [11]. 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 microscopically for cell viability by counting the number of motile cells on an improved Neubauer counting chamber and IC_{50} values of compounds possessing antileishmanial activity were calculated [12].

Gas chromatography-Mass spectrometry 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 0C. 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.

Identification of the compounds

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 and Nist Libraries, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literatures [13,14].

Results

Anticancer activity against HeLa cells.

The results of cytotoxic activity of crude extract and fractions of *Setaria megaphylla* shows significant activity with the hexane fraction exerting highest activity than other fractions and crude extract (Table 1). The potency order was hexane> dichloromethane> ethyl acetate>butanol> acqueous> crude extract.

Table 1: Anticancer activity of crude extract and fractions of leaf of *Setaria megaphylla* against HeLa cells

EXTRACT/ FRACTION	GI_{50}(µg/ml)	LC_{50}(µg/ml)	TGI(µg/ml)
Crude extract	165.0±3.21	244.2±0.52	231.3±3.16
hexane fraction	28.8±0.57	71.3±1.32	44.3±1.41
DCM fraction	37.2±0.56	77.3±1.84	60.3±1.45
Ethyl acetate fraction	40.0±1.15	83.6±1.20	60.6±1.22
Butanol	41.2±0.57	83.3±1.17	46.0±1.38
Aqueous fraction	38.3±1.15	93.5±0.18	65.0±1.18
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

DNA interaction activity

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

Antileishmanial activity.

Crude extract and fractions of ethanolic leaf extract of *Setaria megaphylla* 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 leaf extract of *Setaria megaphylla* 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

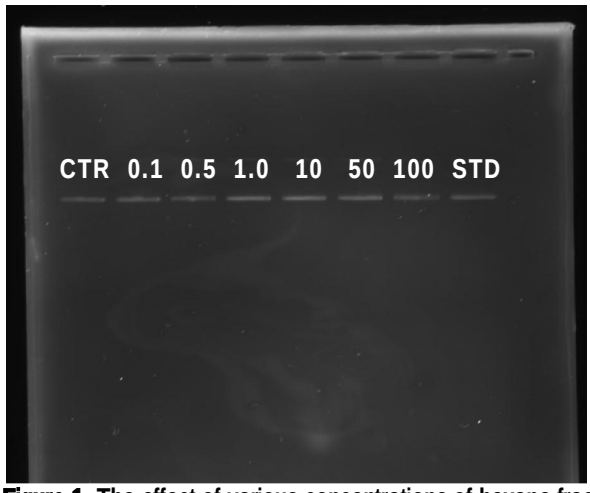

Figure 1: The effect of various concentrations of hexane fraction of *Setaria megaphylla* on DNA interraction using gel electrophoresis.

and macrophages was also recorded and were in dose-dependent manner. The extract produced -5.80 – 53.70 % inhibition in whole blood, 0.00 – 69.50 % in neutrophils when activated with zymosan-A, 49.70 – 80.40 % in neutrophils when activated with PMA and -15.70 – 54.10 % in macrophages (Table 2).

GC-MS analysis

The GCMS analysis of the hexane fraction of *Setaria megaphylla* revealed the presence of 21 bioactive compounds with major and minor ones as represented in Table 4.

Discussion

Setaria megaphylla is use traditionally in the treatment of various ailments and disease especially inflammatory diseases. In this study, the leaf 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, immunomodulatory as well as moderate antileishmanial activity. 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 phytol, Hexadecanoic acid methyl ester, 9,12,15-octadecatrienoic acid ethyl ester (Z,Z,Z)-, 8,11,14-

Table 2: **Cellular antioxidant activity of ethanolic leaf extract of *Setaria megaphylla***

CELL TYPE	Concentration(µg/ml)	%INHIBITION(RLU)
WHOLE BLOOD	1	-5.80 ± 1.04
	10	11.30 ± 3.58
	100	53.70±3.98
NEUTROPHILS (intracellular)	0.5	0.00± 0.00
	5	17.50 ± 2.68
	50	69.50 ±1.32
NEUTROPHILS (extracellular)	0.5	49.70 ± 8.90
	5	64.90 ± 2.25
	50	80.40 ± 2.54
MACROPHAGES	0.5	-15.70 ± 5.43
	5	-11.60 ±6.01
	50	54.10 ± 1.73

Data are represented as mean ± SEM of three independent experiments

Table 3: **Antileishmanial activity of *Setaria megaphylla* (ED_{50})**

EXTRACT/ FRACTION	ED_{50}(µg/ml)
Crude extract	73.40±0.75
Hexane fraction	>100
DCM fraction	>100
Ethyl acetate fraction	84.23±0.34
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).

Table 4 : GC –MS analysis of hexane fraction of *Setaria megaphylla*

S/No.	NAME OF COMPOUND	RI	MOL.WT	CHEMICAL FORMULA
1.	2,4,6-trimethyl octane	311	156	$C_{11}H_{24}$
2.	O-decyl hydroxylamine	376	173	$C_{19}H_{23}NO$
3	2,6,8-trimethyl decane	439	184	$C_{13}H_{28}$
4.	3-methyl undecane	498	170	$C_{12}H_{26}$
5.	6,10,14-trimethyl-2-pentadecanone	580	268	$C_{18}H_{36}O$
6	Hexadecanoic acid	658	256	$C_{16}H_{32}O_2$
7.	2-methyl, Hexadecanoic methyl ester	659	284	$C_{18}H_{36}O_2$
8.	Phytol	717	296	$C_{20}H_{40}O$
9.	9,12-octadecadienoyl chloride (Z,Z)	739	298	$C_{18}H_{31}ClO$
10.	Hexadecatrienoic acid methyl ester	743	264	$C_{17}H_{28}O_2$
11.	Hexadecanoic acid methyl ester	755	284	$C_{18}H_{36}O_2$
12.	9,12,15-octadecatrienoic acid ethyl ester (Z,Z,Z)-	825	306	$C_{20}H_{34}O_2$
13.	8,11,14-eicosatrienoic acid (Z,Z,Z)	805	306	$C_{20}H_{34}O_2$
14	Phthalic acid,diisooctyl ester	907	390	$C_{24}H_{38}O_4$
15.	Vitamin E	1225	430	$C_{29}H_{50}O_2$
16.	-Elemene	1340	204	$C_{15}H_{24}$
17	Urs-12-ene	1498	410	$C_{30}H_{50}$
18	Bicyclogermacrene	1501	204	$C_{15}H_{24}$
19	-muurolene	1504	204	$C_{15}H_{24}$
20	Germacrene- A	1505	204	$C_{15}H_{24}$
21	Guaiol	1595	222	$C_{15}H_{26}O$

eicosatrienoic acid (Z,Z,Z), Vitamin E and mono and sequiterpenes such as -Elemene, Bicyclogermacrene, -muurolene, Germacrene-A which have been implicated in the anticancer activity of plants [15,16]. 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.,[17] to have as antimicrobial, anti-inflammatory, antioxidant, hypocholesterolemic, cancer preventive, hepatoprotective, antiarthritic, antihistimic, antieczemic and anticoronary properties. Moreso, Omega-3-fatty acids have been found to be essential for normal growth and development and may play an important role in the prevention and treatment of coronary artery disease, hypertension, diabetes, and arthritis, other inflammatory and auto immune disorders and cancer [18, 19, 20]. These compounds and others are likely to be involved in the cytotoxic activity of this extract.

The leaf extract was also observed to exhibit significant antioxidative burst activity in whole blood, neutrophils (extracellular and intracellular) and macrophages. This activity may have resulted from the presence of fatty acids, phytol and other monoterpenes and sesquiterpenes as revealed by GCMS analysis. These compounds have been reported to possess antioxidant activity [17, 21]. Although no compound was isolated in this study, these compounds present in the seed and leaf of *Setaria megaphylla* are likely to be present in the leaf extract thereby contributing to the significant antioxidant activity observed. The significant antioxidant activity of this extract explains the prominent anticancer activity of the leaf extract. Generation of reactive oxygen species has been implicated in the pathogenesis of cancer and other diseases [22]. The activities of antioxidant counteract the redox state precipitated intracellularly and hence ensure cytotoxicity. This could possibly be one of the mechanisms of anticancer activity of this extract.

The leaf extract also demonstrated moderate antileishmanial activity. Antimicrobial activities are known to be promoted by proxidant state. In this study, lower doses of the extract have been observed to exhibit pro-oxidant activity. This activity has been reported to enhance antimicrobial activity [23] and may have contributed to the antileishmanial activity observed in this study. 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 [17,

24]. These compounds may have been responsible for the antileishmanial activity observed in this study.

Conclusion

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

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References

[1]. Van Oudtshoorn, F. P. Guide to Grasses of South Africa. Briza Publications, Cape Town. 1999

[2]. Lowe, J. The Flora of Nigeria Grasses (2nd Edition) Ibadan University Press. Ibadan, Nigeria. 1989

[3]. C. Clarkson, V. J. Maharay, N. R. Crouch, O. M. Grace, P. Pillay, M. G. Matsabisa, N. Bhagwandin, P. J. Smith, P. I. Folb. In vitro antiplasmodial activity of Medicinal Plants native to or naturalized in South Africa. *J. Ethnopharm.* 2004;92 (2-3):177-191.

[4]. Okokon JE,Ubulom PM, Udokpoh AE. Antiplasmodial activity of *Setaria megaphylla* .*Phytotherapy Research*. 2007;21(1): 366 – 368.

[5]. Okokon JE,Antia BS. Hypoglycaemic and Antidiabetic effect of *Setaria megaphylla* on normal and alloxan induced diabetic Rats. J*ournal of Natural Remedy.* 2007;1(4):134 – 138.

[6]. Okokon JE, Bassey AL,Nwidu LL. Antidiabetic and hypolipidaemic activity of ethanolic root extract of *Setaria megaphylla*. *International Journal of Pharmacology.* 2007;3(1):91 – 95.

[7]. Okokon JE,Antia BS, Ita BN. Antiiflammatory and antinociceptive effects of ethanolic extract of *Setaria megaphylla* leaves in rodents. *African Journal of Biomedical Research*. 2006; 9(1):229 – 233.

[8]. Helfand S, Werkmeister J, Roder J. Chemiluminescence response of human natural killer cells. I. The relationship between target cell binding, chemiluminescence, and cytolysis. *The Journal of Experimental Medicine* 1982;156(3): 492 – 505.

[9]. Haklar G, Ozveri ES, Yuksel M, Aktan A, Yalcin AS. Different kinds of reactive oxygen and nitrogen species were detected in colon and breast tumors. *Cancer Letters* 2001;165: 219 -224.

[10]. P. Houghton R, Fang I, Techatanawat Steventon G, Hylands PJ, Lee CC. The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity. *Methods* 2007; 42:377-387.

[11]. Tian B, Hua Y. Concentration dependence of prooxidant and antioxidant effects of aloin and aloe-emodin on DNA. *Food Chem*. 2005; 91: 413 – 418.

[12]. Atta-ur-Rahman, M. I. Choudhary, J. T. William. Bioassay techniques for drug development. Harward Academic Publisher, 1997. pp 67- 68.

[13]. Adams RP. Identification of Essential oils by Gas Chromatography Quadrupole Mass Spectrometry. Allured Publishing Corporation, Carol Stream, USA. 2001

[14]. Setzer WN, Stokes SL, Penton AF. Cruzain Inhibitory Activity of Leaf Essential Oils of Neotropical Lauraceae and Essential Oil Components. *Natural Product Communications* 2007;2(12): 1203 – 1210.

[15]. Tan PG, Zhong WJ, Cai WQ. Continuously infused chemotherapy in treatment of malignant brain tumors. *Zhongguo Zhongliu Linchuang* 2001;28: 682 – 684.

[16]. Sarumathy K,Dhana Rajan MS, Vijay T, Jayakanthi J. Evaluation of phytoconstituents, nephro-protective and antioxidant activities of *Clitoria ternatea*. *Journal of Applied Pharmaceutical Science* 2011; 1(5): 164 -172.

[17]. Kumar PP, Kumaravel S, Lalitha C. Screening of antioxidant activity, total phenolics and GC-MS study of *Vitex negundo*. *African Journal of Biochemical Research* 2010;4(7):191-195.

[18]. Simopoulos AP. Omega-3-fatty acids in health and disease and in growth and development. *Am. J. Clin. Nutr* 1991;54: 438 – 463.

[19]. Simopoulos AP ω-3-fatty acids in the prevention management of cardiovascular disease. *Can. J. Physiolo. Pharmacol.* 1997;75:234 – 239.

[20]. Simopoulos AP. Omega -3-fatty acids and anti-oxidants in edible wild plants. *Boil. Res.* 2004; 37:263-277.

[21]. Aderogba MA, McGaw L. J, Bezabih M, Abegaz BM. Isolation and characterisation of novel antioxidant constituents of *Croton zambesicus* leaf extract. Natural Product Research 2011;25(13):1224 -1233.

[22]. Halliwell B, Gutteridge JM. *Free Radicals in Biology* and*Medicine*, 3rd ed. Oxford: Oxford University Press.1999.

[23]. Anderson R, Gatner EMS, van Rensburg CE, Grabow G, Imkamp FH, Kok SK, van Rensburg J. In vitro and In vivo effect of dapsone on neutrophils and lymphocyte functions in normal individuals and patients with lepromatous leprosy. *Antimicrobial Agents and Chemotherapy*.1981;19: 495 – 503.

[24]. Habtemariam S, Gray AI, Waterman PG. A new antibacterial sesquiterpenes from *Premna aligotricha*. *Journal Natural Products* 1993;56: 3864 – 74.