***In Vitro* Antileishmanial, Antitrypanosomal And Cytotoxic Assessment of Extracts of Three Nigerian Medicinal Plants Using Alamar Blue Assay And Cell Counting Methods**

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ABSTRACT

Infections caused by protozoan species are major worldwide health problems resulting in over three million deaths annually in developing countries. Extracts of three selected plants used as medicinal plants by indigenous local communities in Nigeria were screened against Trypanosoma brucei brucei bloodstream isolates and amastigotes forms of Leishmania major and for cytotoxic activity against normal cell lines (macrophages and L929 fibroblasts) and cancer cell lines (Jurkat and SH-SY5Y) using alamar blue assay and conventional cell counting methods. Results demonstrated that two extracts, aqueous extract of Vernonia amygdalina (VA) and methanolic extract of Annona senegalensis (AS) showed significant antileishmanial activity against macrophage infectivity ($\geq 50\%$) without cellular damage. The percentage suppression with VA was significantly higher ($p < 0.05$) while that of AS was comparable to the standard drug, sodium stibogluconate (SSG) (% suppression with VA=65.1; AS=48.4; SSG=40.2). Two extracts showed strong antitrypanosomal activity with EC_{50} and MIC_{10} values of less than $10 \mu\text{g/ml}$ namely hexane extract of AS (2.88 and 3.12) and methanolic extract of VA (8.04 and 6.25) which were more effective than the reference drug Cymelarsan, while other extracts showed moderate to low activity with values ranging from 10 to above $20 \mu\text{g/ml}$. Cytotoxicity studies showed that only methanolic extracts were toxic to L929 fibroblasts and macrophages while no toxicity was observed on the Jurkat and SH-SY5Y cancer cells at this concentration. Phytochemical screening using GC-MS showed the presence of polyacetylenes, ethers, fatty acids, alkaloids, flavonoids, sulphur and alcohol compounds in the active extracts. The findings of this study has shown that extracts of AS and VA contain active compounds which could serve as alternative agents in the development of antileishmanial and antitrypanosomal drugs and warrants further investigation.

Key words: *Trypanosoma brucei brucei, Leishmania major, Nigerian plants, Alamar blue test*

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INTRODUCTION

Protozoan diseases constitute major worldwide health problems especially in developing countries. Leishmaniasis is caused by several species of the flagellated protozoa belonging to the genus *Leishmania*. *Leishmania major* (*L. major*) is the causative agent of cutaneous leishmaniasis with annual incidence of 1-1.5 million cases. In Nigeria it is endemic in the North-central region [1] and transmitted by the phlebotomus sandfly vector. Trypanosomes are protozoan parasites that cause fatal diseases in humans and animals. African trypanosomiasis is endemic in 36 countries with an annual incidence of 2 million and an estimated 300,000 cases of African sleeping sickness occurring in sub-saharan Africa including Nigeria [2]. *Trypanosome brucei brucei* (*T. brucei brucei*) is a haemoprotozoan parasite which is transmitted by the bite of the tse-tse fly. In Nigeria, the endemicity of African trypanosomiasis is localised and it is one of the major constraints to livestock production [3]. Currently, there is high prevalence of these protozoan diseases and their treatment using chemotherapy is limited as a result of high cost, chemoresistance due to misuse, cumbersome mode of treatment and toxicity [4]. This underscores the need to intensify research for safer, less expensive and readily available drugs for the treatment of these diseases.

This need has stimulated a lot of research work into different strategies to identify novel pharmacophores against protozoan parasites. One promising strategy is exploiting alternative modes of treatment especially phytotherapy through the evaluation of natural products from medicinal plants. It is estimated that two-thirds

of the world population rely on traditional remedies due to its availability and affordability [5]. Plant species of the genera *Vernonia*, *Azadirachta* and *Annona* are sources of flavonoids and alkaloids which are exploited for their ethnopharmacological importance. Several reports on their efficacy against single protozoan species like *Trypanosome spp* [6,7], *Leishmania spp* [8], *Plasmodium spp* [9,10] have been documented however, scanty literature exists on Nigerian plants with broad antiprotozoan activities. Following the trend for the documentation of medicinal plants with broad antiprotozoal activities.

The present study intends to evaluate the effects of different extracts (hexane, methanol and aqueous) of three indigenous medicinal plants against two protozoan parasites (*L. major* and *T. brucei brucei*) and their cytotoxic effects on cancer cell lines *in vitro*.

MATERIALS AND METHODS

Plant collection

The three tested plants were collected from Zaria, Kaduna state in northern Nigeria in February. Voucher specimens were identified by the Chief Botanist, Mr Martins Musa and deposited at the herbarium of the Department of Botany at the Ahmadu Bello University, Zaria. Ethnobotanical data of plants are summarised in Table 1.

Drugs

Cymelarsan (Roche, UK) and Sodium Stibogluconate (SSG) [31.1% (w/v) Sb (V)] (Glaxo Wellcome Ltd, Ware, UK) served as the

positive control drugs for the antitrypanosomal and antileishmanial tests respectively.

Extraction of plants

One kilogram of fresh parts of each of the plants was air dried for two weeks, pounded in a mortar and pestle to coarse portions and milled into powder in a Waring commercial blender and sieved (mesh #40) before being extracted in a Soxhlet apparatus successively with different solvents based on their polarity until the solvent in the Soxhlet ran clear. In this study, *n*-hexane (non polar) was used to defat extracts, methanol (semi polar) and water (polar) were used for extraction of more polar compounds. Extracts were evaporated to dryness at reduced pressure at 40°C. The yields are reported in Table 2.

Phytochemical screening

Preliminary screening for the phytochemical components of the plant extracts was carried out using conventional protocol as previously published [11] in the detection of major phytochemical constituents.

Thin Layer Chromatography (TLC) analysis of Extracts

Thin layer chromatography analysis was carried out using silica-gel pre-coated aluminium plates (60F₂₅₄), layer thickness (250 µm), (Merck, Germany) with ethyl acetate-methanol-water (100: 13.5 : 10) as the mobile phase. The migration distance was 5cm and spots were developed by drying with a heat gun, spraying with p-anisaldehyde/sulphuric acid reagent till the colours developed and visualized under ultraviolet (UV) light.

Gas chromatography and mass spectroscopy analysis (GC/MS)

GC-MS analysis was carried out on crude extracts which showed antiprotozoal activity using a GCMS-QP 2010 PLUS (Shimadzu, Japan) comprising AOC-20i autosampler and a gas chromatograph interfaced with a mass spectrometer (GC-MS) equipment with Elite 5MS (5% Diphenyl/95% Dimethyl poly siloxane) fused capillary column (30 x 0.25 µm ID x 0.25 µm df). Helium gas was used as the carrier gas at a constant flow rate of 1.8 ul/min and an injection volume of 3ul was employed (split ratio of 20:1). The injector temperature was maintained at 25°C, the ion source temperature was 200°C while the oven temperature was programmed for 70°C for 1 min and rose to 280°C at the rate of 10°C/min. Mass spectra was taken at 70eV with a scan interval of 0.5 secs and fragments from 20 – 350 Da. The scan speed was 0.84 scans/sec and scanning period of 30 to 700. Solvent delay was 2.5 mins and total running time was 33 mins. Interpretation of mass spectrum GC-MS was conducted using the database of the National Institute Standard and Technology (NIST) which has more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials (extracts) were determined.

Preparation of samples

Stock solutions of the plant extracts were prepared in dimethyl sulphoxide (DMSO) for hexane, and methanol while aqueous extract

was prepared in phosphate buffered saline (PBS). All extracts were dissolved at a concentration of 10 mg/ml and further diluted (1:100) in HMI-9 culture medium to give a top concentration of 100 µg/ml.

Parasite strains and Cell Lines

The bloodstream isolates of *Trypanosoma brucei brucei* (S427) and *L. major* promastigotes (MRHO/SU/598/P) strains were cultured and maintained at 37°C in HMI-9 and RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) respectively. L929 (mouse skeletal fibroblasts), Jurkat and SH-SY5Y cells (human cancer cells) were obtained from the European Collection of Animal Cell Culture (ECACC) Porton Down, UK. Bone marrow macrophages were harvested from the femur of individual healthy mice by injecting 10 ml of bone marrow medium into the isolated bones in a petri dish [12]. The extracted cell suspension was seeded into 24-well plates containing glass coverslips and incubated at 37°C in 5% CO₂/95% air for 7-10 days to enable growth, differentiation and adherence of macrophages. Non-adherent cells were removed by washing with medium. All cells were maintained in Dulbecco's medium (DMEM) supplemented with 10% v/v foetal bovine serum (FBS), 1% w/v penicillin (50 IU/ml) /streptomycin (50 g/ml) and L-glutamine (2 mM) at 37°C in humidified atmosphere of 5% CO₂/95% air.

Antileishmanial activity

The *in vitro* sensitivity of *L. major* to the test compounds was determined as previously

described by [12]. Briefly, bone-marrow macrophages obtained as previously described were seeded at 4x10⁵/well in 24-well plates before infection with *Leishmania major* amastigotes which was made at a ratio 1:20 (macrophage/amastigotes), in a final volume of 0.5 ml of complete medium and incubated for 24 h at 37°C in a 5% CO₂ humidified incubator. Non-phagocytosed parasites were removed by washing and the wells were replenished with 990 µl of complete medium in drug wells and 1000 µl in control wells. Infected macrophages were further incubated for 72 h at 37°C in the presence of 10 µl of solvent used in dissolving the extract (DMSO or PBS) or 10 µl of appropriate concentrations of plant extracts (100 µg/ml) or Sodium Stibogluconate (SSG) used as reference drug at the same conditions described above. Macrophages incubated without drugs were used for control (100% infected). At the end of the assay intracellular parasites were quantified by fixing cells in methanol for 1-2 mins and staining with 10% Giemsa stain. Stain was removed and the coverslips washed with tap water before removal from the wells of the plate. The coverslips were air dried and mounted using DPX mountant onto glass slides (4 coverslips/slide). All experiments were carried out under aseptic conditions and repeated three times. The parasite burden was determined from the percentage of infected cells in 200 random macrophages and the number of amastigotes per infected macrophage in 20 randomly selected infected macrophages on each coverslip in treated and untreated cultures (n=4). The effect of drug treatment was

determined by calculating the suppression in the percentage of cells infected compared to mean control (infected) values. Values are means of two independent assays; each assay was run in triplicates.

Antitrypanosomal activity

The antiparasitic effect of test compounds against the bloodstream form of trypanosomes from axenic culture of *T. brucei brucei* was evaluated using microdilution susceptibility testing performed in flat-bottom 96-well plates containing HMI-9 culture medium (100 l) in each well. Sample solutions (0.2 ml) were serially diluted (two-fold) to give a test concentration range of 100 g/ml to 0.19 g/ml for plant extracts in order to determine the EC_{50} values. Cymelarsan the standard antitrypanosomal drug was used at a fixed concentration of 50 g/ml. The trypomastigotes obtained from axenic culture were counted on a Neubauer haemocytometer, seeded into each well at a concentration of $2-3 \times 10^4$ /ml and incubated for 48 hours in a humidified atmosphere at 37°C in 5% CO_2 /95% air. Sterility and growth controls in the presence of organic solvents employed in the sample preparation were also included. No inhibitory effects were observed in the presence of DMSO at the highest concentration used (0.5% v/v). The viability of the trypomastigotes was evaluated using Alamar Blue assay [13] as an indicator of cell growth. This is based on the detection of metabolic activity in the cells through the reduction of the Redox indicator from blue (oxidised non-fluorescent form) to red (reduced fluorescent form) by growing cells. For this purpose, 20 l of Alamar blue was added to each well and the plates incubated for a further 24

hours. The number of viable cells was determined colorimetrically and more sensitively fluorometrically:

Colorimetric readings: The colorimetric MIC end-points were interpreted as the lowest sample concentration that remained blue (indicating no growth).

Fluorometric readings: This is fluorescence-based, measured at 530 nm excitation wavelength and 590 nm emission wavelength. Absorbance was detected using Wallac Victor II multilabel microplate reader. This was expressed as percentage of the growth control. MIC values indicated less than 10% of the control growth i.e. value that caused 90% inhibition.

Cytotoxicity

Cytotoxicity of the plant extracts was determined on the mammalian cell lines bone-marrow derived macrophages and L-929 (mouse skeletal fibroblasts) as well as on cancer cell lines Jurkat and SH-SY5Y. The cells were seeded into 96-well microtitre plates at a concentration of 1×10^4 cells/well (L929) and 3×10^4 (Jurkat and SH-SY5Y) in Dulbecco's modification of Eagle's minimum essential medium (DMEM) supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 50 IU/ml penicillin and 50 g/ml streptomycin at a final volume of 130 l and in the presence of the extracts at a fixed concentration of 100 g/ml. Plates were incubated for 72 hours at 37°C and 5% CO_2 in a humidified incubator and

appropriate solvent controls were used for comparison. The viability of the cells was evaluated colorimetrically using Alamar Blue assay as described before.

2.10 Statistical analysis

Data on infection rate were expressed as parasite suppression {mean percentage $\pm$ standard error of mean (SEM)} of at least three experiments in which 200 macrophages were analysed for each drug. Parasite suppression was determined for each drug by comparing the mean number of infected

macrophages and parasite burden in treated with the mean values of relevant untreated controls. The Graph-pad prism program was used to fit the data on growth inhibition of *T. brucei brucei* to nonlinear regression and to determine the concentration that inhibits the growth rate to 50% (50% effective concentration EC_{50}). Differences were tested for statistical significance using the ANOVA and Tukey's posthoc test on a Graph Pad Prism program Version 4.03 software.

RESULTS

Table 1: Plants and ethnobotanical information

Plant Name	Voucher number	Family	Common Name	Vernacular Names	Parts used
<i>Azadirachta indica</i> (AI)	900151	Meliaceae	Neem plant	H-Dogon Yaro I-Acha Y-Agbo	Leaves
<i>Annona senegalensis</i> (AS)	900167	Annonaceae	Custard-apple plant	H-Gwanda daji I-Uburu ocha Y-Abo	Stem bark
<i>Vernonia amygdalina</i> (VA)	900675	Asteraceae	Bitter leaf	H-Shuwaka I-Onugbu Y-Ewuro	Leaves

() - code, H-Hausa, I- Igbo and Y- Yoruba

Table 2: Phytochemical screening and Percentage yield of crude plant extracts

Plant	Extract	%Yield	Appearance	Active constituents
<i>Azadirachta indica</i>	Hexane	1.57	Yellow smear	Flavonoids, sterols
	Methanol	25.06	Dark green caramel	Flavonoids, Reducing sugar, terpenoids, saponins
	Water	13.30	Light brown pellets	Reducing sugars, tannins, alkaloids
<i>Annona senegalensis</i>	Hexane	1.63	Yellowish brown smear	Sterols, flavonoids
	Methanol	25.56	Deep brown caramel	Flavonoids, terpenoids, tannins,
	Water	14.30	Golden brown pellets	Alkaloids, flavonoids, glycoside
<i>Vernonia amygdalina</i>	Hexane	1.54	Greenish black smear	Glycosides, flavonoids, sterols
	Methanol	28.69	Deep green caramel	Flavonoids, saponins, tannins
	Water	15.03	Blackish brown pellets	Alkaloids, flavonoids, tannins, glycoside

Table 3: Phytochemical components identified in the active extracts of three Nigerian plants by GC-MS study

RT	Compound	CF	MW	Hexane			MeOH		AQ
				AI	AS	VA	VA	AS	
4.0	2,2dimethyltetrahydro Furan	C ₆ H ₁₂ O	100	+ve	+ve	-ve			
4.2		C ₆ H ₁₂ O	100	+ve	+ve	-ve			
8.4	3-methyl 2-Pentanone	C ₁₀ H ₁₈ O	154	-ve	-ve	+ve			
9.5	2-Decenal (E)	C ₁₀ H ₁₆ O	138	-ve	-ve	+ve			
13.2	2,4-Decadienol (E,E)	C ₁₂ H ₁₄ O ₂	190	-ve	-ve	+ve			
	7-methoxy-2,2-dimethyl								
14.7	2H-1-benzopyran	C ₁₁ H ₁₆ O ₂	180	+ve	+ve	-ve			
16.3	2(4H)-benzofuranone	C ₇ H ₁₂ O ₆	192				-ve	+ve	+ve
16.7	D(-) Quinic acid	C ₁₀ H ₁₇ NO ₆ S	279				-ve	+ve	
18.6	Desulphosinigrin	C ₁₈ H ₃₆ O	268	+ve	-ve	-ve	-ve	-ve	
	Hexahydrofarnesyl acetone								
19.5	Palmitic acid methyl ester	C ₁₇ H ₃₄ O ₂	270	+ve	-ve	-ve	+ve	+ve	
20.1	Palmitic acid	C ₁₆ H ₃₂ O ₂	256	+ve	+ve	+ve	+ve	+ve	+ve
22.7	3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol)	C ₂₀ H ₄₀ O	268	+ve	-ve	-ve			
22.9	Oleic acid	C ₁₈ H ₃₄ O ₂	282	+ve	+ve	+ve	+ve*	+ve	+ve
27.5	9,12-Octadecadienoyl* chlorideZ,Z (Linoleoyl chloride)	C ₁₈ H ₃₇ Cl O	298			+ve*	+ve	+ve	+ve
28.3	Tetracontane		562					+ve	
29.5	Nonacosane*	C ₄₀ H ₈₂ C ₂₆ H ₆₀	408	+ve		+ve*			

indicates that other compounds share the same Retention Time (RT). CF Chemical formula, MW Molecular weight, AI-*Azadirachta indica*, AS-*Annona senegalensis*, VA -*Vernonia amygdalina*, Hex-Hexane, MeOH-Methanol and Aq-Water. +ve indicates presence and -ve indicates absence of compound

Table 4: *In vitro* antileishmanial activity of plant extracts (100 µg/ml) and Sodium Stibogluconate on the amastigote form of *L. major*.

Treatment	% infection of cells	% suppression	No. of parasites per host cell	% suppression of parasites
Control	93.3 ± 2.5	-	14.3 ± 1.9	-
DMSO	91.9 ± 2.5	1.6 ± 1.0	13.9 ± 1.9	3.7 ± 2.5
PBS	92.9 ± 2.0	0.4 ± 1.2	16.5 ± 5.5	0 ± 0
SSG	56.7 ± 3.1	40.2 ± 2.4	4.5 ± 0.3*	75.7 ± 5.1
AI Hexane	84.4 ± 1.9	11.6 ± 1.6	5.5 ± 0.9*	59.9 ± 8.0
AI Methanol	ND	ND	ND	ND
AI Aqueous	82.1 ± 2.1	8.6 ± 1.7	4.4 ± 0.4*	70.5 ± 4.2
AS Hexane	73.9 ± 4.3	22.7 ± 4.9	5.2 ± 0.1*	63.1 ± 2.4
AS Methanol	47.0 ± 1.4*	48.4 ± 2.5	3.2 ± 0.6*	74.6 ± 4.9
AS Aqueous	84.4 ± 7.0	9.1 ± 4.6	7.0 ± 4.0*	59.1 ± 11.9
VA Hexane	74.1 ± 8.4	21.2 ± 7.3	5.4 ± 0.4*	61.3 ± 2.4
VA Methanol	ND	ND	ND	ND
VA Aqueous	31.2 ± 4.4*	65.1 ± 6.9	2.3 ± 0.4*	85.3 ± 0.7

ND indicates not determined due to toxicity

All values are means ± SEM

Symbols indicate significant difference at p<0.001 (*), p<0.01(*), and p<0.05 (*) as compared to the control.

AI, AS and VA stand for *Azadirachta indica*, *Annona senegalensis* and *Vernonia amygdalina* respectively

Table 5: *In vitro* trypanocidal activity of plant extracts and Cymelarsan against *Trypanosome brucei* brucei expressed in ED₅₀ and MIC₁₀

Plant Code	Extract	ED ₅₀ (µg/ml)	CI	R ²	MIC ₁₀ (µg/ml)
AI	Hexane	10.45±0.41	20.53-23.45	0.9989	12.5
	Methanol	21.94±0.99	20.53-23.45	0.9989	25
	Aqueous	ND	ND		100
AS	Hexane	2.88±0.43	2.80-2.97	0.9999	3.12
	Methanol	34.48±0.49	29.91-39.74	0.9996	50
	Aqueous	ND	ND		>100
VA	Hexane	14.77±0.49	0.0066-33045	0.9998	25
	Methanol	8.09±0.27	8.69-9.11	0.9999	6.25
	Aqueous	53.87±0.30	51.68-56.45	0.9998	50
Cymelarsan		10.00±0.04			50
DMSO					100
PBS					>100

MIC indicates the minimum inhibitory concentration as determined by colorimetric endpoint method. It is interpreted as the lowest sample concentration that remained blue (indicating no growth). This result is representative of two tests.

ND indicates not determined due to large values and wide confidence interval (CI)

AI-*Azadirachta indica*, AS-*Annona senegalensis*, VA - *Vernonia amygdala*, DMSO-Dimethyl sulphoxide

Table 6: Cytotoxic activity of plant extracts (100 µg/ml) on different cell lines

Extract (100 µg/ml)	L929	Macrophages	Jurkat	SH-SY5Y
AI Hexane	-ve	-ve	-ve	-ve
AS Hexane	-ve	-ve	-ve	-ve
VA Hexane	-ve	-ve	-ve	-ve
AI Methanol	+ve	+ve	-ve	-ve
AS Methanol	-ve	-ve	-ve	-ve
VA Methanol	+ve	+ve	-ve	-ve
AI Aqueous	-ve	-ve	-ve	-ve
AS Aqueous	-ve	-ve	-ve	-ve
VA Aqueous	-ve	-ve	-ve	-ve

-ve indicates not toxic

+ve indicates toxic

Figure 1: TLC fingerprints of the crude extracts of plants detected under UV 365nm and sprayed with P. anisaldehyde/sulphuric acid reagent. Plate I- Crude extracts of hexane (Lane 1), methanol (Lane 2), aqueous (Lane 3) of VA; methanol (Lane 4), aqueous (Lane 5) and hexane (Lane 6) of AS; standard compound sesquiterpene SST (Lanes 7 and 8). Plate II- Pooled fractions from hexane (Lanes 1,2), methanol (Lane 3) and aqueous (Lanes 4 and 5) extracts of VA. Plate III- fractions from crude extract of AI. Plates I and II showed the presence of high levels of sesquiterpenes in the methanol and aqueous extracts of VA as indicated by the thickness of the bars at the same Rf level with the standard compound used. Methanol extract of AS showed low levels while compound was absent in the extracts of AI.

Figure 2: Bar charts showing the effect of plant extracts against *L. major* amastigotes. Each extract was tested twice at fixed concentration of 100 ug/ml. Sodium Stibogluconate was used as standard drug. The percentage infectivity of macrophages (panel A) and number of amastigotes (panel B) were determined by microscopic counting method. Data which considered the mean of two performed assays from four coverslips for each treatment was analyzed using Graph Pad Prism Version 4.03 software. VA MeOH and AI MeOH were toxic to the macrophages hence was not determined (blank spaces).

Figure 3: Sample slide of A-untreated (control) showing macrophages infected with amastigotes and B-treated (plant extracts and SSG) showing macrophages uninfected with amastigotes. Amastigotes are small round or ovoid structures that occupy parasitophorous vacuoles (black arrows) in the cytoplasm of macrophages. Macrophages are viable with large eccentrically placed nuclei, cytoplasm and pseudopods. (X1000 Giemsa stain).

Figure 4: Sample plate showing results of trypanocidal activity of plant extracts obtained using the colorimetric endpoint determination method. Column G indicates growth control i.e. viability of the parasites, column S indicates sterility control using Cymelarsan a known drug with antitrypanosomal activity. Rows A to F indicate plant extracts: Row A- AI MeOH, Row B-AS MeOH, Row C-VA MeOH, Row D-AI Hexane, Row E- AS Hexane, Row F- VA Hexane, Rows G and H indicate DMSO. Pink colouration indicates growth of parasites while blue-black colouration indicates inhibition of growth i.e. no growth. Extracts inhibited parasite growth by exhibiting blue-black colouration at certain concentrations. Minimum concentration at which growth of parasite was inhibited is the MIC value for each extract.

Figure 5 : GC-MS Chromatograms of active hexane extracts of the three plants against *T.brucei brucei* with that of AS being the most effective and VA least effective. All hexane extracts showed presence of polyacetylenes. AS and AI showed homogeneity (similarity) in the composition of most compounds identified while VA was heterogeneous.

Figure 7 : GC-MS Chromatograms of active methanol and aqueous extracts of plants which showed good activity against *L. major* amastigotes and less activity against *T.brucei brucei*. Extracts contained more polar compounds

Phytochemical screening

Thin Layer Chromatography (TLC) analysis of Extracts

Results from Table 2 showed that methanolic extracts contained significant amount of flavonoids, saponins, triterpenoids, tannins, sterols and reducing sugars hence they had paste-like caramel consistency. Aqueous extracts contained alkaloids, tannins and glycosides in significant amounts while flavonoids occurred in minimal amounts. Hexane extracts contained high level of fatty acids and sterols while flavonoids occurred at minimal levels hence extracts gave an oily consistency. TLC analysis from fig. 1 showed that VA contained bitter principles due to the dark green spots in R_f range of 0.3-0.6 (Plate I). This was not observed in AS fingerprints. Also the aqueous and methanol extracts of VA showed similar pattern to sesquiterpene the reference compound. This was found to be a major component in the pooled fractions of methanolic and aqueous extracts of VA (Plate II). Hexane extracts showed less polar compounds which migrated to the solvent front indicating the presence of essential oils. Fractions from extract of AI which showed little migration with low R_f value of 0.2 in a polar solvent system suggests the presence of saponins (Plate III).

GC-MS Analysis

Phytochemical analysis of the active extracts using GC-MS spectra identified six, eleven and nine compounds from the hexane extracts of AS, AI and VA respectively. Results from table 3 showed that the hexane extracts of the three plants showed similar polyacetylene compounds. In addition, hexane extract of AS and AI which were more effective than VA against *T. brucei brucei* showed presence of

ketone group pentanone and tetrahydrofuran which were absent in VA. Other compounds like pentacosane, phytol, squalene and benzopyran were also heterogeneous. Methanol and aqueous extracts of VA and methanol extract of AS gave five, three and seven compounds respectively including alkaloids and antioxidants like linoenyl chloride, linoleic, quinic and valeric acid. All the active extracts indicated the presence of three major fatty acids namely oleic, stearic and palmitic acids. Erucic acid was heterogeneous. Hexane and methanol extracts of AS showed C35/C37 fatty acid and di-tetrahydrofuran acetogenins and sulphur compound (desulphosinigrin) respectively (Table 3).

Antileishmanial Activity

Nine extracts were screened for *in vitro* antileishmanial activity against *L. major* and from Table 4, only two extracts exhibited good antileishmanial activity (based on the significant reduction in the percentage of infected cells to less than 50%) and was comparable to the reference drug SSG. Results from Fig 2A showed that the most active was the aqueous extract of VA which demonstrated the highest percentage suppression of infected cells (65.1%) and parasite per host cell (85.3%). The other effective extract was methanol extract of AS with approximately 50% and 75% suppression of infected cells and parasite number respectively. Treatments with PBS or DMSO did not affect the infected cells or parasites as compared to the controls. Also, results from Fig 2B showed that all the extracts suppressed the parasite numbers per host cell (>50%). This indicates a direct effect on the parasites by the plant extracts. However, methanolic extracts of VA and AI were toxic to the macrophages as such data could not be determined from their slides. Together these results demonstrate that VA and AS are

effective against the amastigotes forms of *L. major* without toxicity to the macrophages (macrophages were viable with large eccentrically placed nucleus and cytoplasm occupied by parasitophorous vacuoles as in Fig. 3). Their leishmanicidal activity lies in the aqueous and methanolic extracts respectively.

Antitrypanosomal activity

Trypanocidal activity against *T. brucei brucei* was determined colorimetrically and spectrophotometrically. Table 5 shows the minimum inhibitory concentrations (MIC) equivalent to 90% inhibition of growth control of the nine extracts and the reference drug, Cymelarsan. Fig. 4 is an example of a plate showing the MIC results obtained using the colorimetric endpoint determination with Alamar Blue indicator. The indicator is reduced to pink by viable cells undergoing metabolic activity while it remains oxidized as blue colour by non-viable cells indicating inhibition of cell growth or metabolic activity. Overall, hexane extracts showed high selectivity for *T. brucei brucei* particularly AS which showed strong trypanocidal activity (MIC= 3.12 µg/ml) that was over tenfold more effective than the reference drug (MIC= 50 µg/ml). This presents an interesting observation as the standard drug is in its pure form and the plant extract in its crude form are comparable as such further isolation of the active compound from the plant may enhance the antitrypanosomal activity. Bioassays and *in vivo* trials are in progress to determine this. Methanolic extracts showed moderate activity with VA being most effective while aqueous extracts showed weak activity. Based on the EC values, results from Table 4 and Fig. 5 showed that three extracts exhibited strong antitrypanosomal activity with EC₅₀ values comparable to that of the reference drug Cymelarsan. The most active extract was the hexane extract of AS (EC₅₀= 2.88±0.43,

confidence interval: 2.80 to 2.97, $r^2=0.9999$). The other active extract was methanolic extract of VA (EC₅₀ = 8.09±0.27, confidence interval: 8.69 to 9.11, $r^2=0.9999$). Results showed that the lipophilic hexane extracts showed remarkable activity against *T. brucei. brucei* while the more polar methanol and aqueous extracts showed moderate to weak activity.

Cytotoxicity

Regarding the cytotoxicity of the nine extracts, results from Table 5 showed that the methanolic extracts of the three plants were toxic to the mammalian cells corroborating the antiprotozoal tests. However, hexane extract of *Annona senegalensis* was slightly toxic to the L929 fibroblasts but not macrophages while other hexane and aqueous extracts did not show any toxicity to the mammalian cell line. Furthermore, none of the nine extracts exhibited cytotoxicity against the Jurkat and SH-SY5Y cancer cell lines at the tested concentrations.

DISCUSSION

Annona senegalensis is native to Africa and widely cultivated in Nigeria. It is used in ethnomedicinal practices for the treatment of several ailments. In this study, AS showed broad antiprotozoal activity against *L. major* amastigotes and *T. brucei brucei*. The hexane extract exhibited high trypanocidal activity and phytochemical analysis revealed the presence of acetogenins which are C35/C37 long chain fatty acid derivatives (palmitin), and di-tetrahydrofuranic acetogenins commonly found in Annonaceae family. Most of these acetogenins exhibit a broad range of potent biological activities including antiparasitic effects and they may be responsible for the observed activity. In addition all the hexane extracts contained polyacetylenes which are a major group of compounds found in most

hexane extracts and widely distributed in plants and have shown antitrypanosomal activity against *T. brucei brucei* [14]. Antitrypanosomal activity of the hexane extract of AS is reported possibly for the first time and it is worthy of further investigation. Past studies have documented antitrypanosomal activity of the aqueous extract against *T. brucei brucei*, however, in this study, the aqueous stem bark extract was inactive underscoring the need to evaluate all parts of a plant before any generalisation is made on the plant's pharmacological and therapeutic potentials. Also, the methanolic stem bark elicited poor trypanocidal activity similar to the methanolic root bark extract earlier reported against *T. brucei brucei* [17]. These results demonstrated that the active trypanocidal compound is better extracted in hexane. Furthermore, the methanolic extract of AS demonstrated antileishmanial activity on the amastigote form of *L. major* which is a more predictive result than that of the promastigote forms used in previous studies [18]. This is because amastigotes are the mammalian forms responsible for the clinical manifestation of leishmaniasis in humans. Phytochemical analysis revealed the presence of natural antioxidants (quinnic and valeric acids), alkaloids (lineoleic acid chloride), glucosides and thioglycosides (desulphosinigrin). This is in line with past studies that showed the presence of diterpenoids [19] and alkaloids [20] in AS and these may contribute to the antileishmanial activity as these compounds have been implicated in the inhibition of essential antioxidant enzymes like glutathione reductase and trypanothione reductase found in *Leishmania* and *Trypanosome* parasites [21]. This may be responsible for the observed broad antiprotozoal activity with AS.

Vernonia amygdalina (Asteraceae) is native to Nigeria and commonly referred to as bitter leaf. Recent review has shown that VA has numerous phytotherapeutic properties some of

which have been substantiated in scientific literature [22]. Aqueous extract of VA demonstrated strong antileishmanial activity on the amastigote forms as it decreased infection rate of the bone-marrow macrophages and showed direct leishmanicidal effects on the amastigotes by significantly suppressing the number of parasites per host cell without any toxicity to the macrophage. This suggests that it has selective and not general toxicity against the leishmania parasite unlike the methanolic extract which was toxic to the macrophages. This corroborates past works in Ethiopia which demonstrated antileishmanial activity of VA against *L. aethiopica* with amastigotes being more sensitive than promastigotes [23]. It was also potent as an antitrypanosomal plant as all its three extracts showed activity with the methanolic extract being more effective than the hexane extract while the aqueous extract was the least effective. This affirms results from previous studies which demonstrated that the methanolic extract of the leaves showed trypanocidal activity against *T. brucei brucei* and *T. congolense* [15]. These results demonstrated broad antiprotozoan activity with cross activity against several *Leishmania* and *Trypanosome* species. Phytochemical screening of VA revealed the presence of fatty acids like oleic, palmitic stearic, linoleic and eicosanoic acids and their esters. These components have demonstrated high antiprotozoal activity but with toxicity to mammalian cells [24] and may be responsible for the observed toxicity of the methanol extract on intracellular leishmania amastigotes. However, the aqueous extract contained phenolic compounds and was effective against leishmania amastigotes without toxicity. Hexane extract was highly effective against extracellular form of *T. brucei brucei* and showed the presence of terpenes (benzopyran and tetramethyl benzofuranone) and terpene lactone (cyclohexadecanolide). This is in line with previous reports on the bioactive

principles in VA [25,26,27]. Thus its antiprotozoal properties could be attributed to the broad antiparasitic activities of these compounds.

Azadirachta Indica (Meliaceae) though native of Asia is now cultivated throughout Nigeria and commonly known as Neem. AI is reputed to be of great medicinal value and considered a major component in Ayurveda particularly as an antimalarial. The medicinal utilities have been described especially for the leaf, fruit and bark [28]. It showed activity by reducing viability of the amastigotes (suppression of parasite number) but was not effective in suppressing the infectivity of the macrophages. This is in contrast to results of previous studies which showed antileishmanial activity on promastigote forms [29]. Even though amastigote forms which are more predictive of the clinical manifestation was used in this study. Also, it showed high and moderate trypanocidal activities in the hexane and methanolic fractions respectively while the aqueous fraction of the leaves was ineffective. This is in line with previous *in vivo* study on *T. brucei brucei* which showed that treatment with AI significantly increased survival in rats in a dose dependent manner [30]. Its efficacy against Trypanosome, Plasmodium and promastigotes forms of Leishmania parasites but not on amastigotes could be explained in the light of the localization and cellular differences in the parasites in which the amastigotes have a parasitophorous vacuole inside the macrophages [31] while the other parasites are found free in the cytoplasm of cells or extracellular [32]. This suggests a variation in the sensitivity of AI to the different forms of parasites with selectivity for extracellular forms than intracellular. Reason for this observation is unknown. Phytochemical screening of the hexane extract of AI which was most effective showed presence of limonoids (tetrahydro benzofuranone), phytol, squalene, oxirane,

palmitin and fatty acids with their esters. These are biologically active compounds which may be responsible for the trypanocidal effect. It is in line with previous reports on the chemical composition of AI [33] and its antiplasmodial efficacy.

The results of the extracts on the cancer cell lines showed that they were not cytotoxic to the cells at the concentrations used. This conforms to previous work to determine the effect of *Vernonia amygdalina* and *Annona senegalensis* on LPS/H₂O₂ stressed Jurkat cells which showed that the extracts had cytoprotective effect on the cells as they showed antioxidant activity [33]. It is possible that the phytochemical constituents of the extracts are potential antioxidants as such not toxic to the cells.

Conclusion

This study has identified several extracts of Nigerian plants with potential antiprotozoal activity against *T. brucei brucei* and *L. major* thus supporting further investigation of components of these medicinal plants as potential sources for new antiprotozoal agents with cross reactivity against several species. In particular, it revealed that the hexane extracts of AS could be useful in the herbal formulation for treatment of trypanosomiasis while the aqueous and methanolic extracts of VA and AS for the treatment of cutaneous leishmaniasis. This study presents a basis for which further characterisations and isolation of these bioactive compounds could be made.

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