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(RESEARCH ARTICLE)



Anti-trypanosomal evaluation of *Ximenia americana* root bark and chromatographic-mass spectrometric profile

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Abstract

Medicinal plants are the richest bio-resource of drugs for traditional systems of medicine, modern medicines, food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs. Human African Trypanosomiasis (HAT) is a challenging and deadly disease due to its complex epidemiology and clinical presentations. This study was conducted to investigate anti-trypanosomal action of *Ximenia americana* root bark on *Trypanosoma brucei brucei* using various solvent extracts and to develop thin layer (TLC-MS) and liquid chromatography-mass spectrometric (LC-MS) profiles of the plant. Soxhlet extraction was used to obtain acetone, 70% ethanol total extracts in addition to n-hexane, dichloromethane, ethyl acetate and methanol fractions by sequential extraction. The inhibitory activity of the various extracts was compared by testing against *T. b. brucei* using isometamidium chloride as standard drug. The most active extract was separated by solid-phase extraction (C18 stationary phase) to obtain fractions which were profiled by TLC-MS (+ESI) and LC-MS. It was observed that anti-trypanosomal activity of acetone (16.83% yield) and 70% ethanol (18.23% yield) were comparable. However, methanol extract exhibited the highest activity with 99.18%, 97.5% and 87.50% inhibition at 3 h incubation (room temperature) using 1000 µg, 500 µg and 250 µg concentrations respectively. The activities at 1000 µg for methanol extract and isometamidium chloride were comparable with 95% CI [-1.10, 1.77]. TLC-MS and LC-MS analyses suggested gallic acid, 2,3,4,5-tetrahydroxybenzoic acid, 2',5-dimethoxyflavone, quercetin, dihydroquercetin and sesquiterpene when compared with literature database. This study presents data that could be useful in standardisation and preparation of alternative medicine in the treatment of African trypanosomiasis.

Keywords: Trypanosomiasis; *Ximenia americana*; Isometamidium chloride; Thin-layer chromatography; Mass spectrometry

1. Introduction

Human African Trypanosomiasis (HAT) is a challenging and deadly complex disease due to its complex epidemiology and clinical presentations. Presently, there is no vaccine against sleeping sickness because of the ever-changing variable surface glycoproteins (VSG) which prevent effectiveness of the host's immune response [1-2]. The drugs used for treatment of human and animal trypanosomiasis are limited with adverse effects [3]. Trypanosomes have shown resistance to current drugs used for the treatment of trypanosomiasis [4]. This present predicament in combination with adverse drug effects stimulates research studies on alternative therapy from plant products. Ethno-botanical records of various plants indicate promising results using active medicinal plants in the development of cheaper and lesser toxic drugs [5]. Some investigators have reported that some medicinal plants have anti-trypanosomal activity [6-7]. In Nigeria, about 90 plants have been identified, with 54 compounds as potential active agents against

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trypanosomiasis [8]. Some of these plants include *Khaya senegalensis*, *Moringa oleifera*, *Vitex simplicifolia*, *Tridax procumbens*, *Monodor amyristica* amongst others. *Ximenia americana* used in this study is one of the eight (8) species of the genus *Ximenia* which belongs to the family Olacaceae [9]. It is commonly known as “wild olive” in English and Yellow Plum or Sea Lemon in Australia and Asia; in Nigeria; Tsada (Hausa), Chabbuli (Fulani), Anomadze (Tiv), Igo (Yoruba) [10-11]. Ethno-medicine research on the plant revealed that the leaves have antibacterial activity [12] and has been used in the treatment of fever, tuberculosis, stiffness, onchocerciasis, tooth decay, leprosy, syphilis, dysentery, and wounds [13]. James *et al.* [14] and Maikai *et al.* [15] investigations reported that methanolic root extract of the plant was active against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus vulgaris*, *Candida albicans*, *Bacillus subtilis*, *Salmonella typhi* and *Shigella flexineri*.

Phytochemical screening of methanol extract of *X. americana* revealed presence of alkaloids, anthraquinones, cardiac glycosides, flavonoids, phlobatannins, saponins, tannins and terpenoids while the aqueous extract showed presence of flavonoids, saponins, tannins and terpenoids [16]. The antimicrobial activity of the plant extracts could be attributed to the presence of these secondary metabolites in *X. americana*. Le *et al.* [17] isolated sambunigrin, gallic acid, gallotannins, β -glucogalline, 1,6-diagalloyl- β -glucopyranose, quercitrin, quercetin, avicularin, quercetin-3-o- β -d-oxylopyranoside, quercetin-3-o-(6"-galloyl)- β -glucopyranoside and kaempferol-3-o- β -oxylopyranoside from *X. americana* leaves. Biological and chemical understanding of any natural product is important in medicinal chemistry. Therefore, this study was conducted to investigate anti-trypanosomal action of *Ximenia americana* root bark on *Trypanosoma brucei brucei* using various solvent extracts and to develop TLC-MS and LC-MS profiles of the plant.

2. Material and methods

2.1. Plant collection, authentication and extraction

The plant material was collected in Jos, Plateau State, Nigeria, and authenticated after which a voucher specimen was kept in the Pharmacognosy department. The plant part was pulverized and 70 g was sequentially extracted with the Soxhlet apparatus using hexane, dichloromethane, ethyl acetate, methanol and water. 70% ethanol and acetone were used separately so as to have ethanol and acetone total extracts. Each extract was filtered, evaporated to dryness using Bibby vacuum rotary evaporator (RE 100), and stored in an air tight container until further analysis.

2.2. Biological assay

2.2.1. Extract preparation

10 mg of each extract was weighed and dissolved in 1 mL of 0.1 M phosphate buffer saline (PBS) at pH 7.4 to obtain 10 mg/mL stock solution. Concentrations of 1 mg/mL, 0.5 mg/mL and 0.25 mg/mL were prepared from the stock by serial dilution with PBS.

2.2.2. Trypanosome

Trypanosoma brucei brucei (Federe strain) was obtained from Nigerian Institute for Trypanosomiasis (and Onchocerciasis) Research (NITR), Kaduna, Nigeria and inoculated intraperitoneally into two mice which served as donor animals. They were properly monitored for 72 h to ascertain presence of trypanosome. One of the animals was humanized (according to the animal management guidelines of NITR to get infected blood into sample bottle containing ethylenediamine tetra-acetic acid (EDTA). The blood was diluted with phosphate buffer saline-glucose (PBSG) as described by Atawodi *et al.* [18] to have approximately 15.8×10^7 parasites/mL. Parasite count was estimated using the rapid matching method of Herbert and Lumsden [19].

2.2.3. In-vitro test

100 μ L was measured from each of the prepared 1 mg, 0.5 mg, 0.25 mg plant extracts and transferred into 98-well plate to give final concentrations of 1000 μ g/mL, 500 μ g/mL and 250 μ g/mL respectively. 50 μ L of infected blood (15.8×10^7 parasites/mL) was added into each well to have total volume of 150 μ L and incubated at room temperature. Microscopic examination was carried after 1 h and 3 h of incubation at room temperature using microscopic magnification X40. This was done in triplicate pattern for certainty. Isometamidium chloride (Samorin®) (Merial – 29, avenue Tony Garnier – 69007 Lyon – France) was used as standard drug.

Percentage (%) inhibition was calculated as;

$$\frac{\text{Total Number of parasite count in control} - \text{Total Number of parasite count in test extract}}{\text{Total Number of parasite count in control}}$$

2.2.4. Statistical analysis

The group mean \pm SD was calculated for each analyte by analysis of variance (ANOVA), post-test analysis was done using Newman-Keuls comparisons. All statistical analysis was performed using GraphPad Prism software package (version 5).

2.2.5. Chromatography and spectrometry

Solid-Phase Extraction (SPE) was carried out using reversed phase (C18 Sep-Pak); 200 mg sorbent pore size 125Å with methanol: water mobile phase to obtain fractions labeled as A1-50:50; A2-60:40; A3-70:30; A4- methanol; A5-ethyl acetate. Thin layer chromatography (TLC) was done on gel 60ÅF254 TLC analytical aluminum plate (Merck KGaA, 64271 Darmstadt, Germany) with 7 cm developing distance in methanol: water: ethyl acetate: formic acid (25:5:2:0.5; v/v) and sprayed with anisaldehyde/sulfuric acid. Crude extract (C) and rutin (R) were spotted along the various fractions for similarity. Thin-layer chromatography – Mass spectrometry (TLC-MS) adopted in this study involved direct elution of a given band from the chromatographic plate with the use of TLC-MS interface (Advion Expression LCMS), which enabled an introduction of a given band to mass spectrometer. In this study, elution was carried out at capillary temperature of 150°C using acetonitrile: water, 95:5 v/v with 0.1% formic acid (flow rate 0.2 mL/min). The eluent was directly introduced to the Expression LCMS m/z 2000 model mass spectrometer (Advion) and the samples were analyzed in the electro spray ionization; ESI mode (Full ESI-MS scan, positive ionization, drying gas temperature 350°C, capillary voltage 150V). Liquid Chromatography-Mass spectrometry (LC-MS) was carried out using Thermo Scientific Accela LC system with PDA detector controlled by Xcalibur software and coupled to Orbitrap Q-exactive Focus mass spectrometer.

3. Results and discussion

3.1. Extraction and biological activity

Acetone and 70 % ethanol showed comparable yield of 16.83 % and 18.23 % respectively meanwhile methanol had the highest yield of 19.53 % (Table 1) showing that percentage yield obtained was independent of the solvent used for extraction; however, tannins which were shown to be present in the plant material are reported to be efficiently extracted with acetone. Anti-trypanosomal activity of acetone and 70% ethanol were observed to be comparable while methanol extract exhibited the highest activity with 99.18%, 97.5% and 87.50% inhibition at 3 h incubation (room temperature) using 1000 µg, 500 µg and 250 µg concentrations respectively (Table 2). It was observed that inhibitory ability of the extracts increased with time. The results obtained showed that at 1000 µg methanol extract and isometamidium chloride had comparable activity with 95% CI [-1.10, 1.77] and [0, 0] respectively. There was no significant difference ($P < 0.05$) in activity between methanol extract of *X. americana* root bark when compared to isometamidium chloride suggesting that the extract contains secondary metabolites which are active against *Trypanosoma brucei brucei*. However, there was significant difference ($P < 0.05$) in 70% ethanol, acetone and ethyl acetate compared with isometamidium chloride (Table 2) similar to an earlier report by Maikai *et al.* [20]. Plants are known to contain a myriad of complex chemical compounds which could be essential health-wise to humans and animals. Complete elimination of parasites or reduction in motility when compared to the control could be taken as indices of activity [6]. Anti-trypanosomal activity observed in methanol extract against *T. brucei brucei* could be linked to presence of polyphenolic, tannins and saponins constituents which can cause cessation, drop in motility and total clearance of trypanosome *in vitro*. Flavonoids are known to exert antioxidant effect thereby reducing oxidative reaction (free radicals) in the bloodstream [21-22] and saponins are reported to interact with parasite membrane, protein and phospholipids, thereby causing parasite apoptosis [23]. Moreover, flavonoids are naturally occurring phenols, which possess numerous biological activities such as anti-inflammatory, antifungal, antibacterial and vasoprotective effects [14]. *X. americana* has been used as alternative medicine for treatment of malaria, leprotic ulcers and infectious diseases of mixed origin by natives in Ethiopia, Guinea, Sudan and the Northern part of Nigeria [24]. This study however reports the *in vitro* anti-trypanosomal activity of different solvent extracts derived from *X. americana* root bark against *T. b. brucei* and the chromatographic profiles of the active fraction. However, *in vivo* study is required to ascertain toxicity level of the extracts and their ability to cross cell membranes (blood brain barriers).

Table 1 Percentage yield of different solvents

Solvent	Acetone	70% Ethanol	Hexane	Dichloromethane	Ethyl acetate	Methanol
Amount of biomass (g)	70	70	70	70	70	70
Amount of extract recovered (g)	11.78	2.8	0.5	1.5	4.3	13.7
% yield	16.83	18.23	0.71	2.14	6.14	19.53

Table 2 Biological activity of *X. americana* root bark against *T. brucei brucei* after 3 h incubation at room temperature

	Extract	Concentration ($\mu\text{g/mL}$)		
		1000	500	250
Acetone	Parasitemia	36.67 \pm 1.53	37.00 \pm 1.00	40.00 \pm 0.00
	% Inhibition	8.33	7.50	-
	Significant difference	***	***	***
70% EtOH	Parasitemia	34.67 \pm 0.58	37.67 \pm 0.58	40.00 \pm 0.00
	% Inhibition	13.33	5.83	-
	Significant difference	***	***	***
EtOAc	Parasitemia	34.33 \pm 0.58	37.00 \pm 1.00	40.00 \pm 0.00
	% Inhibition	14.18	7.50	-
	Significant difference	***	***	***
MeOH	Parasitemia	0.33 \pm 0.58	1.00 \pm 0.00	5.00 \pm 1.00
	% Inhibition	99.18	97.50	87.50
	Significant difference	Ns	Ns	Ns
Positive Control	Parasitemia	38.67 \pm 0.58	40.00 \pm 0.00	39.67 \pm 0.00
	% Inhibition	-	-	-
	Significant difference	***	***	***
Isometamidium Chloride	Parasitemia	-	-	4.67 \pm 1.16
	% Inhibition	100	100	88.33

Parasitemia values are mean \pm standard deviation; n = 3; significant different at P<0.05 compared with isometamidium chloride (***) = extremely significant; Ns = Not significant; - = Nil) EtOH = Ethanol, EtOAc = Ethyl acetate, MeOH = Methanol

3.2. Thin layer chromatography (TLC) analysis of solid-phase extraction (SPE) fractions

Fractions obtain from SPE were examined on TLC plate (normal phase; analytical silica gel 60Å F₂₅₄ plate) with methanol: water: ethyl acetate: formic acid (25:5:2:0.5 v/v) as mobile phase. Fractions labeled as A1, A2, A3 and crude extract (Fig. 1a&b) were similar with R_f values of 0.35, 0.34, 0.34 and 0.35 respectively. After spraying with sulphuric acid/anisaldehyde reagent and viewing at 365nm with the UV lamp, it was observed that methanol extract of *X. americana* root bark is rich in saponins, tannins and flavonoids. However, the TLC finger print alone cannot be used to ascertain the specific compound without coupling to other spectroscopic techniques but it can be used to establish chemical markers in the plant material. The duplicate developed TLC plate without spray was used for TLC-MS analysis. The fractions labeled as A1-A5 were further analyzed with NMR but the results obtained indicated a need for better separation and purification processes.

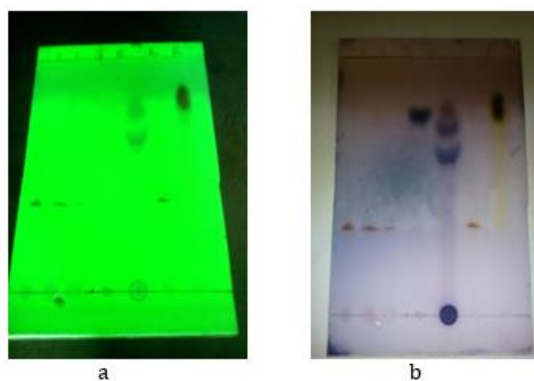


Figure 1 (a) TLC plate viewed at 254nm with UV lamp; (b) TLC plate 365nm with UV lamp after spraying and heating at 100°C for 5mins

3.3. Thin layer chromatography-Mass spectroscopic (TLC-MS) analysis

In an attempt to determine molecular weight to charge ratio (m/z) of various bands observed on TLC plate, the different peaks at a given retention time as shown in the TLC-ESI/MS chromatogram were considered (Fig. 3a). It's a known fact that the process of isolation and characterization using other column methods is tedious, expensive and generates large solvent wastes. The coupling of analytical technologies such as TLC-MS and TLC-NMR is a cost-effective alternative to online coupling of HPLC-MS/NMR or other column methods which leads to reduced organic solvent usage and waste solvent disposal systems [25]. TLC is useful in phytochemical analyses, as heat sensitive components remain mostly unaffected and components devoid of UV absorbing groups can be detected after post chromatographic derivatization though it is HPTLC that is recognized in the pharmacopoeias [Camag Bibliographic Service (CBS) 118, Planar chromatography in practice: Comparison of conventional TLC and HPTLC for identity testing of herbal medicinal extracts, https://www.camag.com/en/tlc_hptlc/customer_magazine_cbs/camag_bibliography_service_cbs.cfm?id=42H622Q4-CBS_118; Last accessed on 25/07/2018]. This method could also aid the de-replication process where fewer resources are spent isolating and characterizing well known bioactive compounds. The scans at different retention time between 200 and 1000 m/z could serve as chromatographic fingerprint for identification of the bioactive components in the plant part. The low - resolution mass spectrometer used for the TLC-MS technique did not show strong correlation with the data obtained for LC-MS which was coupled to a high resolution Orbitrap mass spectrometer. However, high-resolution MS and the elemental pattern of the compound at various peaks with nuclear magnetic resonance are required to characterize the compounds. The spectrum for RT 15.85 – 16.01 shows a moderate peak at 289.1 (Fig. 3d) which suggests $(M+H)^+$ for quercetin similarly isolated from a study on *X. americana* leaves reported by Le *et al.* [17]. The flavonol has been established to have high radical scavenging capacity, thus anti-trypanosomal activity of *X. americana* root bark may be attributed possibly to antioxidant activity. Moreover, Maria *et al.* [26] observed that quercetin directly induces death of *Trypanosoma brucei gambiense* without affecting normal cell viability. Peak 792.1 m/z (Fig. 3b) suggests large molecule trimers of a sesquiterpene (MW 264; $C_{15}H_{20}O_4$). Voss *et al.* [27] isolated this compound from ethanol extract of *X. americana* stem using EI-MS but was reported not to be active against human leukemia and human breast cancer.

3.4. Liquid chromatography-Mass spectroscopic (LC-MS) analysis of crude extract and solid phase extraction fractions

Liquid Chromatography-Mass spectrometric analysis of crude methanol extract of *X. americana* root bark was carried out in analytical reversed phase column with mobile phase gradient shown in Table 3. Some peaks were observed in blank which are similar to some peaks in sample chromatogram (Fig. 2a). Appearance of these peaks could be attributed to carryover effects from column or solvent impurities. LC-MS UV spectra and high-resolution mass spectrometer data in Fig. 2a & b using Fourier Transform Mass Spectrometer with positive ion mode ESI showed peak at retention time 16.43 to give molecular ion at peak 282.2790 with dimer at peak 563.55. This suggests 2',5-dimethoxyflavone (MW 282.1; $C_{17}H_{14}O_4$) with 304.2609 $(M+Na-22.0019)$ -Na adduct [Massbank, Japan High quality Mass Spectral Database, <http://www.massbank.jp/peaksearch/>, Last accessed 13/02/2017]. A sesquiterpene (MW 264.25; $C_{15}H_{20}O_4$) was also suspected based on compounds reported in literature. A very intense signal at 187.0968 negative ion mode ESI-MS spectrum retention time 10.39 can be seen, which possibly originates from the (gallic acid - H) 171.1195 m/z on the spectrum by hydroxylation to form 2,3,4,5-tetrahydroxybenzoic acid (MW 186.1189; $C_7H_6O_6$). One of the fractions derived from solid-phase extraction chromatography (A1) was subjected to mass spectrometric analysis using

electrospray ionization in positive ionization mode. A very intense signal was observed at 283 m/z which suggested the presence of 2',5-dimethoxyflavone and its dimeric form at 564 m/z.

Table 3 Gradient system of Liquid Chromatography

Xa' 1. 10		-esi_02				
Accela Pump						
Method creator:		Thermo				
Last Modified:		10/24/2016	9:46:43 AM			
Instrument:		Accela Pump				
Common settings:						
Pressure Units:		Bar				
Pressure Stability		10.00				
Pump 1 Setting:						
Name:		Pump 1				
Comment:						
Solvent A		Ethyl Acetate				
Solvent B		0.1% HCOOH				
Solvent C		MeOH				
Solvent D		CAN				
Start Settings:		Accela.AS injection logic				
Method finalizing:		First line conditions				
Operating mode:		Low pressure (0 ~ 7000 PSI)				
Min pressure:		0.00				
Max pressure:		1000.00				
Pump 1 gradient table						
No.	Time	A%	B%	C%	D%	μL/min
0	0.00	0.0	80.0	20.0	0.0	400.0
1	5.00	0.0	50.0	50.0	0.0	400.0
2	10.00	0.0	50.0	50.0	0.0	400.0
3	15.00	0.0	5.0	95.0	0.0	400.0
4	18.00	0.0	5.0	95.0	0.0	400.0
5	18.50	0.0	80.0	20.0	0.0	400.0
6	23.00	0.0	80.0	20.0	0.0	400.0

Peak 304.2609 represents hydroxylation of quercetin to form 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychroman-4-one known as dihydroquercetin (C₁₅H₁₂O₇) in comparison with peak database by Massbank, Japan; High quality Mass Spectral Database. Sesquiterpene (MW 264.25; C₁₅H₂₀O₄) was identified as (MW 265.25; M⁺ +H) as earlier mentioned. A very intense signal at 187.0968 negative ion mode ESI-MS spectrum retention time 10.39 can be seen, which possibly originates from the (gallic acid – H) 171.1195 m/z on the spectrum by hydroxylation to form 2,3,4,5-tetrahydroxybenzoic acid (MW 186.1189; C₇H₆O₆). One of the fractions derived from solid-phase extraction chromatography (A1) was subjected to mass spectrometric analysis using positive ion mode electrospray ionization. Very intense signal was observed at 283 m/z which confirmed presence of 2',5-dimethoxyflavone and its dimers form at 564 m/z as earlier mentioned.

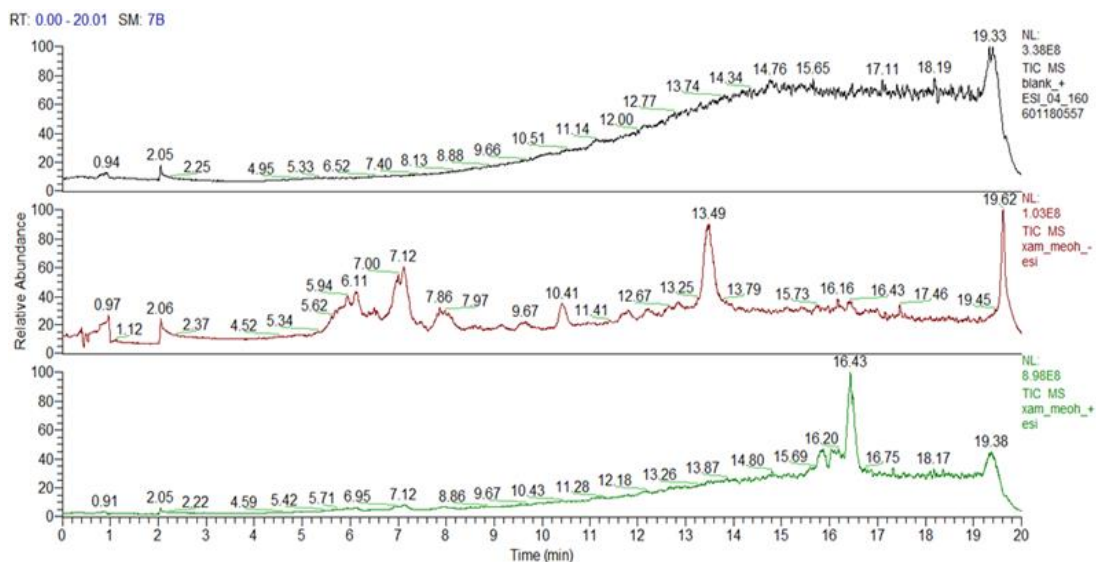


Figure 2a LC-MS UV scan of *X. americana* methanol extract showing blank run and peaks at ESI negative and positive ionization modes

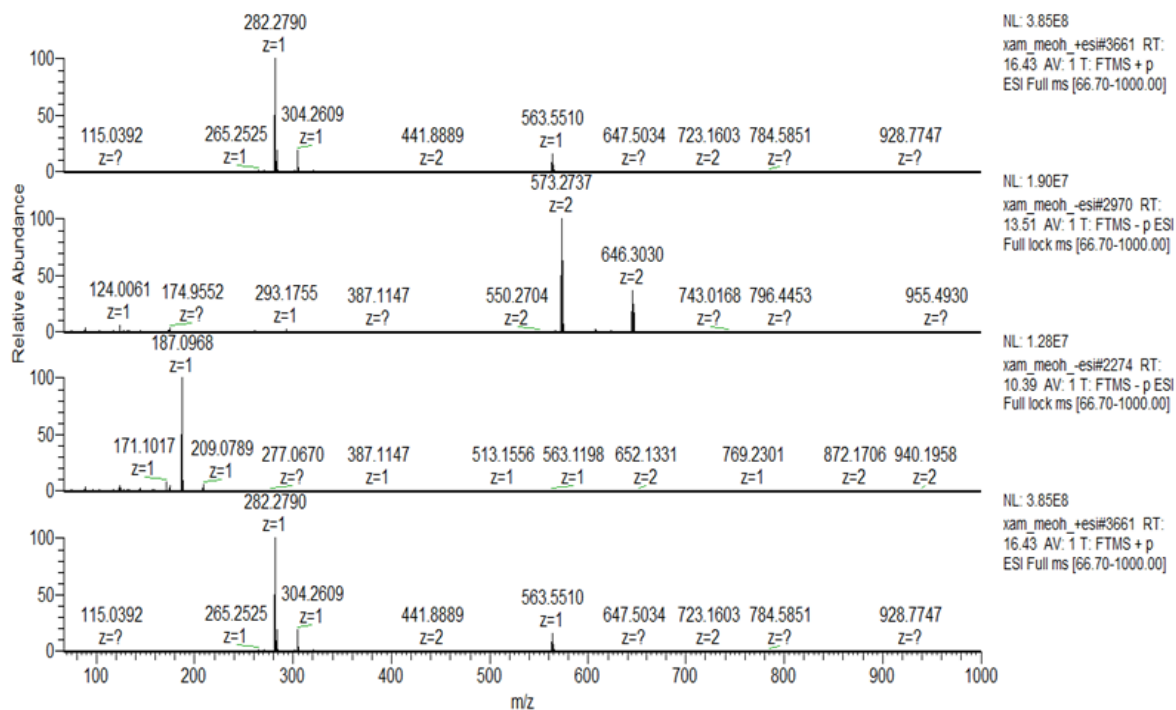


Figure 2b High Resolution Mass spectrum of *X. americana* methanol extract

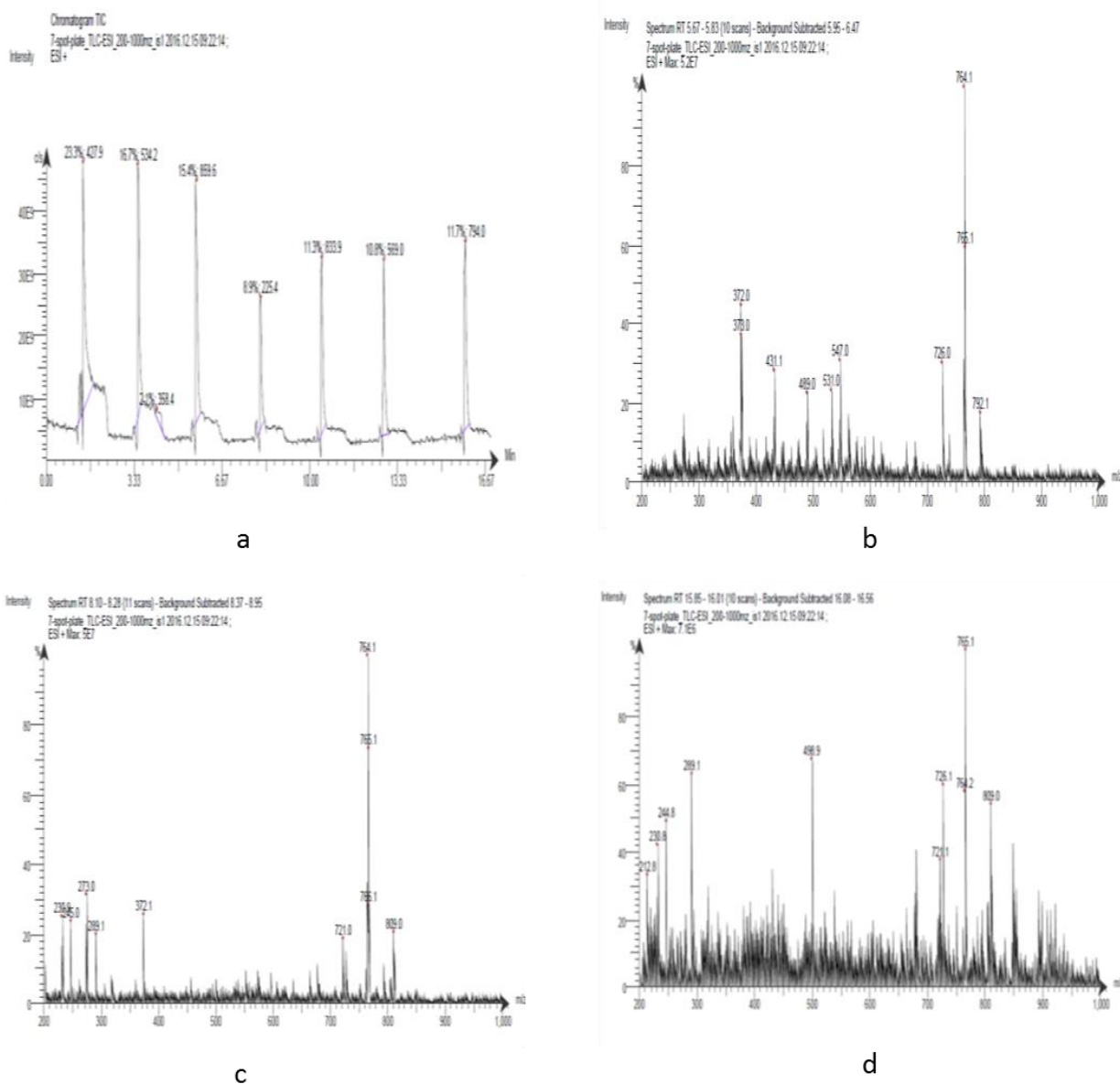


Figure 3(a) TLC-MS scan of *X. americana* methanol extract using Advion Mass Spectrometer in +ESI. (b) TLC-MS spectrum (RT 5.67-5.83) using Advion Mass Spectrometer in +ESI. (c) TLC-MS spectrum (RT 8.10-8.28) using Advion Mass Spectrometer in +ESI. (d) TLC-MS spectrum (RT 15.85-16.01) using Advion Mass Spectrometer in +ESI

4. Conclusion

Human African trypanosomiasis remains one of the neglected tropical diseases (NTDs) with limited therapeutic agents to combat the disease. It was observed in this study that total extraction yield of *Ximenia americana* root bark is independent of different solvents used (acetone and 70% ethanol). The plant part (root bark) possesses secondary metabolites such as tannins, saponins, flavonoids and terpenes. Methanol extract derived from sequential Soxhlet extraction exhibited inhibitory effect on *Trypanosoma brucei brucei* when compared with the standard drug (Isometamidium chloride) at the same concentration. Chromatographic and spectroscopic techniques used in this study provided data similar to some library data for gallic acid, quercetin, dihydroquercetin, 2,3,4,5-tetrahydrobenzoic acid and 2',5-dimethoxyflavone. Thus, results presented will be useful in further analysis of plant materials. Elucidation of active compound(s) from natural products sometimes requires application of multiple separating techniques. Therefore, purification processes such as flash chromatography and preparative HPLC could be used to obtain purer substance(s). Moreover, *in vivo* study on anti-trypanosomal potency of *X. americana* root bark is required to ascertain the toxicity of the active agent(s) and its trypanocidal effect.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest

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