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Full Length Research Article

EVALUATION OF *IN VITRO* ANTI-TRYPANOSOMAL ACTIVITIES OF LEAVES, STEM BARK AND ROOT BARK EXTRACTS OF *ACACIA NILOTICA* (L) WILLD EX DEL., *GUIERA SENEGALENSIS* J. F. GMEL AND *ZIZIPHUS ABYSSINICA* HOCHST EX A. RICH

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ABSTRACT
Currently, the control and treatment of African trypanosomiasis are limited by the number of chemotherapeutic drugs with associated side effects. Consequently, there is an urgent need for a non-toxic herbal treatment for African trypanosomiasis. Leaf, stem, and root bark extracts of *Acacia nilotica* L. and *Guiera senegalensis* J. F. Gmel and *Ziziphus abyssinica* Hochst ex A. Rich were sequentially extracted using hexane, ethyl acetate, methanol and water as solvents and evaluated for *in vitro* anti-trypanosomal activities against *Trypanosoma brucei*, and phytochemical contents. Results revealed that out of the 36 extracts, Methanol leaf extracts of *G. senegalensis,* aqueous leaf extract of *G. senegalensis,* methanol leaf extract of *A. nilotica* and methanol leaf extract of *Z. abyssinica* leaf extract (MIC 3.93±2.88, 10.98±3.21, 16.91±3.21 and 18.88±3.44 µg/ml respectively), gave the best *in vitro* anti-trypanosomal activity against *T. b. brucei* compare to the control. The quantitative phytochemical analysis of the 4 most trypanocidal plant extracts revealed the presence of alkaloids, flavonoids, terpene, quinines, saponins and tannins, with alkaloids and flavonoids having the highest concentrations (4.3.13±0.05 mg/100 g and 4.5±0.02 mg/100 g respectively) in the methanol leaf extract of *G. senegalensis* and quinone with the lowest concentration (0.1±0.07 mg/100 g).The methanol leaf extracts of *G. senegalensis* were found to have the most *in vitro* anti-trypanosomal activities (MIC of 3.93±2.88 ug/ml), possibly due to the high content of alkaloids and flavonoids. The results of this study revealed the potential of *G. senegalensis* for the treatment of African trypanosomiasis. Consequently, further studies are needed with this plant to evaluate its *in vivo* anti-trypanosomal potential, the structures of the bioactive compounds responsible for its activity, and its other medicinal properties.

Keywords: African trypanosomiasis, medicinal plants, *in vitro* and chemotherapeutic drugs.

INTRODUCTION

African trypanosomiasis is a parasitic disease caused by single-celled protozoan parasites of the class *Trypanosoma,* which are transmitted by infected tsetse fly bites. The disease weakens both human and animal health, which has a negative impact on the economy of sub-Saharan Africa (D'Archivio *et al*., 2018). *Trypanosoma brucei rhodensiense* in eastern Africa and *Trypanosoma brucei gambiense* in western Africa are the pathogens responsible for human African trypanosomiasis (HAT), also known as "sleeping sickness (WHO, 2019). There are some overlaps between the two trypanosomes in Central Africa. The illnesses caused by protozoan parasites are mostly responsible for socioeconomic threats, affecting millions of people in affected areas (Monzote and Siddi, 2011). Current medications against trypanosomiasis protozoans have been in use for more than 50 years. Several factors limit the use of existing drugs in resource-poor settings, such as high cost, poor compliance, drug resistance, low efficacy, and poor safety. Consequently, there is a constant demand for the use of natural products as anti-trypanosomal medications (Monzote and Siddi, 2011).

Herbs and medicinal plants have been used by humans as food and medicine since time immemorial. Native plants such as *Acacia nilotica*, *Guiera senegalensis*, and *Ziziphus abyssinica* (Figures 1-3), have been utilized for medicinal purposes among rural dwellers, some of which were depicted in Egyptian papyrus works that involve therapeutic plants for over 3,000 BC (Shuaiba *et al*., 2008; Eldahshan and Abdel-Daim, 2015). In this study, the *in vitro* anti-trypanosomal activities and phytochemical screening of the native plants from Nigeria was carried out.

Figure 1: Acacia *nilotica* with branches showing fruits and leaves

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Figure 2: *Guiera senegalensis* with branches showing flowers and leaves

Figure 3: *Ziziphus abyssinica abyssinica* with branches having flowers and leaves

MATERIALS AND METHODS

Collection of Plant Materials

Guiera senegalensis was collected from a farmland in Zaria, Kaduna State, whereas *Acacia nilotica* and *Ziziphus abyssinica* were collected from a farmland in the Toro Local Government area of Bauchi State. A plant taxonomist verified the authenticity of all the three plants using voucher numbers. ABU001 for *Zizyphus abyssnica*; ,
ABU0698 for *Acatia nilotica*, and ABU01823 for *Guiera senegalensis*. Voucher samples were kept at the Herbarium Unit of the Biological Sciences Department, Ahmadu Bello University, Zaria.

Extraction of the Plant Materials

All three freshly collected plant materials were dried for three weeks in an aerated laboratory at room temperature. A mill with a 2 mm sieve was used to pulverize the air-dried plant materials into a fine powder. Two hundred grams (200 g) of each dried powdered plant material was weighed and sequentially extracted at room temperature for 72 h using hexane, ethyl acetate, methanol, and water. Briefly, 2 litres of hexane was dispensed in a round-bottom flask with a reflux condenser. The powdered plants were added, mounted on a shaker, and allowed to macerate for 72 h. The mixture was filtered through Whatman filter paper No. 1, and the filtrate was concentrated under reduced pressure by rotary evaporation (BUCHI Rotavapor R-200, Switzerland) at temperatures below 50°C. The Mac recovered from the hexane extract was air dried at room temperature (27°C) and refluxed exhaustively in 2 litres ethyl acetate for 72 h [4,5]. In addition, the Mac recovered from the ethyl acetate extracts was air dried at ambient temperature (27°C) and refluxed exhaustively in 2 litres methanol for 72 h [4,5] five millilitres (5 ml) of glacial acetic acid was added to the methanol filtrate before evaporation to prevent loss of the active component (Olajide and Adekemi, 2020).. The same procedure was used for the aqueous extract, using 2 litres of distilled water, after 24 h, the filtrate was concentrated in a water bath at 50°C. The resulting crude extracts were concentrated *in vacuo*, and the dry extracts were weighed and stored at -20°C until required for further studies, that is, *in vitro* anti-trypanosomal assay and phytochemical tests.

Trypanosome stock

Trypanosoma brucei (Federe strain) was obtained from stabilates maintained in cryogenic tanks at the Nigerian Institute for Trypanosomiasis Research (NITR), Kaduna State, Nigeria. The parasites were maintained in the laboratory by continuous passaging in three tines to give parasitemia of approximately log10^6 parasites/ml on days 4-8 for *T. brucei* inoculation.

Preparation of Culture Medium and Determination of *in vitro* Activity of Crude Extract

The culture medium for the cultivation of the bloodstream forms consist of 70% of Dubellco Modified Eagles Medium (DMEM), 20% of foetal calf serum, 2% of D-glucose, (1g NaHCO3, and 0.5 mg non-essential amino acids) stirred gently on a magnetic stirrer. The stock medium was further supplemented with 0.2 mM 2-mercaptoethanol, 2 mM sodium pyruvate, 0.1 mM hypoxanthine, 100 µl/ml of antibiotics i.e. gentamicin tetracycline or streptomycin. The whole mixture was further stirred thoroughly, and the pH of the medium was adjusted to 7.8 by gradual addition of HCl to the medium until the desired pH was achieved (Hussein *et al*., 2017). Before performing the assay, all solutions of crude plant extracts were freshly prepared. The lipophilic extracts were first dissolved in 10% dimethyl Sulphur Oxide (DMSO). One milligram of each extract was dissolved in 1 ml of trypanosome culture medium to obtain a concentration of 1 mg/ml (stock solution). Each plant extract was tested twice in duplicate in 96 well microtiter plate (Costar, USA) in four-fold serial dilutions using DMEM stock media of DMEM to obtain concentrations of 1000, 250, 62.5, 15.6, 3.9, 0.98 and 0.2 µg/ml. Fifty microliters of the prepared medium containing each extract was dispensed into a well of the microtiter plate in duplicate using a micropipette, followed by inoculation with 50 µl containing approximately 10^6 trypanosomes per well to give a final volume of 100 µl. Positive and negative control wells were also included (Rosas *et al*., 2007)
After incubation at 27 °C for 12 h in the presence of 5% CO_2 generated using a 5CG-Desicator (Bulus *et al.,* 2016)

The Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration of crude plant extract, in which no trypanosomes with normal morphology or motility could be found, was determined microscopically by viewing several fields

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of a drop of each concentration under X 40 objectives (Roses *et al*., 2017; Jose *et al*., 2023).

Phytochemical screening

The quantitative phytochemical screening was carried out according to the following methods: The total flavonoids, tanins, steroids, saponin and alkaloid content were estimated (Agbafor *et al.,* 2011).

Determination of Phenolic and Flavonoid Concentrations in the most active Plant leaf extract

The phenolic content of the four plant extracts was determined using the Folin-Ciocalteu method (Agbafor *et al*., 2011) Folin-Ciocalteu solution (100 µl of Folin-Ciocalteu solution was added to 500 µl of each extract and placed in an incubator in the dark at room temperature (22-25°C). After 15 min of incubation, 2500 µl of saturated sodium carbonate was added and incubated for 30 min at room temperature. The absorbance of the solution was read at 760 nm using a spectrophotometer (GeneSyn 20, Thermo Fisher Scientific, USA), and the concentration was determined from the standard curve constructed using gallic acid as the standard. The concentrations of phenolic compounds were estimated in triplicate. The results were recorded as mean values and expressed as mg of gallic acid equivalent (GAE)/100 g of dry extract (Agbafor *et al.,* 2011) The flavonoid content was quantified using the aluminum chloride colorimetric method (Agbafor *et al*., 2011) with some modifications. Each extract (1 ml) in methanol was separately mixed with 1 ml of methanol, 0.5 ml of 1.2% aluminum chloride, 0.5 ml of 0.12 M potassium acetate and 2.8 ml of distilled water. The mixtures were incubated at room temperature for 30 min, and the absorbance of the reaction mixture was measured at 415 nm using a spectrophotometer (GeneSyn 20, Thermo, USA). The total flavonoid content of the extract was expressed as milligram equivalents of quercetin per 100 g (mg QE/100 g) dry weight of the extract (Agbafor *et al*., 2011).

Statistical analysis:

The data obtained from the study were expressed as mean ± standard deviation (SD). Data analysis was performed using the Statistical Package for Social Science (SPSS), version 20.0 (Inc. Chicago IL, USA).

RESULTS

***In vitro* anti-trypanosomal activities against *T. b. brucei* (Federe strain)**

The results obtained from the *in vitro* anti-trypanosomal activity of the 36 extract from the 3 plants (Tables 1 and 2) showed that, four extracts (11%) showed strong activity (MIC values 3.93±2.88 – 18.88±3.25 µg/ml), seventeen extracts (47 %) showed moderate activity (MIC values 24.09±1.56– 54.68±3.11 µg/ml), fourteen extracts (39%) showed mild activity (MIC values 56.35±1.12 – 109±0.82) and one extracts (2.8%) was inactive. The methanol and aqueous leaf extracts of *G. senegalensis* (MIC 3.93 and 10.98 µg/ml) respectively, the methanolic leaf extract of *A. nilotica* (MIC 16.91 µg/ml) and methanolic leaf extract of *Z. abyssinica* (MIC 18.88 µg/ml,) showed the best anti-trypanosomal activity against *T. b. brucei*.

Table 1: *In vitro* Anti-trypanosomal Activity (methanol & water)

Plant Species	Plant	MIC (µg $\bar{X}$/ml±SC)	
		MeOH	H_2O
G. senegalensis.	L	3.93±2.88	10.98±3.21
	SB	15.13±1.03	16.14±2.15
	RB	10.31±1.28	15.18±3.21
A. Nilotica	L	16.91±3.18	41.02±1.10
	SB	32.14±3.18	43.02±3.1
	RB	37.31±0.18	45.12±0.28
Z. abyssinica	L	18.88±1.08	47.19±1.28
	SB	38.19±0.18	50.01±1.19
	RB	40.12±0.21	52.61±1.13

Table 2: *In vitro* Anti-trypanosomal Activity (hexane with ethyl acetate)

Plant Species	Plant	MIC ($\bar{X}$µg /ml±SC)	
		HEXANE	ETACT.
G. senegalensis.	L	40.01±2.1	59.02±2.11
	SB	51.21±1.10	70.06±4.1
	RB	54.68±3.11	80.05±2.38
A. Nilotica	L	56.35±1.12	67.17±2.18
	SB	58.19±3.1	70.12±2.18
	RB	60.18±0.19	71.28±1.99
Z.. *abyssinica*	L	100.1±1.10	90.21±0.19
	SB	106±0.82	94.07±1.71
	RB	N.A	98.92±1.19

Quantitative Photochemical Components of the most Active Extract

Tables 3, 4, 5 and 6 show the phytochemical screening of the four most active extracts, which revealed the presence of alkaloids, saponins, flavonoids, terpenes, tannins, steroids, anthraquinone, and quinones. Alkaloid and flavonoid had the highest concentrations (4.3±0.05 and 4.5±0.02 mg/100 g respectively) which was found in the methanol leaf extract of *G. senegalensis* while quinone has the lowest concentration (0.09 mg/100 g) found in the aqueous extract of *G. senegalensis*.

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Table 3: Quantitative Photochemical Components of methanol leaves extract of *G. senegalensis*

Phytochemicals	Concentration (mg/100g)
Alkaloid	4.32 0.11
Flavonoid	4.51±0.02
Terpenes	2.91±0.03
Tannines	2.11±0.04
Saponins	3.17±0.04
Quinones	0.11±0.00
Anthraquinones	1.17±0.03
Steroids	3.13±0.02

Data in duplicate: mean ±S.D

Table 4: Quantitative Photochemical Components of aqueous leaves extract of *G. senegalensis*

Phytochemicals	Concentration (mg/100g)
Alkaloid	3.20 0.13
Flavonoid	3.10±0.07
Terpenes	2.12±0.06
Tannines	1.19±0.01
Saponins	2.13±0.03
Quinones	0.09±0.00
Anthraquinones	1.13±0.01
Steroids	3.14±0.01

Data in duplicate: mean ±S.D

Table 5: Quantitative Photochemical Components of methanol leaves extract of *A. indica*

Phytochemicals	Concentration (mg/100g)
Alkaloid	2.91 0.15
Flavonoid	2.83±0.05
Terpenes	2.10±0.06
Tannines	4.15±0.04
Saponins	2.90±0.06
Quinones	1.2±0.03
Anthraquinones	1.93±0.01
Steroids	2.11±0.03

Data in duplicate: mean ±S.D

Table 6: Quantitative Photochemical Components of methanol leaves extract of *Z. abyssinica*

Phytochemicals	Concentration (mg/100g)
Alkaloid	2.11 0.11
Flavonoid	1.92±0.03
Terpenes	2.17±0.04
Tannines	4.15±0.04
Saponins	2.11±0.07
Quinones	1.97±0.01
Anthraquinones	2.31±0.01
Steroids	2.31±0.07

Data in duplicate: mean ±S.D

DISCUSSION

One of the molecular actions of most phytochemicals is to complex proteins through non-specific forces, such as hydrogen bonding, hydrophobic effects, and covalent bond formation. The mechanisms of trypanocidal activity include the formation of aldehyde-thiol adducts with sulfur-containing components, thereby decreasing the buffering of agents that can create oxidative stress in cells (Ifijen *et al*., 2019) and the oxidation of glutathione and pyruvic acids (Mbaya *et al*., 2007).

The methanol and aqueous leaves extract of *G. senegalensis*, methanol leaf extract of *A. indica* and *Z. abyssinica* crude extracts had shown significant *in vitro* anti-trypanosomal activities with the methanol leaves extracts of *G. senegalensis* showing the most *in vitro* anti-trypanosomal activities compared to the control. The high activity observed with the methanolic extracts could be associated with the high dielectric constant of methanol in comparison with hexane, ethyl acetate, and water, which exhibited lower activities, possibly due to their low dielectric constant. This unique property of methanol enables it to extract highly polar, neutral, basic, and acidic compounds; amino acids; nucleotides; sugars; and polysaccharides (Atawodi and Shehu, 2010). The observed *in vitro* anti-trypanosomal activity of the active extract could also be attributed to either the individual class of compounds present in the extract or to the synergistic effect exerted by each class of compounds on the observed activity (Zongo *et al.,* 2019). Natural products can generate free radicals that cause oxidative damage and DNA damage (Omar and Khan, 2017). This extract may exert its trypanocidal activity through peroxidative and DNA damage, which is consistent with previous studies (Mann *et al*., 2010; Feyera *et al*., 2014).

The quantitative phytochemicals used in the present study indicated high concentrations of alkaloids, flavonoids, saponins, tannins, terpenes, and steroids as the phytochemicals present in most active extracts, which may have contributed to their high *in vitro* trypanocidal activity (Pelttari *et al.,* 2002).
This finding agrees with a previous study by Zongo *et al*. (2019), who reported that *G. senegalensis* leaf extract contains tannins, saponins, alkaloids, flavonoids, steroids, quinones, terpenes, and anthraquinone (Zongo *et al.,* 2019).

Alkaloids, one of the major active phytochemicals observed in the present study, can inhibit protein biosynthesis, intercalate DNA, interrupt membrane fluidity, inhibit microtubule formation, and induce programmed cell death in the bloodstream forms of trypanosomes (Debela *et al*., 2022). A class of alkaloids, known as spermidine alkaloids, present in some plants has been reported to exert high anti-trypanosomal activity (Debela *et al*., 2022). The mechanism of action of this compound involves the presence of an aryl moiety in the spermidine molecule, which interacts with the hydrophobic region of trypanothione reductase so that spermidine adopts a non-extended bound conformation. This could be responsible for the observed effects of the alkaloid (Debela *et al*., 2020). The spermicidine alkaloid resembles the chemical structure of pentamidine, which binds to nucleotides in DNA and RNA by promoting cleavage of the parasite circular DNA in a manner similar to that of topoisomerase II inhibitors, which may have been responsible for the trypanocidal activity (Venugopal and Liu, 2012). Flavonoids, another important group of compounds found in the extracts, can cause a significant reduction in the parasite when administered. The mode of action could be interference with replicating forms of trypanosomes, which are completely dependent on glycolysis for energy production and membranes of the parasites (Muhammad and Sajid, 2014). Similarly, sterols such as stigmasterols, vernoguinosterol, and vernoguinoside have been reported to have soapy characteristics that enable them to precipitate and coagulate red blood cells (Debela *et al.,* 2020).

Saponins with detergent properties can dissolve in biomembranes and disturb the fluidity and function of membrane proteins of parasites (Mann *et al*., 2010). Saponins are also known to inhibit the development of protozoa by interacting with cholesterol present on the parasite cell membrane, thus leading to parasite death (Wang, 2000).

Tannins are a group of compounds with high medicinal activity; they are water-soluble plant polyphenols that precipitate proteins and can form chelates with metal ions, particularly iron (Debela *et al.,* 2020). Tannins have also been reported to prevent the development of microorganisms by precipitating microbial proteins and making nutritional proteins unavailable to them (Debela *et al.,* 2020).

Numerous *in vitro* anti-trypanosomal studies conducted on some classes of compounds listed in the present study have shown the potential of each class of these compounds to kill or inhibit the growth of a wide range of trypanosomes (Zongo *et al*., 2019; Debela *et al*., 2020)

The present study showed that the leaf extract of *G. senegalensis* has potential anti-trypanosomal activity, which corroborates previous reports and justifies the traditional use of G. senegalensis in the treatment of trypanosomiasis (Zongo *et al*., 2029).

Ethics Approval and Informed Consent
Ethical approval for this study was obtained from the Animal Use Ethics Committee of the Nigerian Institute for Trypanosomiasis Research Kaduna (reference number NITR/FMST/2019/5613). All participants were informed of the objectives of the study and the protocol for sample collection.

Authors Contribution
MA. conceptualized the study. TJO and KYA designed this study. AUO participated in the fieldwork, data collection, and data analysis and interpretation. AUO prepared the first draft of the manuscript and reviewed it by MA, TJO, and KYA. All the authors contributed to the development of the final manuscript and approved its submission.

Conflict of Interest
There is no Conflict of interest

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