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Sesquiterpene Lactone-Rich Extract of *Tithonia diversifolia* (Hemsley) A. Gray (Asteraceae) suppresses *Trypanosoma brucei brucei* in both *In Vivo* and *In Vitro* Experimental Models

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ABSTRACT

Tithonia diversifolia has continued to play vital roles in phytomedicine due to its ethnopharmacological relevance, medicinal properties and agricultural applications. Its richness in sesquiterpene lactones has also continued to generate interest due to its diverse pharmacological activities, structure-activity relationships, and pharmacokinetics. The study evaluated the anti-trypanosomal and cytotoxic activities of the sesquiterpene lactone (STL) constituents of *Tithonia diversifolia* using *in vivo* mouse and *in vitro* experimental models. The *T. diversifolia* methanol extract was fractionated by solvent-solvent partitioning in *n*-hexane, EtOAc, and *n*-butanol and further separated by the vacuum liquid chromatographic (VLC) method. The *in vitro* anti-trypanosomal and cytotoxic activities were evaluated using resazurin-based cell viability assays. The *in vivo* anti-trypanosomal activity was determined using a hematocrit-based packed cell volume analysis and rapid matching counting of *Trypanosoma brucei brucei* inhibition methods in a mouse model. The two STL-enriched sub-fractions (VLC-3 and VLC-4) obtained from the VLC separation displayed significant ($p < 0.05$) *in vitro* activity against *Trypanosoma brucei* with IC_{50} values of 0.88 and 0.52 µg/mL respectively and SI of 44 and 165 respectively. In the *in vivo* mouse model, a 400 mg/kg dose of VLC-3 and VLC-4 elicited a 100% clearance of the parasites within 16 and 14 days post-induction of parasitemia compared with a similar effect of the positive control drug, diminazene aceturate on day 12. The strong *in vivo*/*in vitro* anti-trypanosomal activity of *T. diversifolia* STL represents a promising starting point for discovering potent trypanocidal agents against *T.b. brucei*, the major cause of animal African trypanosomiasis.

Keywords: Animal trypanosomiasis, Cytotoxicity, Sesquiterpene lactones, *Tithonia diversifolia*

Introduction

Animal African trypanosomiasis (AAT) is a parasitic disease of animals caused by the *Trypanosoma congolense*, *Trypanosoma vivax*, and *Trypanosoma brucei brucei*.[1] In sub-Saharan Africa, the debilitating effects on livestock production are enormous and this has resulted in the death of millions of livestock with over 50 million cattle at risk.[2] A dense tsetse-trypanosome burden has a high socio-economic impact on food security. Trypanosomiasis also affects crop yield by hampering the availability of draft animals to plough fields and provide manure for fertilizer.[3] In Nigeria, cattle and its products meet different needs of people; however, AAT threatens its economic potential. Lack of surveillance, tsetse fly redistribution and reservoir host have constrained the government's control efforts.[2] More so, no drug against AAT has been discovered in the past twenty years and research on vaccine development is still ongoing.[4] Therefore, chemotherapy remains the most effective strategy for the control of AAT; and plants-based compounds have remained the mainstay.

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Diminazene, quinapyramine, homidium, and isometamidium have been in use but resistance, toxicity, disposition, and efficacy problems have limited their benefits.[1,4,5] Several plants of the Asteraceae family have shown promising alternatives.[6]

Tithonia diversifolia (Hemsley) A. Gray (Asteraceae) has maintained its vital roles in phytomedicine due to its medicinal, agricultural, and ethnopharmacological relevance.[7-19] In folk medicine, it is claimed that the weed serves as forage for livestock and medicine for nagana in North-Central and South Western Nigeria.[20] This claim has been scientifically validated in different reports.[20,21] Like many other plants in the Asteraceae family, its richness in sesquiterpene lactones (STLs) has continued to generate a prospect as well as diverse pharmacological activities, structure-activity relationships, and pharmacokinetics.[22-24] Studies have shown that through non-selective off-targets binding as a Michael acceptor, the interaction of the thiol (-SH) of proteins and enzymes with the nucleophilic alpha-methylene-gamma-lactone of STLs could be responsible for their diverse activities.[22,24] This uniqueness compared with other secondary metabolites has continued to serve as the major focus for the progression and translation of STLs and their diverse pharmacological activities to clinical trials.

The efficacy of plant-based products or preparations has continued to attract attention in drug discovery, development, therapy, and monitoring. In addition, cytotoxic activity studies on the bioactive preparations, extracts, or molecules from plants during the discovery lifecycle constitute significant steps in the development of safe medicines.[25] In a recent study, it was hypothesized based on the

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phytochemical constituents and ethnomedicinal uses that STLs previously characterized from *T. diversifolia* could possess strong anti-trypanosomal activity considering their strong binding when docked to *Trypanosoma brucei* ornithine decarboxylase target in an *in silico* experimental model.[21] To track the phytoconstituents of *T. diversifolia* responsible for the anti-trypanosomal activity, this study evaluated the anti-trypanosomal and cytotoxic activities of the STL-rich fractions of *T. diversifolia* using *in vivo* and *in vitro* models to provide further insights into the discovery of safe and potent STLs for the management of animal African trypanosomiasis.

Materials and Methods

Plant material

The aerial part of *T. diversifolia* used for this study was collected in Nsukka (Location: N 7 43' 50", E 8 32' 10"), Nigeria by Mr. Felix Nwafor, a taxonomist at the Institute of Plant Science of the University of Nigeria Nsukka, in January 2021. A voucher specimen of the authenticated plant (ID: PCG/UN/2021/Atd) was stored at the herbarium of the Institute of Pharmacognosy and Environmental Medicine of the same University. The plant material was air-dried under shade at 25 ºC for 14 days before further processing.

Experimental animals

The mice (weighing 22.5 ± 4.5 g) used for the anti-trypanosomal activity studies were of either sex. They were procured from and housed in the animal house of the Veterinary Teaching Hospital, University of Nigeria Nsukka. They were kept for 7 days in standard conditions for acclimatization with free access to standard feed and water. The protocol for and the permission to use the mice for this study was reviewed and granted by the University of Nigeria ethical committee (Reference No.: FPSRE/UNN/2021/00015) for research.

Parasites and cultures

Bloodstream forms trypomastigotes of *Trypanosoma brucei brucei* (strain s427) were used for this study. The *T.b. brucei* used for the *in vitro* study were cultured and passaged every 72 h in supplemented HMI-9 at 37 ºC in a vented flask (5% carbon dioxide atmosphere). For the *in-vivo* study, the parasite was passaged in albino rats every 48 h. The human foreskin fibroblast (HFF) cells used for the cytotoxic activity evaluation were preserved in a culture of 500 mL Dulbecco's Modified Eagle's Medium (DMEM), 5 mL penicillin/streptomycin, 50 mL New-born Calf Serum (NBCS), and 5 mL L-glutamax (200 mM). Incubation of the cells was maintained at 37 ºC/5% CO_2 and the cells were passaged in vented flasks at 80 - 85% confluence.

Phytochemical studies

Extraction of plant material

The dried aerial part of *T. diversifolia* was pulverized to a coarse powder and 2 kg was cold-macerated twice in fresh 95% methanol (5 L each) successively for 48 h with intermittent agitation as previously reported.[21] The filtrate was concentrated at 45 ºC using a rotary evaporator. The yield of the dried extract (TDE) was 5.6% w/w of the coarsely powdered sample. A 100 g of the extract was dispersed in 250 ml aqueous methanol (10% v/v) using magnetic stirring and made up to 500 ml in a separating funnel. The dispersion was partitioned in equal volumes each of *n*-hexane (n-Hex), ethyl acetate (EtOAc), and *n*-butanol (n-But) to yield n-Hex (6.2 g), EtOAc (58.4 g) and n-But (19.8 g) fractions after evaporation under reduced pressure.

Vacuum liquid chromatographic separation

A vacuum liquid chromatographic (VLC) method was adopted for the enrichment of STLs in the EtOAc fraction.[22] A 50 g of EtOAc fraction was dissolved in 10 ml solvent comprising dichloromethane (DCM): EtOAc (1:1) and introduced to a 45 x 5 cm glass column containing silica gel (G 100-200 mesh size), sample to silica gel ratio 1:50 at room temperature. The applied fraction was eluted under pressure with a gradient of DCM in EtOAc (1.5 L) each, starting with 100% DCM, 0% EtOAc and gradually increasing the amount of EtOAc to 20, 40, 60, 80, and 100% to obtain six sub-fractions VLC-1 to VLC-6 respectively.

Thin-layer chromatographic analysis

A pre-coated silica gel 60 F_{254} plates, 20 × 10 cm, and a mobile phase system composed of DCM and EtOAc (1:1) at room temperature were used for TLC analysis of the extract, n-Hex, EtOAc, and n-But fractions as well as VLC-1 to VLC-6 sub-fractions. The developed plates were observed at 254/365 nm under UV light and/or with an anisaldehyde-sulphuric acid reagent.[25]

Qualitative phytochemical analysis

The extract, fractions (n-Hex, EtOAc, and n-But), and sub-fractions (VLC-1 to VLC-6) were subjected to qualitative phytochemical analysis following standard protocols.[26] Specifically, a terpenoid analysis was performed as follows: a 0.5 ml each of 1 g, 0.5 g, and 0.25 g per mL of extract, fractions, and sub-fractions respectively was evaporated to dryness. The dried sample was heated with 3 ml of concentrated H_2SO_4 for 10 min in a water bath.[27]

Biological assays

Induction of parasitemia

Induction of parasitemia in the mice followed the previously described protocol.[21,25,28] A 0.2 ml titer of normal saline-diluted blood (containing 2.5 x 10^5 *Trypanosoma brucei brucei* per ml) was administered intraperitoneally to infect the mice. The mice were monitored until a $>10^7$ *T. b. brucei* per ml level of parasitemia was obtained but not lethal to the mice. Thereafter, the baselines for the packed cell volume (PCV), weight, and parasitemia were established appropriately. Parasitemia was measured using a rapid matching counting method in each mouse's tail blood smear examination.[21,25,28]

In vivo anti-trypanosomal activity assay

The mice were randomly divided into 30 groups of 5 mice per group and treated as follows: Groups 1-3: infected and treated with 200, 400 and 600 mg of n-Hex fraction per kg body weight of mice, respectively; Group 4-6: infected and treated with 200, 400 and 600 mg of EtOAc fraction per kg body weight of mice, respectively; Groups 7-9: infected and treated with 200, 400 and 600 mg of n-But fraction per kg body weight of mice, respectively; Groups 10-12: infected and treated with 100, 200 and 400 mg of VLC-1 sub-fraction per kg body weight of mice, respectively; Groups 13-15: infected and treated with 100, 200 and 400 mg of VLC-2 sub-fraction per kg body weight of mice, respectively; Groups 16-18: infected and treated with 100, 200 and 400 mg of VLC-3 sub-fraction per kg body weight of mice, respectively; Groups 19-21: infected and treated with 100, 200 and 400 mg of VLC-4 sub-fraction per kg body weight of mice, respectively; Groups 22-24: infected and treated with 100, 200 and 400 mg of VLC-5 sub-fraction per kg body weight of mice, respectively; Groups 25-27: infected and treated with 100, 200 and 400 mg of VLC-6 sub-fraction per kg body weight of mice, respectively; Groups 28-29: infected and treated with 5 ml normal saline, and 3.5 mg of diminazene aceturate per kg body weight of mice respectively; Group 30: uninfected and untreated. All the treatments commenced after infection on the 5th day and the observations lasted for 16 days. The PCV (hematocrit method) and parasitemia (rapid matching counting method) were determined on days 0, 5, 10, and 15 post-infection.[21,25,28]

In vitro anti-trypanosomal activity assay

The *in vitro* test for anti-trypanosomal activity of the extract, fractions, and VLC-1 to VLC-6 against *T.b. brucei* was performed following the standard protocol.[6,29-31] For the drug sensitivity assay, *T.b. brucei* cells (adjusted to 2.5 x 10^5 cells/ml) were exposed to 10 doubling dilutions of test samples starting from 400, 200, and 100 µg/ml of the extract, fractions, and VLC sub-fractions, respectively. Diminazene aceturate and pentamidine served as positive controls. Thereafter, the plate was incubated at 37 ºC/5% carbon dioxide atmosphere for 48 h, followed by the addition of resazurin solution and final incubation for a further 24 h. The fluorescence was measured using OPTIMA fluorimeter at 530/590 nm excitation/emission wavelengths

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In vitro cytotoxic activity assay

A standard resazurin-based assay was adopted for the cytotoxic activity determination.[29,31] The HFF cells (density 4 x 10^5 cells/ml) were suspended at 100 µL in a 96-well plate. The plate was incubated for 24 h at 37 ºC and a 5% carbon dioxide atmosphere to allow for cell adhesion. Serially diluted extract, fractions, and VLC-1 to VLC-6 were prepared in a separate sterile plate and 100 µL was transferred to the wells containing the cells. Phenylarsine oxide was used as a positive control. Thereafter, the plate was incubated at 37 ºC/5% carbon dioxide atmosphere for 30 h, followed by the addition of resazurin solution and final incubation for a further 24 h. The fluorescence was measured using OPTIMA fluorimeter at 530/590 nm excitation/emission wavelengths.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM), (n=5). One-way analysis of variance (ANOVA) was performed to test for the significant difference between the means of treatments and control at $p < 0.05$ by post-hoc using 2-sided Dunnett's test. In all cases, a $p < 0.05$ was considered to be significant.

Results and Discussion

Many indigenous medicinal plants are still useful today due to their effectiveness in the treatment of various parasitic infections including animal African trypanosomiasis (AAT). The efficacy of the STLs-rich extracts against AAT-causing animal trypanosomiasis complements several other pharmacological properties of *T. diversifolia* towards discovering potent phyto-molecules.[7-19,21,23,28] This study identified the abundance of STLs in the extract of *T. diversifolia* and examined their potential as a promising source of anti-trypanosomal leads.

Phytochemical evaluation

The VLC separation of 50 g of EtOAc fraction of *T. diversifolia* yielded six sub-fractions: VLC 1 to VLC-6 with variable physicochemical characteristics (Table 1). A TLC analysis revealed uniformly distributed phytoconstituents in sub-fractions VLC-3 and VLC-4. A qualitative phytochemical test for the presence of terpenoids showed a heavy grey colour indicating the abundance of terpenoids in sub-fractions VLC-3 and VLC-4.

Biological activity studies

In vivo anti-T.b. brucei activities of solvent and VLC fractions

The effects of *T. diversifolia* fractions on parasitemia and PCV levels of *T.b. brucei* infected mice are shown in Figures 1 and 2. Generally, the anti-*T.b. brucei* effect of the fractions was largely dose-dependent. There was a dose-dependent increase in the inhibitory activity of the fractions post-infection. The 600 mg of EtOAc fraction per kg of mouse caused a complete clearance of the parasites in the mice's bloodstream within the 16th day of the treatment compared to the similar effect of diminazene aceturate on the 12th day (Figure 1A). The slight decline in the PCV of the infected mice was also restored to the pre-treatment levels by the EtOAc fraction within the same period (Figure 1B).

The inhibitory effect of the VLC fractions followed similar trends to other fractions. The VLC-3 elicited 100 % inhibition of the *T.b. brucei* within 16 days. The VLC-4 also caused a 100% clearance of the parasites after day 14 which relapsed briefly before a total clearance on the 20th day (Figure 2C). the effect on PCV followed similar trends (Figure 2D).

In vitro anti-T.b. brucei and cytotoxic activities

The extract, fraction, and VLC-1 to 6 showed a varying degree of trypanocidal activity (Table 2). The extract showed *in-vitro* anti-trypanosomal activity of 1.18 µg/ml, selectivity index (SI) > 84. The sensitivities of the fractions showed that the EtOAc fraction was the most active (IC_{50} 0.92 µg/ml, SI > 77. Both n-Hex and n-But fractions showed IC_{50} of >20 µg/ml and were regarded as inactive. Of the six VLC sub-fractions, only VLC-3 and VLC-4 showed improved and significant anti-trypanosomal activity compared with the extract and other sub-fractions (Table 2). The results of the cytotoxicity study showed that the TDE, n-Hex, n-But, VLC-1, VLC-2, and VLC-6 exhibited high cytotoxic IC_{50} > 100 µg/ml while EtOAc, VLC-3, VLC-4, and VLC-5 showed cytotoxic IC_{50} < 100 µg/ml.

Table 1: Characteristics of VLC sub-fractions of *T. diversifolia* extract

Sample	Weight (%w/w)	Colour	hRf of constituents	Suspected phytoconstituents
TDE	5.60**	Brown	-	Terpenoids
EtOAc	2.92**	Brown	-	Terpenoids
VLC-1	22.2*	Yellow	93.5, 87.4, 72.1, 70.2	Terpenes
VLC-2	8.40*	Dark red	72.6, 67.9, 68.5, 61.7	Terpenes, steroids
VLC-3	15.4*	Brown	72.5, 65.2, 58,6, 50.4	Terpenoids
VLC-4	11.2*	Brown	72.8, 66.0, 58.6, 59.4	Terpenoids
VLC-5	17.8*	Wine red	52.7, 47.7, 32.1	Flavonoids
VLC-6	13.0*	Wine red	24.6, 20.5, 18.2	Flavonoids, tannins

calculated based on the *50 g EtOAc fraction and **2 kg of dried coarse powder; hRf = relative retention factor (Rf x 100)

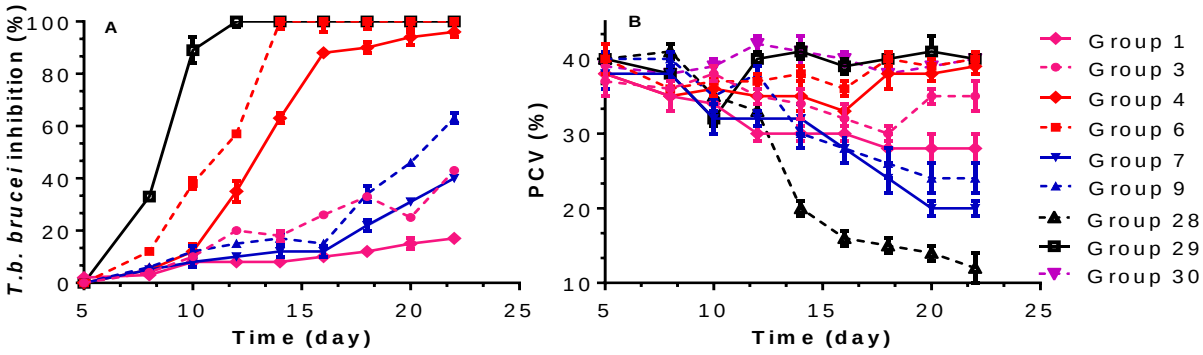

Figure 1: Effect of *T. diversifolia* fractions on parasitemia (A) and PCV (B) of *T.b. brucei* infected mice. Only two extreme doses (200 and 600 mg/kg) of the *n*-hexane, EtOAc and *n*-butanol fractions were plotted. Data are expressed as mean ± SD, n=5; p < 0.05 compared to the untreated group are considered significant.

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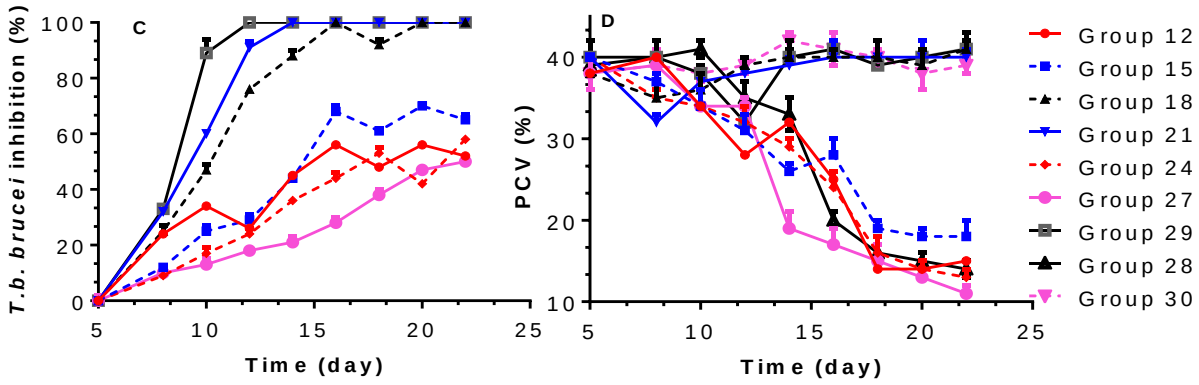

Figure 2: Effect of *T. diversifolia* VLC fractions on parasitemia (C) and PCV (D) of *T.b. brucei* infected mice. Only the groups administered with 400 mg/kg dosage of VLC-1 to VLC-6 were shown in the plots. Data are expressed as mean ± SD, n=5; $p < 0.05$ compared to the untreated group are considered significant.

Table 2: *In vitro* anti-*T.b. brucei* and cytotoxic activities of *T. diversifolia*

Tested sample	*T.b. brucei*	HEK	S.I.
	IC_{50}, µg/mL	IC_{50}, µg/mL	
TDE	1.18 ± 0.29	>100	>84.7
n-Hex fraction	21.80 ± 2.63	>100	>4.58
EtOAc fraction	0.92 ± 0.03	70.85 ± 7.45	77.01
n-But fraction	36.02 ± 0.11	>100	>2.77
VLC-1	>50	>100	n.d
VLC-2	18.15 ± 0.95	>100	>5.50
VLC-3	0.88 ± 0.03	38.95 ± 0.62	44.26
VLC-4	0.52 ± 0.12	86.03 ± 0.78	165.44
VLC-5	10.64 ± 0.25	54.21 ± 6.60	5.09
VLC-6	48.24 ± 0.02	>100	>2.07
[1]Pentamidine	4.25 ± 0.06	n.d	n.d
[1]Diminazene	56.23 ± 0.08	n.d	n.d
[1]Phenylarsine oxide	n.d	107.6 ± 3.8	n.d

Data represent the mean of three independent determinations and are expressed as Mean ± SD, [1]IC_{50} values are given in nM, SI = IC_{50} (host cell) / IC_{50} (target cell), n.d means not determined.

Of the separated three fractions of TDE, the EtOAc fractions 600, 400, and 200 mg/kg body weight elicited 57, 42, and 37% clearance of *T.b. brucei* compared with the 100% by diminazene aceturate in 12 days in experimental mice. Correspondingly, the *in vitro* assay resulted in the IC_{50} of 0.92 µg/mL, and SI of 77. Further separation yielded two bioactive sub-fractions. The VLC-3 and VLC-4 caused 72 and 90% inhibition at 400 mg/kg doses in 16 and 14 days, respectively in the *in vivo* mouse model as well as IC_{50} values of 0.88 and 0.52 µg/mL, respectively with corresponding SI values of 44 and 165 in the *in vitro* assay. The use of *T. diversifolia* in folk medicine for the treatment of protozoal diseases is well known and this has also been validated.[15,20,21] The present findings were in agreement with the previous study on the *in vivo* activity of TDE, however, the previous findings did not attribute the activities to any known phytochemicals.[20,21,32] The *in vivo* and *in vitro* activities of the fractions and sub-fractions were found to be strongly correlated as both VLC-3 and VLC-4 showed strong activities in both models. However, a state of aparasitemia was achieved in a lesser time of 12 days than the 14 days in TDE with no sign of relapse in either treatment. The slow onset of action observed in this study has been reported to confer an advantage to newly discovered trypanocidal agents similar to the slow trypanocidal onset of activities of standard diamidines.[25,30] The higher activity observed in the EtOAc fraction and the mid-VLC sub-fractions (VLC 3 and VLC-4) was an indication that the anti-trypanosomal activity of *T. diversifolia* resided in the moderately polar phytoconstituents. To further identify the phytochemicals responsible for this activity, the extract, fractions, and sub-fractions were subjected to phytochemical tests. A qualitative phytochemical analysis showed predominantly terpenoids, steroids, terpenes, tannins, and flavonoids in the VLC sub-fractions. The qualitative terpenoid test showed a heavy grey colour indicating the presence of terpenoids in the most active sub-fractions VLC-3 and VLC-4. An *in-silico* study hypothesized the critical roles of the *T. diversifolia* STLs in the inhibition of the *T. brucei* ODC.[21] This study, however, has validated the hypothesis and further identified the specific class of phytochemicals responsible for the strong anti-trypanosomal activity

The *in vitro* cytotoxicity test of the extract and fractions displayed a level of cytostatic activity as opposed to cytotoxic effect in the lower microgram per mL range. Apart from the EtOAc fraction, VLC-3, VLC-4 and VLC-5 sub-fractions which showed IC_{50} < 100 µg/mL against HFF cells, TDE and other fractions/sub-fractions showed IC_{50} > 100 µg/mL. Generally, only VLC-3 showed IC_{50} < 50 µg/mL, an indication that the STL-rich sub-fraction could be cytostatic rather than cytotoxic. Whereas the results obtained here appear to be highly promising, the decline in the IC_{50} values of TDE, EtOAc fraction, and VLC-3 from >100 to 38.95 µg/mL on separation is a major concern in the cytotoxicity of the STLs on eventual isolation. This could mean that further purification might increase both anti-trypanosomal and cytotoxic activities. Another concern is that low *in vitro* IC_{50} (HFF) may not necessarily translate to high *in vivo* toxicity considering the complexity of the whole organism and the many varied tissues.[33] However, the overall cytostatic activity of the extract and fractions of *T. diversifolia* in the *in vitro* model and the corresponding anti-trypanosomal activity in the *in vivo*/*in vitro* models have further validated the usefulness of this plant in folk medicine across Africa.

In a biological activity-guided fractionation and separation of the methanol extract of *T. diversifolia*, the study identified the two STL-rich VLC sub-fractions responsible for the tested activities. This class of phytochemical constituent of plants has been implicated to possess anti-trypanosomal activities against different strains of *Trypanosoma* species.[23,24] However, the present study did not isolate, quantify or characterize the STLs from the VLC sub-fractions responsible for the moderate cytostatic and strong anti-trypanosomal activities against human foreskin fibroblast and *Trypanosoma brucei brucei* respectively. To further confirm the consistency in the strong anti-trypanosomal activity of the STL constituents of *T. diversifolia*,

however, would require the isolation, quantification, and characterization of the compounds(s) responsible for the activities.

Conclusion

Sesquiterpene lactone content of *T. diversifolia* demonstrated strong anti-trypanosomal activity against *T.b. brucei* in both *in vivo* mouse and *in vitro* experimental models. The study has also shown that the STLs could be a major source of lead molecules against *T.b. brucei*. Apart from the cytostatic activity, all the tested substances were not cytotoxic against human foreskin fibroblast cells. This has provided a promising starting point for the discovery of trypanocidal STLs from *T. diversifolia.*

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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