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Molecular identification of *Trypanosoma brucei brucei* and in vitro anti-trypanosomal activity of different parts of methanolic extract of *Senna occidentalis*

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Abstract

Objective: The present study investigated the anti-*Trypanosoma brucei brucei* activity of methanolic extract of *Senna occidentalis* roots, leaves, stem bark, and seed in vitro and bioactive components of the most active plant part.

Materials and methods: *Trypanosoma brucei brucei* was identified by PCR, cultured in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37 °C and 5% CO₂. Subsequently, the cultured *T. brucei brucei* were incubated with 500 µg/ml concentration of different parts of *S. occidentalis* plant and the most potent fraction was identified and subjected to Gas Chromatography-Mass Spectrometry (GC-MS).

Results: Amplicons of the rRNA gene of *T. brucei brucei* detected had a size of 1200 bp. The anti-trypanosomal activities indicated that the root extract of the plant was the most active at 500 µg/ml with inhibitory activity of 88.89% and fractions (2 and 5) the most active with IC₅₀ values of 1.49 and 1.16 µg/ml respectively.

Conclusion: Results from this study insinuates that phenolic and simple aliphatic compounds might play key role in the anti-trypanosomal activity of *S. occidentalis* roots.

Keywords: *Senna occidentalis*, Anti-trypanosomal activity, Polymerase chain reaction, *Trypanosoma brucei brucei*, Gas chromatography-mass spectrometry

Introduction

African trypanosomiasis has been estimated to render 50 million people and 48 million cattle at risk leading to low productivity and death [1]. The disease is responsible for major health and economic problems in rural sub-Saharan Africa and caused mainly by an extracellular protozoa belonging to the genus, *Trypanosoma* with species such as *Trypanosoma brucei brucei* [2].

Treatment option using vaccine is difficult owing to antigenic variation exhibited by the parasite and as such,

majority of the treatment against the disease are basically therapeutic and prophylactic. However, the available drugs for the treatment of trypanosomiasis are toxic, expensive, difficult to administer, less effective, cause severe adverse reactions, and face the problem of drug resistance [3, 4]. Due to these shortcomings, it is imperative to explore more effective plant-derived therapeutic agents against African animal trypanosomiasis. This is because the search for effective, less toxic, cheaper plant-derived drugs for the treatment of animal trypanosomiasis is growing and is being encouraged, particularly in developing countries of the world, where orthodox medicines are usually too expensive for the majority of the population [5]. In fact, approximately 80% of the

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world population uses traditional medicine for primary health care and medicinal plants have been very instrumental to pharmaceutical drug discovery and industry [6].

Senna occidentalis (L.) Link, formally known as *Cassia occidentalis* L. [7], is a member of the *Senna* genus and belongs to the family Fabaceae. It is a wild flowering plant found in many tropical countries with roots, leaves, flowers, and seeds used in herbal medicine around the world. Extracts of different parts of the plant are known to have antidiabetic [8], analgesic [9], antibacterial [10]), antipyretic [9], antianxiety and antidepressant activities [11]. The plant extract was also proven to have effective antimalarial [12–14], anti-inflammatory [15] and antioxidant /hepatoprotective activities [16, 17].

Based on a previous report [18], the crude extract of the leaves of *S. occidentalis* demonstrated in vitro activity against *T. brucei brucei* and caused a complete cessation of parasites' motility within 10 min post incubation. Furthermore, the extract also showed in vivo anti-trypanosomal activity and consequently ameliorated trypanosome-induced anaemia, hepatomegaly and splenomegaly in infected rats. However, the in vitro screening of the plant in the study involves counting the parasites at 10 min interval for 1 h which might not be adequate for drug-parasite interaction. Moreover, microscopy was used to detect the *T. brucei brucei* which cannot effectively eliminate the presence of other species of trypanosomes in addition to other inherent limitations of the basic microscopy. Therefore, in the present study, PCR was used to detect the presence of the *T. brucei brucei* DNA in the blood of infected rats. Additionally, other parts of the plant have not been investigated for the anti-trypanosomal activity and possible bioactive antitrypanosomal components have not been identified. Hence, this study was designed to evaluate the in vitro anti-trypanosomal efficacy of the methanolic root, leaf, seed and stem bark extracts in addition to the molecular identification of the parasite and identification of bioactive antitrypanosomal agents.

Materials and methods

Chemicals and reagents

Sodium dihydrogen phosphate, disodium hydrogen phosphate, methanol, ethyl acetate, glucose, HEPES salt, Ethylene diamine tetraacetic acid (EDTA), ethidium bromide, were products of British Drug House (BDH) Chemicals Ltd., Poole England, Dulbecco Minimum Essential Medium (DMEM) from Sigma Chemical Company (USA), diminal® from Eagle Chemical Company LTD, Ikeja, Nigeria, Silica Gel 60–200 mesh was from Qualikems fine Chem Pvt. Ltd., India, Silica gel coated Aluminum TLC plates 60F254 were products of Merck, India.

Plant collection and extraction

Senna occidentalis leaves were collected from farmlands in Unguwan Rimi, Kaduna State. The plant was

identified and authenticated at the herbarium in the Department of Biological Science, Nigeria Defence Academy, Kaduna and voucher number issued (voucher number: 1627). Plant leaves were air dried at room temperature (28 °C–30 °C) and stored in air-tight polythene bags. A total of 135 g of powdered *S. occidentalis* root, 150 g of *S. occidentalis* leaves, 55 g of *S. occidentalis* stem bark and 200 g of *S. occidentalis* seeds were weighed and individually soaked in 350 ml absolute methanol. This was shaken intermittently and left for 72 h. The solvent was decanted and filtered using Whatman filter paper No .1. The respective extracts were then concentrated using a rotary evaporator (Model RE52A, China) at 40 °C.

Trypanosome parasites

Trypanosoma brucei brucei (Federe strain) was obtained from Nigerian Institute for Trypanosomiasis and Oncocerciasis Research (NITOR), Kaduna state. Parasites were harvested at peak parasitemia (10^9 parasites/ml) from the blood of a donor rat, grown in healthy laboratory white albino rats and maintained in the laboratory by serial passage in rats.

At peak parasitemia, the rat was sacrificed and blood collected by cardiac puncture into EDTA container. Phosphate buffer saline was added in the ratio of 1:1 and centrifuged at 4000 rpm for 10 min. The buffy coat layer and portion slightly above the buffy coat layer was collected and centrifuged at 8000 rpm for 5 min. The supernatant was discarded leaving behind the concentrated parasite suspension.

Extraction of DNA from concentrated *T. brucei brucei* suspension and PCR detection

Trypanosome DNA extraction was carried out according to the instruction of GeneJET genomic DNA extraction kit (Thermo Scientific, Germany). Briefly, 200 µl of parasite suspension was lysed by adding 400 µl of HEPES-triton lysis buffer solution and 10 µl of proteinase K and vortexed to obtain a homogenous suspension. These were incubated at 65 °C for 1 h with vortexing after every 20 min. Thereafter, 400 µl of phenol-chloroform was added to the prepared lysate, vortexed for 15 s and centrifuged at 14,000 rpm for 10 min. Supernatant was removed into another tube followed by the addition of 1000 µl absolute ethanol and 40 µl of 3 M sodium acetate. The tube was inverted and incubated overnight at –20 °C followed by a subsequent centrifugation at 14000 rpm for 10 min and the supernatant was discarded. Then, 400 µl of 70% ethanol was added and supernatant once again discarded. The eluent of DNA collected was air dried and 50 µl of deionized water was added. Finally, the DNA collected in the micro-centrifuge tube was stored at -20 °C until needed for processing.

The PCR detection of the parasites was conducted based on the ITS-1 primer sets to validate the presence of trypanosome DNA. The forward and reverse primers used were CCG GAA GTT CAC CGA TAT TG and TTG CTG CGT TCT TCA ACG AA respectively. The PCR was carried out in a final volume of 11 μ l containing 4 μ l phusion flash high fidelity PCR master mix, 1 μ l each of forward and reverse primers, 2 μ l of extracted DNA and 3 μ l of PCR grade water. Initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, annealing at 52 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 10 min were employed. At the end of the PCR, the product was electrophoresed through 2% agarose containing 0.5 μ g/ml ethidium-bromide and the voltage was set at 60 V for 30 min. The product was visualized using a gel documentation system (Gel-Doc 2000, Bio-Rad).

In vitro cultivation of *Trypanosoma brucei brucei*

Cultivation of the prepared *T. brucei brucei* was initiated by placing 6.6×10^6 parasites/ml parasite density in a test tube containing 5 ml of DMEM supplemented with 10% horse serum, 25 mM HEPES salt, and 0.1% glucose. This was maintained at 37 °C using 5% CO₂ in an incubator for 6 hrs. The parasites were counted using a New Improved Neubauer counting chamber [19] and the parasite density was calculated using the formula below:

Density (cells/ml) = Number counted \times 50 \times 1000 \times Dilution Factor employed.

In vitro study with methanolic crude extracts of different parts *Senna occidentalis*

Exactly 500 μ g/ml of the methanol extract from different parts of *S. occidentalis* plant (leaves, stem bark, roots and seeds) were incubated with cultured *T. brucei brucei*. For each experiment, 50 μ l of parasite suspension (1.6×10^6 parasites/ml) and 50 μ l of the extract were dispensed into 100 μ l of the constituted medium in wells of microtitre plates and incubated in a CO₂ incubator for 1 h. Wells containing 50 μ l of standard drug (diminal®) instead of plant extract served as the positive control while those containing no plant extract served as negative control. The parasite density was determined every hour for the period of 6 h.

In vitro anti-trypanosomal activity of root extract of *S. occidentalis*

Based on the results of the above experiment, different concentrations (1,10,50,100,500,1000 and 2000 μ g/ml) of the root extract that had the best activity were incubated with cultured *T. brucei brucei* and the parasite density was calculated as described earlier.

Column chromatography of Methanolic extract of *S. occidentalis* root

A column (30 \times 4 cm) was packed with 100 g of silica gel (mesh size 60–200) to a height of 12 cm. About 5 g of the extract was dissolved in small amount of methanol then mixed with a small amount of activated sand and allowed to dry. The dried mixture was gently loaded on the packed column which was eluted with 100% ethyl acetate, ethyl acetate: methanol (2:1), ethyl acetate: methanol (1:1), and ethyl acetate: methanol (1:2) as the mobile phase. One hundred fractions were collected in 50 ml aliquots at a flow rate of 3 ml/min. The contents of the fractions were examined by TLC and fractions with similar TLC pattern were pooled together into five fractions labelled fraction 1,2,3,4 and 5. Different concentrations (1 – 2000 μ g/ml) of each fraction was incubated with the cultured *T. brucei brucei* and screened for anti-trypanosomal activity as described earlier. The most active fractions were further subjected to gas chromatography- mass spectrometry analyses.

Gas chromatography- mass spectrometry analyses

The GC-MS analysis of fractions 2 and 4 were conducted with an Agilent 6890 N GC interfaced with double-focusing 5973 mass spectrometer (MS). Helium was used as the carrier gas with a flow rate of 1.0 ml/min and a linear velocity of 37 cm/s. The MS operating conditions were: Ionization voltage of 70 eV and ion source of 250 °C. The GC was fitted with a 30 m \times 0.32 mm fused capillary silica column coated with DB-5. Identification of the compounds was done by comparing retention indices, matching fragmentation patterns and mass spectral data with those in the National Institute of Standards and Technology (NIST) library.

Statistical analysis

All experiments were carried out in three replicates. Data were analyzed with a statistical software package (SP for Windows, version 18, IBM Corporation, NY, USA) using Tukey's-HSD multiple range post-hoc test. Values were considered significantly different at $P < 0.05$.

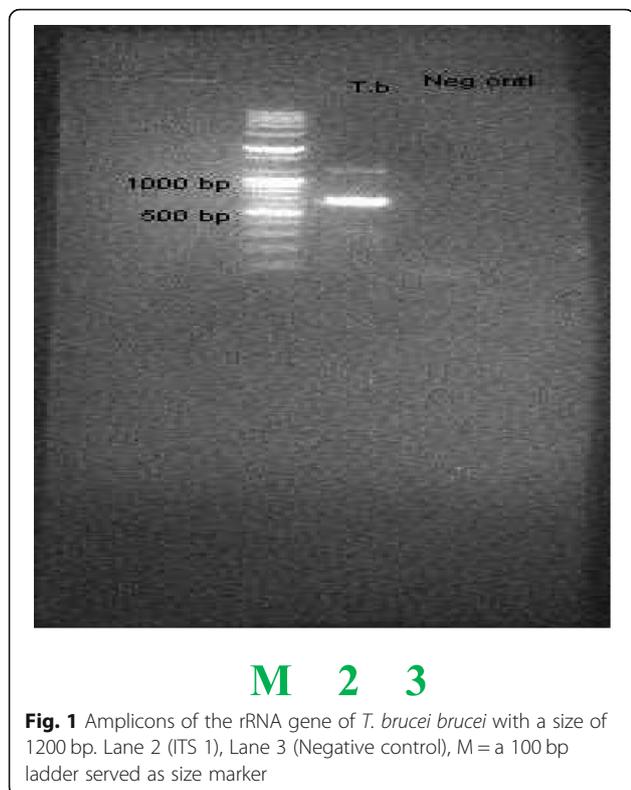
Results

PCR detection of *Trypanosoma brucei brucei*

The PCR detection based on ITS-1 primer sets detected the presence of *T. brucei brucei* DNA in the blood of infected rats as shown in Fig. 1. The *T. brucei brucei* PCR product was approximately 1200 bp.

Percentage yield and in vitro anti-trypanosomal activity of methanolic extracts of different parts of *Senna occidentalis*

The yield and anti-trypanosomal activity of different parts of *S. occidentalis* extracted with methanol is



presented in Table 1. The leaves and stem bark of *S. occidentalis* showed a higher yield compared to the roots and seed. The anti-trypanosomal activities indicated that the root extract at 500 µg/ml produced highest inhibitory activity (88.89%) against the parasites than other extracts while for diminazine aceturate (50 µg/ml), trypanosomes were not detectable for the 5 h experimental duration. Hence, the root extract was selected for further studies.

In vitro anti-trypanosomal activities of different concentrations of Methanolic extracts of *S. occidentalis* roots

From Fig. 2, the results for the in vitro anti-trypanosomal activity of different concentrations of the root fractions showed 100% inhibition at concentration of 500 µg/ml while 100 µg/ml was found to have 74.55% inhibition after 6 h incubation. The standard drug, diminazine aceturate showed complete inhibition at a concentration of 50 µg/ml (data not shown).

Table 1 Percentage yield and in vitro anti-trypanosomal activity of different parts of *Senna occidentalis*

| Methanolic Extracts of different plant parts | % Yield | % inhibition of <i>T. brucei brucei</i> |
|--|---------|---|
| <i>Senna occidentalis</i> Leaves | 10.30 | 38.89 |
| <i>Senna occidentalis</i> Stem bark | 6.58 | 58.33 |
| <i>Senna occidentalis</i> Roots | 3.28 | 88.89 |
| <i>Senna occidentalis</i> Seeds | 3.28 | 58.33 |

Moreover, of the five fractions tested, fractions 2 and 5 demonstrated excellent anti-trypanosomal activity with IC_{50} of 1.49 and 1.16 µg/ml respectively against *T. brucei brucei* while fractions 1, 3 and 4 exhibited lower anti-trypanosomal activity with IC_{50} of 5.65, 2.83 and 2.72 µg/ml respectively (Table 2).

GC-MS analysis

The most active fractions (2 and 5 showed a number of compounds from the GC chromatogram which were identified from the NIST library and presented in Tables 3 and 4. The compounds comprised mainly oxygenated and aromatic hydrocarbons.

Fraction 2 contained aromatic hydrocarbon comprising of cyclohex-3-enyl methyl heptyl ester, hydrocarbon (cyclohexyl methyl ester, cyclo hex-3-enyl methyl propyl ester, cyclo hex-3-enyl methyl hexyl ester, tricyclo {4.3.1.1(3,8)} undecan-1-amine and predominantly furan-2-yl-(2,2,4-trimethyl-4-phenyl-3,4-dihydro-2H-quinolinyl)-methanone containing phenols with percentage peak area of 22.22.

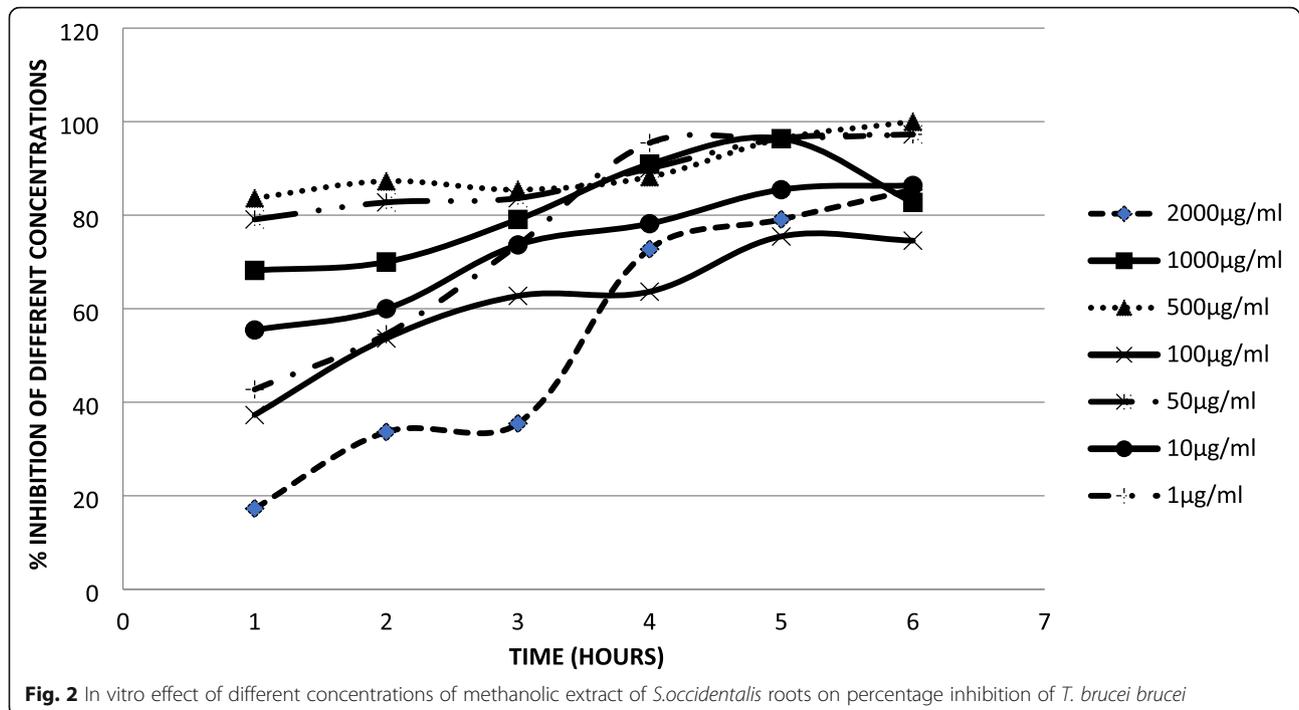
Fraction 4 showed the presence of mainly aromatic hydrocarbons such as (di {cyclo hex-3-enylmethyl} ester, tricyclo {4.3.1.1(3,8)} undecan-1-amine, Cyclo hex-3-enyl methyl hexyl ester, oxygenated hydrocarbons like (2H-pyrrol-2-one, N-(3-trifluoro methyl benzyl)-2-(4-methyl-4H-1,2,4-triazol-3-yl thio)-octa decanedioic acid and predominantly 10-chloro-2-methyl-2 azatricyclo {4.3.1.0(3,8)} decane with high percentage peak area of 17.55.

Discussion

Previous studies demonstrated the in vitro anti-trypanosomal effects of only the leaves of *S. occidentalis* extract against *T. brucei brucei* within the span of 1 h [18], however the root, seed and stem bark extracts have not been evaluated. In this current study, we evaluated the in vitro anti-*T. brucei brucei* activity of leaf, root, seed and stem bark extracts of *S. occidentalis* within the period of 6 h allowing for sufficient drug-parasite interaction. Subsequently, the most active extract was also fractionated and further investigated for bioactive agent(s).

Unlike microscopy which cannot effectively eliminate the presence of other species of trypanosomes, this study employed identification of *T. brucei brucei* using PCR detection based on ITS primer which eliminates the presence of other species of trypanosomes [20, 21]. This finding detected 1200 bp amplicon size of the rRNA gene in *T. brucei brucei* which agrees with Ahmed et al. [22] who identified same species of trypanosomes using ITS primer.

Although reports of Ibrahim et al. [18] showed that crude ethanolic extract of *S. occidentalis* leaves exhibited anti-trypanosomal activity against *T. brucei brucei* as it completely eliminated the parasites' motility within 10 min post incubation with 6.6 mg/ml of effective extract



concentration, this study showed 100% and 74.55% inhibition after 6 h incubation at 500 µg/ml and 100 µg/ml concentration of root extract respectively. The disparity in anti-trypanosomal activities among the different parts could be due to differences in concentration and composition of bioactive constituents present in the plant part which is responsible for the separate roles they play in the plant.

While the previous reports of Ibrahim et al. [18], employed shorter time (1 h) for the studies, this investigation involved the cultivation of trypanosome in vitro for longer period of time (6 h) in order to allow for adequate drug-parasite interaction, thereby avoiding wrong or misleading interpretations. Our selection of the root extract for subsequent study was based on the classification according to Bero et al. [23] where extract with $IC_{50} < 20 \mu\text{g/ml}$ and $IC_{50} > 100 \mu\text{g/ml}$ are considered very good and ineffective, respectively. Owing to this classification, result from the present study of the methanolic extract of *S. occidentalis* root with IC_{50} of 18 µg/ml suggest that the extract have a good anti-trypanosomal activity. Prior to this study,

Table 2 IC_{50} *T. brucei brucei* of various fractions tested

| Fractions tested | IC_{50} <i>T. brucei brucei</i> (µg/ml) |
|------------------|---|
| Fraction 1 | 5.65 |
| Fraction 2 | 1.49 |
| Fraction 3 | 2.83 |
| Fraction 4 | 2.72 |
| Fraction 5 | 1.16 |

no attempt has been done to demonstrate the in vitro activities of methanolic extract of *S. occidentalis* root against *T. brucei brucei*.

The observed in vitro anti-trypanosomal activity of fractions of *S. occidentalis* methanolic root extract is not astounding owing to a report Ibrahim et al. [18] which demonstrated that ethanolic leaf extract of the plant possessed anti-trypanosomal activity. Using IC_{50} as index for measuring anti-trypanosomal activity, fractions 2 and 5 exhibited excellent antitrypanosomal activity as opposed to the other three (3) fractions.

The GC-MS analysis of the fractions 2 and 5 identified a number of compounds. A total of 29 different chemical compounds were identified. Anti-trypanosomal activity of both fractions might be attributed to the presence of phenolic compounds which have been reported to show significant anti-trypanosomal activity as reported by Oluwatosin and Clement [24] and Ibrahim et al. [25]. The predominant phenolic compound detected in fraction 2 is furan-2-yl-(2,2,4-trimethyl-4-phenyl-3,4-dihydro-2H-quinolinyl)-methanone. Additionally, 8-azabicyclo {3.2.1} oct-2-ene in the fractions can also be attributed to the anti-trypanosomal activity of the fractions owing to the reports of Oluwatosin and Clement [24] in which simple aliphatic compounds were observed to possess anti-trypanosomal property in vivo. Therefore, it could be concluded that the aforementioned bioactive compounds present in the methanolic root extract of *S. occidentalis* might be responsible for its anti-trypanosomal activity in vitro.

Table 3 Bioactive components identified in fraction 2 of methanol root extract of *S. occidentalis* by GC-MS

| S/NO | Compounds | Retention time | Peak area (%) |
|------|---|----------------|---------------|
| 1 | Bicyclo {3.3.1} nonan-2-one | 33.358 | 1.69 |
| 2 | Tricyclo {4.3.1.1 (3,8)} undecan-1-amine | 25.964 | 3.86 |
| 3 | 8-azabicyclo {3.2.1} octane-3,6-diol | 29.227 | 1.46 |
| 4 | Cyclohex-3-enyl methyl heptyl ester | 29.901 | 6.31 |
| 5 | 2H-pyrrol-2-one | | 0.88 |
| 6 | 8-azabicyclo {3.2.1} oct-2-ene | 31.646 | 1.63 |
| 7 | Glycidyl palmitate | 31.859 | 3.80 |
| 8 | Fumaric acid | 31.846 | 2.73 |
| 9 | Cyclo hex-3-enyl methyl hexyl ester | 33.719 | 5.61 |
| 10 | Cyclo hex-3-enyl methyl propyl ester | 33.390 | 5.61 |
| 11 | Furan-2-yl-(2,2,4-trimethyl-4-phenyl-3,4-dihydro-2H-quinolinyl)-methanone | 34.10 | 22.22 |
| 12 | Cyclohexyl methyl ester | 34.346 | 3.64 |
| 13 | Glutaric acid | 29.942 | 2.97 |

Table 4 Bioactive components identified in fraction 4 of methanol root extract of *S. occidentalis* by GC-MS

| S/NO | Compound | Retention time | Peak area (%) |
|------|---|----------------|---------------|
| 1 | Bicyclo {3.3.1} nonan-2-one | 26.321 | 1.69 |
| 2 | Camphor-10-sulfonic acid | 29.591 | 3.96 |
| 3 | 2H-pyrrol-2-one | 29.591 | 3.96 |
| 4 | Fumaric acid | 29.841 | 4.01 |
| 5 | Di (cyclohex-3-enyl methyl) ester | 24.418 | 4.01 |
| 6 | Glutaric acid | 29.942 | 2.04 |
| 7 | Cyclo hex-3-enyl) methyl cyclohexyl methyl ester iso sativene | 29.942 | 2.04 |
| 8 | Tricyclo {4.3.1.1 (3,8)} undecan-1-amine | 35.748 | 3.66 |
| 9 | Cyclo hex-3-enyl methyl hexyl ester | 34.356 | 7.28 |
| 10 | Cyclo hex-3-enyl methyl decyl ester | 30.476 | 2.31 |
| 11 | Borazine | 30.325 | 2.23 |
| 12 | Cyclo hex-3-enyl methyl heptyl ester | 34.259 | 2.73 |
| 13 | Glycidyl palmitate | 31.850 | 3.75 |
| 14 | N-(3-trifluoro methyl benzyl)-2-(4-methyl-4H-1,2,4-triazol-3-yl thio)-octa decanedioic acid | 31.850 | 3.75 |
| 15 | Cyclo hex-3-enyl methyl ester | 30.195 | 1.20 |
| 16 | Di (cyclo hex-3-enyl methyl) ester | 33.733 | 2.97 |
| 17 | 8-azabicyclo {3.2.1} octan-3-ol | 34.092 | 3.26 |
| 18 | 8-azabicyclo {3.2.1} octan-3,6-diol | 34.092 | 3.26 |
| 19 | 10-chloro-2-methyl-2-azatricyclo {4.3.1.0 (3,8)} decane | 34.092 | 17.55 |
| 20 | 19-cyclo-27-norlanostan-25-one | 35.491 | 0.88 |

Conclusions

This study insinuates that the root of *S. occidentalis* has potent anti-trypanosomal activities and phenolic and simple aliphatic compounds might play key role in the anti-trypanosomal activity. However, further studies will investigate the in vivo anti-trypanosomal activity of the suggested bioactive compounds.

Abbreviations

GC-MS: Gas chromatography- mass spectrometry; DMEM: Dulbecco's Modified Eagle Medium; ITS: Internal transcribed spacer; IC50: Half maximal Inhibitory concentration; PCR: Polymerase Chain Reaction; rRNA: Ribosomal ribonucleic acid; NIST: National Institute of Standards and Technology; EDTA: Ethylenediaminetetraacetic acid

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Authors' contributions

ZAA performed all the in vitro studies, analyzed and interpreted the data and was a major contributor in writing the manuscript. MSA performed cultivation of the prepared *T. brucei brucei* and preparation of the culture medium. MAI conducted the selection, collection and preparation of plant extract fractions and also contributed to the writing of the manuscript. TB carried out GC-MS analysis. ABS conducted part of the molecular identification of the parasite. All authors read and approved the final manuscript.

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Availability of data and materials

All the data was analyzed with a statistical software package (SP for Windows, version 18, IBM Corporation, NY, USA) using Tukey's-HSD multiple range post-hoc test.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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