Full Length Research Paper

In vitro anti-trypanosomal activities of crude extracts, β-sitosterol and α-sulphur from *Buchholzia coriacea* seed

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The seeds of *Buchholzia coriacea* Engler are traditionally used as a febrifuge in Southeast Nigeria. The bioassay-guided fractionation of the crude extracts was carried out using *in vitro* cultures of *Trypanosoma brucei brucei* S427. On soxhlet extraction, methanol gave the highest yield (5.44 %) followed by hexane (3.34%) and ethyl acetate (0.66%). The ethyl acetate extract showed the highest anti-trypanosomal activity followed by the hexane and methanol extracts. Beta-sitosterol and α–sulphur were isolated from *B. coriacea* seed extracts by preparative chromatography and identified by nuclear magnetic resonance and x-ray crystallography, respectively. Beta-sitosterol and α–sulphur showed modest anti-trypanosomal activities against *T. b. brucei* S427 *in vitro* with minimum inhibitory concentration (MIC) values of 12.5 and 25 µg/ml respectively.

Key words: *Trypanosoma brucei brucei, Buchholzia coriacea,* bioassay-guided fractionation.

INTRODUCTION

The seeds of *Buchholzia coriacea* Engler (Capparaceae) are folklorically used in Eastern Nigeria to treat feverish conditions (Nweze et al., 2009). The seeds are commonly called elephant or wonderful colas. They are chopped and soaked overnight in the local gin and the infusion is drunk for the cure of such ailments as malaria and sleeping sickness that cause fever.

Trypanosomosis has continued to be a problem in Africa, south of the Sahara. This is more so with the development of resistance to the frequently used trypanocides like diminazene aceturate (Berenil[®]) and isometamidium chloride (Jamal et al., 2005). Other drugs like Suramin and Homidium have also been affected by parasite resistance (Codja et al., 1993). Most of the common trypanocides have been in use for more than half a century. These few trypanocides are old, toxic and very expensive for the rural poor (Legros et al., 2002).

Even in cases where treatment is effective often relapse of infection after treatment. There is an urgent need for new, effective and inexpensive drugs for the chemotherapy of trypanosomosis.

In an *in vivo* study, the ethanol extract of *B. coriacea* seed was shown to have antitrypanosomal activity in mice experimentally infected with *Trypanosoma brucei* (Nweze et al., 2009). At the dose of 1000 mg/kg body weight intraperitoneally, the ethanol extract was able to clear trypanosomes from peripheral blood circulation. Other workers have reported the anthelmintic activity of the leaves (Ajaiyeoba et al., 2001) and seeds (Nweze and Asuzu, 2006) of *B. coriacea.* The antimicrobial activity of the fresh seed and its extracts against some food borne pathogens has been reported (Ezekiel and Onyeoziri, 2009). Fractions prepared from the methanol extract of *B. coriacea* stem bark have shown both antibacterial and antifungal activities (Ajaiyeoba et al., 2001).

This study was designed to identify the active compound(s) responsible for the observed anti-trypanosomal activity of this plant through bioassay-

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guided fractionation of the crude extracts.

MATERIALS AND METHODS

Plant material

Mature seeds of *B. coriacea* Engler were collected in February, 2009 and identified by a taxonomist Mr A. O. Ozioko of the Bioresources Development and Conservation Centre (BDCP), Nsukka where voucher specimens were also deposited. The seeds were pulverised into fine powder in a mill. The powdered plant materials were stored in sealed cellophane bags in order to protect it from light.

Extraction

The plant material (500 g) was placed in a soxhlet apparatus and extracted successively with hexane, ethyl acetate and methanol (HPLC grade, Sigma, UK) at 40°C for 72 h for each solvent. All the extracts were concentrated in a rotary evaporator (BUCHI labortechnik AG, Switzerland) under reduced pressure at 40°C. Methanol extracts were in addition freeze-dried using an Edward's freeze drier (Edward's high vacuum, Crawley, England). The extracts were stored at -20°C before use.

Separation and isolation of compounds

Thin layer chromatography (TLC) of the plant extracts was carried out by using pre-coated silica gel plates (0.063 to 0.020 mm, Kieselgel 60 PF^{254}, Merck No 5554). The mobile phase was 10% ethyl acetate in hexane. Following development and drying, chromatograms were examined under short (254 nm) and long (366 nm) UV light. Spots on TLC were visualized using Anisaldehyde-sulphuric acid spray. Column chromatography of the hexane and ethyl acetate extracts was done using column grade silica gel (silica gel 60, Merck) in glass columns and eluted gradient wise starting with 100% hexane and ending in 40% ethyl acetate in hexane. Eluates collected were monitored by TLC and bulked as appropriate. Preparative TLC was used to separate and purify active fractions in order to get pure compounds according to the method of Kirchner (1978).

Structural elucidation

Characterisation of the pure compound obtained was by nuclear magnetic resonance (NMR) while characterisation of the element was by x-ray crystallography. The ^{1}H NMR spectra were run in a JEOL Eclipse (400 MHz) while the ^{13}C NMR spectra were run in a Bruker spectrophotometer (600 MHz). Deuterated chloroform was used as solvent. The ethyl acetate fraction (E1) formed colourless crystals. Also, the hexane fraction (Hex 1) formed yellow crystals. Their structures could not be determined using NMR. Data for E1 and H1 crystal structures were measured at 123 K with graphite monochromated Mo$_{k\alpha}$ radiation (λ = 0.71073 Å) using a Bruker Appex II CCD instrument. All non hydrogen atoms were refined anisotropically. All structures were refined to converge against F^2 using the SHELXL-97 program.

In vitro determination of antitrypanosomal activity

An Alamar blue® assay was used to determine the *in vitro* anti-trypanosomal activity of *B. coriacea* seed crude extracts, fractions and isolated compounds. This was done according to the method of Raz et al. (1997). The samples were prepared as stock solutions in DMSO at a concentration of 10 mg/ml. The concentration of DMSO in the wells was always below 10 %. The concentration of *T. b. brucei* S427 was 2-3 × 10^4 trypanosomes/ml.

Initial screen at one concentration

A tenfold dilution was made by adding 5 μl of the extracts and fractions to 45 μl of HMI-9 media in a round-bottomed 96 well micro titre plate. Four control wells (1E – H) with 5 μl of DMSO and 45 μl of HMI-9 media were also set up. This dilution plate was placed on a micro titre shaker for about 1 min in order to thoroughly mix the contents. Four micro-litres of the contents of the dilution plate were transferred to the corresponding wells of a flat bottomed 96 well micro-titre assay plate. HMI-9 media (96 μl) was added to all the wells to make them up to 100 μl each, except the column 12 wells which received 80 μl of the media only. Column 12 wells served as the positive control wells which received 20 μl of Suramin. 100 μl of the blood stream form of trypanosomes were added to each well and was incubated at 37°C, and 5% CO_2 in a humidified atmosphere for 48 h. After incubation for 48 h, 20 μl of Alamar blue (REDOX indicator, Abdserotec, UK) was added. The assay plate was incubated under previously described conditions for a further 24 h. Fluorescence was determined by using the Wallac Victor apparatus at an excitation wavelength of 530 nm and emission wavelength of 590 nm.

Determination of minimum inhibitory concentration (MIC)

Briefly, 4 μl of the test samples were added in duplicate to the column 2 wells of a flat bottomed micro-titre plate. HMI-9 medium (196 μl) was added to the same wells to give a volume of 200 μl. All the other wells received 100 μl each except those on column 12 which got 80 μl. Serial double dilutions were carried out from column 2 to11. A solution of Suramin (Sigma, UK) was prepared and filter sterilized to give a concentration range of 1 to 0.008 μM. 20 μl of each concentration was added to the corresponding well of column 12. 100 μl of a suspension of the blood stream form of *T. b. brucei* S 427 was added to each well. The assay plate was incubated and the results read as aforementioned.

RESULTS

Hexane, ethyl acetate and methanol gave yields of 3.34, 0.66 and 5.44% respectively. Beta-sitosterol isolated from hexane fraction 69-102, ethyl acetate fractions E16-24 and E25-39 was a white solid which gave a pink colour on TLC after treatment with anisaldehyde. It was identical to the standard sample (Sigma, UK). The compound was not visible under UV light (254 or 365 nm). ^{13}C NMR spectrum (400 MHz CDCl$_3$) showed a carbon bearing hydroxyl group at 71.8 ppm (C-3) and two olefinic carbons at 140.8 ppm (C-5) and 121.7 ppm (C-6). ^{1}H NMR spectrum displayed a doublet at 5.36 ppm (J = 5.3 Hz) attributed to H-6 while the signal at 3.53 ppm was assigned to the axial proton at C-3. Verification of this compound as β-sitosterol came from comparison with NMR spectrum published in the Aldrich Atlas of NMR spectral data (Pouchert and Behnke, 1993). Hexane fraction 1 crystallized out as pure yellow crystals while
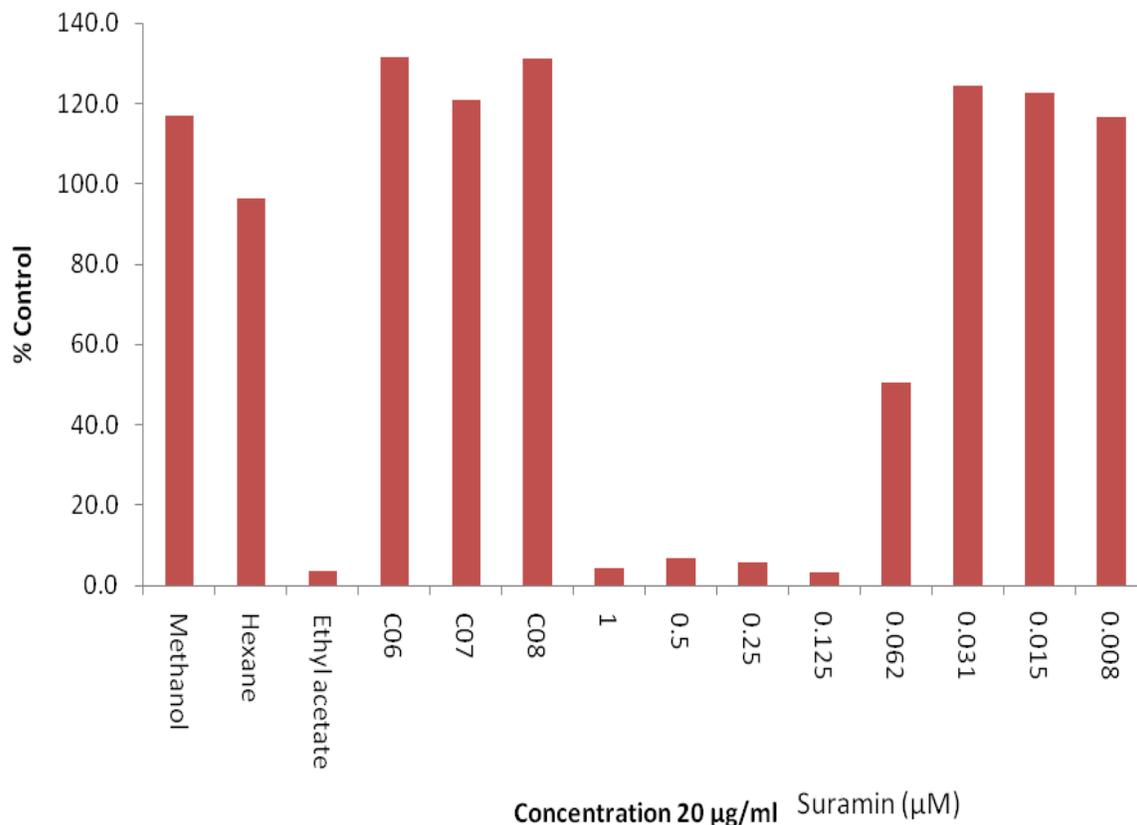
Figure 1. *In vitro* screen of crude extracts of *B. coriacea* seed for antitrypanosomal activity against *Trypanosoma brucei brucei* S427. C06 - C08 are untreated control wells.

ethyl acetate fraction 1 gave colourless crystals which were identified as α-sulphur and γ-sulphur respectively using x-ray crystallography.

The result of the screening of the crude extracts of *B. coriacea* seed using *in vitro* culture of *T. b. brucei* S427 is shown in Figure 1. The crude ethyl acetate extract showed the highest antitrypanosomal activity followed by the hexane and methanol extracts. The result of the screening of the hexane and ethyl acetate fractions for *in vitro* antitrypanosomal activity is shown in Figures 2a and 2b. Hexane fractions 1 and 69-96 and ethyl acetate fractions 16 to 24 and 25 to 39 showed antitrypanosomal activity. The MIC values of the hexane and ethyl acetate active fractions are shown in Figure 3. Hexane fraction 69-96 had an MIC value of 12.5 µg/ml. Ethyl acetate fractions 16 to 24 and 25 to 39 had MIC values of 12.5 and 25 µg/ml respectively. After separation of the active fractions using preparative TLC, trilinolein, beta-sitosterol and oil were obtained. They were further screened for antitrypanosomal activity and the result is shown in Figure 4. Only beta-sitosterol showed *in vitro* anti-trypanosomal activity. Figure 5 shows the result of the MIC test for beta-sitosterol and hexane fraction 1 (which was identified to be α-sulphur). Their MIC values were 12.5 and 25 µg/ml respectively.

DISCUSSION

On extraction, methanol gave the highest yield followed by hexane and ethyl acetate. The plant yield increased with increasing polarity of the solvents used. This might be due to the increased extraction of polar substances like sugars and tannins. This trend was also observed by Ezekiel and Onyeoziri (2009). In their work, hexane and methanol gave yields of 1.3 and 23.3%, respectively when used to extract *B. coriacea* seeds. The differences in obtained yields could be due to the length and sequence of extraction.

The methanol extract however, showed the least anti-trypanosomal activity. This suggests that perhaps the concentration of the active substances in this extract was too low for it to show any activity. The crude ethyl acetate extract was the most active of all the extracts followed by the hexane extract. Beta sitosterol was obtained on preparatory chromatography from both the hexane and ethyl acetate fractions. There was no lupeol found in all the seed extracts. Ajaiyeoba et al. (2003) found both lupeol and β-sitosterol in the methanolic extract of *B. coriacea* leaves.

Other sterols that have been reported to have anti-trypanosomal activities are vernoguinosterol and
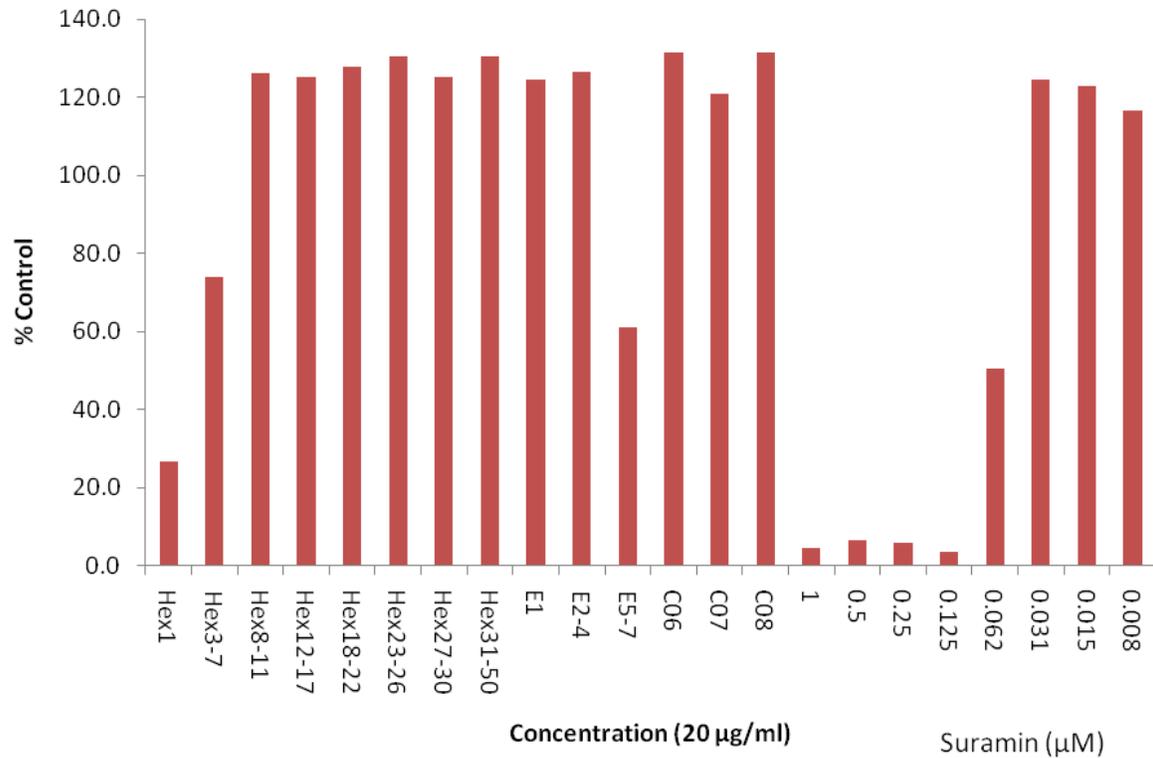
Figure 1a. *In vitro* screen of the fractions of *B. coriacea* seed for antitrypanosomal activity against *Trypanosoma brucei brucei* S427. C06 –C08 are untreated control wells; Hex 1 – Hex 50 are hexane fractions; E1 – E7 are ethylacetate fractions.
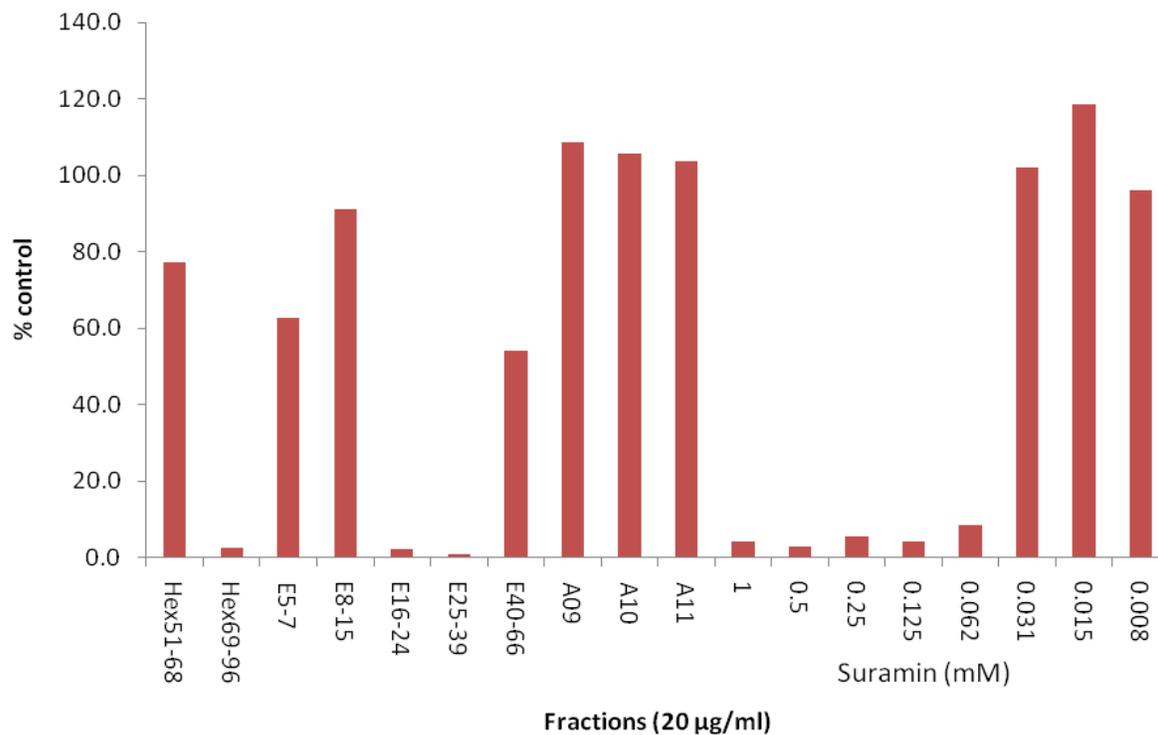
Figure 2b. *In vitro* screen of *B. coriacea* seed for antitrypanosomal activity against *Trypanosoma brucei brucei* S427. A09 – A11 are untreated control wells; Hex 51 – 96 are hexane fractions; E7 – E66 are ethylacetate fractions.
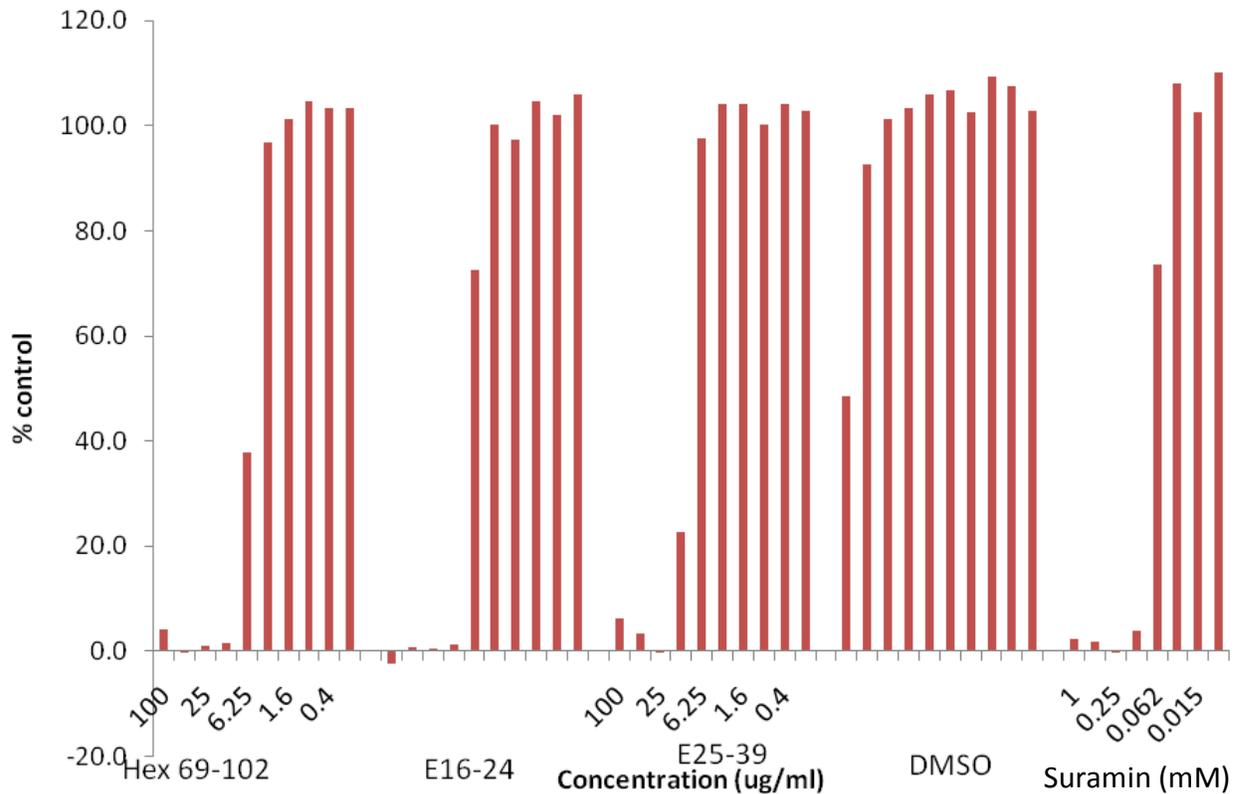
Figure 2. Dose-effect curve of active fractions of *B. coriacea* seed against *Trypanosoma brucei brucei* S427.
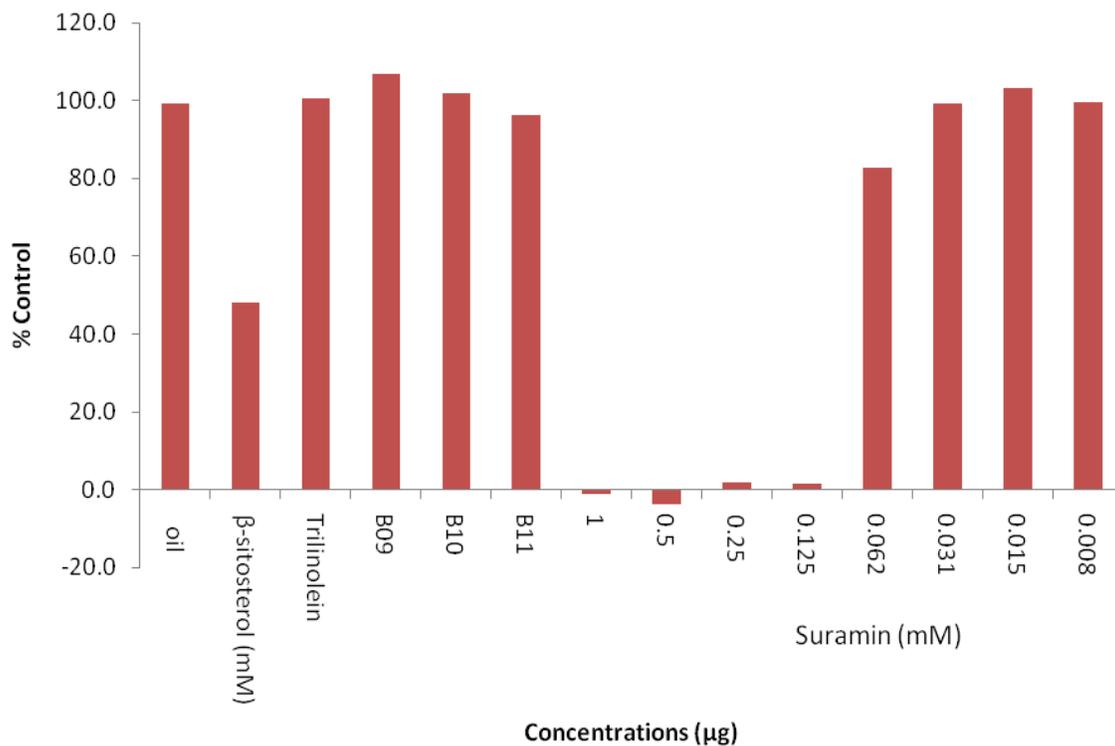
Figure 3. *In vitro* screen of compounds isolated from B. coriacea seed against Trypanosoma brucei brucei S427. B09 – B11 are untreated control wells.
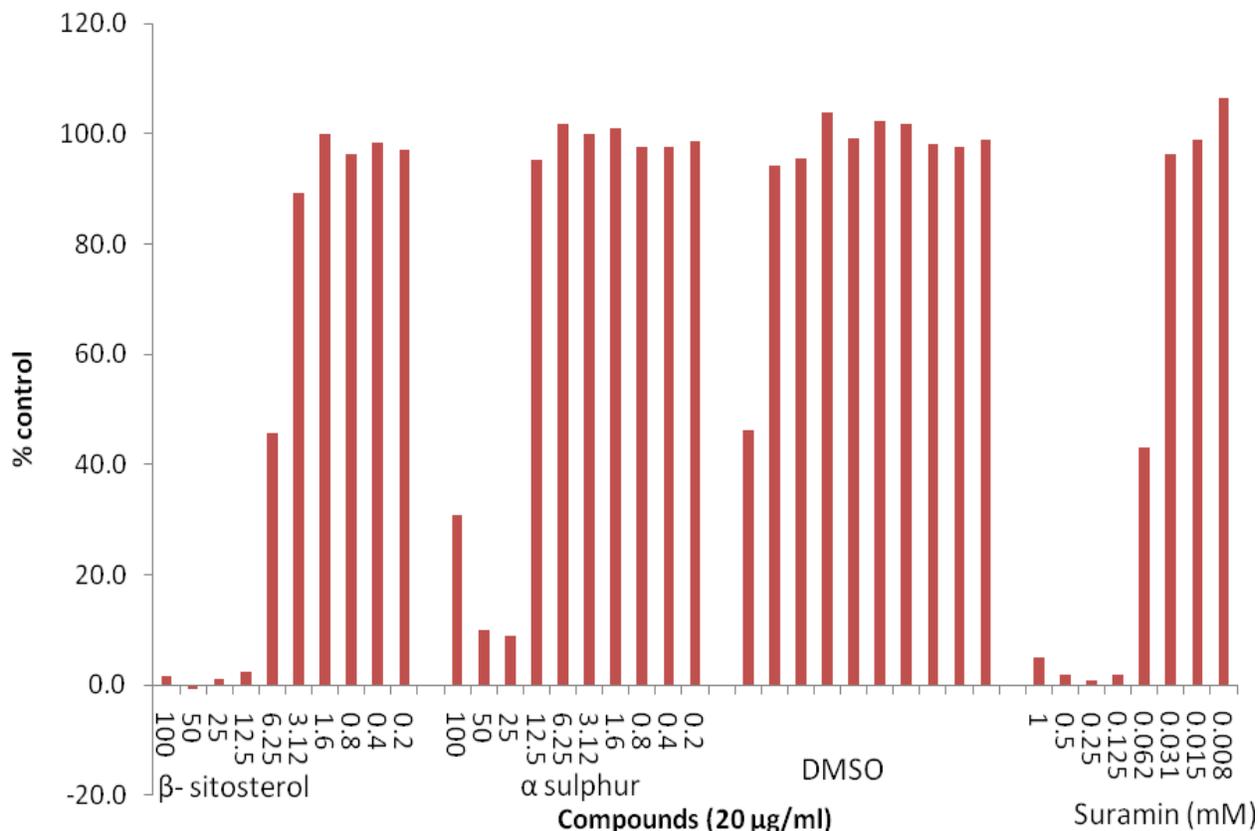
Figure 3. Dose-effect curve of active compounds from *B. coriacea* seed against *Trypanosoma brucei brucei* S427.

vernoguinoside isolated from the stem bark of *Vernonia guineensis* (Tchinda et al., 2002). They were shown to have significant inhibitory activity against the bloodstream trypomastigote forms of *T. b. rhodesiense* with IC$_{50}$ values between 3-5 µg/ml. Also, fractionation of the lipophilic extracts of the leaves of *Strychnos spinosa* yielded two sterols namely, saringosterol and 24-hydroperoxy-24-vinylcholesterol which were found to possess *in vitro* anti-trypanosomal activities against *T. b. brucei* (Hoet et al., 2007). Their IC$_{50}$ values were 7.8 ± 1.2 and 3.2 ± 1.2 µM respectively. In the same report, the above authors found that one of the main compounds isolated from their active fractions was β-sitosterol which they found to be inactive against *T. b. brucei.* They however reported the weak anti-trypanosomal activity of stigmasterol and clerosterol.

The dichloromethane extract of the stem bark of *Acacia nilotica* showed high *in vitro* anti- trypanosomal activity against *T. b. brucei* STIB 345 (Aderbauer et al., 2008). It also had low toxicity both *in vivo* and *in vitro* but an unexpected immunosuppressive effect *in vivo.* One of the constituents isolated from *A. nilotica* was β-sitosterol (Prakash and Garg, 1981).

The second element isolated which showed activity against the bloodstream forms of *T. b. brucei* was α-sulphur. The hexane and the ethyl acetate fractions

yielded the alpha and gamma types of sulphur crystals respectively. The former were yellow crystals while the latter were colourless crystals. They were absent from the methanolic extract. This is the first report of the presence of sulphur in *B. coriacea* seeds. α-Sulphur crystals showed anti- trypanosomal activity against *T. b. brucei* S427 bloodstream forms *in vitro* with an MIC value of 25 µg/ml.

Different sulphur containing aliphatics originally isolated from garlic bulb (*Allium sativum*) have been studied for their anti trypanosomal activities (Hoet et al., 2004). Diallyl trisulfide is a chemically stable final transformation product of allicin that can be synthesized and is used in China to treat bacterial, fungal and parasitic infections in man. This product exhibits IC$_{50}$ values in the range of 10-15 µM when tested *in vitro* on bloodstream forms of *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense* and 30 µM when tested on *T. congolense* bloodstream forms. For up to 140 µM, no noticeable morphological changes could be observed on fibroblasts (Lun et al., 1994). A fraction of the oily extract from garlic bulb, which apparently contained mainly diallyl disulfide cured mice infected with *T. b. brucei* in four days when given intraperitoneally at a dose of 120 mg/kg per day. Nok et al. (1996) showed that the extract interferes with the parasites' synthesis of membrane lipids.

The active compound responsible for the observed *in vitro* anti-trypanosomal activity of *B. coriacea* seed against *T. b. brucei* S427 bloodstream forms was identified to be beta sitosterol. α- Sulphur also exhibited modest lethal effects on *T. b. brucei* S427. Further work on the crude ethyl acetate extract which showed high anti-trypanosomal activity and on the alpha sulphur needs to be carried out to confirm their anti-trypanosomal activities.

ACKNOWLEDGEMENT

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