ANTITRYPANOSOMAL POTENTIAL AND PHYTOCHEMICAL CONSTITUENTS OF AQUEOUS AND METHANOLIC FRACTIONS OF *Combretum molle IN-VITRO*

Supported by tetfund TERTIARY EDUCATION TRUST FUND

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Received: May 23, 2016 **Accepted:** August 26, 2016

Abstract: In this study, aqueous and methanolic fractions of *Combretum molle* were investigated *in vitro* for antitrypanosomal potentials against Trypanosomes. The test involved incubating the parasites, *Trypanosoma brucei brucei*, with (10 mg, 5 mg/ml) of the fractions in 96-well microtitre plate. Cessation or drop in the parasite motility, determined microscopically, was taken as a measure of efficacy of the extract against control wells of Diminazene aceturate at (3.5 mg/kg). The test organisms were immobilized within 40-50 minutes of incubation. The *in vitro* activity of the extract at (10 mg/kg and 5 mg/kg) for both methanolic and aqueous fractions containing saponins and flavonoids, respectively were also determined. The phytochemical constituents of the crude extracts revealed the presence of tanins, saponins, flavonoids, glycosides, cardiac glycosides, anthraquinones and carbohydrates, while alkaloids were not detected hence a need for continued and sustained research in ethnomedicinal plants.

Keywords: Antitrypanosomal, *Combretum molle*, aqueous and methanolic extracts, nagana

Introduction

The scourge of trypanosomiasis has remained a serious health challenge and constitutes a setback to the economic and social wellbeing of sub-Saharan Africa (Atawodi & Alafiatayo 2007). Trypanosomes are protozoan parasites responsible for Human African Trypanosomiasis (HAT) and "nagana" in cattle and are transmitted by the bite of an infected tsetse fly (*Glossina* spp) (Mbaya & Ibrahim 2011). *Trypanosoma brucei brucei*, the causative agent of 'nagana' is closely related to *Trypanosoma brucei rhodesiense* which is the agent of HAT in East to South Africa and *Trypanosoma brucei gambiense* found in West and Central Africa.

Sleeping sickness currently affects about half a million people in sub-Saharan Africa and an estimated 60 million people are at risk of contracting this disease, which is fatal if untreated (WHO, 1998; Berret, 1999). However, the currently available treatments are far from being ideal. The few registered trypanocides are frequently toxic, required lengthy parenteral administration, lack efficacy and are unaffordable for most of the patients (Keiser *et al.*, 2001; Legros *et al.*, 2002). Therefore, there is an urgent need for new, safe, effective and cheap compounds and for new leads with new mechanisms of action. This paper reports the antitrypanosomal effects of *Combretum molle* extracts.

Materials and Methods

Plant collection and authentication

The leaf and twigs of *Combretum molle* were collected around Zaria Local Government Area of Kaduna State Nigeria. The plant materials were taken to the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria for authentication with voucher number (900191) and specimen deposited in the herbarium for reference.

Plant preparation and extraction

The collected plant materials were carefully washed under running tap water (to remove dust and any other foreign materials) and were allowed to drain off. The plant material was spread on the laboratory bench to air-dry at room temperature for two weeks. This was then pulverized in a mortar and finely sieved. Exactly 100 grams was soaked in 500 millilitres of distilled water and methanol separately for 24 h; this was then filtered using Whatman filter paper (No. 1). The aqueous filtrate was evaporated to dryness on steam at 60°C in a water bath while the methanolic extract was allowed to evaporate at room temperature. The extract was stored in cupboard at room temperature until needed for use Trease and Evans (1989).

Phytochemical screening of crude extracts

The screening techniques of Harborne (1973), Trease and Evans (1989) and Sofowora (1993) were adopted.***Alkaloids*:** Approximately 0.5 g of the powdered plant material was stirred with 5 ml of 10% aqueous hydrochloric acid in a steam bath. This was filtered and 1 ml of the filtrate was treated with a few drops of Mayer's reagent, another 1 ml portion was treated with Dragendorff's reagent. Turbidity or precipitate with either of the reagents gives a positive test for alkaloids.

Saponins

Approximately, 0.1 g of the powdered plant material was boiled in 10 ml of distilled water for 5 min, and decanted while still hot; the filtrate was used for the following tests:

a. **Frothing test:** Approximately, 1 mg of filtrate was diluted with 4 ml of distilled water and the mixture shaken vigorously and observed on standing for stable froth.
b. **Emulsion test:** This was done by addition of drops of olive oil to the frothing solution and shaken vigorously to observe for emulsion (Trease and Evans, 1989).

Test organism

Trypanosoma brucei brucei was obtained from Nigerian Institute for Trypanosomiasis Research (NITR), Kaduna. The parasites were maintained in the laboratory by continuous passage in Trypanosome-free mice intraperitoneally throughout the period of the study.

Determination of parasitaemia

Blood from the tail of mice was used to determine parasitaemia in wet mount. Trypanosome count was estimated by examination of the wet mount microscopically using the "rapid matching" method of Herbert and Lumsden (1976) at x400 magnification. The method involved microscopic counting of parasites per field in pure blood or blood approximately diluted with buffered phosphate saline (PBS; pH 7.2).

In vitro* antitrypanosomal activity of fractionated extracts of *Combretum molle

In vitro assessment of the antitrypanosomal activity of the crude extract of *Combretum molle* was performed in duplicates in 96 wells microtitre plates. Approximately 10 µl blood of infected mice in heparin blood was mixed with 50 µl of serial dilution of stock solution of extracts. The controls were a serial dilution of 25 mg/ml diaminazene aceturate and blood mixed with 50 µl Phosphate buffered saline (PBS) containing 10% dimethylsulfoxide (DMSO) for positive and negative controls, respectively. The plates were incubated in a moist chamber at room temperature (29°C) for 60 min. Antitrypanosomal activity was determined by microscopic examination of a drop of the mixture in a microslide at x400 magnification Herberts & Lumsden (1976). Complete cessation of motility or reduction in parasite count per ml was taken as significant activity of the extracts.

Fractionation of crude extracts using thin layer chromatography (TLC)

The aqueous and methanolic crude extracts were fractionated by Thin Layer Chromatography (TLC). The modified method of (Woo *et al.,* 2004) was adopted, it is described as follows:

The crude samples were soaked in 250 ml of Diethyl Ether and 100 ml of distilled water added; this was allowed for 24 h for the fractions to elute. After 24 h, 250 ml N-Butanol was used to flood the extracts in the presence of 100 ml dilute Hydrochloric acid (HCl). This was allowed to fractionate Saponins; afterwards 1% Potassium hydroxide (KOH) was added to neutralize the HCl and Flavonoid fraction was obtained. This was carried out for both aqueous and methanolic crude extracts separately.

Result and Disscusion

The phytochemical screening of the methanolic and aqueous crude extracts of *Combretum molle* revealed the presence of active phytochemical constituents like saponins, tannins, glycosides, cardiac glycosides, anthraquinones, carbohydrates and flavonoids were present while alkaloids were absent (Table 1). The *in-vitro* activity of crude/fractions of *Combretum molle* showed that flavonoidsfraction from methanol extract at 10mg/kg inactivated the parasites at 40 min post incubation. The aqueous fraction at 5 mg/kg however immobilised the parasites at 50 min post incubation. Similarly, the saponin fraction from methanol extract at 10 mg/kg inactivated the parasites 60 min post incubation. The same trend occurred upon incubation with the aqueous fraction at 5 mg/kg, where the parasites were inactivated one hour post incubation. The positive control Diminazene aceturate at 3.5 mg/kg cleared the parasites 30 min post incubation. The parasites were still active after one hour upon incubation with the negative control phosphate buffered salinePBS) (Table 2).

Table 1: Phytochemical constituents of aqueous and methanolic crude extracts of *Combretum molle*

Test for carbohydrates	Methanol	Aqueous
Mollish test	+	+
Fehling test	+	+
Test for glycosides		
Fehling test	+	+
Ferric chlorides test	+	–
Test for anthraquinones		
Free anthraquinones	+	–
Combined anthraquinones	+	–
Test for cardiac glycosides		
Kella-killiani Test	+	+
Kedda test	+	+
Salkowsk test	+	+
Test for saponins		
Frothing test	+	+
Steroid & triterpenes	+	+
Test for flavonoids		
Shinoda test	+ (orange)	+ (red)
Sodium hydroxide test	+	+
Test for tannins		
Lead sub-acetate test	+	+
Ferric chloride test	+	+
Bromine water test	+	+
Test for alkaloids		
Meyers test	–	–
Wagners test	–	–

+ = Presence of phytochemical constituent; – = Absence of constituent.

The preliminary phytochemistry of *Combretum molle* showed the presence of active constituents like saponins, tanins, glycosides, cardiac glycosides, anthraquinones, flavonoids as present in both aqueous and methanolic crude extracts (Table 1). It is a common occurrence that these phytochemicals are usually reported in most plants screened for ethnomedicinal properties, as previous studies have shown that different parts of the same plant could show varying level of antitrypanosomal activity (Atawodi *et al*., 2003; Shuaibu *et al*., 2008). Various phytochemicals such as alkaloids, flavonoids, diamidines, and lypophylic amines have also been reported to possess antitrypanosomal activity (Maikai *et al*., 2011). However, the absence of alkaloids in both crude aqueous and methanolic extracts is quite striking as its presence enhances the trypanocidal activity of the plant being analysed. Similarly, the quantity of these active constituents in any plant confers higher activity on the plant (Shuaibu *et al*., 2008). In this current study, only preliminary screening and fractionation was conducted, hence we can at best speak of the function of these constituents within the context and scope of our study.

Table 2: Activity of *Combretum molle* fractions *invitro* monitored by motility after 1 h of incubation at 37°C

Conc. of Plant Extracts/ Diminazene (mg/kg)	Survival of Trypanosomes in Minutes						
	0	10	20	30	40	50	60
Diminazene (3.5)	++++	++	+	–	–	–	–
Flavonoid (MeOH 10)	++++	+++	++	+	–	–	–
Flavonoid (Aqueous 5)	++++	+++	+++	++	+	–	–
Saponin (MeOH, 10)	++++	+++	+++	++	++	+	–
Saponin (Aqueous 5)	++++	+++	++	++	++	++	+
Negative Control (Distilled water & PBS)	++++	++++	++++	++++	++++	++++	+++

++++ = Very actively motile, +++ = Moderately motile, ++ = Slightly motile, + = Weakly motile, – = Non-motile,

Based on previous study describing the phytochemistry of the species of this family Combretaceae and the medicinal value of plants is believed to lie in the chemical substances that produce a physiological change in the human body (Edeoga *et al*., 2005). Phytochemical study carried out in the genus *Combretum* have demonstrated the occurrence of many classes of constituents, including triterpenes, flavonoids, lignins and non-protein amino acids, among others (Petrovski *et al*., 2006). This finding is in consonance with this present study where these phytochemical constituents were detected.

The *in vitro* antitrypanosomal activity of *Combretum molle* was demonstrated in this study at different concentrations of the fractions and the extracts. The flavonoid fraction of the methanolic extract at 10 mg/kg and aqueous extracts at 5 mg/kg immobilized the parasites 40^{th} and 50^{th} minutes respectively, this is an important finding as this corroborates with other *in vitro* findings (Atawodi *et al.,* 2003; Maikai *et al*., 2011) where the extracts inactivated the trypanosomes at the 25^{th} and 30^{th} min post incubation. The saponin fractions on the other hand brought about a reduction in motility of the parasites 50 min post incubation. That parasite motility could be a measure of viability among most zooflagelate parasites has long been established (Peter *et al*., 2009; Mbaya *et al*., 2011). The simple techniques employed in this study uses the motility of trypanosome as indicator of parasite viability, this was earlier reported that the technique correlated well with other *in vitro* methods (Atawodi *et al*., 2007).

This study has shown that *Combretum molle* possesses moderate antitrypanosomal activity *in vitro* activity in mice infected with *Trypanosoma brucei brucei* strains. We therefore conclude that the trypanocidal activity of the methanolic and aqueous fractions of flavonoid and saponin at various concentrations of 10 mg/kg and 5 mg/kg *in vitro* was established in this present study. This is an important finding because it presents *Combretum molle* as a potential candidate for drug development against trypanosomiasis.

Conclusion

This study has shown that *Combretum molle* extracts possesses moderate antitrypanosomal activity *in vitro*, hence a potential candidate for drug development against trypanosomiasis. However, serious attention be paid to such diseases termed less profitable diseases for research to be conducted to stem them.

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