In Vivo Activity of Fractions of Combretum Molle R. and Haematological Profile of Trypanosoma Brucel Brucei Infected Mice

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Citation

Abstract
The in vivo antitrypanosomal potentials of Combretum molle were investigated. The in vivo activity of the fractions at (15mg/kg and 10mg/kg) were compared with standard diminazene aceturate (Diminal) at (3.5mg/Kg), both methanolic and aqueous fractions containing saponins and flavonoids respectively were also fractionated and used for intraperitoneal treatment of mice infected with Trypanosoma brucei brucei federe strain. The Lethal Dose (LD₅₀) were (28.98mg/kg) and (14.14mg/kg) respectively for both methanolic and aqueous crude extracts. The haematological profile showed a significant difference (t-test =9.116; p-value =0.001) between the pre-infection and post infection and treatment values of packed cell volume (PCV) of the infected mice. However, there was no significant difference in the values of neutrophils, lymphocytes as well as the total white blood cell (WBC) counts. The results showed that Combretum molle had mild in vivo activity, hence can serve as a promising candidate for drug development against trypanosomosis.

1. Introduction

Trypanosomes are protozoan parasites responsible for Human African Trypanosomiasis (HAT) and Animal African Trypanosomosis (AAT) “nagana” in cattle and are transmitted by the bite of an infected tsetse fly (Glossina spp). 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 gambiensense 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 [1, 2]. 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 [3, 4]. Therefore, there is an urgent need for new, safe, effective and cheap compounds and for new leads with new mechanisms of action. Currently, the administration of chemotherapeutic agents constitutes the principal method of control as the development of vaccines against African Animal
Trypanosomiasis (AAT) is still in progress. Trypanosome infections are known to cause immunosuppression responsible for the host’s inability to eliminate the trypanosomes even after administration of trypanocidal drugs [5, 6]. Diminazene aceturate and isomethanidium chloride are the most currently used trypanocides, used for both prophylactics and curative purpose for the control of the disease in cattle [1]. Unfortunately, the parasites have developed resistance to these drugs [7, 8, 9] which makes the search for efficacious chemotherapeutic agents from locally available ethnomedicinal plants for their use as trypanocidal agents necessary.

Previous studies had documented trypanocidal effects of plants [10, 11]. Combretum molle R. Br. ex G. Don commonly called ‘Velvet bush willow’ is a shrub or small graceful deciduous tree 3 – 13 m high; belonging to the family combretaceae, the trunk is crooked or leaning occasionally swollen at the base up to 30 cm in diameter. Bark is grey and smooth when young, grey-brown to almost black rough and flaking when older, twigs often with reddish hairs – branching heavily and dropping – giving a round or flat-round sometimes oval-crown. It may be evergreen deciduous and yields a gum. Leaves are opposite, simple leathery 5 – 17 cm long, 2.5 – 9 cm wide. The name molle was coined from “Mollis” in Latin meaning soft because of the softness of the leaves [12, 13]. Studies have been performed on the biological activities of Combretum molle. Ojewole and Adewole [14] reported the analgesic, anti-inflammatory and cardiovascular effects of mollic acid glucoside isolated from Combretum molle leaves.

### 2. Materials and Methods

#### 2.1. 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 was deposited in the herbarium of the same Department for reference purpose.

#### 2.2. 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 pulverised and finely sieved. Exactly 100 grams was soaked in 500 millilitre of distilled water and methanol separately for 24 hours; 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.

#### 2.3. Fractionation of Crude Extracts Using Thin Layer Chromatography (TLC)

![Schematic chart for the Fractionation of Flavonoids and Saponins](Image)

Key: KOH=Potassium hydroxide, HCL=Hydrochloric acid.

#### 2.4. Experimental Mice

A total of 50 Swiss Albino Mice with an average weight of (16-25g) were obtained from the Animal unit of the Department of Pharmacognosy and Clinical Pharmacy, Ahmadu Bello University Zaria, Nigeria. The animals were kept in plastic cages to acclimatise in the laboratory unit of the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria Nigeria for 2 weeks before the commencement of the study. The mice were fed with commercial pelleted animal feed and water ad libitum.

#### 2.5. 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.

#### 2.6. 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 [16] 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).

#### 2.7. Inoculation of Study Mice

Parasite infected blood was obtained from the tail of donor mice at high parasitaemia (10⁸) and was used to maintain a parasite suspension in phosphate buffered saline (PBS pH 7.2), the number of parasite per millilitre of suspension was determined using rapid matching method of [16]. Each mouse was challenged with 1 x 10⁸ parasites intraperitoneally in 0.2 ml blood/PBS solution by serial dilution.
2.8. Distribution of Test Albino Mice (Prophylactic/Curative Group)

Thirty (30) of the test mice weighing between 16-30g were randomly distributed into six (6) groups A – F of five (5) mice per group.

Group A: uninfected and untreated (normal control).
Group B: infected and treated with methanolic extracts in duplicates.
Group C: infected and treated with aqueous extracts in duplicates.
Group D: infected and treated with standard drug Diminazene aceturate (positive control).
Group E: infected and untreated (negative control)
Group F: Prophylactic group (this group were administered the extract before infection and treatment).

Note: All infections and treatment of the experimental mice were via intraperitoneal route.

2.9. Lethal Dose (LD<sub>50</sub>) Determination

The method of [17] was adopted. The method is briefly described as follows: In the first phase nine (9) mice were divided into three (3) groups of three (3) mice each. The first group was given 10mg/kg, second group 100mg/kg and third group 1000mg/kg of the crude extracts (Methanolic and Aqueous). Based on the results obtained in the first phase, using a standard chart the second phase was carried out in groups of four with two mice per group for the two extracts.

2.10. In Vivo Administration of the Standard Trypanocidal Drug

Diminazene aceturate was administered at 3.5 mg/kg of body weight once intraperitoneally.

2.11. In Vivo Administration of Extracts/Therapeutic Monitoring

The plant extracts were administered intraperitoneally for 5 days at 15mg/kg, 10mg/kg body weight daily for both methanolic and aqueous extracts from day 3 post-infection and treated groups were monitored for relapse. Mice were checked daily during the 5 days after the first treatment to estimate number of trypanosomes in their tail blood in a wet blood film. The absolute number of parasites per millilitre of blood was taken as the logarithm using the rapid matching method for estimating parasitaemia described by[16]. This was achieved by matching microscopic fields of wet blood against standard charts.

2.12. Haematological Profile of Trypanosoma Brucei Brucei Infected Swiss Albino Mice

The packed cell volume (PCV), haemoglobin concentration (Hb), total and differencial white blood cells (WBC) were carried out once a week from day zero (pre-infection), through the study period as well as post treatment profile, similarly weight assessment was done pre-infection and post-infection.

2.13. Statistical Analysis

The computer statistical software, Statistical Programme for Social Science version 17.0 (SPSS v 17.0) for Windows® (SPSS Inc., Chicago, IL, USA), was used for the statistical analysis of the data. Student t-test was used to compare the in vivo antitrypanosomal activity of the crude extracts and the standard drug, while lethal dose (LD<sub>50</sub>) of the crude extracts was determined by probit analysis. The haematological indices was analysed using Duncan multiple range test.

3. Results and Discussion

The various quantity of the different fractions at concentrations of 18.39mg and 16.23mg for both saponin and flavonoid were pooled Table (1) by Thin Layer Chromatography (TLC) Using Diethyl Ether and N-Butanol as solvents. The lethal dose LD<sub>50</sub> of the plant Combretum molle in Aqueous and Methanol were (14.14mg/kg) and (28.93mg/kg) respectively Table (2). There was no significant difference in vivo activity of flavonoid and saponin fractions from aqueous and methanolic extracts at 15mg/kg and 10mg/kg as determined by monitoring parasitaemia and the length of time the mice survived. However, in comparison with positive control Diminazene aceturate (Diminal) at 3.5mg/kg, there was significant difference (p<0.001) where the parasites were cleared and the mice survived for a longer time vis-à-vis the Combretum molle treated extracts (fractions) Table (3). In the prophylactic group (pretreated with the extracts before infection) there was no significant difference as parasitaemia was not improved, neither was there any protection prior to infection and treatment; on the other hand in the negative control group (infected and untreated) all the mice died day six post infection Table (3). In the haematological profile of the infected mice in this study a striking trend was observed in the packed cell volume (PCV), total white blood cell (WBC) as well as the differential leucocytes counts. The pre-infection/treatment PCV indices of the mice showed that there was a significant difference (t-test= 9.116, p-value= 0.001**) compared to the post infection/treatment PCV values. However, there was no significant difference (t-test= 1.117, p-value= 0.278) between the pre-infection/treatment and post infection/treatment total white cell (WBC) count Figure (2). No significant difference in the pre- and post-treatment values of neutrophils, lymphocytes, and monocytes in the Albino mice Figure (3).

| Table 1. Fractions of Saponins and Flavonoids pooled by Thin Layer Chromatography (TLC). |
|-----------------|---------|--------------|----------------|
| Extracts       | Solvents| Fractions    | Quantity       |
| Methanolic     | Diethyl Ether | Saponins   | 18.39g        |
| Aqueous        | N-Butanol    | Flavonoids  | 16.23g        |

Table 2. Lethal dose (LD$_{50}$) of Combretum molle.

<table>
<thead>
<tr>
<th>Solvent Of Extraction</th>
<th>Extract Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQUEOUS</td>
<td>14.14mg/kg</td>
</tr>
<tr>
<td>METHANOLIC</td>
<td>28.98mg/kg</td>
</tr>
</tbody>
</table>

Table 3. Antitypansomal activity of various fractions of crude extracts at 10 mg/kg and 15 mg/kg, and Diminazene aceturate positive control at 3.5mg/kg.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Mean ± SEM</th>
<th>Aqueous (10 mg/kg)$^a$</th>
<th>Methanolic (15 mg/kg)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoid</td>
<td>7.27 ± 0.26$^a$</td>
<td>7.12 ± 0.19$^a$</td>
<td></td>
</tr>
<tr>
<td>Saponin</td>
<td>7.33 ± 0.38$^a$</td>
<td>7.30 ± 0.27$^a$</td>
<td></td>
</tr>
<tr>
<td>Prophylactic group</td>
<td>7.16 ± 0.33$^a$</td>
<td>7.11 ± 0.31$^a$</td>
<td></td>
</tr>
<tr>
<td>Positive control (3.5 mg/kg)</td>
<td>5.52 ± 0.12$^a$</td>
<td>5.52 ± 0.12$^a$</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>6.98 ± 0.58$^a$</td>
<td>6.98 ± 0.58$^a$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ F = 10.372; p < 0.001**
$^b$ F = 12.504; p < 0.001**

Values with different superscripts in the same column are significantly different. Mean values were separated using Duncan’s multiple range test.

Figure 2. Haematological profile (Pre-infection) of in vivo Trypanosoma infected albino mice.

Packed Cell Volume: (P < 0.001*)
Haemoglobin concentration (Hb): (P < 0.001*).

Figure 3. Haematological profile (Post-infection) of in vivo Trypanosoma infected albino mice.

White blood cell (WBC): (P < 0.278)
Lymphocyte: (P < 0.671)
In this study the in vivo antitrypanosomal activity of Combretum molle was done by the intraperitoneal (i.p) inoculation of the T. b. brucei into mice with the flavonoid and saponin fractions of the methanolic and aqueous crude extracts at 15mg/kg and 10mg/kg concentrations.

Both flavonoid and saponin extracts/fraction in this study exhibited mild antitrypanosomal activity in vivo at 15mg/kg and 10mg/kg, this was monitored by parasitaemia determination and the length of time the mice survived compared to the Diminazene aceturate (positive control) which cleared the parasites with no relapse unlike the fractions of Combretum molle which did not completely clear the parasites in the peripheral blood of the infected mice this showed a statistically significant difference (p<0.001). However no statistically significant difference in vivo for prophylactic group was noticed. The absence of outstanding in vivo activity of the extracts has also been observed and may be attributed to degradation or xenobiotic metabolism of the active principle through various metabolic processes in the animal host, or to the toxicity of high levels of the crude extract required for therapeutic efficacy [18, 19]. The high level of parasitaemia in the infected mice could also be a factor, and on the other hand, it is possible for the animal’s metabolic processes to inactivate a compound with little or no activity in vivo [20]. Many natural products exhibit their trypanocidal activity by virtue of their interference with the redox balance of the parasites acting either on the respiratory chain or on the cellular defenses against oxidative stress. This is because natural products possess structures capable of generating radicals that may cause peroxidative damage to enzymes that are very sensitive to alterations in redox balance. Some agents also act by binding with the kinetoplast DNA of trypanosomes [21, 22]. Different phytochemical constituents might also be responsible for the antitrypanosomal activities and some saponins tested for antitrypanosomal activity did not show any activity [23].

The haematological profiles of the infected mice as seen in this present study showed a striking trend especially in the packed cell volume (PCV) and Haemoglobin concentration (Hb) where statistically significant difference exist between the pre-infection/treatment and post-treatment values.

It has been established that the measurement of anaemia gives a reliable indication of the disease status and productive performance of trypanosome infected animals [24]. Trypanosome infection may cause anaemia as a result of massive erythrophagocytosis by an expanded and active mononuclear phagocytic system (MPS) of the host [25, 22].

The haematological profile obtained in this study agree with earlier studies by [26, 27]. The low PCV observed in the infected group may be as a result of acute haemolysis due to growing infection. Previous studies have also shown that infection with trypanosomes resulted in increased susceptibility of red blood cell membrane to oxidative damage probably as a result of depletion of reduced glutathione on the surface of the red blood cell [28]. Severity of anaemia usually reflects the intensity and duration of parasitaemia as reported by [29, 27] have also ascribed acute anaemia in trypanosomosis to proliferating parasites. The lower counts of WBC, lymphocytes and neutrophil observed in the post treatment group may be attributed to the immunosuppressive actions of trypanosome infection [30].

4. Conclusion

This study has shown that both Saponin and Flavonoid fractions of Combretum molle possesses antitrypanosomal activity in-vivo in mice infected with Trypanosoma brucei brucei strains. The trypanocidal activity of the methanolic and aqueous fractions of flavonoid and saponin at various concentrations in vivo at 15mg/kg and 10mg/kg 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. This study also showed a significant difference in the haematological indices of the infected mice, where haemoglobin concentration (Hb) and the Packed Cell Volume (PCV) at preinfection were higher than post infection indices.

References


