Anti-trypanosomal, antioxidant and antimicrobial activities of the fruiting bodies of *Ganoderma lucidum* (W. Curt.: Fr) (Ganodermataceae) aqueous extract

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Abstract
Human African Trypanosomiasis (HAT) infection is caused by sub species of *Trypanosoma brucei*. The clinically licensed drugs have unacceptable toxicities and variable efficacies. The prognosis is influenced by the level of circulating free radicals and opportunistic infections. Many plants have been screened for activity in different models of HAT but the same is not true of mushrooms. This study screened the aqueous extract of the fruiting bodies of *Ganoderma lucidum* for trypanocidal, antioxidant and antimicrobial activities. Fruiting bodies of the mushroom were extracted sequentially with n – hexane, ethyl acetate, absolute ethanol and distilled water. The extracts were screened for phytochemical constituents and in vitro trypanocidal activity, the most active of which was further subjected to in vivo trypanocidal, anti-oxidant and anti-microbial screening. Alkaloids, carbohydrates, flavonoids, saponins and anthraquinones were present. *In vitro* trypanocidal screening showed the aqueous extract as the most active (IC$_{50}$ = 14.65 µg/µl). It also dose-dependently inhibited parasitaemia and prolonged survival in parasite - infected mice (50, 100, 200 and 400 mg/kg). DPPH radical scavenging activity gave an IC$_{50}$ of 131.00 ± 0.03 mg/ml. The extract demonstrated broad spectrum antimicrobial activity at 250 mg/ml. The fruiting bodies of *G. lucidum* is a potent source of trypanocidal compounds.

Keywords: *Ganoderma lucidum*; Mushrooms, Trypanocidal; *Trypanosoma brucei brucei*.

INTRODUCTION
Human African trypanosomiasis (HAT, sleeping sickness) is a parasitic disease caused by sub - species of the haemoflagellate *Trypanosoma brucei*, namely *Trypanosoma brucei gambiense* (west African trypanosomiasis) and *Trypanosoma brucei rhodensiense* (East African trypanosomiasis) [1], transmitted by haemophagous tsetse flies [2]. It is a neglected tropical disease (NTD) which affects nearly 30 million people in sub – Saharan Africa [3]. The infection causes a wide range of peripheral and central symptoms including anaemia, obtundation due in part to trypanosome – induced oxidative stress and secondary microbial infections [4,5].

Chemotherapy, with synthetic drugs, is the main stay of management using the early
phase drugs suramin and pentamidine, and the latter phase drug melarsoprol. However, increasing reports of drug resistance, toxicities, complex administration regimen and variable efficacies of these drugs create an urgent need for medicines with better safety profile and efficacy [6,7]. Unfortunately, the resources needed for the development of newer chemotherapeutic agents against this disease are scarce, largely because the resource constraints of the affected population do not provide guaranteed returns on any investments made in the drug discovery process [8]. Most patients therefore have to resort to traditional remedies [9]. It is well documented that traditional medicine is a vital part of the health care delivery system for many African communities [10] and a first resort before orthodox treatment is sought.

Many plants are used indigenously for the traditional management of HAT and a good number have been scientifically screened and the claims validated [1,11]. Mushrooms, like plants, are comparatively endowed with a plethora of medicinally useful bioactive constituents [12,13]. However, there are only a limited number of reports on the screening of mushrooms against trypanosomiasis. Polysaccharides from the mushroom *Lentinus strigosus* showed some activity against *Trypanosoma cruzi*, the causative organism responsible for south American trypanosomiasis (Chagas disease) [14,15], while in another study, mushroom extracts did not show any activity against *Trypanosoma congoles* [16]. In view of the foregoing, there is a need to further evaluate mushrooms for activity against *Trypanosoma brucei brucei* to clarify their potential trypanocidal effects.

*Ganoderma lucidum* (W. Curt.: Fr.) (Ganodermataceae) is reported to possess antibacterial, antifungal and antiviral properties, due to identified compounds such as colossolactone, ganoderic acid and ganoderiol [21]. However, reports on the antiparasitic property of extracts from *Ganoderma* species are limited to scanty publications on their antiplasmodial activity [21]. There are none on their trypanocidal activity. The objective of this study therefore was to screen different extracts of the fruiting bodies of *Ganoderma lucidum* (W. Curt.: Fr.) for possible trypanocidal, antimicrobial and antioxidant activities. Our experimental design was to first determine the most active of the extracts in the *in vitro* trypanocidal screening and then use this in the *in vivo* trypanocidal, anti-oxidant and anti-microbial studies. The data from this study would help the scientific community in its search for new leads against trypanosomiasis.

**EXPERIMENTAL METHODS**

**Mushroom collection and preparation of extracts.** The fruiting bodies of *Ganoderma lucidum* were collected from the wild in Kunwur village in North Central Nigeria between the months of June and September, 2013. The Samples were identified by Mr. Valentine Trevor (botanist) of the Africa Centre of Phytomedicine Research and Development, University of Jos. The fruiting bodies were cleaned of debris, dirt and shade dried over a period of 21 days and then reduced to coarse powder in a mortar and pestle. Seven hundred and fifty grams (750 g) of the coarse powder was sequentially and serially macerated in 1500 ml of cold n-hexane, ethyl acetate, absolute ethanol and distilled water, respectively for 72 h. The resulting extracts were filtered and evaporated to dryness at 40°C in vacuo.

**Animals.** Thirty albino mice (22 – 25 g) of mixed sex were sourced from the Animal Experimental Unit of the University of Jos. They were kept in standard polypropylene cages (72 cm x 36 cm x 44 cm) under 12-12
hour daylight-dark cycle with access to water and food *ad libitum*. They were allowed to acclimatize for two weeks before commencement of the experiments. Animal experiments were conducted in strict accordance with institutional guidelines on animal use and welfare of the University of Jos, for which ethical approval was sought for and obtained (Ethics clearance number is F17 – 00379). The parasite used in this study was the virulent strain of *Trypanosoma brucei brucei* (Federe Strain) obtained from the Nigeria Institute for Trypanosomiasis Research, Vom Plateau State, Nigeria.

**Phytochemical analyses.** The extracts were evaluated for their phytochemical constituents according to standard protocols described by Sofowora [21].

**Trypanocidal studies**

*In vitro trypanocidal screening*. The different extracts were evaluated for *in vitro* trypanocidal activity in a 200 µl 96-well microtitre plate. Forty microlitres of each extract (50 µg/µl) was serially diluted across the wells with phosphate buffered saline – PBS - (NaCl 0.8, KCl 0.02, Na₂HPO₄ 0.14, KH₂PO₄ 0.024 % w/v) giving a concentration range of 0.97, 0.19, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00 and 50.00 µg/µl. Forty microlitres of massively parasitized blood (500.00 x 10⁶ parasites per ml of blood) obtained retrobulbally from a *T. brucei brucei* infected rat was transferred into each test well and the content mixed. The positive control wells contained diminazine aceturate (0.97, 0.19, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00 and 50.00 µg/µl). Forty microlitres of massively parasitized blood (500.00 x 10⁶ parasites per ml of blood) obtained retrobulbally from a *T. brucei brucei* infected donor mice. Treatments were administered intraperitoneally as a single dose immediately parasitaemia was established, as observed microscopically. The mice were observed daily for microscopic estimation of parasitaemia by wet film preparation using a drop of caudal blood. In all cases the degree of parasitaemia was estimated and expressed as number of motile trypanosomes per ml of blood.

**Antioxidant test.** The 2,2-Dipheny-1-picrylhydrazyl (DPPH) radical scavenging test was conducted using protocol reported by Brand-Williams et al [22] in order to evaluate the anti – oxidant activity of the aqueous extract of *G. lucidum*. Briefly, triplicate concentrations (3.91, 7.81, 15.63, 31.25, 62.50, 125.00 mg/ml) of the aqueous extract of *G. lucidum* was prepared in methanol to yield a final volume of 3 ml to which was added 1 ml of 0.1 mM of DPPH solution in methanol. Gallic acid and rutin solutions were similarly prepared. The reaction tubes were incubated for 30 minutes, protected from light after which the absorbances of the solutions were taken at 517 nm with a UV spectrophotometer. The percentage inhibition was calculated according the formular below:

\[
\text{Percentage inhibition} = \left(\frac{A_c - A_t}{A_c}\right) \times 100
\]

Where:  
- \(A_c\) is the absorbance of the control
- \(A_t\) is the absorbance of the extract

IC₅₀ is the concentration of the test that produced a 50 % inhibition.
**Determination of antimicrobial activity.** The cup diffusion method was used to screen the effect of the aqueous extract of *G. lucidum* (62.5 – 250.0 mg/ml) against *P. aeruginosa*, *S. aureus*, *Bacillus subtilis* and *Candida albicans*, according to procedures described by Usman et al [23]. Briefly, Mueller – Hinton agar was aseptically prepared and seeded separately with the isolates. Seven millilitres was poured into pre-labeled sterilized glass petri dishes. After the agar had set, 4 holes were bored into the plates with a sterilized 9 mm cork borer. Thereafter, 0.2 ml of the extract was introduced into the wells and incubated at 37 °C for 24 hours, at the end of which the plates were examined for clear zones of growth inhibition. The experiment was carried out in triplicates. The bacterial specimen used in this study were clinically derived isolates which were identified using standard biochemical tests. Microscopy and Gram staining were first performed. *Pseudomonas aeruginosa* was confirmed by the oxidase and urase tests which identifies cytochrome oxidase producing organisms [24]. *Staphylococcus aureus* was identified using the catalase and triple sugar tests. The former is based on identifying organisms that produce the hydrogen peroxide metabolizing enzyme known as catalase [25]. *Bacillus subtilis* was confirmed using the indole test which gives a positive result with tryptophanase producing organisms [24].

**Statistical analysis.** Treatment groups were compared with vehicle control groups and results were analyzed by simple t – test and analysis of variance (ANOVA). Differences at p < 0.05 were deemed statistically significant.

**RESULTS**
Phytochemical screening of the extracts of *G. lucidum* showed the presence of alkaloids, carbohydrates, flavonoids, saponins and anthraquinones (Table 1). Evaluation of the *in vitro* trypanocidal activity of the different extracts of the fruiting bodies of *G. lucidum* showed that the aqueous extract demonstrated the greatest activity with an IC_{50} value of 14.65 µg/µl (Table 2) while the least active was the n – hexane extract (IC_{50} = 1500 µg/µl). Both ethyl acetate and ethanol extracts had modest level of activity. Subsequent investigation of the most active extract (aqueous) against an *in vivo* model of *T. brucei brucei* infection showed a dose dependent trypanocidal activity. The extract reduced the rate of increase of parasitaemia and increased duration of survival of infected mice at a dose range of 50 - 400 mg/kg (Figure 1) compared to the vehicle control group in which there was a steep rise in the number of trypanosomes and 100 % mortality on the 5th day post-inoculation. The moderate DPPH – scavenging property of the aqueous extract of *G. lucidum* compared to rutin is shown in Table 3 while the antimicrobial activities are presented in Table 4. At a concentration of 250 mg/ml, the extract demonstrated broad spectrum activity against Gram positive and Gram-negative bacteria.

| Table 1: Phytochemical Constituents of the Different Extracts of Fruiting Bodies of *G. lucidum* |
|-----------------------------------------------|
| **n-Hexane** | **Ethyl acetate** | **Ethanol** | **Water** |
| Alkaloid | - | - | +++ | - |
| Saponins | - | + | + | - |
| Tannin | - | - | - | + |
| Flavonoid | - | - | - | ++ |
| Carbohydrate | + | + | + | - |
| Anthraquinone | - | - | + | - |
| Glycoside | - | - | - | - |

- = Absent; + = Present
Table 2: *In vitro* Trypanocidal activity [IC₅₀ (µg/µl)] of the Different Extracts of Fruiting bodies *G. lucidum*

|          | n-Hexane | Ethyl acetate | Ethanol | Water | Diminazine aceturate |
|----------|----------|---------------|---------|-------|----------------------|
| *G. lucidum* | 1500.92 ± 56.34 | 27.51 ± 3.41 | 83.12 ± 4.31 | 14.65 ± 2.54 | 0.98 ± 0.02 |

Values are mean ± S.E.M.  N = 5

Figure 1: Effect of the aqueous extract of the fruiting bodies of *G. lucidum* on duration of survival and level of parasitemia in mice infected with *T. brucei brucei*

Table 3: DPPH scavenging activity of the aqueous extract of the fruiting bodies of *G. lucidum*

| Test substance | IC₅₀ (mg/ml) |
|----------------|-------------|
| Gallic Acid    | 3.00x10⁻² ± 1.00x10⁻² |
| Rutin          | 6.00x10⁻² ± 1.00x10⁻² |
| *G. lucidum*   | 1.31x10⁻² ± 0.29x10⁻¹ |

Values are mean ± SEM  N = 3

Table 4: Effect of the aqueous extract of the fruiting bodies of *G. lucidum* on bacterial and fungal zones of growth inhibition

| Treatment          | Concentration (mg/ml) | *P. aeruginosa* | *S. aureus* | *Bacillus subtilis* | *Candida albicans* |
|--------------------|-----------------------|-----------------|-------------|--------------------|--------------------|
| Distilled water    | 0.2 ml                | 0               | 0           | 0                  | 0                  |
| *G. lucidum*       | 62.5                  | 10.83 ± 0.40*   | 0           | 0                  | 0                  |
| *G. lucidum*       | 125.0                 | 15.17 ± 0.79*   | 12.33 ± 0.33* | 0                  | 11.67 ± 0.56*     |
| *G. lucidum*       | 250.0                 | 22.83 ± 0.31*   | 18.67 ± 0.80* | 19.00 ± 0.57*     | 18.00 ± 0.68*     |
| Gentamicin         | 0.08                  | 30.00 ± 1.29    | 34.50 ± 1.45 | 32.17 ± 0.83      | -                  |
| Ketoconazole       | 0.02                  | -               | -           | -                  | 18.83 ± 0.83      |

Values are mean ± S.E.M.  N = 6  * p < 0.05

**DISCUSSION**

The result of the phytochemical screening of different extracts of the fruiting bodies of *G. lucidum* showed the presence of secondary metabolites such as carbohydrates, alkaloids, saponins, glycosides and flavonoids. These secondary metabolites occur in relatively small amounts compared to primary metabolites, yet they are of pharmacological and medicinal significance [26]. Alkaloids, carbohydrates and saponins are common constituents of the fruiting bodies of mushrooms and this agrees with results of published phytochemical studies of some
mushrooms [27,28]. The data presented also agree with the report of Okwulehie and Ogoke [29] who found a high alkaloid concentration in the fruiting bodies of some mushroom species. They observed that the caps had the highest amounts of alkaloids. The present study however, did not assay the relative distribution of alkaloids in the different parts of the mushroom.

The presence of phenolics (flavonoids, tannins) in the aqueous extract of *G. lucidum* agrees with the report by Abugri and Mcelhenney [30] who found a significant amount of flavonoids in both cultivated and wild mushrooms. Flavonoids are antioxidants and this property is a major health benefit of mushrooms; a relationship between increased intake of flavonoids and decreased incidence of adverse cardiovascular events has been reported [31]. Furthermore, antioxidants have a direct benefit in trypanosomiasis by mitigating free radical – induced lysis of red blood cells thereby improving the overall prognosis in infected animals [4,5]. The aqueous extract of *G. lucidum* used in this study was the terminal product of the sequential extraction process.

Table 2 shows the *in vitro* trypanocidal effects of the different extracts of *G. lucidum*, estimated as the inhibitory concentration 50 (IC$_{50}$). The IC$_{50}$ in this study is the extract concentration that reduced the population of motile forms of the trypanosomes by half. It gives a measure of the potency and efficacy of the extracts, and offers an objective basis of comparing their activities. The degree of extract or drug activity is directly proportional to the population of exposed motile forms of the trypanosomes. The lower IC$_{50}$ value of the aqueous extract suggests that it was the most active. The results further suggest the strong likelihood that the active principle(s) responsible for the observed trypanocidal activity may be hydrophilic in nature. This agrees with the findings of other investigators who reported that the aqueous extracts of plants parts demonstrated the greatest trypanocidal activity compared to other solvents [32,33,34]. Previous reports of the trypanocidal activity of the *G. lucidum* were not found, however, extracts of the fruiting bodies of *G. lucidum* have been reported to possess activity against bacteria and fungi, a property attributed to their content of phenolic compounds. The values obtained in the current *in vitro* trypanocidal studies are lower than those reported by Abedo et al [16] who reported values in the milligram per milliliter range. A number of factors may be responsible for this difference. Those authors used *T. Congolese* as against *T. brucei brucei*. It is possible therefore that the mushroom screened in this present study has greater potency against the organism used relative to the mushrooms investigated elsewhere.

The aqueous extract was found to be the most potent from the *in vitro* trypanocidal study and it was further evaluated for *in vivo* trypanocidal activity as well as anti – oxidant and anti – microbial actions. Our hypothesis was to screen extracts that demonstrated *in vitro* trypanocidal activity for *in vivo* trypanocidal, anti-oxidant and antimicrobial actions. Evaluating only the most active extract from the *in vitro* tests eliminated the need to subject experimental animals to extracts that were deemed ineffective. This is in line with the guidelines of 3R (replacement, reduction and refinement) which prescribe that use of experimental animals be only when absolutely necessary, and in as limited number as possible, without compromising the robustness and reproducibility of the experiment [35].

The effect of increasing doses of the extract in infected mice on the level of parasitaemia, expressed as the number of motile trypanosomes per ml of blood showed a slowing of the rate of increase of parasitaemia and a concomitant increase in the duration of survival of infected mice (Figure 1). This contrasted with the vehicle control group which showed a steep rise in parasitaemia and
death of all mice in the group by the 5th day. The expected outcome of an ideal trypanocidal agent is seen in the diminazine treated group. The reference drug diminazine aceturate (an aromatic diamidine) used in this experiment is an approved trypanocide. It can be observed that it killed the parasites and rendered the mouse aparasitaemic by the 5th day post–inoculation. With undetectable parasites in the thin film, the animals survived for up to 30 days (not shown), in excess of both extract -treated and vehicle control groups. Although the precise mechanism of action diminazine is not known, it is thought to inhibit kinetoplast function by arresting cell cycle and the processing of DNA [36].

The reproduction of haemolymphatic trypanomastigotes is by an efficient binary fission process [37] which explains the rapid rise in parasitaemic levels in untreated infected mice. The ability of the extract to retard the buildup of parasitaemia indicates a trypanocidal effect. This is corroborated by the in vitro effect of the aqueous extract against T. brucei brucei parasites (Table 2). The strain of T. brucei brucei (Federe strain) used in the present study is highly virulent and the prolongation of the duration of survival of animals in the extract treated group is an important finding. In addition to retarding the rate of blood stream trypanosome multiplication, the observed increase in duration of survival may also be due to an indirect effect of the extract in enabling the animals cope with the burden of infection. Such coping mechanisms include immunomodulatory and anti-inflammatory activities of the extract (Unpublished data), properties now known to be possessed by diminazine [36]. The murine model of Human African Trypanosomiasis (HAT) is a robust and very useful experimental tool for screening of potential trypanocidal agents, especially in resource constrained settings. It makes possible direct estimation of parasitaemia from a peripheral blood smear and offers a quick evaluation of the effect of test substances [38]. This model was combined in this study with an in vitro model to provide evidence of the trypanocidal action of this aqueous extract.

This observed trypanocidal activity of the aqueous extract of the fruiting bodies of G. lucidum in T. brucei brucei infected mice has not previously been reported. The constituents of the aqueous extract were polyphenolic compounds (Table 1) and the trypanocidal activities of flavonoids have severally been reported [39,40]. Though the mechanism of action of the extract was not determined, flavonoids have been reported to interfere with mitochondrial function in trypanosomes [41].

The antioxidant property of the aqueous extract of G. lucidum is beneficial in trypanosomiasis because the infection imposes an oxidative stress on infected animals due to the generation of free radicals. This oxidative stress is an important aetiologic factor in the haematological symptoms of infection and antioxidant therapy has been reported to mitigate trypanosomiasis – induced anaemia [42]. Antioxidant properties of natural products are attributable to their polyphenolic compounds such as flavonoids and tannins which stabilize free radicals by donating H+ from the hydroxyl groups [43,44].

The aqueous extract of G. lucidum, within a concentration range of 62.5 – 250.0 mg/ml inhibited the growth of Gram positive and Gram-negative bacteria, and Candida albicans in the agar diffusion in vitro test (Table 4). Immune suppression is a well-documented complication of human African trypanosomiasis that makes the patient susceptible to infections by opportunistic pathogens [45,46]. The broad-spectrum action of the extract suggests its potential benefit as adjunctive therapy in the management of trypanosomiasis. Antimicrobial drugs act via a number of mechanisms, including inhibition of cell wall, protein and nucleic acid syntheses. Though the exact mechanism of action of the observed antimicrobial effect of the extract
was not investigated, the antimicrobial actions of flavonoids have been widely reported [47,48].

On the whole, the extract showed promise for further investigation and development into leads that could be used in the adjunctive management of HAT. These activities observed from the screening of extracts of a mushroom in this study indicates the benefit inherent in bioprospecting as a model of sourcing potentially useful leads from local biodiversity. Mushrooms can become important and substantial local sources of bioactive constituents due to their ease of cultivation from lignocellulosic wastes.

In conclusion, the aqueous extract of the fruiting body of G. lucidum demonstrated trypanocidal action and prolonged the duration of survival of mice infected with T. brucei brucei. It shows promise for the development of products useful in the management of human African trypanosomiasis.

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