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Phytochemical screening and *in vitro* evaluation of the antitrypanosomal action of the methanolic leaf extract of *Corymbia torelliana*

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ABSTRACT

Background: African trypanosomiasis is a parasitic disease that affects both human and livestock across the sub-Saharan Africa. Chemotherapy of the disease remains far from being satisfactory. **Aim**: The aim of this study is to explore an alternative source of antitrypanosomal agent from the leaves of *Corymbia torelliana.* **Methods**: The dried leaves were pulverized and extracted with methanol by maceration. Phytochemical investigation was carried out and subsequent *in vitro* studies of the extract on the survival of *Trypanosoma congolense*. Motility assessment of trypanosomes maintained in Phosphate Ringer glucose saline was carried out after exposure to the extract over a period of 105 minutes. Observations were made at 0 minutes, and subsequently at intervals of 15 minutes. **Results**: The phytochemical assay revealed the presence of saponins, tannins, flavonoids, cardiac glycosides, steroids and terpenes. The extract showed *in vitro* anti-trypanosomal effect against the parasite concentrations at 100 mg/ml, 0.2 mg/ml, 0.03 mg/ml and at 0.00005 mg/ml. The highest effective dose was at 100 mg/ml and the lowest at 0.00005 mg/ml. **Conclusion**: This study shows that the methanolic leaf extract of *Corymbia torelliana* is rich in phytochemical components and has great potentials for the treatment of trypanosomiasis. The presence of these secondary metabolites in this plant might be responsible for the antitrypanosomal activity exhibited by its extracts.

Key words: *Corymbia torelliana*, trypanosomiasis, phytochemistry, *Trypanosoma congolense,* melarsoprol, suramin

INTRODUCTION

Trypanosomiasis is a neglected tropical disease which consists of a group of important animal and human diseases caused by parasitic protozoa of the genus *Trypanosoma.*[1] African trypanosomiasis which is caused by different species of trypanosomes is a severe parasitic disease and has devastating effects on both

humans and livestock.[1,2] Human African trypanosomiasis (HAT) which is commonly called sleeping sickness occurs in 36 sub-Saharan African countries with *Trypanosoma brucei gambiense* accounting for more than 98% of the reported cases.[3,4] Trypanosomiasis and other neglected tropical diseases constitute great disease burden especially to the developing countries of the world.[1,2,5,6,7,8] There are two distinct stages of sleeping sickness. The first or early stage of the disease, also known as the haemolymphatic phase, is defined by the restriction of the trypanosomes to the blood and lymph system. The symptoms of this stage are fever, headaches, joint pains and itching. The second or late stage of the disease, which is also known as the neurological phase, is characterised by the presence of the parasites in the cerebrospinal fluid.[4] This stage describes the manifestation of typical signs of the disease as: confusion, disturbed sleep pattern, sensory disturbances, extreme lethargy, poor condition and coma. If left untreated, sleeping sickness patients die within months when infected with *Trypanosoma brucei rhodesiense* or within years when infected with *Trypanosoma brucei gambiense*. The disease affects mostly poor population living in remote rural areas. Travellers also risk becoming infected if they venture through regions where the insect is common.[3,4] The disease constantly threatens to reach epidemic proportions, as was the case at the beginning of the 20th century.[4] Due to topographic and security challenges, the prevalence/incidence and location of undetected and unreported cases, including areas with little or no epidemiological knowledge as a result of lack of surveillance and accessibility, is difficult.[4] "African Animal trypanosomiasis (AAT) remains a major constraint to health and productivity of cattle and other domestic animals in tsetse infested areas of tropical Africa".[9] AAT is a major obstacle to economic development of affected rural areas, and is a serious obstacle to human welfare because of the serious nutritional and economic problems it causes.[4,10] The disease reduces milk and meat protein, carcass weight, calving rate, hides and skin production, there is increase in abortion, infertility, stillbirth and cost of treatment of livestock.[11]

The use of drugs for the prevention and treatment of trypanosomiasis has been important for many decades, although trypanosomes have developed resistance to each drug introduced.[12,13,14] Pathogenic trypanosomes like *T. congolense* infections in cattle have been reported to increase trypanocidal drug use and consequently accelerated the development of resistance against trypanocidal drugs.[14] Presently, there is no vaccine against African Trypanosomiasis. This is due to the problem of antigenic variation.[15]The recommended drugs used for the treatment of Human African Trypanosomiasis are melarsoprol, suramin and pentamidine, DL-alpha-difluoromethylornithine (DFMO) and for African Animal Trypanosomiasis, the salts of homidium, quinapyramine, diminazene aceturate (Berenil), isometamedium chloride (samorin) and homidium chloride (Novidium).[16,17,18] they show variable efficacy, resistance, serious side effects, toxicity, and can require long-term treatment.[19,20]

"Plants are the oldest source of pharmacologically active substances and have provided humans with many medically useful compounds".[21] A medicinal plant is one which any of its parts contains substances of for therapeutic potential or which are precursors used in the synthesis of drugs.[22] An analysis into the source of new drugs from 1981 to 2007 reveals that almost half of the drugs approved, were based on natural products.[23] During the years 2005–2007, thirteen natural products-related-drugs were approved[23] Natural products like plant extracts are sources of new drug formulation.[24]

Plants have been reported to be useful in the management of various diseases in complementary and alternative medicine. In our modern society today, research emphasis on the characterization and evaluation of plants and plant constituents against a number of diseases are based on traditional claims of the plants.[25] The isolation and characterization of plant substances are derivable from phytochemistry.[25]

Corymbia torelliana belongs to the Myrtaceae family with at least 133 genera and more than 3,800 species and is believed to have originated from Australia.[26] The tree grows in almost all

tropical and subtropical areas and is cultivated in many other parts of the world, It is large and grows up to 30 m high; the common English names are blood-leaf gum.[26] It is one of the most distinctive and unusual eucalypts and one of the few that occurs naturally in and around rain forests.[26] The tree is readily propagated from seeds, which germinate in one to two weeks.[27] It is cultivated for its timber, oil, gum, pulp, medicinal and aesthetic value.[27] Essential oil found in its foliage is very important and finds extensive use in food, perfumery and pharmaceutical industry.[27] The oil possesses a wide range of biological activities including anti-microbial, fungicidal, insecticidal/insect repellent, herbicidal, acaricidal and nematicidal effects.[28]

In Nigeria, *C. torelliana* is used in the management of gastrointestinal diseases associated with *Helicobacter pylori* infections, such as gastric and duodenal ulcers.[29] Also, a decoction of the leaves has been reported to be useful in the treatment of sore throat and other bacterial respiratory and urinary tracts infections.[30] In addition, the leaves has been reported to be useful in the management of malaria and typhoid fevers in some northern parts of Nigeria.[30,31] The poultice of the leaves is useful in wounds and ulcers management.[31] Its volatile oil has been documented to exhibit potent inhibitory activities against four human tumor cell lines.[32]

Our interest in *C. torelliana* in the search for new trypanocides stems from the various claims for its use in alternative medicine, and findings on its potential anticancer properties. This is hinged on the fact that antitumour drugs have been screened for trypanocidal action,[33] and trypanocidal drugs have been screened for anti-cancer activity.[34,35] This is perhaps due to the fact that protozoan parasites, such as those of malaria, trypanosomiasis and leishmaniasis, have a number of features in common with the proliferating cells of cancer and some forms of heart disease.[36] Phytochemical analysis and *in vitro* studies are very important in the process of searching for new drugs. Due to the increasing importance of both human and animal trypanosomiasis in public health and possible re-emergence of the Human African Trypanosomiasis, this study was conducted to explore the antitrypanosomal potential from *C. torelliana* leaf extracts, and its use as an alternative medicine.

METHODOLOGY

Study area

The study was conducted at the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Jos, Plateau State Nigeria; located at latitude 9.43^0 N, longitude 8.47^0 E, bordering Nasarawa and Kaduna States to the south and north respectively. Cattle-keeping pastoralists are attracted to the area due to the absence of animal trypanosomiasis. However, during the past two decades animal trypanosomiasis has become a significant problem for livestock keepers. Therefore, the problem of trypanosomal drug resistance, toxicity and non-availability of adequate have necessitated in our view, the search for alternative and new trypanocides that maybe easily accessible and sustainable by the local community.

Plant materials

Corymbia torelliana leaves were obtained in the paddock of the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Jos, Plateau State. The plant was authenticated byAzila J.J of the Department of Horticulture, Federal College of Forestry, Jos, Nigeria. A Specimen Voucher (FHJ 028) was deposited in the Herbarium, The leaves were air-dried to constant weight under shade, pulverized into a fine powder with mortar and pestle and then stored in a plastic container for use when required.[37,38]

Preparation of the methanolic extract of *Corymbia torelliana*

Three hundred grams of the powdered material was dissolved in 1 litre of 98 % methanol. The mixture was dissolved to stand for 48 hours. The marc was separated from the solvent by decanting and filtrations using a clean piece of muslin cloth and subsequently with Whatman filter paper No. 1. Additional fresh solvent was added to the marc, it was occasionally agitated and the extraction process continued daily for five days for the maximum extraction of the chemical constituents. The extract was concentrated at 35^0C.[37,38]

Phytochemical screening
The extract was screened for the presence of phytochemicals – saponins, tannins, cardiac glycosides, anthraquinones, flavonoids, alkaloids, terpenes, and steroids.

Test for saponins
About 0.5 g of the extract was dissolved in 0.5 ml of distilled water in a test tube and shaken well. The formation of frothing which persisted on warming indicated the presence of saponins.[39]

Test for tannins (reduction test)
About 0.5 g of the extract was stirred with 1.0 ml of distilled water and filtered, ferric chloride solution was added to the filtrate. The formation of a blue-black, green or blue-green precipitate indicated the presence of tannins.[40]

Test for cardiac glycosides (Keller Killiani test)
About 0.1 g of extract was dissolved in 1.0 ml of glacial acid containing one drop of ferric chloride solution. A 1.0 ml of concentrated sulphuric acid was added gently by the side of the test tube. A brown ring formed at the interphase indicated the presence of deoxy sugar characteristic of cardenolides.[40]

Test for anthraquinones (Borntrager's test)
About 0.5 g of extract was put in a test tube and 5 ml of chloroform was added and shaken for 5 minutes. The extract was filtered, and the filtrate shaken with equal volume of 100 % ammonia solution. Pink, violet or red colour in the ammonical layer indicated the presence of free anthraquinones.[40]

Test for flavonoids
About 0.5 g of the extract was completely detanned with acetone on a water bath. The mixture was filtered while hot. The filtrate was cooled and used for the following lead acetate test.[41] Lead acetate solution was added to 5 ml of detanned water extract. A yellow coloured precipitate indicated the presence of flavonoids

Test for alkaloids
About 0.5 g of extract was stirred with 3 ml of 1 % aqueous hydrochloric acid on a steam bath and filtered, 1 ml of the filtrate was treated with few drops of Dragendorff's reagent, Picric acid solution and Mayer's reagent. The formation of turbidity or precipitate indicated the presence of alkaloids.[40]

Test for terpenes (Salkowski test)
About 1 g of extract was dissolved in 2 ml of chloroform and 3 ml of concentrated sulphuric acid was carefully added. A reddish brown colour at the interface indicated the presence of terpenoids.[42]

***In vitro* studies**
An albino rat (*Rattus norvegicus)* raised at the Animal House of the Nigerian Institute for Trypanosomiasis Research (NITR) and heavily infected with *Trypanosoma congolense was* sacrificed and the whole blood was collected for trypanosome suspension preparation in a beaker. A suspension of trypanosomes was prepared in normal saline with the addition of Ethylene Diamine Tetra-acetic Acid (EDTA) and the concentration was adjusted to about 1 x 10^6 organisms per ml.

The amount, 0.1 ml of the suspension of Ringer's solution, and 0.1 ml of suspension of trypanosomes were dispensed into 9 tubes (A-I). 0.1 ml of the extract concentrations 0.00005 mg/ml, 0.03 mg/ml, 0.2 mg/ml, and 100 mg/ml was added to the first 4 tubes (A-D), while 0.2ml of the extract concentrations 0.00005 mg/ml, 0.03 mg/ml, 0.2 mg/ml, and 100 mg/ml was added to tubes (E-H) respectively. The ninth tube (I) was an untreated control (no extract added). The tubes were then incubated at 37˚C. The contents of the tubes were each examined at time 0 minutes and subsequently observed at intervals of 15 minutes for 105 minutes by aspirating small amounts using a Pasteur pipette onto clean slides then covered with cover slips and checking for the presence and motility of the parasites under the microscope (X 40 objective lens). [43,44]

Ethical statement
The animals were maintained and used at the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, following the guidelines of NITR Ethical Committee. The study was carried out in accordance with the principles of Laboratory Animal Care (National Institute of Health Publication No. 86.23, revised 1985).

Statistical analysis

The data was subjected to analysis of variance (ANOVA) using DSAASTAT version 1.101. There was significant difference ($p<0.05$) between the treatments (the extract concentrations) and the control (without extract), but no significant difference ($p>0.05$) among the varying concentrations of the extract.

RESULTS

Table 1 shows the phytochemical content of the plant extract. This revealed the presence of saponins, tannins, flavonoids, cardiac glycosides, steroids as well as terpenes. Alkaloids and anthraquinones were not detected.

The *in vitro* studies results are shown in table 2. The cessation of the movement of the parasites was observed to be concentration dependent. The higher the concentration of the extract, the less time it took to inhibit parasite motility. At a concentration of 0.03 mg/ml of the extract, the trypanosomes were completely immobilized after 15 minutes and at a concentration of 0.00005 mg/ml, there was complete inhibition after 60 minutes. There was no cessation of the movement of the parasites in the control throughout the period of the experiment.

DISCUSSION

The present research work has shown that *Corymbia torelliana* contains an array of phytochemicals, which may have potentials for the treatment of some disease conditions with special reference to trypanosomiasis and cancer. Phytochemical investigations revealed that the leaf extract contains saponins, tannins, flavonoids, cardiac glycosides, steroids and terpenes. This agrees with the observation of earlier authors who found tannins, cardiac glycosides and saponins in the methanolic extract of the leaves of *Corymbia torelliana*.[45] However, anthraquinones, which was not detected in the present study, has earlier been reported by the same workers.[45] This is suggestive that phytochemical constituents of plants can vary depending on several factors such as climate, habitat, and soil nutrient, time of harvest, stress and physiological age of the plant.[46] Several authors have identified the presence of flavonoids, saponins, tannins, cardiac glycosides in plants that showed trypanocidal activities,[47,48,49,50] which could also be responsible for the antitrypanosomal activity observed in this study.

Table 1: Phytochemical Constituents of Methanolic Leaf Extract of *Corymbia torelliana*

Constituents	Determination
Saponins	+
Alkaloids	-
Tannins	+
Anthraquinones	-
Flavonoids	+
Cardiac glycosides	+
Steroids	+
Terpenes	+

Key: + Detected; - Not detected

Table 2: Motility assessment of the Crude Leaf Extract on *Trypanosoma congolense*

Conc. (mg/ml)	Extract (ml)	Motility Rate Time (minutes)							
100	0.2	0	15	30	45	60	75	90	105
100	0.1	++++	-	-	-	-	-	-	-
0.2	0.2	++++	-	-	-	-	-	-	-
0.2	0.1	++++	-	-	-	-	-	-	-
0.3	0.2	++++	-	-	-	-	-	-	-
0.3	0.1	++++	1/60	1/40	1/20	-	1/60	-	-
0.00005	0.2	++++	-	-	1/7	-	-	-	-
0.00005	0.1	++++	3/7	1/10	1/1	1/15	-	-	-
Control	++++	++++	+++	+++	+++	++	++	++	++

Key: ++++ very active; +++ active; ++ sluggish; + very sluggish; (-); motility not detected; 1/60 = 1 parasite in 60 fields; 3/1 = 3 parasites in 1 field; 1/15 = 1 parasite in 15 fields.

In the *in vitro* studies, cessation or decrease in the parasite motility was taken as a measure of the anti-trypanosomal effect of the crude extract. This was observed even at a very low concentration of 0.00005 mg/ml which compare favorably with the works of Igweh and others.[44]] Natural products possess structures capable of generating radicals that may cause peroxidative damage to trypanothione reductase which is very sensitive to alterations in redox balance.[51] Furthermore, Atawodi and his colleagues[52] suggest that 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 defences against oxidative stress. It has also been proposed that iron chelation is an effective way of killing trypanosomes and the prime target is the enzyme ribonucleotide reductase whose activity is central to DNA synthesis prior to cell division as depicted in trypanosome infection.[53 Although the mechanism of the present *in vitro* anti-trypanosomal activity of the leaf extract of *Corymbia torelliana* is not known, the direct contact of the trypanosomes with the extract might also result in the penetration of the extract into the parasite and possibly the disruption of the functions of some important organelles in the trypanosome.[54,55] Moreover, observations by previous workers indicate that after *in vitro* exposure to potential drugs/agents such cells/organisms might not multiply by division and initiate infection in a host, due to the fact that the drugs/agents and /or extracts, one way or the other block glycolysis and cell division.[43,56] Similar *in vitro* antitumor activity of the volatile oils of *C. torelliana* has been reported showing its potent inhibitory activities against four human tumor cell lines.[32]

The independent observations on the leaf extract and the volatile oils, might help to give credence to the assertions that trypanocidal agents can be tried for antitumor activities, as earlier reported by Barret and colleague as well as Ivan and others.[34,35] and antitumor agents can also be tried for antitrypanosomal activities.[33] Nevertheless, our work was limited to the effects of the extracts on the rate of parasite motility.

Study limitation

The content of the tubes were not inoculated into rats or mice after the incubation period. This was due to the fact that we considered the use of large number of animals as not necessary. Besides, this would have involved weighing the animals, and observation that may be for a longer period of time, which is beyond the scope of the present study.

CONCLUSION

The results of our findings indicate that the leaf extract of *C. torelliana* is rich in phytochemical components, which might have potentials for

antitrypanosomal activity, as revealed by the *in vitro* antitrypanosomal activity of the leaf extract, even at very low concentration. Hence, a high potential for affordable and locally available alternative plant medication for trypanosomiasis infections in humans and animals that is affordable and accessible by the local population and possibly, other areas known to be endemic for the diseases. This study has contributed and extended the frontiers of knowledge in that it has combined the search for antitrypanocidal agent into the realm of materials traditionally claimed as anti parasitic and anticancer agents. Our findings will also stimulate our research team and other researchers to carry out further works on this plant material which may eventually result in new drug sourced from natural products that are locally available and easily accessible/affordable by the local population

RECOMMENDATIONS

Further research should be carried out to access the *in vivo* antitrypanosomal activity of the leaf extract of *C. torelliana.* Studies should be conducted as well to elucidate the structures of the active principles.

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