sciendo

herba polonica
International journal edited by the Institute of Natural Fibres and Medicinal Plants
Vol. 65 No. 4 2019

Received: 2019-01-21
Accepted: 2019-11-21
Available online: 2019-12-31

DOI: 10.2478/hepo-2019-0024

EXPERIMENTAL PAPER

Antiprotozoal investigation of three *Combretum* species (*Combretaceae*) growing in Nigeria

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Summary

Introduction: *Combretum* species has been utilised for decades in African indigenous medical practices for the treatment of several parasitic infections.

Objectives: This study aims at investigating the antileishmanial, antiplasmodial and antitrypanosomal properties of *Combretum racemosum, Combretum platypterum* and *Combretum zenkeri*.

Methods: The leaf extracts of the plants were screened against two strains of *Plasmodium falciparum* using Plasmodium lactate dehydrogenase (pLDH) assay; promastigote and amastigote forms of *Leishmania donovani*; and *Trypanosoma brucei brucei* using Alamar Blue assay. Cytotoxicity screening were also carried out on *African green monkey kidney* cell line (Vero) and human monocytic leukemia (THP-1) cell lines.

Results: *C. racemosum* was active against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum* (IC_{50} of 25.6 and 26.7 μg/ml, respectively) and exerted significant antiprotozoal activities against *T. brucei brucei* (IC_{50} = 18.44 μg/ml). The extract of *C. platypterum* displayed a slightly lower

antiplasmodial activity when compared to *C. racemosum,* while *C. zenkeri* was inactive against the parasites. In addition, the extracts failed to display significant inhibitory activity on the proliferation of *L. donovani*.

Conclusions: This study supports the ethnomedicinal use of *C. racemosum*. Further research needs to be carried out to identify the antiprotozoal compounds in *C. racemosum,* as this could be explored for possible antiprotozoal drug development.

Key words: ***Combretum racemosum, Combretum platypterum, Combretum zenkeri, antiprotozoal assay, infectious diseases***

Słowa kluczowe: ***Combretum racemosum, Combretum platypterum, Combretum zenkeri, działanie przeciwpierwotniakowe, choroby zakaźne***

INTRODUCTION

During the past decades, there has been an upsurge in the worldwide morbidity and mortality caused by infectious diseases, particularly in undeveloped countries. In fact, infectious diseases contribute to almost 40% of all deaths in these regions [1] and 20% of annual mortality in developed nations [2]. Leishmaniasis, Human African trypanosomiasis (HAT) and onchocerciasis transmitted by female sand fly, tsetse fly and black fly, respectively, have been included in the World Health Organization's list of seventeen neglected tropical diseases (NTDs) [3]. Despite the low public awareness associated with NTDs, especially in developed countries, NTDs affect residents of several low-income countries of Africa, Latin America and Asia, with over 1.4 billion people worldwide – including approximately 800 million children [4-6]. Due to the climatic condition that favours the existence of the protozoan vectors which transmit these diseases, the mortality, morbidity and socio-economic burden caused by these vector-borne protozoan diseases is higher in the tropical and subtropical regions when compared with the temperate zones, and also the poor hygienic conditions associated with low-income tropical countries [7].

Although significant milestones have been reached in microbiology, as exemplified in the discovery and application of several potent anti-infective agents for the treatment and control of these disease-causing organisms, the growing number of incidences of drug-resistant microorganisms and the emergence of new pathogenic organisms have continued to pose a threat to global public health. In addition, the genetic complexity of *Plasmodium* spp. which hindered the development of effective vaccine, antigenic variations in protozoan parasites that stimulate the evasion of host immune system as well as the toxicity of some currently used chemotherapeutic agents – all these are limitations in attempts of eradicating these protozoan diseases. These factors have stimulated the research in the discovery of new antimicrobial agents [8-10].

Plant-based medicine provides remedies against several ailments including infectious diseases. The population of most developing countries, especially those residing in rural settlements, utilise herbal medicine to meet their healthcare needs [11]. Medicinal plants have been reported to be useful in the management of protozoan infections and investigative work on these plants have led to the discovery of potent antiprotozoal agents such as quinine and artemisinin [12].

The *Combretaceae* family comprises of 20 genera, the largest of which is *Combretum*, with approximately 370 species. *Combretum* species are widely distributed in southern and western Africa, and widely used in Africa folk medicine for the treatment of several diseases such as constipation, diabetes, diarrhoea, digestive disorders, fever, haemorrhage, heart diseases, inflammation, jaundice, malaria, mental problems and toothache [13-15]. Although the antibacterial, antifungal, antiprotozoal and antiviral properties of several *Combretum* species from many African countries have been well investigated [15-19], the antiprotozoal activities of *Combretum* species indigenous to Nigeria have not been well studied. Therefore, this study was designed to address this gap by investigating the antiprotozoal activities of three *Combretum* species growing in Nigeria.

In this work, the methanol leaf extracts of *Combretum platypterum* (Welw.) Hutch. and Dalz., *Combretum racemosum* P. Beauv and *Combretum zenkeri*

Engl. & Diels (*Combretaceae* family) were screened against chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum*, *Leishmania donovani* (intracellular and extracellular forms) and *Trypanosoma brucei brucei*.

MATERIAL AND METHODS

Plant collection and preparation of extracts

The leaves of *C. platypterum*, *C. racemosum* and *C. zenkeri* were obtained from their natural habitat in the University of Ibadan (Nigeria). Plant identification and authentication was carried out at the Forest Herbarium Ibadan where Forestry Research Institute of Nigeria (FRIN), Ibadan, by Dr. Osiyemi who assigned the voucher specimen numbers for the plant materials as Forest Herbarium Ibadan (FHI) 109988, FHI 109989 and FHI 111997 for *C. racemosum*, *C. platypterum* and *C. zenkeri*, respectively. Voucher specimens were herbarium-mentioned in the preceding text. The plants were air-dried, pulverised, and extracted in methanol at a room temperature (25–29°C) for 72 h. The crude extracts obtained were concentrated *in vacuo* at 40°C and stored in a refrigerator at 4°C prior to use.

Antiplasmodial assay

The antiplasmodial potentials of the extracts were estimated using the Plasmodium lactate dehydrogenase (pLDH) activity measurement, as earlier reported [20]. Erythrocytes infected with either chloroquine-sensitive (D6, Sierra Leone) or chloroquine-resistant W2 strains (Indochina) of *Plasmodium falciparum* (2% parasitemia and 2% hematocrit) were briefly prepared in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% human serum and 60 μg/ml amikacin. The assay was carried out in a 96-well microplate which contained several concentrations (10 μl each) of the extract and 200 μl of the erythrocyte-infected preparation. Single concentration of 15.9 μg/ml of the extracts were tested for the primary antiplasmodial assay. Extracts that displayed more than 50% inhibition in the primary assay were subjected to secondary screening with treatment concentration ranging from 5.3 to 47.6 μg/ml. Artemisinin and chloroquine (Sigma-Aldrich, St. Louis, USA) were used as the positive standards, while dimethylsulfoxide (DMSO)-containing medium served as negative control. The selectivity indices (SI) of the extracts were estimated by measuring their cytotoxicity on the African green monkey kidney cell line (Vero) following a method described in [21]. The SI was estimated by dividing the IC_{50} in Vero cells over the IC_{50} in *P. falciparum*. The experiment was carried out in triplicate and the IC_{50} values were statistically calculated from a dose-response curve obtained from the GraphPad® software.

Antileishmanial assay

The *in vitro* antileishmanial property of the extract was determined using Alamar Blue assay following an earlier described method [22]. The assay was performed on cell cultures of *Leishmania donovani* promastigotes and axenic amastigotes. The parasites were cultured in optimum growth conditions before commencement of treatment. The promastigotes were cultured in RPMI *1640* medium supplemented with 10% fetal bovine serum (FBS) and maintained at pH 7.4 and 5% CO_2 at 26°C, while the axenic amastigotes were cultured in RPMI *1640* medium supplemented with 4-morpholineethanesulfonic acid (MES) (4.88 g/l), L-glutamine (298.2 mg/l) and 10% FBS and maintained at 37°C and humidified atmosphere of 5% CO_2, with the medium pH fixed at 5.5. Single concentration (20 μg/ml) of the extract was used in the primary assay to treat the parasites with 72 h incubation period at 26°C and 37°C, for promastigotes and axenic amastigotes, respectively. However, no secondary assay was carried out due to little or no activity displayed by the extract in the primary assay.

Antitrypanosomal assay

A method described recently [23] was modified and used for this test. Briefly, in obtaining 5000 parasites/ml, two-days-old culture of *Trypanosoma brucei brucei* was diluted in 96-well microplates, containing Iscove's Modified Dulbecco's medium (IMDM). The culture was at maintained 37°C in a humidified atmosphere of 5% CO_2. For the primary antitrypanosomal assay, 4 μl of the extract, at a single concentration of 20 µg/ml, was added into each well of the microplate containing 196 μl of the IMDM-parasite culture to obtain a final volume of 200 μl. The incubation of the plates was carried out at 37°C in 5% CO_2 for 48 h. Thereafter, 10 μl of Alamar Blue was added into each

well of the plates and incubated for 24 h. At the expiration of the incubation period, the fluorescence could be measured in the microplate fluorometer FLUOstar Galaxy (BMG LabTechnologies GmbH, Offenburg, Germany) at 544 nm excitation and 590 nm emission. The parasite culture was subjected to a secondary treatment using the method described above, except for the treatment concentration that ranged from 10 to 0.4 μg/m. Pentamidine and amphotericin B were used as a positive standard. The IC_{50} values were determined from the dose-response analysis curve obtained from XLfit ver. 5.2.2.

Cytotoxicity assay

For the determination of the cytotoxicity of the extracts, four-day-old culture of transformed human monocytic cell line (THP-1) were cultured in RPMI *1640* medium to obtain a working cell concentration of 2.5×10^5 cells/ml. Phorbol 12-myristate 13-acetate (PMA; 25 ng/ml) was added to the THP1 cell culture to transform the cells to adherent macrophages [24]. 200 μl of cell suspension was added into each well of a 96 well plates and allowed to undergo overnight incubation at 37°C in 5% CO_2 humidified atmosphere. At the expiration of the incubation period, the old medium was replaced with a new one and the extracts were dispensed into the wells of the 96-well plates. Thereafter, the plates were incubated for 48 h at 37°C and 5% CO_2. Then, 10 μl of Alamar Blue solution was incorporated into each well of the plates and incubated for 24 h. Standard fluorescence was measured on a fluorometer at 544 nm excitation and 590 nm emission. The experiment was carried out in triplicate and the percentage inhibition of the extract was calculated using standard procedure.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS

Antiplasmodial assay

The methanol extract of *C. platypterum* and *C. racemosum* displayed >50% growth inhibition *P. falciparum* strain D6 with values of 52% and 64%, respectively (tab. 1) and therefore subjected to secondary antiplasmodial screening. In the secondary assay, the extracts displayed significant antiplasmodial activity against *P. falciparum* strains D6 and W2 with IC_{50} values lower than 50 μg/ml. However, they displayed low SI with values ranging between 1.3 and 1.9 (tab. 1). *C. zenkeri* could not proceed beyond primary assay due to lack of significant inhibitory activity (< 50% inhibition).

Antileishmanial and antitrypanosomal assay

In the primary antileishmanial assay, none of the three *Combretum* species had any significant inhibitory activity (≥50%) on both the promastigote and axenic amastigote of *L. donovani*. Thus, there was no need for any further secondary assay. However, only *C. racemosum* (54%) had a significant inhibitory activity against *T. brucei brucei* in the primary assay and was therefore subjected to secondary screening. *C. racemosum* showed good antitrypanosomal activity in the secondary assay with IC_{50} value of 18.44 μg/ml (tab. 2).

Cytotoxicity assay

All the three *Combretum* species exhibited less than 50% inhibition on the THP1 cells with the highest

Table 1.

In vitro antiplasmodial activity of the methanol extract of three *Combretum* species against *P. falciparum* strains

Plant extract	Primary assay [% inhibition]	Secondary assay IC_{50} [μg/ml]			SI	
	D6	D6	W2	Vero	D6	W2
C. zenkeri	40	NT	NT	NT	NT	NT
C. platypterum	52	27.279	36.293	>47.600	>1.7	>1.3
C. racemosum	64	24.578	26.674	>47.600	>1.9	>1.8

NT – not tested; SI – selectivity index; D6 – chloroquine-sensitive strains of *Plasmodium falciparum*, W2 – chloroquine-resistant strains of *Plasmodium falciparum*; Vero – *African green monkey kidney* cell line

Table 2.

In vitro antitrypanosomal and antileishmanial activities of methanol extract of three *Combretum* species against *L. donovani* and *T. brucei brucei*

S/No	Plant extract	Primary assay [percentage inhibition, %]					Secondary Assay [IC_{50}, μg/ml]				
		L. donovani			*T. brucei*	THP1	*L. donovani*			*T. brucei*	THP1
		P	A	AT			P	A	AT		
1.	*C. zenkeri*	0	23	1	14	11	>20	>20	>20	>20	>20
2.	*C. platypterum*	0	27	0	16	11	>20	>20	>20	>20	>20
3.	*C. racemosum*	0	3	0	**54**	0	>20	>20	>20	18.44	>20

THP-1 – transformed human monocyte cell line; P – promastigote; A – amastigote; AT – amastigote/THP

percentage inhibition of 11% (tab. 2). However, *C. platypterum* and *C. racemosum* showed moderate cytotoxicity on Vero cells with IC_{50} values >47 μg/ml.

DISCUSSION

Natural products obtained from medicinal plants have been utilised for many years by diverse communities for the management of many diseases. Historically, the earliest written report of the use of natural products in health care are from Mesopotamia (2600 B.C.), which recorded several plant-derived substances on tablets of clay, including the oils of *Cupressus sempervirens* L. (*Cupressaceae*), *Glycyrrhiza glabra* L. (*Leguminosae*) and *Papaver somniferum* L. (*Papaveraceae*) [25]. Natural medicinal agents have contributed immensely to drug discovery as nearly three quarter of medicines used in today's drug industry are models of natural products [26]. Many *Combretum* species are used in traditional African medicines for the treatment of several conditions including worm infections, abdominal pain, Hansen's disease and trypanosomiasis [19]. In view of this, three *Combretum* species indigenous to Nigeria were investigated for their *in vitro* antiprotozoal activity against *Plasmodium falciparum, Trypanosoma brucei brucei* and *Leishmania donovani.*

Among the plant extracts examined in this study, *C. racemosum* extract showed the most significant antiplasmodial activity with IC_{50} values of 25.6 μg/ml and 26.7 μg/ml, respectively, against P. falciparum strains D6 and W2. The extract of *C. platypterum* displayed slighter lower antiplasmodial activity, when compared to *C. racemosum,* with IC_{50} values of 27.3 μg/ml and 36.3 μg/ml, respectively, against D6 and W2 strains of *P. falciparum*. In this study, the antiplasmodial activity shown by *C. racemosum* and *C. platypterum* extracts is in line with a recent study where the extracts demonstrated higher antiplasmodial activity than chloroquine using the β-hematin inhibition assay [27]. It is noteworthy that *C. zenkeri* had no significant activity using pLDH assay in this work, however, in previous study it had moderate β-hematin inhibitory activity [27]. Another earlier study reported the antiplasmodial activity of *C. racemosum* leaf extract against chloroquine-resistant and pyrimethamine-resistant strains of *P. falciparum* which was determined using a modified [3H]-hypoxanthine incorporation assay [28]. In addition, *C. platypterum* methanol extract displayed a better selectivity towards the chloroquine-sensitive *P. falciparum* strain (SI = >1.7), as compared to the chloroquine-resistant *P. falciparum* strain (SI = >1.3). A similar observation was noted for the methanol extract of *C. racemosum.* Although *C. racemosum* and *C. platypterum* displayed significant antiplasmodial activity in this work, their low selectivity indices (SI <2) may be an indication of a narrow safety margin in therapeutic use. To the best of our knowledge, this is the first report on the antiplasmodial activity of *C. platypterum*.

The antitrypanosomal potential displayed by *C. racemosum* in this work (IC_{50} value = 18.44 μg/ml) stays in agreement with previous findings of Eze *et al.* [29] where the leaf extract of *C. racemosum* immobilised *T. brucei brucei* and reduced parasitaemia in infected mice. Similarly, an earlier study reported the antitrypanosomal activity of *C. racemosum* leaf extract against *T. brucei rhodesiense* with IC_{50} value of 24 μg/ml [28]. It is notable that although *C. racemosum* was active against *T. brucei brucei*, it lacked significant activity on the promastigote and amastigote forms of *L. donovani.*

C. racemosum, commonly called '*ogan pupa*' by the Yoruba ethnic group of southwestern Nigeria,

is a shrub used in Nigerian traditional medicine for the treatment of anaemia, cholera, diabetes, haemorrhoids, ulcer, trypanosomiasis, worm infections, haematuria, convulsion, cough, toothache, male sterility and tuberculosis [29, 30]. The phytochemical constituents of *C. racemosum* leaf extract includes alkaloids, steroids, cardiac glycosides, saponins and tannins [31]. Various pharmacological studies have validated the ethnomedicinal use of the plant in the management of several ailments including cancer, ulcer, inflammation, microbial infections, spasms, trypanosomiasis and termination of unwanted pregnancy [29, 32-37]. In a recent study, several pentacyclic triterpenoids including combregenin, combreglucoside, arjungenin, arjunolic acid, chebuloside II and bellericaside B were isolated from the roots of *C. racemosum* [37]. In the same study, 3-*O*-*β*-acetyl-ursolic acid, betulinic acid, and quadranoside II displayed significant cytotoxic activity against some carcinoma cell lines with IC_{50} values, while arjungenin, terminolic acid and 3-*O*-*β*-acetyl-ursolic acid showed moderate antimicrobial activity against some Gram-positive bacteria [37].

CONCLUSION

The present study demonstrates that the methanol extract of *Combretum racemosum* leaves possesses remarkable antiplasmodial and antitrypanosomal activities but lacks in significant antileishmanial properties. In addition, the antiplasmodial potential of the methanol extract of the leaves of *Combretum platypterum* is reported for the first time. However, it was observed that the extract of *Combretum zenkeri* lacked significantly in the activity against *Plasmodium falciparum*, *Leishmania donovani* and *Trypanosoma brucei brucei*. The phytochemical variations that exist among species of the same genus could account for this variation in the antiprotozoal activities of these three *Combretum* species. This work may provide supporting evidence for the ethnomedicinal use of *C. racemosum* leaves in the treatment of malaria and parasitic infections in African traditional medicine. Therefore, further analysis of this plant is needed in order to identify the molecules that could be responsible for the antiplasmodial and antitrypanosomal activity, as this could provide lead compounds for drug discovery and development to combat these tropical diseases.

ABBREVIATIONS

D6 – chloroquine-sensitive strains of *Plasmodium falciparum*
DMSO – dimethylsulfoxide
FHI – Forest Herbarium Ibadan
FRIN – Forestry Research Institute of Nigeria
HAT – human African trypanosomiasis
NTDs – neglected tropical diseases
PLDH – plasmodium lactate dehydrogenase
RPMI – Roswell Park Memorial Institute
SI – selectivity index
THP-1 – transformed human monocyte cell line
Vero – African green monkey kidney cell line
W2 – chloroquine-resistant strains of *Plasmodium falciparum*
WHO– World Health Organization

ACKNOWLEDGEMENTS

We acknowledge the National Centre for Natural Products Research, School of Pharmacy, University of Mississippi, USA, for providing facilities and materials for the *in vitro* antiprotozoal assays.

Conflict of interest: Authors declare no conflict of interest.

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