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Determination of IC_{50} and IC_{90} Values of Ethanolic Extracts of Some Medicinal Plants against *Trypanosoma brucei brucei*

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Abstract

Background: *In vitro* cultivation of salivarian species of trypanosomes requires the use of CO_2-incubator, expensive equipment which relies solely on steady electricity supply. A simple culture system which uses glass desiccator gassed with laboratory-prepared carbon dioxide (5CG-Desiccator) was found to effectively sustain trypanosomes growth in vitro and so the technique was recommended for in vitro antitrypanosomal screening of compounds and plant extracts. This paper reports an attempt to evaluate the utility of this system for the determination of IC_{50}, IC_{90} values of ethanolic extracts from some medicinal plants against *Trypanosoma brucei brucei* using Diminazine Aceturate (DA) as standard trypanocide.

Methods findings: Parasites were grown in Eagle's Medium in the presence of varying concentrations of each plant extract in a 96-well micro-titer plate using 5CG-Desiccator culture method. IC50 and IC90 values were extrapolated from two graphical approaches. Comparative analysis of IC90 values showed that extracts from four plants (*Cymbopogon spp, Moringa oleifera, Vernonia amygdalina and Allium sativum*) had IC_{90} values less than 200 g/ml, five (*Azadirachta indica, Khaya senegalensis, Carica papaya, Eucalyptus spp* and *Aloe vera*) had values between 200 g/ml and 1000 g/ml and one (*Mitracarpus scaber*) had value greater than 1000 g/ml.

Conclusion: The results obtained using two different approaches permitted the comparative assessment of the trypanocidal potentials of the extracts. Suggestions on practical ways to improve this technique for IC_{50} determination of values of synthetic and plant-derived compounds/fractions were discussed.

Keywords: 5CG-Desiccator; IC_{50} and IC_{90} determination; Plant extracts; *Trypanosoma brucei brucei*

Introduction

Several species of trypanosomes are the causative agents of African Trypanosomiases, a disease responsible for death and morbidity of man and his domestic animals in the tropical Africa. According to World Health Organization [1], approximately 300,000 to 500,000 people are affected by Human African Trypanosomiasis (HAT) with an estimated $ 4.5 billion annual economic losses accrued to animal trypanosomiasis. To date no effective vaccine, against any species of African trypanosomes, is in commercial circulation [2] and the trypanocides in use are faced with several challenges including high cost of purchase, toxic side effects associated with intake and the growing incidence of drug resistance developed by various species of the parasite [3].

In an attempt to source for easily available and safer agents, different synthetic and plant-derived compounds are being screened for their antitrypanosomal potentials [3-6]. *In vitro* screening procedures of substances against trypanosomes require the parasite to be cultured axenically. Today, there are several long term culture techniques for the cultivation of various species of trypanosomes which have been adapted and used in many research laboratories [4-9]. The practice involves culturing organisms under 5% CO_2 concentration in a CO_2 incubator, equipment that is expensive and not readily available in most laboratories in the developing countries of the world. In addition, the equipment relies on steady supply of electricity which is usually a problem to researchers in such countries. As a result of this challenge, scientists in developing countries had employed *in vitro* cultivation time for as short as an hour while screening extracts for their antitrypanocidal potentials [5,10,11]. Even though some measure of success was recorded, the equipment had the limitation of using only drop in parasite motility as index for parasite mortality. Even though motility may reflect parasite viability, sometimes, immotile organisms could retain their infectivity and in such case, motility could give misleading *in vitro* results, thereby questioning the reliability of data generated. Again, since, no sufficient time was given for parasite-extract interaction, it was impossible, by this approach; to determine the IC_{50} or IC_{90}

values of the plant extracts and so preclude any comparative study of screened antitrypanocidal principles. Review of literature revealed that studies on the African medicinal plants against trypanosomes are few [12].

Recent effort to source for alternative field-adapted culture system to CO_2-incubator utilized a glass desiccator gassed with laboratory prepared CO_2 [13]. This study will explore the possibility of using the 5CG desiccator for in vitro micro-assessment of the trypanocidal potentials of ethanolic extracts of *Cymbopogon spp, M. oleifera, V. amygdalina and A. sativum,* medicinal plants indigenous to northern Nigeria against *Trypanosoma brucei brucei*.

Materials and Methods

Plants collection and identification

Fresh leaves samples of ten plants (*Alium sativum* (AS), *Aloe vera* (AV), *Azadirachta indica* (AI), *Carica papaya* (CP), *Cymbopogon spp* (CS), *Eucalyptus spp* (ES), *Khaya senegalensis, Moringa oleifera* (MO), *Mitracarpus scaber* (MS) and *Vernonia amygdalina*) were collected within Kaduna town and taken to the Department of Biological Sciences, Kaduna State University, and Kaduna Nigeria for identification.

Experimental animals

Rats and mice raised at the animal colony of the Department of Biochemistry, Kaduna State University, Nigeria, were used for parasite maintenance and as source of parasites for the in vitro culture experiments. All animals were kept in accordance with accepted standards.

Parasite

The parasite, *T. b. brucei*, was obtained from the Nigerian Institute for Trypanosomiasis Research (NITR), Kaduna and was maintained by serial passage in mice and rats.

Chemical/reagents

Diethylaminoethyl cellulose (DE52) and Tris base were purchased from Sigma Chem. Co. (USA). Amino acids, Sodium Chloride, Sodium Dihydrogen Phosphate ($NaH_2PO_4.2H_2O$), Disodium Hydrogen Phosphate ($Na_2HPO_412H_2O$), Glucose, Antibiotics (Ampicillin, Streptomycin), Ethylenediamine Tetraacetic acid (EDTA), Chloroform, Glycerol, Giemsa powder, Calcium Carbonate (Ca_2CO_3) were all of analytical grade.

Preparation of plants extracts

Leafy part of each Plant was collected fresh and chopped into pieces using a sterile knife. Exactly 100 g of the chopped plant was transferred into the blender, and 250 ml of ethanol was added before blending. The blended plant material was transferred into a clean 500 ml glass bottle and allowed to extract by macerations for 12 hrs. Thereafter, it was filtered with cheese cloth and then with double layers of Whatman filters paper. The filtrate was collected and concentrated using rotary evaporator. The concentrate was transferred into a cleaned weighed crucible and placed in a water bath set at 45°C until the residual solvent is fully removed. The dried extract was then stored in a refrigerator at 4°C until required.

Preparation of goat serum

Fresh blood sample was collected from young apparently healthy goat into sterile 10 ml centrifuge tubes and centrifuged at 3000 rpm for 10 minutes. The serum was heat-inactivated at 55°C for 1 hour and stored in aliquots of 10 mls in 10 ml centrifuge tubes at -20°C.

Constitution of the culture medium for *in vitro* studies

The culture medium, Eagle minimum essential medium, was manually prepared using composition defined by Eagle [14]. The amino acids, vitamins, inorganic salts, phenol red and the antibiotic filtered components of the medium were separately prepared and stored in aliquots of 1 ml at -20°C. Prior to experiments, aliquots were taken and reconstituted to the required concentrations of the Eagle's medium. Goat serum (prepared from freshly collected blood) and glucose were added to the medium to achieve a 20% and 1% concentration respectively as previously described [15].

In vivo growth and Isolation of *T. b. brucei* from infected animals

At log phase parasitaemia, infected rats (weighing between 100-150 g) were sacrificed after chloroform anesthesia and through cardiac puncture, blood were collected into syringes containing 1% (w/v) EDTA solution in 100 mM phosphate buffer saline (0.2 ml of EDTA: 2 ml of blood). Collected blood were pooled into 10 ml centrifuge tubes, concentrated (3000 rpm for 10 minutes) and the parasites which aggregate a white cloudy buffy coat were carefully picked using pasteur pipette into a sterile microfuge tube containing phosphate saline glucose (100 mM PSG, pH 7.2, 0.2% (w/v) Glucose) and then subsequently freed of all erythrocytes and leucocytes contaminants via passage through ion exchange chromatographic column as described by Lanham and Godfrey [16]. Briefly, DE52 cellulose matrix was dissolved in phosphate buffer saline glucose and loaded on a 20 ml syringe column whose outlet was blocked with little cotton wool. The buffy coat parasites were applied on the column pre-equilibrated with phosphate buffer saline glucose (pH 8.0). By this procedure, the residual red cells were attached to the matrix while the parasites run down the column as a thick white suspension. The column is continually washed until eluent is transparent again. The suspension was then centrifuged at 2500 rpm for 10 minutes, the supernatant was discarded and the parasites pellet washed thrice with PBS (pH 7.2) before they were finally re-suspended in the cultivation medium and the parasite density determined using haemocytometer.

Counting of parasite in suspension and culture medium

Parasite densities in the culture media were determined using New Improved Neubauer counting chamber [13] described previously [15].

In vitro micro-assessment studies

Stock extract solutions were prepared by dissolving 1 mg of each plant extract with 1 ml of the culture medium containing 1% D-glucose, buffered with 25 mM Hepes to pH 7.4 and supplemented with 20% heat-inactivated goat serum. One hundred micro liters of this stock extract-medium and the various dilutions made from it were dispensed in triplicates into wells of 96 wells micro titer plate producing a range of concentrations from 1000 g/ml to 7.8 g /ml. To each well, 20 l of the parasite suspension (containing approximately 10000 parasites and producing parasite density of 8 × 10^4 parasite/ml) was added and the plate was then covered and put into a glass desiccator and the carbon dioxide gas (in amount that is equivalent to 5% of the capacity of the desiccator) was immediately released into the desiccator at 5% concentration (from where the 5 CG acronym was derived). The details of the preparation of the carbon dioxide have been described elsewhere [15]. The effective concentrations produced were from 995 to 6.5 g/ml. To establish the extent of extracts activities, parasite in the micro litre plate were incubated at 27°C for 24 hrs in the presence of the extracts and the parasite densities at the end of the incubation period were determined using a haemocytometer and compared with densities obtained from Negative control (parasite cultured in 100 l of the medium without extract or standard drug) and Standard drug control (parasite cultured in 100 l of 1000 g/ml of Diminazine Aceturate) wells. For each extract or drug the concentration which inhibit parasite population by 50% (IC_{50}) and 90% (IC_{90}) were determined by interpolation method of Hills as explained by Huber and Koella [17] and used by Scory and Steverding [9].

Results

The mean IC_{50} and IC_{90} values of all the extracts were determined using two separate graphical methods. In the first approach the parasite population (%) was plotted on the Y-axis directly against extract concentration on the X-axis (**Figure 1**). From this graph, it was difficult to extrapolate IC_{50} values for concentration less than 50 mg/ml due to clustering of the curves around this region. The IC90 values determination, however, did not pose any challenge since the use of logarithmic parameter on the X-axis gave rise to well-spaced-out plots (**Figure 2**) around portions leading to IC_{50}/ IC_{90}.

Discussion

The IC_{50} and IC_{90} values of ethanol extracts of ten medicinal plants were evaluated using a 5CG-Desiccator culture technique. This is of great significance because despite reports on the antitrypanosomal activities of extracts from local plants, the IC_{50} values of most of them remain unknown [12], providing limited opportunity for comparative analysis of their trypanocidal potentials.

The 5CG-Desiccator culture technique earlier developed in this laboratory and reported [15] was used successfully to culture the trypanosomes.

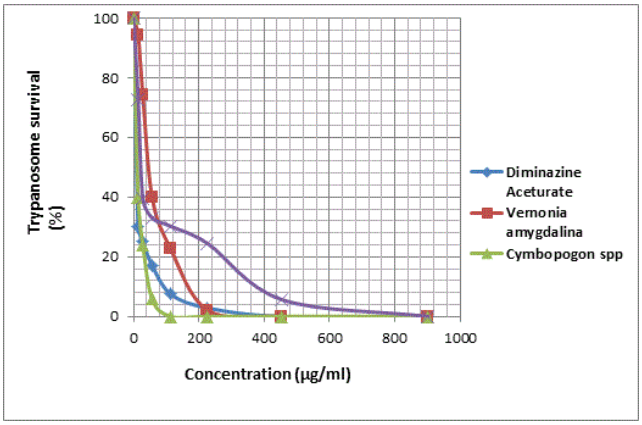

Figure 1 Relationship between parasite survival and extract concentration of three representative plants and Diminazine Aceturate (standard compound)

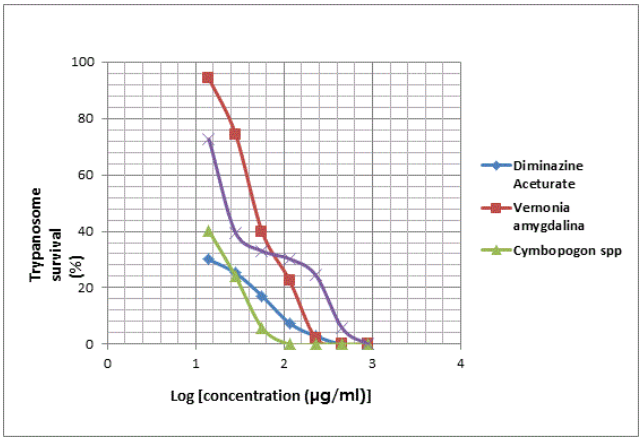

Figure 2 Relationship between parasite survival and logarithm of extract concentration of three representative plants and Diminazine Aceturate (standard compound)

It proved effective for the screening of all the extracts tested. The trypanosomes remained alive and active *in vitro* for the 24 hr duration required for the micro-assessment of extracts' activities, thus confirming the previous observation [15].

Extrapolation of the IC_{50} values from the two graphical methods employed (**Figures 1 and 2**) using data of some representative extracts was difficult. This difficulty was attributed to the absence of extract dilutions below 14.2 g/ml. There is a need to have lower concentrations (as low as 0.1 g/ml) as part of the series of dilutions employed, in order to have values less than 1 on the Logarithm (concentration) scale.

Table 1 presents the comparative results obtained for IC_{50} and IC_{90} of all the ethanolic extracts using the two methods earlier mentioned. Mean values obtained were fairly consistent irrespective of the method adopted. The IC_{90} values were identical for both methods of extrapolation. Higher values appeared to be associated more with the logarithmic plot than with the direct concentration plot.

Table 1 The IC50 and IC90 Values of the ethanolic extracts extrapolated using two approaches.

Plant /Drug	Method-I Concentration vs % Survival		Method-II Log Concentration vs % Survival	
	IC50 values (µg/ml)	IC90 Values (µg/ml)	IC50 values (µg/ml)	IC90 values (µg/ml)
Diminazine Aceturate	DE†	90	DE	89.13
Azadirachta indica	300	420	316.23	416.87
Khaya senegalensis	50	680	50.12	602.56
Carica papaya	DE	820	ND	891.25
Cymbopogon spp	12	40	ND	41.68
Eucalyptus spp	DE	370	21.88	380.19
Moringa oleifera	85	190	95.4	199.52
Mitracarpus scaber	>1000	>1000	>1000	>1000
Vernonia amygdalina	40	160	41.68	158.49
Allium sativum	DE	140	ND	151.35
Aloe vera	100	540	100	562.34

† Values are either difficult to estimate (DE) by the method employed or not determined (ND) at all.

The results showed that four of the plant extracts (*Cymbopogon spp, M. oleifera, V. amygdalina* and *A. sativum*) had IC90 values less than 200 µg/ml, five (*A. indica, K. senegalensis, C. papaya, Eucalyptus spp* and *A. vera*) had values between 200 µg/ml and 1000 µg/ml and one (*Mitracarpus scaber*) had value greater than 1000 µg/ml.

Several incubation periods are used to determine the IC50 values of various compounds [4-6,18,19]. Most importantly, IC_{50} values are generally been used to compare different biological responses under the effect of different drugs/ extracts. Results from the present study were obtained using 24hr incubation period since the duration is sufficient to allow for several rounds of parasite growth cycle and parasite-drug interaction [15].

It seems, however, that extrapolation from graph of (parasite survival vs logarithm of the concentration) will be much easier as the lines were more stretched out and better spaced apart (**Figures 1 and 2**).

For the determination of IC_{90} values both graphical approaches were effective (**Table 1**). Therefore, the comparative trypanocidal analysis of the studied extracts was based on the IC_{90} results presented in **Table 1**. Extract of *Cymbopogon spp* was adjudged most trypanocidal (having IC90 value of about 42 g/ml) while *Mitracarpus scaber* extract was considered least trypanocidal (with IC90 value above 1000 g/ml).

A number of workers have studied both the *in vitro* and *in vivo* antitrypanosomal properties of most of the plants under investigation [3,20,21] with varying outcome. Unfortunately, standard *in vitro* trypanocidal studies (involving the determination of IC_{50}) were not carried out for most of these plants by local researchers in the tropical countries were the plants grow in their natural habitats. Inability to culture trypanosomes for up to 24 hrs was a major hindrance requiring carbon dioxide which the 5CG-desiccator culture has partly addressed.

As a way of optimizing the utility of this culture method, we recommend that the IC_{50} values should be determined in phases: the first phase should be devoted to testing concentration from 0.01 to 100 g/ml while subsequent phase (from 100 to 1000 g/ml or less than 0.01 g/ml) should be left for extracts whose IC_{50} values are visibly well above 100 g/ml or well below 0.01 g/ml. The phase two experiment should only be carried out if the IC_{50} could not be found by the phase one experiment.

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Competing and Conflicting Interests

The authors do not have any conflicting interest whatsoever.

Ethical Approval

The study was approved by the Institutional Research Ethics Committee.

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