Evaluation of Plant Extracts for Antileishmanial Activity using a Mechanism-Based Radiorespirometric Microtechnique (RAM)

Maurice M. Iwu[1,2,4], *Joan E. Jackson*[3], *John D. Tally*[3], *and Daniel L. Klayman*[2]

[1] NRC Senior Research Associate; on leave of absence from the University of Nigeria, Nsukka, Nigeria
[2] Department of Medicinal Chemistry, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, D.C. 20307, U.S.A.
[3] Department of Parasitology, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, D.C. 20307, U.S.A.
[4] Address for correspondence

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Abstract

Extracts of eleven plants used in Nigerian traditional medicine have been evaluated for possible antileishmanial activity using a radiorespirometric microtest technique based on *in vitro* inhibition of catabolism of $^{14}CO_2$ from a battery of ^{14}C-substrates by promastigotes. Of 13 methanol extracts tested, 5 from *Gongronema latifolia, Dorstenia multiradiata, Picralima nitida, Cola attiensis*, and *Desmodium gangeticum*, were active at concentrations of 50 μg/ml or less against a visceral *Leishmania* isolate.

Key words

Leishmaniasis, *Gongronema latifolia, Dorstenia multiradiata, Picralima nitida, Desmodium gangeticum, Cola attiensis.*

Introduction

Infections due to protozoa of the genus *Leishmania* are a major world wide health problem, with high endemicity in developing countries. The global prevalence of leishmaniases in man is about 12 million cases, with an estimated incidence of 2–3 million cases per annum (1, 2). Approximately 350 million people within 80 countries are threatened by the disease worldwide. The pathological effects of the disease are complex, manifesting in various forms. They include self-healing cutaneous lesions, recurrent leishmaniasis recidivans, disfiguring mucocutaneous and diffuse cutaneous diseases, and visceral leishmaniasis (kala azar). In the advanced form of the latter, the reticuloendothelial system is infected with the resultant toll on the spleen, liver, bone marrow, lymph glands, and, often, some degree of intestinal tract disfunction. The mucocutaneous, diffuse cutaneous, and visceral disease forms can be fatal if untreated.

Clinical drug intervention is presently limited to the use of pentavalent antimonials (SbV), sodium stibogluconate, and *N*-methylglucamine antimonate, and secondarily, amphotericin B or pentamidine (2, 3). These antileishmanials require parenteral administration with clinical supervision or hospitalization during treatment because of the severity of possible toxic side-effects that include cardiac and/or renal failure (2). Treatment with these agents is not consistently effective particularly for the most virulent leishmanial disease forms (2, 4–7). The World Health Organization has reported large scale resistance of kala azar to antimonials, which are the preferred chemotherapy for treatment of most forms of leishmanial disease (7). In some endemic regions, it has been observed that prolonged medication (2 months or more) with SbV is required to effect a clinical cure (3). Long term SbV therapy, however, is not usually advocated due to the aforementioned toxicity of pentavalent antimonials. There is, therefore, a need for the development of more effective, less toxic and orally-active antileishmanial agents.

Development of a new drug for the treatment of leishmaniasis has been impeded by the lack of a simple, rapid and universally applicable (i.e., to the various *Leishmania* species/strains infecting humans) drug evaluation system (8, 9). The lack of progress in the development of new antileishmanial agents is evident by the fact that all the clinically useful drugs were developed between 1947 and 1959 (9). Current methods used for the screening of potential antileishmanial agents generally utilize intracellular amastigotes (the mammalian intracellular form) since promastigotes (monoflagellate forms found within the insect vector and culture *in vitro*) are reported "insensitive" within *in vitro* assays to SbV compounds used for human leishmaniases (9). Since there is no system yet available for culturing amastigotes extracellularly, except re-isolation from infected tissues and macrophage cultures, their mass culture is rather limited (8, 9), making them unsuitable for primary screening of potential antileishmanial agents.

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An *in vitro* radiorespirometric microtest (RAM) using promastigotes has been developed in our laboratories which relies on drug inhibition of parasite production of $^{14}CO_2$ from a battery of ^{14}C-substrates by promastigotes to detect drug-mediated parasite damage at low drug concentration within a short time (10, 11). The test is quantitative, rapid, consistent, and conducted in a serum-free chemically-defined medium in which prior adaptation is not necessary to cultivate the so-called "difficult to grow" species. The method has been shown to correlate well with patient response to SbV therapy (11).

Visceral leishmaniasis is endemic to the central Nigerian highlands, and zoonotic cutaneous leishmaniasis, prevalent in the northern half of this country (12). Because of limited supply, expense and high toxicity of commercial antileishmanials, traditional herbal therapy is frequently utilized in many leishmanial endemic regions of Nigeria.

In this study, we have evaluated extracts of 11 plants used in Nigerian folk medicine as antiparasitic remedies for possible antileishmanial activity using the radiorespirometric microtest, RAM.

Materials and Methods

Plant materials

Plants were selected from a collection made as part of a Salvage Ethnography Project, Institute of African Studies, University of Nigeria, Nsukka. Samples were authenticated by Dr. C. O. Okunji of the Dept. of Pharmacognosy, University of Nigeria, Nsukka and Mr. F. Ozioko of the Department of Botany of the same University. Voucher specimens have been deposited in the Pharmacy Herbarium of the University of Nigeria, Nsukka. The plants investigated are shown in Table 1.

Table 1 List of plants evaluated for antileishmanial activity.

Species	Plant Family	Plant Part	Solvent	Test Code
Afromomum danielli	Zingiberaceae	Rhizome	MeOH	ADF
Cola attiensis	Sterculiaceae	Seed	CH_2Cl_2	CT-1
Cola attiensis	Sterculiaceae	Seed	MeOH	CT-2
Crescentia cujeta	Bignoniaceae	Fruit	MeOH	CCX
Desmodium gangeticum	Fabaceae	Leaf	MeOH	SM
Dorstenia multiradiata	Moraceae	Leaf	MeOH	DL
Dracaena mannii	Agavaceae	Leaf	MeOH	DM
Garcinia kola	Guttiferae	Seed	MeOH	GKX
Gongronema latifolia	Asclepiadaceae	Leaf	MeOH	GG
Picralima nitida	Apocynaceae	Seed	CH_2Cl_2	HB
Picralima nitida	Apocynaceae	Seed	MeOH	PN
Rothmania withfieldii	Loganiaceae	Fruit	MeOH	RQ
Schumaniophyton magnificum	Loganiaceae	Leaf	MeOH	SCM

Extraction procedure

Two hundred grams of powdered material from each plant were percolated for 24 hours with 80% methanol or dichloromethane and concentrated to a sticky gum under reduced pressure. The extracts from the seed materials were partitioned between chloroform and water and the two fractions were submitted to bioassay. The list of extracts prepared is shown in Table 1.

Leishmania species/strains

A clinical isolate of visceral *Leishmania* (*Leishmania chagasi*, MHOM/BR/84/BA-13) was used for this study. This isolate was selected because its sensitivity to SbV was previously determined using RAM. MHOM/BR/84/BA-13 is sensitive to Pentostam, sodium antimony gluconate, at 6 μg/ml Sb (20 μg/ml drug); and to Glucantime, *N*-methylglucamine antimonate, at 80 μg/ml Sb (286 μg/ml drug).

Cultivation medium

Promastigotes of *L. chagasi* were grown in a serum-free, defined medium, MM2 (10). The MM2 medium contained 120 μg/ml protein (10 μg/ml human transferrin, 10 μg/ml human insulin, 100 μg/ml defatted bovine albumin), plus 10 μg/ml low density bovine lipoprotein. Previous research demonstrated the need for low protein-serum-free medium because serum protein : drug association reduces *in vitro* antiparasite activity (10). Cultures were maintained at 25 °C during growth and incubation with drug.

^{14}C-Substrates

The ^{14}C-labelled substrates are (numerical code) ^{14}C-substrates: (2) L-arginine (guanidino-^{14}C), (3) L-aspartic acid (4-^{14}C), (4) L-asparagine (U-^{14}C), (5) L-glutamic acid (U-^{14}C), (6) L-glutamine (U-^{14}C), (7) glycine (U-^{14}C), (9) L-isoleucine (U-^{14}C), (10) L-leucine (1-^{14}C), (12) L-methionine (1-^{14}C), (13) L-ornithine (1-^{14}C), (15) L-proline (U-^{14}C), (17) taurine (U-^{14}C), (18) L-threonine (U-^{14}C), (20) tyramine (7-^{14}C), (24) L-fucose (1-^{14}C), (25) D-galactose (1-^{14}C), (28) D-mannose (1-^{14}C), (42) orotic acid (carboxyl-^{14}C), (44) succinic acid (1,4-^{14}C), (46) Na-butyric acid (1-^{14}C), (49) D-glucosamine (1-^{14}C), (52) Na-glycocholic acid (1-^{14}C), (53) L-methionine (methyl-^{14}C). All ^{14}C-substrates were selected with specific activities as close to 50 mCi/mM/carbon atom as obtainable from commercial sources. The sources are as reported in reference (10). A "U" in the ^{14}C designation indicates a "uniform" ^{14}C-label at each carbon atom in the molecule. For use in radiorespirometry, the ^{14}C-substrates were diluted to a final concentration of 100,000 disintegrations per minute (dpm)/25 μl using a phosphate buffered balanced salt solution (PBSS: NaCl 6.58 g, KCl 0.4 g, $CaCl_2$ 0.14 g, KH_2PO_4 0.06 g, $MgSO_4$ 0.05 g, sodium phosphate 0.01 M, made up to 1 l with sterile, glass-distilled H_2O, final pH 7.4). The ^{14}C-substrates were filter sterilized (0.22 μm Acrodisc® filter, Millipore Corporation, Bedford, Massachusetts) into sterile screwcap vials and stored at 4 °C until use. Subsequent to sterilization, ^{14}C-substrate vials were opened only within a laminar hood.

Radiorespirometric procedure

Promastigotes were maintained in log phase growth for 3 successive transfers (48–72 h apart) prior to radiorespirometric testing. Test extracts (or PBSS plus drug solvent [DMSO], for parallel control cultures) were added 24 h after the third promastigote transfer to fresh growth medium. Incubation in the presence of plant extracts was continued for 96 additional hours while the parasites remained in mid-log phase growth. The rest of the radiorespirometric procedure was conducted as previously described (10). Briefly, to each well of a microtiter tray (Biospherics Type T010 + C010, Universal Plastics & Engineering Company, Rockville, Maryland) were added 25 μl of a single ^{14}C-substrate (100,000 dpm). The tray was covered with a friction-fit lid to prevent evaporation while the promastigotes were being 3 × centrifugally (700 × G, 10 min, 4 °C) washed free of nutrient medium and drug using PBSS. The final organism pellet was resuspended to a concentration of 1×10^9 organisms/ml in PBSS. After the addition of 25 μl of organism suspension to each well (total volume per well, 50 μl: ^{14}C-substrate + promastigote suspension), the wells were immediately covered with a filter paper disc (22 mm, # 410, Schleicher & Schuell, Inc., Keene, New

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Hampshire) which had been premoistened with one drop of saturated $Ba(OH)_2$ solution. The trays were recovered with the lid. If during the 30 min incubation at 33 °C, the *Leishmania* metabolize the ^{14}C-substrates to $^{14}CO_2$, the radioactive gas was collected as a $Ba^{14}CO_3$ precipitate on the filter paper discs. After the incubation, the filter discs were removed from the trays, dried using an infrared lamp, and the ^{14}C quantity determined using an argon : methane (P10 mixture, argon : methane 9 : 1 v/v, respectively) gas-flow proportional counter (Model 5110, Tennelec, Inc., Oak Ridge, Tennessee). Data (dpm corrected for background, 1 count per minute; and machine efficiency) were electronically sent to a computer for analysis and graphic presentation.

A quantitative replicate test variability was determined in a previous study (10). Tests were initially repeated in duplicate on 4–5 separate days (8–10 tests/drug concentration/organism). The mean dpm/^{14}C-substrate had a linear relationship to the magnitude of the standard deviation (SD) (10). It was established from the analysis of previous data on the test system that the percent coefficient variation is estimated to be approximately 19% and 15% at counting levels of 100 dpm and 20,000 dpm, respectively (10). Therefore testing was only repeated in duplicate for each test extract with parallel duplicate drug vehicle control tests (unless otherwise indicated).

Drug test procedure

The procedure was conducted as described earlier (10). The extract concentration of 50 µg/ml was used for the tests. Drug activity was based on determining the ^{14}C-substrate(s) (Table 2) for which $^{14}CO_2$ release was decreased in the drug-treated parasites compared to the effect on the control group, i.e., parasites treated with phosphate buffered balanced salt solution and the solvent vehicle (PBSS + DMSO).

Each experiment consisted of parallel: (a) duplicate tests of drug-treated parasites; plus (b) duplicate tests of drug vehicle control-treated parasites; plus (c) one "nonbiological" sterility control. The nonbiological control consisted of each ^{14}C-substrate (one substrate per microtiter tray well), and PBSS (the same PBSS batch used to wash, to suspend the parasites, and to make drug solution). Since there were no parasites in the nonbiological control, any $^{14}CO_2$ detected was attributed either to biologic contamination (or, less likely, chemical contamination) of the ^{14}C-substrates resulting in breakdown of the ^{14}C-substrates. If radioactivity above background (10 disintegrations per minute, dpm) was detected in the nonbiological control, the suspect solution(s) was replaced and the experiment was repeated.

Table 2 Antileishmanial activity of the extracts.

Extract Designation	Concentration	Activity (^{14}C-Substrate Catabolism)
CT-1	50 µg/ml	Active (10*/21 Suppression)
	85 µg/ml	Active (10/21 Suppression)
CT-2	50 µg/ml	No Activitiy
DL-55	5 µg/ml	Active (1/18 Suppression)
	25 µg/ml	Active (1/18 Suppression)
	50 µg/ml	Active (11/19 Suppression)
RQ-2	50 µg/ml	No Activitiy
PNF-5A	50 µg/ml	No Activity**
PN-04	100 µg/ml	No Activity
HB-3	50 µg/ml	Active (7/16 Suppression)
SM-7	50 µg/ml	Active (9/17 Suppression)
GG-8	50 µg/ml	Active** (12/17 Suppression)
ADF	50 µg/ml	No Activity
SCM	50 µg/ml	No Activity
DM-6	50 µg/ml	No Activity
GKX-1	50 µg/ml	No Activity
SB-2	50 µg/ml	No Activity**
CCX-1	50 µg/ml	No Activity**

* The numerator represents the number of ^{14}C-substrates for which catabolism was lower for drug treated parasites than catabolism from corresponding drug vehicle-treated (= control) parasites. The demoninator is the total number of different ^{14}C-substrates tested in each experiment.

** n = 1; unstarred results, n = 2.

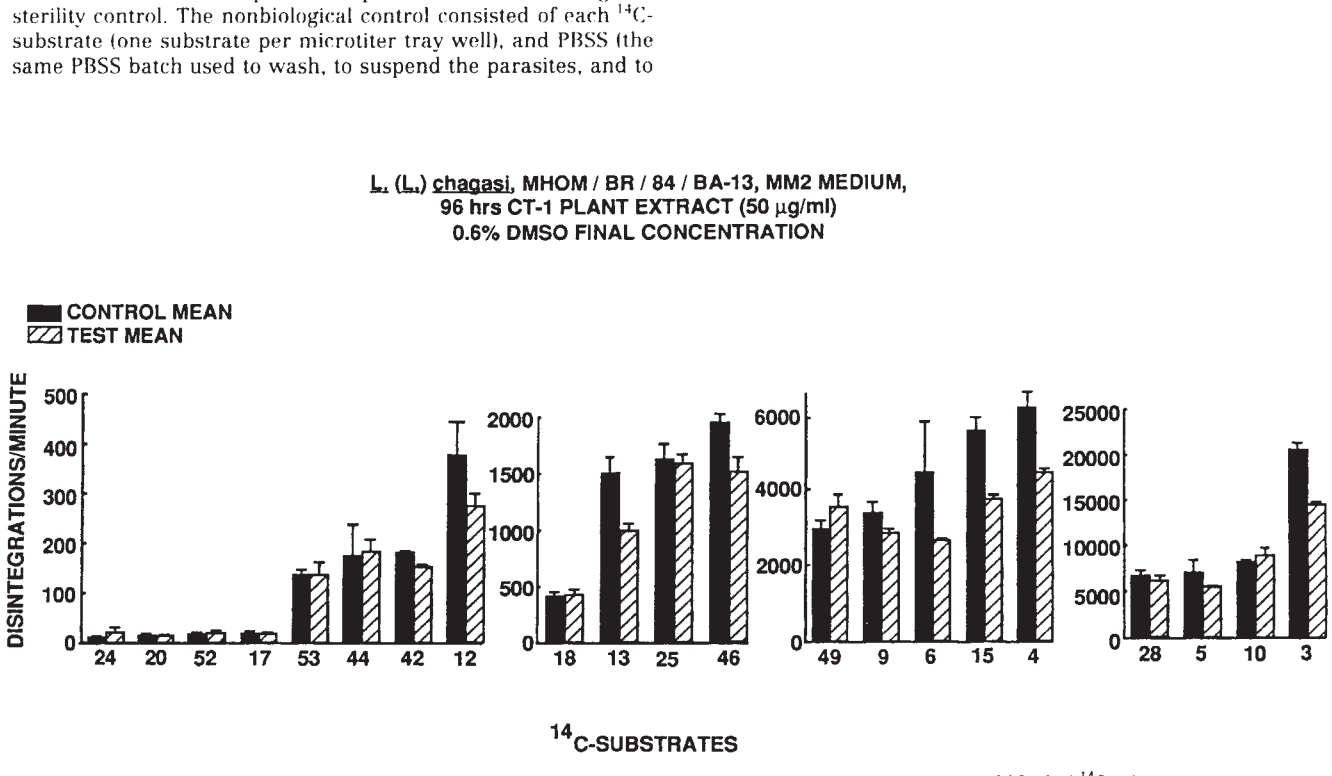

Fig. 1 Test results for the plant extract, CT-1, from *Cola attiensis*. At 50 µg/ml, suppression of parasite catabolism of 10 of 21 ^{14}C-substrates occurred. Cultures of control parasites were treated with the same quantity of drug solvent solution (= drug vehicle control) minus drug as corresponding drug-treated (= test) parasites. Drug solvent was a mixture of balanced salt solution plus dimethyl sulfoxide (DMSO).

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Results

At a concentration of 50 μg/ml, 5 of the 11 plant extracts tested inhibited the catabolism of two or more of the substrates to CO_2 (Table 2). *C. attiensis* extract (CT) inhibited parasite catabolism of 10 of the 21 substrates used in the assay, with the strongest activity observed on the disintegration of L-ornithine, L-proline, L-asparagine, and L-aspartic acid (Fig. 1). *G. latifolia* (GG) displayed strong inhibition of the catabolism of succinic acid, D-galactose, D-mannose, L-aspartic acid, L-glutamine, and D-glucosamine, as well as L-proline, L-ornithine, and L-glutamic acid (Fig. 2). For *P. nitida* extract (HB), the strongest activity was observed against Na butyric acid, with the drug treated parasite cultures showing a suppression of more than 90% when compared with the values observed for the controls. An inhibition rate of 40 % or more was observed for succinic acid, glycine, and L-glutamine. Strong activity was also noted for L-aspartic acid, L-glutamic acid, and ornithine (Fig. 3). No significant inhibition occurred in the catabolism of tyramine, taurine, and L-fucose at the dose of HB tested.

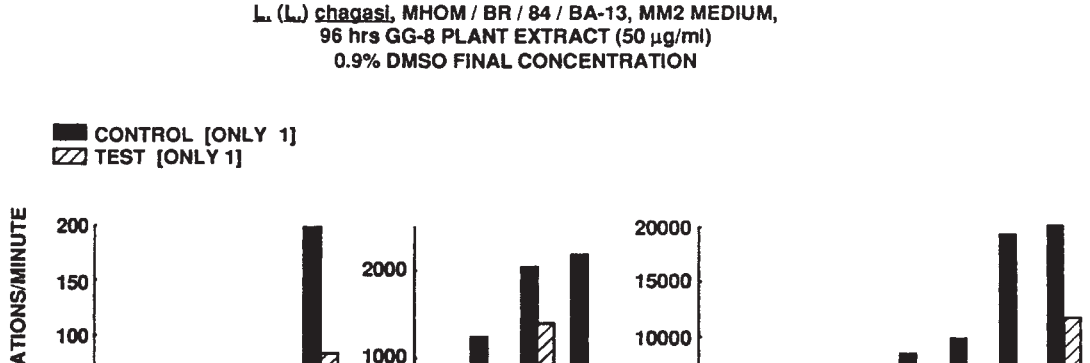

Fig. 2 Test results for the plant extract, GG-8, from *Gongronema latifolia*. At 50 μg/ml suppression of parasite catabolism of 12 of 17 ^{14}C-substrates occurred.

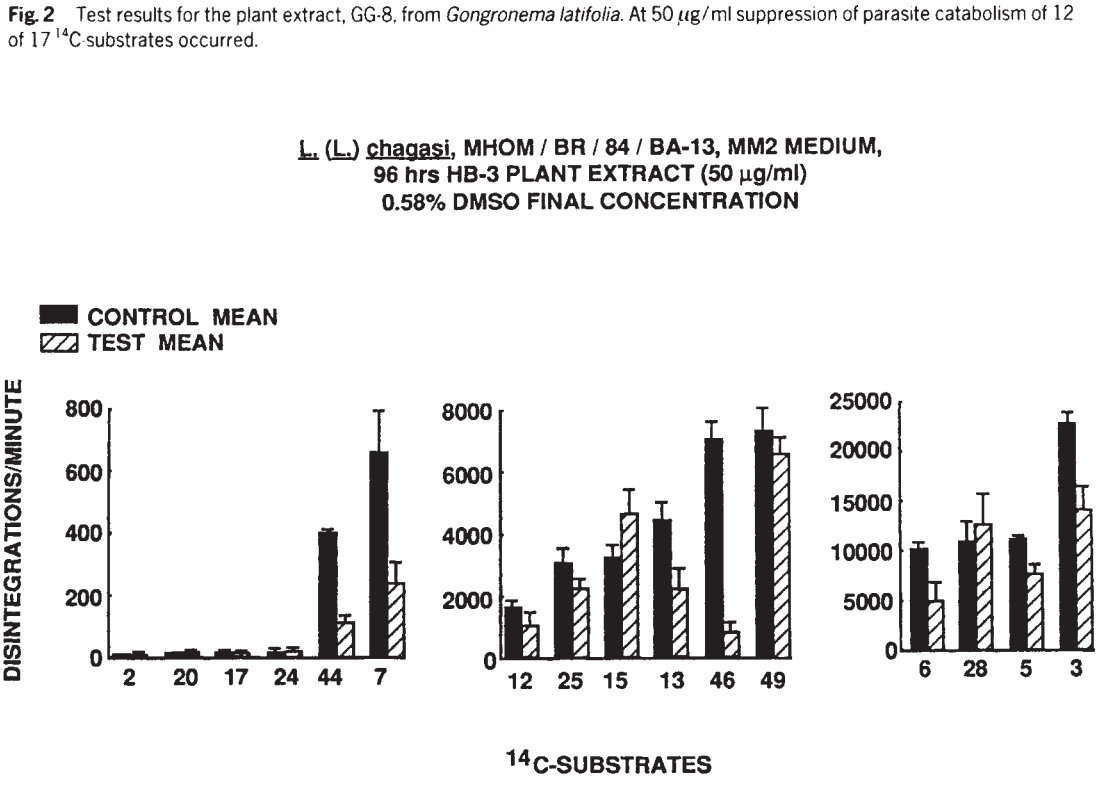

Fig. 3 Test results for the plant extract, HB-3, from *Picralima nitidia*. At 50 μg/ml suppression of parasite catabolism of 7 of 16 ^{14}C-substrates occurred.

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The extract of *D. multiradiata* (DL) strongly inhibited the catabolism of L-ornithine, butyric acid, and L-proline (Fig. 4). Moderate inhibition was observed on L-aspartic acid, L-asparagine, D-mannose, and D-galactose. *D. gangeticum* extract (SM) showed strong inhibition of 5 of the 17 substrates used in the study, with the strongest inhibition observed against L-proline and L-glutamine.

Discussion

The results show that the extracts could be explored as sources of leads for new antileishmanial agents. The extracts displayed varied inhibition patterns which suggests different mechanisms in their mode of action.

Three of the extracts, CT, DL, and SM, appear to be more active against amino acid catabolism, whereas HB and GG showed preferential inhibition against sugars and fatty acids.

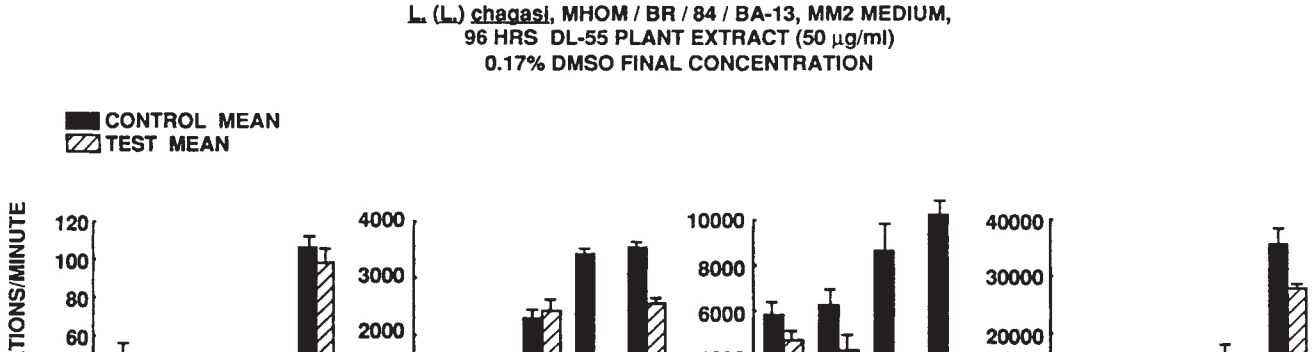

Fig. 4 Test results for the plant extract, DL-55, from *Dorstenia multiradiata*. At 50 µg/ml suppression of parasite catabolism of 11 of 19 ^{14}C-substrates occurred.

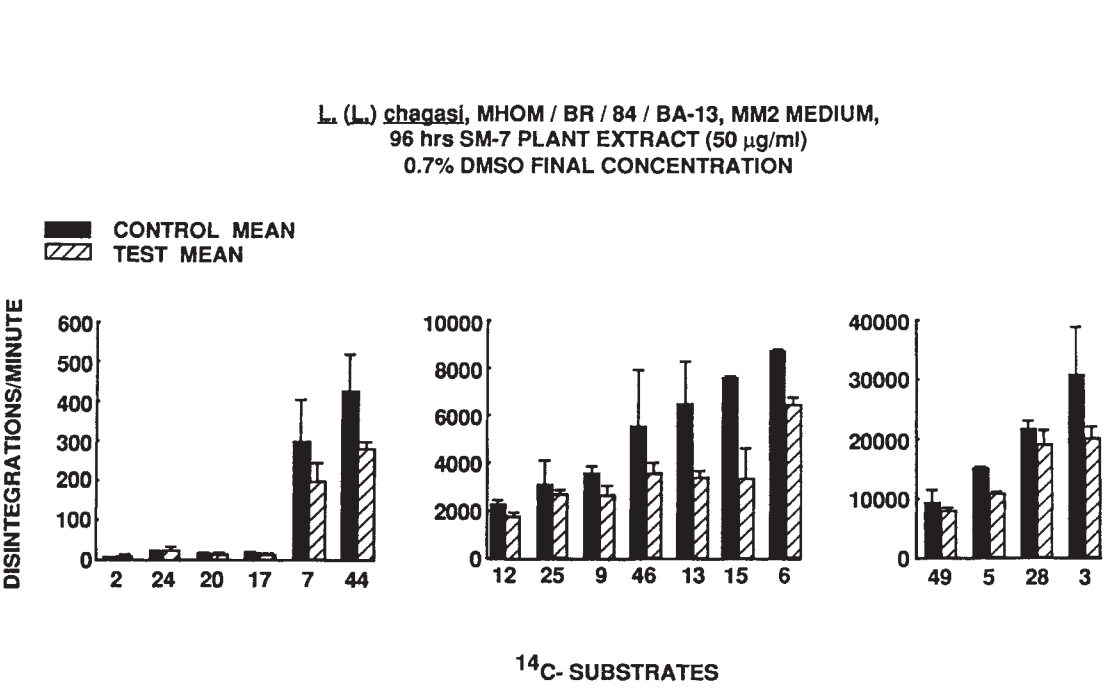

Fig. 5 Test results for the plant extract, SM-7, from *Desmodium gangeticum*. At 50 µg/ml suppression of parasite catabolism of 9 of 17 ^{14}C-substrates occurred.

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One of the plants investigated, *C. attiensis*, is used among other things for the treatment of migraine, bronchitis, and catarrh. *P. nitida* has been employed in the treatment of malaria, African sleeping sickness, and bacterial infections. *D. gangeticum* is reputed in folk medicine as a very effective antifungal agent, antiviral, anti-inflammatory, and as an oral remedy for various parasitic skin infections. Aqueous decoction of *D. multiradiata* is used as an antiviral agent and as a local anti-inflammatory. *G. latifolia* is valued as a bitter tonic, and the alcoholic infusion is dispensed for bilharzia, viral hepatitis, and as a general antimicrobial agent.

Pentavalent antimonials have a serum half-life of 2 hours with the maximum achievable serum level of approximately 20 μg/ml Sb (or approximately 73 μg/ml drug) (13, 3). It is interesting to note that even as crude mixtures, the 5 active plant extracts (Table **3**, Fig. **1–5**) were active at 50 μg/ml and one, DL-55, retained antileishmanial activity to 5 μg/ml. The observation that the crude extracts exhibited antileishmanial activity, at drug concentrations comparable to SbV, seems to indicate high potential for the active drug principles as new antileishmanials.

The plants are presently being analyzed for their chemical constituents. A literature search, however, revealed that the plants vary widely in their constituents. *P. nitida* contains the indole alkaloids picraline, akuammiline, akuammicine, akuammidine, pseudoakuammigine, picraphylline, and echitamine as the major components (14), *D. gangeticum* yields β-carbolines and phenylethylamines (15). There is no available report on any previous chemical analysis of *C. attiensis, G. latifolia*, or *D. multiradiata*.

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