ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1988, p. 1725–1729
0066-4804/88/111725-05$02.00/0

Vol. 32, No. 11

Use of Microdilution To Assess In Vitro Antiamoebic Activities of *Brucea javanica* Fruits, *Simarouba amara* Stem, and a Number of Quassinoids

COLIN W. WRIGHT,[1]* MELANIE J. O'NEILL,[1] J. DAVID PHILLIPSON,[1] AND DAVID C. WARHURST[2]

Department of Pharmacognosy, The School of Pharmacy, University of London, 29-39, Brunswick Square, London WC1N 1AX,[1] and Department of Medical Protozoology, London School of Hygiene and Tropical Medicine, London WC1E 7HT,[2] United Kingdom

Received 16 November 1987/Accepted 11 August 1988

A microdilution technique for the assessment of in vitro activity against *Entamoeba histolytica* was devised and validated with metronidazole. The test was used to detect the antiamoebic activities of plant extracts prepared from the traditional remedies *Brucea javanica* fruits and *Simarouba amara* stems. The activity was associated with quassinoid-containing fractions. The 50% inhibitory concentrations for some quassinoids against amoebae were determined by using the microdilution method. These concentrations ranged from 0.019 $\mu g \cdot ml^{-1}$ for bruceantin, the most active quassinoid, to >5 $\mu g \cdot ml^{-1}$ for glaucarubol, the least active compound tested. These results are discussed with reference to the known activities of these compounds against *Plasmodium falciparum*. Overall, the activities of the quassinoids against both protozoa are similar. The microdilution technique will be useful in the search for novel antiamoebic drugs.

An estimated 12% of the world's population harbor *Entamoeba histolytica*, and as a result amoebic dysentery is common in the tropics and subtropics. If left untreated, this condition may lead to amoebic liver disease and other serious complications (8). The nitroimidazole, metronidazole (Fig. 1), is a highly effective amoebicide and is considered by many clinicians to be the drug of choice for treating acute amoebiasis. However, metronidazole has mutagenic effects in bacteria and is carcinogenic to rodents (12). The drug is relatively ineffective against asymptomatic infections in the intestinal lumen ("cyst-passers") (12), and adverse effects, especially severe nausea, and interactions with alcohol may reduce the level of patient compliance. In addition, amoebae may develop resistance to metronidazole. Clearly, there is a need for alternative antiamoebic agents.

Previously published methods used to assess antiamoebic activity in vitro have utilized techniques such as the observation of clonal growth of amoebae in semisolid medium to determine viability (4) or the counting of amoebae grown in flat-sided tissue culture tubes (6). These techniques are time-consuming, produce variable results, and require relatively large quantities of test compounds and materials. A micromethod has been described that is based on measuring the incorporation of [^{3}H]thymidine into *E. histolytica* grown in microtiter plates (3). We have developed a simple micromethod which does not require the use of radiolabeled compounds or special gas mixtures, thus making it suitable for use in laboratories where scintillation counting and other facilities are not available.

Many plant species are used in traditional medicine to treat dysentery; these plants should be analyzed to determine their efficacy (and toxicity) and thus their potential as sources of new antiamoebic agents. These plants include species of the family Simaroubaceae, e.g., *Brucea javanica* (L.) Merr. and *Simarouba amara* Aubl. *B. javanica*, a tree which grows in The People's Republic of China and elsewhere in the Far East, produces fruits and seeds which have long been used in the treatment of dysentery (7). The barks of *S. amara* and of the related species *Simarouba glauca* are also used in traditional medicine; fluid preparations made from them have been marketed for the treatment of amoebiasis (13). In the present study, we assessed the new microdilution procedure for evaluating plant extracts and purified compounds for antiamoebic activity. In particular, extracts of *B. javanica* fruits and *S. amara* stems, along with some quassinoids (the bitter constituents of these plants), were tested by this technique.

MATERIALS AND METHODS

Plant material and extraction procedure. *B. javanica* fruits from Thailand were kindly supplied by P. Tantivatana, D. Ponglux, and N. Supavita. *S. amara* stems were collected in the Colon region of Panama by M. P. Gupta and J. D. Phillipson. Plant material was air dried, ground, and successively extracted with petroleum ether (fraction 1), methanol (fraction 2), and water (fraction 3) as described previously (9). The methanol extract was partitioned between chloroform and water, and this aqueous phase was further partitioned with butanol to yield a chloroform extract (fraction 4), a butanol extract (fraction 5), and an aqueous extract (fraction 6). This fractionation procedure allows some separation of the constituents of *S. amara* stem according to their relative polarities. Highly lipophilic material is extracted into petroleum ether (fraction 1). Constituents which have a medium polar nature pass into the methanol extract (fraction 2) and then into chloroform (fraction 4) or butanol (fraction 5). Highly polar constituents will be extracted into the aqueous fractions 3 and 6. Extracts were evaporated to dryness under reduced pressure (fractions 1, 2, 4, and 5) or freeze-dried (fractions 3 and 6).

Test compounds. Metronidazole was obtained from May and Baker, Ltd. Quassin was a gift from Bush, Boake, and Allen, Ltd. Glaucarubol was kindly supplied by M. Suffness, National Cancer Institute, Bethesda, Md. Bruceines A, B, C, and D, as well as bruceantin and yadanzioside F, were available from earlier extractions of *B. javanica* fruits (9).

* Corresponding author.

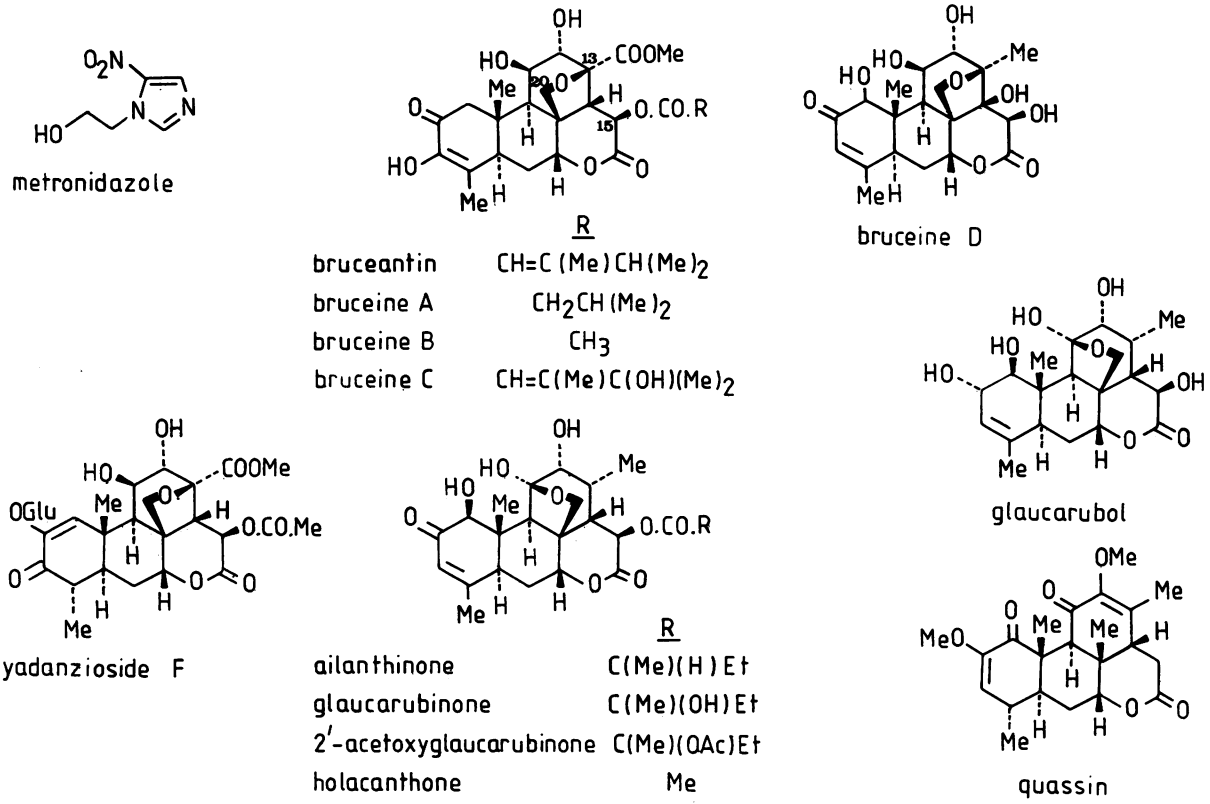

FIG. 1. Structures of metronidazole and the quassinoids.

Glaucarubinone, 2'-acetylglaucarubinone, ailanthinone, and holacanthone were available from previous extractions of *S. amara* fruits (11). All quassinoids have been identified previously by their spectroscopic characteristics (9, 11).

Culture of test organism. *E. histolytica* NIH 200 was cultured in Falcon flasks (25 cm^2; Nunc) by using Diamond TPS-1 medium as described previously (6), except for the following modifications. Trypticase was replaced by casein digest peptone 94072 (BBL Microbiology Systems), and the basic medium was sterilized by pressure cooker heating for 10 min at 15 lb · in^{-2} rather than by filtration. Inactivated bovine serum (Difco Laboratories, Inc.) replaced inactivated horse serum. When the amoebic growth was confluent, subculturing was carried out by chilling the flask on ice to detach the amoebae and by diluting the resulting suspension 2 to 3 times with fresh culture medium.

Test procedure. Ethanol (50 μl) was added to samples (ca. 10 mg) of plant extracts or pure compounds (ca. 1 mg), followed by enough culture medium to obtain concentrations of 10 or 1 mg · ml^{-1}, respectively. Samples of plant extracts or pure compounds were dissolved or suspended by mild sonication in a Sonicleaner bath (Ultrasonics, Ltd.) for a few minutes and then further diluted with culture medium to concentrations of 1 or 0.1 mg · ml^{-1}, respectively. The maximum concentrations of ethanol in the test did not exceed 0.25%, at which level no inhibition of amoebal growth occurred. Twofold serial dilutions were made in the wells of 96-well microtiter plates (Linbro; Flow Laboratories, Inc.) in 170 μl of culture medium. Each plate included metronidazole as a standard amoebicidal drug, control wells (culture medium plus amoebae), and a blank (culture medium only). A suspension of amoebae was prepared from a confluent culture by pouring off the medium, adding 2 ml of fresh medium, and chilling the culture on ice to detach the organisms from the side of the flask. The number of amoebae per milliliter was estimated with a hemacytometer, and trypan blue exclusion was used to confirm viability. Fresh culture medium was added to dilute the suspension to 10^5 organisms per ml, and 170 μl of this suspension was added to the test and control wells in the plates so that the wells were completely filled (total volume, 340 μl). An inoculum of 1.7 × 10^4 organisms was chosen so that confluent, but not excessive, growth took place in control wells. Plates were sealed with expanded polystyrene (ca. 0.5 cm thick), secured with tape, placed in a modular incubating chamber (Flow Laboratories), and gassed for 10 min with nitrogen before incubation at 37°C for 72 h.

Assessment of antiamoebic activity. After incubation, the growth of amoebae in the plates was checked with a low-power microscope. The culture medium was removed by inverting the plates and shaking them gently. Plates were then immediately washed once in sodium chloride solution (0.9%) at 37°C. This procedure was completed quickly, and the plates were not allowed to cool in order to prevent the detachment of amoebae. The plates were allowed to dry at room temperature, and the amoebae were fixed with methanol and, when dry, stained with aqueous eosin (0.5%) for 15 min. Stained plates were washed once with tap water and then twice with distilled water and allowed to dry. A 200-μl portion of 0.1 N sodium hydroxide solution was added to each well to dissolve the protein and release the dye. The optical density of the resulting solution in each well was determined at 490 nm with a microplate reader (MR-700; Dynatech Laboratories, Inc.). Optical density readings and numbers of amoebae were correlated by setting up two plates, each containing serial dilutions of a suspension of

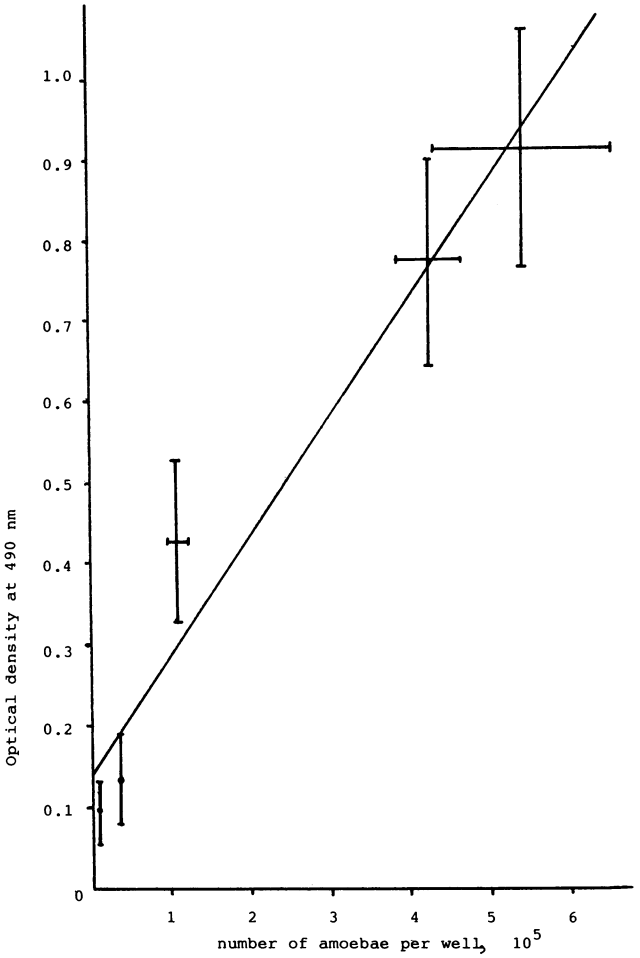

FIG. 2. Correlation of optical density with numbers of amoebae. Each point represents the mean values of three optical density readings and three hemacytometer counts. Standard deviations are shown. Linear regression analysis was used to determine the best-fitting line. The correlation coefficient was 0.98.

amoebae. After incubation for 24 h, plates were counted with a hemacytometer (for one plate) or by staining with eosin and measuring the optical density (for the other plate). Triplicate counts and optical density readings were obtained for each concentration. The relationship between the optical density at 490 nm and the numbers of amoebae is illustrated in Fig. 2. Under the conditions of the assay, the amoebae adhered firmly, provided that overgrowth did not occur. Thus the assay results indicate the inhibition of amoebal growth. Drugs which affect adhesion will also show activity, and this characteristic may be advantageous for a screening test. The percent inhibition of amoebal growth was calculated from the optical densities of the control and test wells and was plotted against the logarithm of the dose of the drug or extract being tested. Linear regression analysis was used to determine the best-fitting straight line, as illustrated in Fig. 3 for metronidazole. At least two experiments were performed for each compound or extract tested.

RESULTS AND DISCUSSION

This microdilution technique is selective for antiamoebic drugs (14). Metronidazole had a 50% inhibitory concentration (IC_{50}) of 0.320 $\mu g \cdot ml^{-1}$, which is close to the previ-

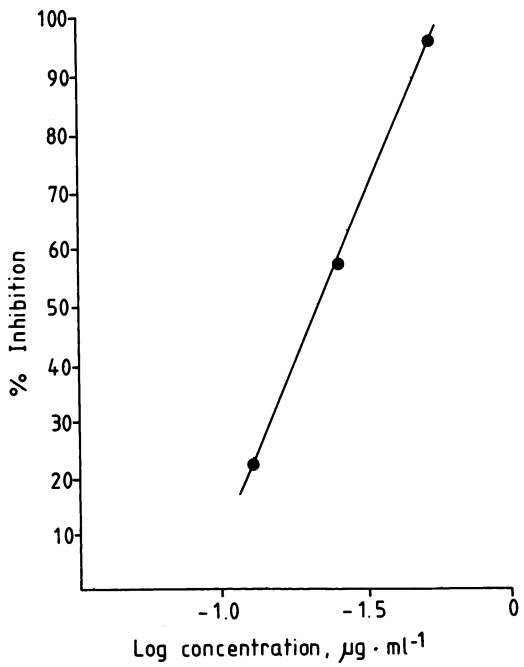

FIG. 3. Amoebicidal activity of metronidazole against *E. histolytica* NIH 200 in vitro.

ously reported IC_{50} of 0.22 $\mu g \cdot ml^{-1}$ obtained against the same strain of *E. histolytica* by counting amoebae grown in flat-sided tissue culture tubes (6). The selectivity of the test when applied to plant extracts is also underlined by our finding that the extracts of many plant species are inactive.

The antiamoebic activities of the plant extracts prepared from *B. javanica* fruits and the *S. amara* stem are shown in Table 1. Little activity was present in the petroleum ether extracts of either species, but the methanol extracts possessed marked antiamoebic properties. The aqueous extract (fraction 3) of *B. javanica* fruits was much less active than the methanol extract, and the same fraction from the *S. amara* stem was only slightly more active than the petroleum ether extract. When the methanol extract from *B. javanica* was partitioned between chloroform, butanol, and water, most of the activity passed into the chloroform phase, with low but significant activity remaining in the butanol (fraction 5) and aqueous (fraction 6) phases. Similarly, the chloroform extract of *S. amara* was highly active, but the activity of fractions 5 and 6 favored fraction 5, the butanol phase.

When the extraction procedure described above is followed, the major quassinoid constituents present in these

TABLE 1. In vitro activities of extracts from *B. javanica* fruits and from *S. amara* stem against *E. histolytica* NIH 200

Fraction	Antiamoebic activity (IC_{50}, $\mu g \cdot ml^{-1}$) of extract from:	
	B. javanica fruits	*S. amara* stem
1, Petroleum ether	282 (232–332)[a]	114 (86.6–150)
2, Methanol	10.7 (8.97–12.8)	6.20 (4.21–9.13)
3, Aqueous	52.5 (30.9–74.1)	89.8 (72.5–111)
4, Chloroform	0.45 (0.35–0.55)	2.90 (2.38–3.54)
5, Butanol	38.0 (26.0–50.0)	6.89 (6.22–7.63)
6, Aqueous	28.2 (15.6–40.8)	52.5 (47.9–57.6)

[a] 95% Confidence interval is indicated in parentheses.

TABLE 2. In vitro activities of some quassinoids against *E. histolytica* NIH 200 and *P. falciparum* K1

Quassinoid	Antiamoebic activity		Antimalarial activity (IC_{50}, $\mu g \cdot ml^{-1}$) (reference)
	IC_{50}, $\mu g \cdot ml^{-1}$	CC[a]	
Bruceine A	0.097 (0.090–0.105)[b]	0.993	0.011 (9)
Bruceine B	0.306 (0.175–0.437)	0.804	0.011 (9)
Bruceine C	0.279 (0.241–0.347)	0.932	0.005 (9)
Bruceantin	0.019 (0.018–0.021)	0.996	0.0008 (9)
Bruceine D	0.386 (0.334–0.446)	0.992	0.015 (9)
Yadanzioside F	2.33 (1.38–3.94)	0.866	5.0 (9)
Glaucarubinone	0.168 (0.140–0.201)	0.973	0.004 (11)
2′-Acetylglaucarubinone	0.155 (0.134–0.180)	0.982	0.008 (11)
Ailanthinone	0.063 (0.055–0.071)	0.948	0.015 (11)
Holacanthone	0.162 (0.157–0.167)	0.999	0.007 (11)
Glaucarubol	>5		0.41 (10)
Quassin	>50		>25 (5)
Metronidazole	0.320 (0.318–0.322)	0.995	

[a] CC, Correlation coefficient.
[b] 95% Confidence interval is indicated in parentheses.

species are concentrated in the chloroform extract (9). *B. javanica* contains the quassinoids bruceines A, B, and C, which pass into the chloroform extract together with the minor constituent, bruceantin. These quassinoids were highly active against amoebae and account for the high potency of the chloroform extract (Table 2). The polar fractions 3, 5, and 6 are devoid of the quassinoids listed above, but they do contain other more polar quassinoids, including quassinoid glycosides (9). One of the nonglycosides from the polar fractions, bruceine D, had antiamoebic activity comparable to that of bruceines B and C, whereas the glucoside, yadanzioside F, was much less active. The antiamoebic activity of the polar fractions is thus presumably due to the presence of polar quassinoids. Polar quassinoids have not yet been detected in *S. amara*, although the related species, *S. glauca*, produces 15-*O*-glucosides of glaucarubol and glaucarubolone in the seeds (1). The presence of polar constituents may be important with respect to the way in which the plants are used in traditional medicine. Often, herbal teas are prepared which tend to contain the more water-soluble quassinoids rather than the lipophilic quassinoids (which are extracted with chloroform).

Quassinoids are bitter terpenoids with a polycyclic skeleton (Fig. 1) that is modified in various ways so that, among the Simaroubaceae, a number of different types occur. The principal difference between quassinoids found in *B. javanica* and those present in *S. amara* is the location of the methyleneoxy bridge; the oxygen is attached to C-13 in *B. javanica* and to C-11 in *S. amara*, and the carbon is attached to C-8 in both. However, the position of the methyleneoxy bridge does not seem to affect biological activity significantly. Quassinoids of both types are potent antiamoebic agents, and both types have antimalarial (10) and antileukemic (2) activities. The antiamoebic activities of the compounds are shown in Table 2, and for comparison their in vitro antimalarial activities against a multidrug-resistant strain (K1) of *Plasmodium falciparum* (5, 9–11) are also given.

The major constituent quassinoids of *B. javanica*, bruceines A, B, and C, were active against *E. histolytica*. Bruceine A had an IC_{50} of 0.097 $\mu g \cdot ml^{-1}$, a value 3 times greater than those for bruceines B and C, which were similar to metronidazole (IC_{50}, 0.32 $\mu g \cdot ml^{-1}$). Bruceantin (IC_{50}, 0.0192 $\mu g \cdot ml^{-1}$) was, however, 10 to 15 times more potent than bruceines B and C. These compounds differ only in the nature of the ester function at C-15 in the quassinoid skeleton. It is interesting that bruceines B and C have similar activities and yet differ substantially in the nature of their C-15 ester groups. Bruceine B has a simple acetate at C-15, whereas bruceine C possesses a 3′,4′-dimethyl-4′-hydroxypent-2′-ene-oic moiety at this position (Fig. 1). On the other hand, bruceantin, which is considerably more active than bruceine C, differs from it only in the lack of the 4′-hydroxy group on the C-15 chain. Bruceine D has in vitro antiamoebic and antimalarial properties similar to those of bruceines B and C but differs structurally from the latter two compounds; its C-15 is unesterified, its C-13 bears a methyl rather than a carbomethoxy unit, and its substituents in the A-ring are arranged differently. The glucoside, yadanzioside F, is markedly less active than the other quassinoids examined.

The four quassinoids from *S. amara* had significant antiamoebic activity, with ailanthinone (Fig. 1) being twice as potent as the others. In contrast to the series of *Brucea* quassinoids, variation in the C-15 ester group of the *S. amara* quassinoids does not seem to affect antiamoebic activity markedly. Glaucarubol (Fig. 1) from *S. glauca* was inactive against amoebae. This compound does not possess an ester function at C-15, nor does it have the α,β-unsaturated ketone in the A-ring, which is present in all the highly active quassinoids investigated here. Quassin itself (Fig. 1), which is a constituent of *Quassia amara* and *Picrasma excelsa* was also inactive against amoebae.

Some of the quassinoids evaluated here for antiamoebic activity have been examined earlier for activity against a different strain (HM1, isolated at the Institute of Medical and Social Security, Mexico) of *E. histolytica* by Gillin et al. (4), who used a method in which viability is determined by observing the clonal growth of amoebae. Bruceantin was reported to be highly active (IC_{50}, 0.018 $\mu g \cdot ml^{-1}$), and this result is very close to our own value. However, bruceine B was reported to be inactive at 2 $\mu g \cdot ml^{-1}$, whereas in our study it had an IC_{50} of 0.306 $\mu g \cdot ml^{-1}$. The activities found by Gillin et al. for ailanthinone (IC_{50}, 0.068 $\mu g \cdot ml^{-1}$) and glaucarubinone (IC_{50}, 0.14 $\mu g \cdot ml^{-1}$) were similar to our own findings (0.063 and 0.168 $\mu g \cdot ml^{-1}$, respectively). Glaucarubol was found to be inactive at 2 $\mu g \cdot ml^{-1}$ by Gillin et al. and was inactive at 5 $\mu g \cdot ml^{-1}$ in our study. In contrast, holacanthone was reported to be inactive at 2 $\mu g \cdot ml^{-1}$ by Gillin et al., but it was markedly active in our own study (IC_{50}, 0.162 $\mu g \cdot ml^{-1}$).

Quassin was tested by Keene et al. (6) using a method in which amoebae were grown in flat-sided tissue culture tubes and was found to be active (IC_{50}, 0.5 $\mu g \cdot ml^{-1}$), but in the present study quassin was inactive at 50 $\mu g \cdot ml^{-1}$. Clearly, there are some differences between our findings and those of other researchers. Thus it may be worthwhile to consider the in vitro antimalarial activities published for the quassinoids (Table 2). Antimalarial activity was measured as the inhibition of [^{3}H]hypoxanthine incorporation into *P. falciparum*. Of the compounds isolated from *Brucea* and *Simarouba* spp., bruceantin was the most active quassinoid and yadanzioside F was the least active quassinoid against both *E. histolytica* and *P. falciparum*, and, while there is not an exact parallel, the antiamoebic and antimalarial activities follow this same general trend. This correlation between antiamoebic and antimalarial activities for quassinoids lends

support to the findings of the present study that quassin is inactive and that bruceine B and holacanthone are active antiamoebic agents in vitro. Some quassinoids are known to inhibit protein synthesis in mammalian cells (2). However, their mode of action against *E. histolytica* remains to be investigated.

ACKNOWLEDGMENTS

We thank Jenny Blackwell of The Department of Tropical Hygiene, London School of Hygiene and Tropical Medicine, for the use of her microplate reader. We thank A. Cavanagh for drawings.

Financial support from The Pharmaceutical Society of Great Britain and from the Medical Research Council is acknowledged. D. C. Warhurst is supported by the Public Health Laboratory Service, London, United Kingdom.

LITERATURE CITED

1. **Bhatnagar, S., J. Polonsky, T. Prange, and C. Pascard.** 1984. New toxic quassinoid glucosides from *Simarouba glauca*. Tetrahedron Lett. **25**:299–302.
2. **Cassady, J. M., and M. Suffness.** 1980. Terpenoid antitumor agents, p. 201–269. *In* J. M. Cassady and J. D. Douros (ed.), Anticancer agents based on natural product models. Academic Press, Inc., New York.
3. **Cedeno, J. R., and D. J. Krogstad.** 1983. Susceptibility testing of *Entamoeba histolytica*. J. Infect. Dis. **148**:1090–1095.
4. **Gillin, F. D., D. S. Reiner, and M. Suffness.** 1982. Bruceantin, a potent amoebicide from a plant, *Brucea antidysenterica*. Antimicrob. Agents Chemother. **22**:342–345.
5. **Guru, P. Y., D. C. Warhurst, A. Harris, and J. D. Phillipson.** 1983. Antimalarial activity of bruceantin *in vitro*. Ann. Trop. Med. Parasitol. **77**:433–435.
6. **Keene, A. T., A. Harris, J. D. Phillipson, and D. C. Warhurst.** 1986. *In vitro* amoebicidal testing of natural products. Part 1. Methodology. Planta Med. **1986**:278–284.
7. **Key, J. D.** 1976. Chinese herbs: their botany, chemistry and pharmacodynamics. Charles E. Tuttle Co., Rutland, N.J.
8. **Martinez-Palomo, A. (ed.).** Human parasitic diseases, vol. 2. Elsevier Publishing Co., Cambridge.
9. **O'Neill, M. J., D. H. Bray, P. Boardman, K. L. Chan, J. D. Phillipson, D. C. Warhurst, and W. Peters.** 1987. Plants as sources of antimalarial drugs. Part 4. Activity of *Brucea javanica* fruits against chloroquine-resistant *Plasmodium falciparum in vitro* and against *Plasmodium berghei in vivo*. J. Nat. Prod. **50**:41–48.
10. **O'Neill, M. J., D. H. Bray, P. Boardman, J. D. Phillipson, D. C. Warhurst, W. Peters, and M. Suffness.** 1986. Plants as sources of antimalarial drugs: in vitro antimalarial activities of some quassinoids. Antimicrob. Agents Chemother. **30**:101–104.
11. **O'Neill, M. J., D. H. Bray, P. Boardman, C. W. Wright, J. D. Phillipson, D. C. Warhurst, W. Peters, M. Correya, and P. Solis.** 1987. The activity of *Simarouba amara* against chloroquine-resistant *Plasmodium falciparum in vitro*. J. Pharm. Pharmacol. **39**(Suppl.):1–80.
12. **Reynolds, G. E. F. (ed.).** 1982. Martindale, the extra pharmacopoeia, 28th ed., p. 968–970. The Pharmaceutical Press, London.
13. **Steck, E. A.** 1971. The chemotherapy of protozoan diseases, vol. 1, p. 3.71–3.74. Walter Reed Army Institute of Research, Washington, D.C.
14. **Wright, C. W., M. J. O'Neill, J. D. Phillipson, and D. C. Warhurst.** 1987. A microdilution assay for determination of *in vitro* activity against *Entamoeba histolytica*. J. Pharm. Pharmacol. **39**(Suppl.):1–105.