Potent Anti-amoebic Effects of Ibogaine, Voacangine and the Root Bark Alkaloid Fraction of *Tabernaemontana arborea*

Authors
Julio César Carrero[1], Violeta Curay-Herrera[2], Lysette Chacón-Niño[1], Felix Krengel[3], Silvia-Laura Guzmán-Gutiérrez[4], Mayra Silva-Miranda[4], Luisa-Carolina González-Ramírez[5], Raúl J. Bobes[1], Clara Espitia[1], Ricardo Reyes-Chilpa[6], Juan-Pedro Laclette[1]

Affiliations
1 Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad de México, México
2 Escuela Profesional de Ciencias Biológicas, Facultad de Ciencias, Universidad de Piura, Piura, Perú
3 Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, Universidad Nacional Autónoma de México, Ciudad de México, México
4 CONACyT-Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad de México, México
5 Grupo de Investigación "Análisis de Muestras Biológicas y Forenses", Carrera Laboratorio Clínico, Facultad de Ciencias de la Salud, Universidad Nacional de Chimborazo, Riobamba, Ecuador
6 Departamento de Productos Naturales, Instituto de Química, Universidad Nacional Autónoma de México, Ciudad de México, México

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Correspondence
Prof. Dr. Julio César Carrero
Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México
Av. Universidad 3000, 04510 Ciudad de México, México
Phone: + 52 55 56 22 92 20
carrero@unam.mx

Correspondence
Prof. Dr. Ricardo Reyes-Chilpa
Departamento de Productos Naturales, Instituto de Química, Universidad Nacional Autónoma de México
Av. Universidad 3000, 04510 Ciudad de México, México
Phone: + 52 55 36 68 62 94
chilpa@unam.mx

ABSTRACT

Plants of *Tabernaemontana* species have several pharmacological activities including antimicrobial effects. Amoebiasis continues to be a public health problem, with increasing evidence of resistance to metronidazole. In this study, we assessed the effect of the alkaloid fraction of *T. arborea* root bark and the alkaloids ibogaine and voacangine on the viability and infectivity of *Entamoeba histolytica* trophozoites. Cultures were exposed to 0.1–10 µg/mL for 24, 48 and 72 h, and viability was then determined using a tetrazolium dye reduction assay and type of cellular death analyzed by flow cytometry. Results showed that the alkaloid fraction, but mainly ibogaine and voacangine alkaloids, exhibited potent dose-dependent anti-amoebic activity at 24 h post-exposure (IC_{50} 4.5 and 8.1 µM, respectively), comparable to metronidazole (IC_{50} 6.8 µM). However, the effect decreased after 48 and 72 h of exposure to concentrations below 10 µg/mL, suggesting that the alkaloids probably were catabolized to less active derivatives by the trophozoites. The treatment of trophozoites with the $IC_{50\,s}$ for 24 h induced significant morphological changes in the trophozoites, slight increase in granularity, and death by apoptonecrosis. The capacity of *T. arborea* alkaloids to inhibit the development of amoebic liver abscesses in hamsters was evaluated. Results showed that even when the treatments reduced the number of amoebic trophozoites in tissue sections of livers, they were unable to limit the formation of abscesses, suggesting their rapid processing to inactive metabolites. This work leaves open the possibility of using *Tabernaemontana* alkaloids as a new alternative for amoebiasis control.

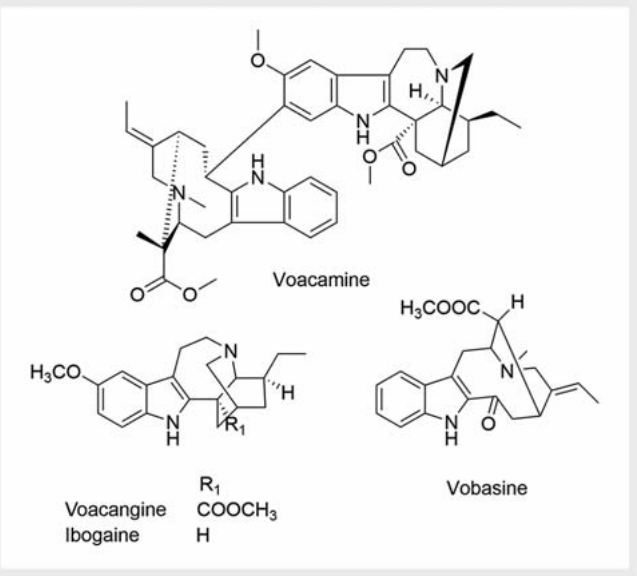
Introduction

Amoebiasis caused by the enteric protozoan parasite *Entamoeba histolytica* is a cosmopolitan disease that infects millions of people, making it a leading cause of diarrhoea, mainly in developing countries, and estimated to cause the death of more than 55 000 people each year [1–3]. In a global burden of diseases study from 1990 to 2010, worldwide deaths due to amoebiasis were reported to be around 1 per 100 000 people, causing a loss of disability life-years (DALY) of 32 years on average [4]. Amoebic trophozoites invade the intestinal mucosa, producing dysentery, and sporadically migrate to the liver resulting in abscesses, which is the main cause of death by this parasite [3]. Nitroimidazole derivatives such as metronidazole (MTZ) are the drugs of choice for treatment. However, MTZ is inefficient against the transmission stage of cysts, and it has the disadvantage of inducing many side effects that may result in the abandonment of therapy before eradication of the infection [5]. Moreover, genotoxicity in human cells, mutagenicity in bacteria, and carcinogenicity in rodents have been reported for MTZ [6, 7]. In addition, *in vitro* resistance to MTZ by *E. histolytica* trophozoites has been observed when parasite cultures are exposed to increasing concentrations of the drug [8, 9]. These results increased awareness about the development of MTZ resistance in field conditions when this drug is indiscriminately used [3, 10].

Therefore, it is imperative to identify or develop novel anti-amoebic compounds with low toxicity in humans and at low cost, since producing a new drug can be prohibitive, exceeding one billion dollars [11]. In this sense, plant extracts are considered a natural and affordable source of new compounds with powerful anti-parasitic activity and safe use in humans [12].

Tabernaemontana (Apocynaceae) genus includes nearly 100 species with pantropical distribution, and are found in Asia, Africa, Australia, North and South America, as well as in a variety of oceanic islands [13, 14]. The plants are evergreen shrubs and small trees that grow between 1 and 15 m tall. The leaves are opposite, 3 to 25 cm long, with milky sap, commonly called "milk wood". The flowers are fragrant, white and 1 to 5 cm in diameter [13]. Plants within the *Tabernaemontana* genus are characterized by a high alkaloid content, usually displaying several commonly known pharmacological activities including antimicrobial, antioxidant, anti-inflammatory, anticancer, antidiabetic, antihypertensive, antineurodegenerative, antivenom, wound healing, analgesic, and others [13–15].

México presents a particularly high number (18) of *Tabernaemontana* species, such as *T. arborea* Rose ex J. D.Sm. [14]. To the best of our knowledge, *T. arborea* has not been recorded to have any ethnomedical application in México, but other species of this genus are used in Latin America and other countries in traditional medicine, and active extracts and compounds have been reported. Therefore, we followed a chemotaxonomic approach. For instance, *T. alba* Mill., a species found from the south of México to Panama, is used in traditional medicine to treat skin conditions due to their anti-inflammatory, analgesic, and anti-microbial properties [15]. In addition to the well-known antibacterial effect of extracts and alkaloid fractions of several *Tabernaemontana* species plants [16–21], anti-parasitic effects have also been reported,



▶ **Fig. 1** Alkaloids of *T. arborea* from the alkaloid fraction of the root bark methanolic extract.

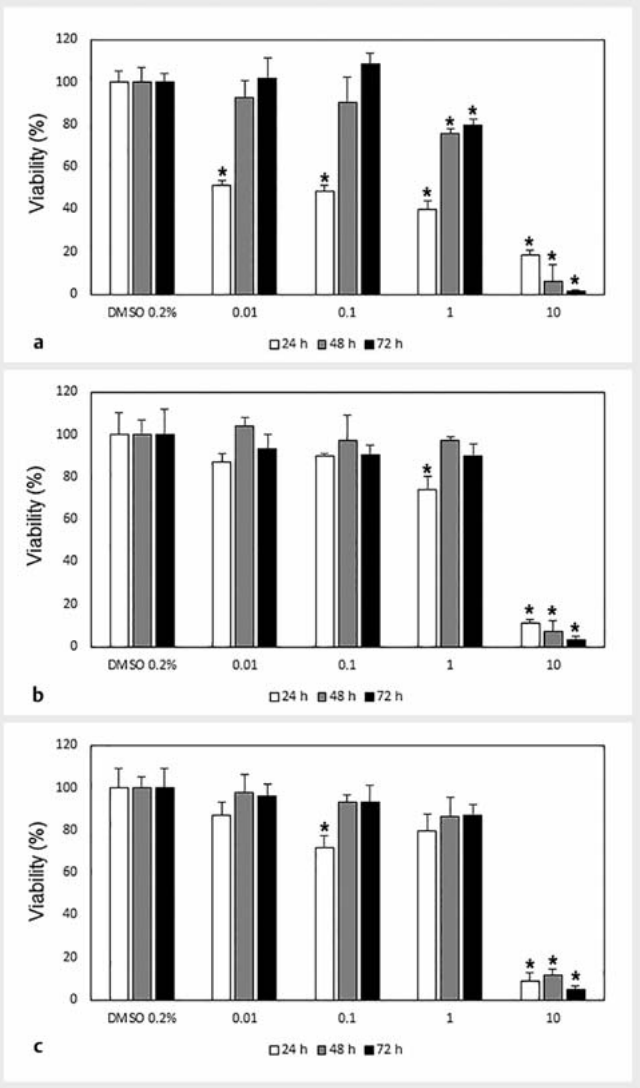
including extracts and alkaloids from *T. citrifolia* on the gastrointestinal nematode *Haemonchus contortus* [22], *T. elegans, T. pachysiphon, T. peduncularis* and *T. macrocarpa* on the malarial protozoan *Plasmodium falciparum* [23–26], *T. pandacaqui* on the protozoan *Trypanosoma cruzi*, causal agent of Chagas disease [27] and *T. longipes* on *Trypanosoma brucei*, the causal agent of African sleeping sickness [28]. Previous phytochemical research has shown *T. arborea* root bark to be particularly rich in monoterpenoid indole alkaloids (MIAs), including ibogaine, voacangine, and vobasine, as well as the bis-indole type alkaloid voacamine [21, 29].

Due to the clear need for new drugs to treat amoebiasis, this study was aimed at determining the effect of the alkaloid fraction of *T. arborea* root bark, and its ibogaine and voacangine MIAs (▶ **Fig. 1**), on the viability of *E. histolytica* trophozoite cultures and their effect on the development of amoebic liver abscesses in hamsters.

Results

Details on the identification and isolation of ibogan-type alkaloid from root bark of *T. arborea* methanol extract was previously reported by our group [21]. This alkaloid fraction contains three MIAS – ibogaine, voacangine and vobasine – identified by GC-MS with 33.74, 53.58, and 62.67 mg/g, respectively. It also includes the bis-indole type alkaloid voacamine, detected only by HPLC-UV probably due to its high molecular weight, and it was not quantified (▶ **Fig. 1**).

The *T. arborea* alkaloid fraction and pure alkaloids ibogaine and voacangine were tested for their amoebicidal effect in cultures of 24, 48 and 72 h of exposure. Results showed that the alkaloid fraction significantly decreased the viability of *E. histolytica* trophozoites after 24 h of exposure at all concentrations tested in a



▶ **Fig. 2** Effect of *T. arborea* alkaloids on the amoebae viability. *E. histolytica* trophozoites (10^5/mL) were cultured in the presence of 0.01 to 10 µg/mL of alkaloid fraction of the root bark methanolic extract (**a**) and isolated ibogaine (**b**) and voacangine (**c**) alkaloids, for 24, 48, and 72 h at 37 °C. Viability was determined by MTT measuring the absorbance at 595 nm. Vehicle treatment controls (DMSO 0.2%) that do not affect viability are also included. Values are presented as means ± SD of three independent experiments. The asterisks mark the conditions where a significant difference was found (* $p < 0.01$).

dose-dependent manner (▶ **Fig. 2a**; $p < 0.05$). When amoebae were exposed for 48 and 72 h to the alkaloid fraction, only the higher concentrations tested, of 1 and 10 µg/mL, significantly affected the viability, killing almost all parasites at 10 µg/mL (▶ **Fig. 2a**; $p < 0.01$). Like the alkaloid fraction, ibogaine and voacangine significantly decreased the amoebic viability in all cultures, but only at the highest concentration of 10 µg/mL (▶ **Fig. 2b** and **c**; $P < 0.01$). No clear differences in the anti-amoebic activity between the two alkaloids were observed. In agreement, the half-maximal inhibitory concentration (IC_{50}) values for the alkaloid fraction and both alkaloids were quite similar over cultures treated for 24 h, ranging between 1.4 µg/mL (4.5 µM) for ibogaine and 3.0 µg/mL (8.1 µM) for voacangine (▶ **Table 1**). IC_{50} median values obtained were very close to the metronidazole IC_{50} of 1.17 µg/mL (6.8 µM) previously reported by us [30]. Considering the CC_{50} values on VERO cells previously reported by our group [21], the SI obtained for the alkaloid fraction of *T. arborea* root bark and the isolated alkaloids ibogaine and voacangine were of 10.14, 252.8 and 13.6, respectively (▶ **Table 1**).

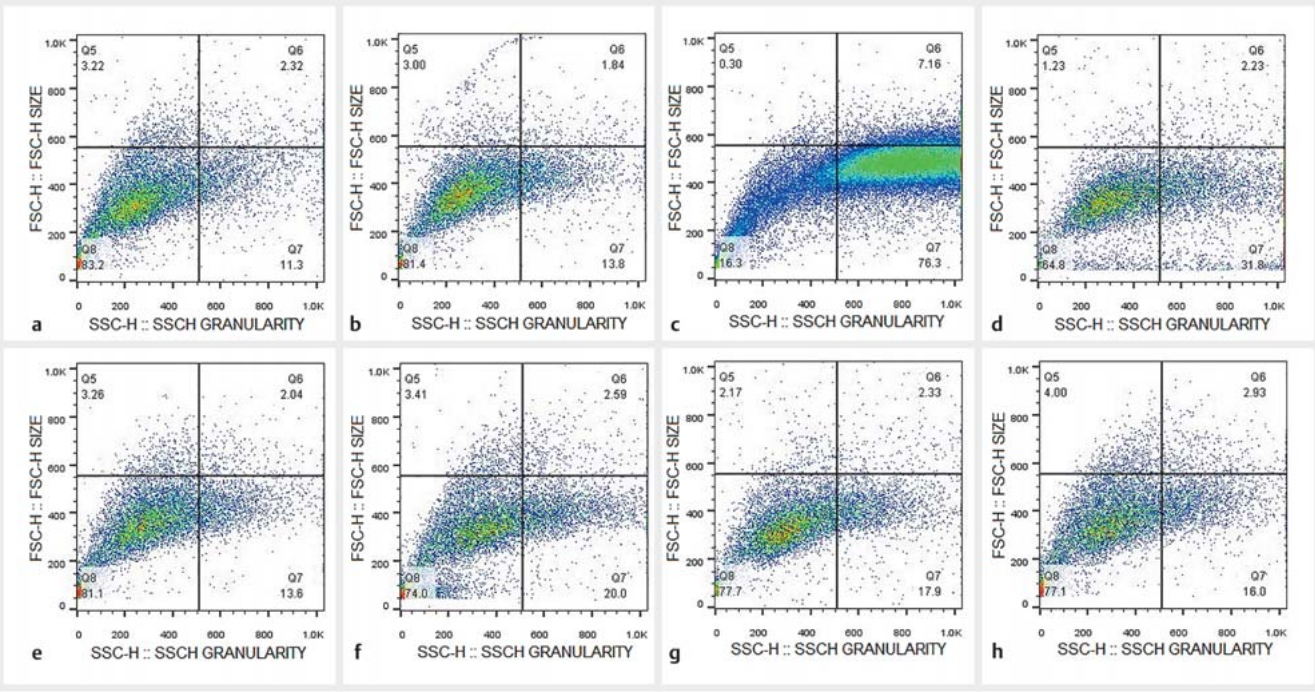
To determine the changes on the amoebic morphology and the type of death induced by the treatments, we analyzed amoebic trophozoites exposed for 24 h to the IC_{50}. ▶ **Fig. 3** shows that trophozoites treated with the *T. arborea* alkaloid fraction or the pure alkaloids exhibited a slight increase in granularity when compared to the DMSO-treated control (from 13% to 16–20%; ▶ **Fig. 3e** vs. **f–h**). This increase was small when compared to the increase in granularity induced by the heat and metronidazole treatments (76 and 31.8%, respectively; ▶ **Fig. 3c** and **d**). On the other hand, increase in cellular size was only observed in heat-treated trophozoites (▶ **Fig. 3c**). No differences in size and granularity were observed between non-treated amoebae (stained with Annexin or propidium iodide; ▶ **Fig. 3a** and **b**, respectively) and the vehicle control (DMSO; ▶ **Fig. 3e**). ▶ **Fig. 4** shows that trophozoites treated with the alkaloid fraction exhibit a significant percentage of cells dying by apoptonecrosis (late apoptosis) and necrosis (11.5 and 7.35%, respectively; ▶ **Fig. 4f**) when compared to the DMSO treated control (0.84 and 1.10%; ▶ **Fig. 4e**), and even when compared with the heat and metronidazole treated amoebae (▶ **Fig. 4c** and **d**). In contrast, the treatment of trophozoites with the pure alkaloids did not induce a clear pattern of cell death (▶ **Fig. 4g** and **h**). However, ibogaine induced the same level of apoptonecrosis that metronidazole (around 1.8%), suggesting a similar mechanism of action (▶ **Fig. 4g** vs. **d**).

Considering that the alkaloid fraction from *T. arborea* and ibogaine and voacangine showed a potent *in vitro* anti-amoebic activity comparable to that of metronidazole, we decided to evaluate its effect *in vivo* on the development of amoebic liver abscesses in hamsters, an animal model widely used for this purpose [31]. Intraperitoneal administration to hamsters of each treatment one day previous, and two days after the intraportal challenge did not inhibit the development of liver abscesses in any animal (▶ **Fig. 5**). In detail, the 15 treated hamsters (5 per group) developed numerous large abscesses throughout the four liver lobules, indistinguishable from those of non-treated, but infected, animals. No differences were also scored by weighing the complete livers nor evaluating the abscesses dissected from them (data not shown). However, histopathological analysis on tissue sections from treated hamsters revealed lower number of trophozoites in the alkaloid fraction-treated and alkaloids-treated animals compared to the control DMSO-treated animals (Compare ▶ **Fig. 5a** vs. **b–d**). Despite this, tissue sections of all treated hamsters showed large necrotic zones surrounded by a ring of intense inflammatory infiltrate mainly composed of neutrophils, lymphocytes and few macrophages, a pattern like that observed in the tissue sections of untreated, but infected, animals (▶ **Fig. 5**).

▶ **Table 1** IC_{50} values of *T. arborea* alkaloid fraction and alkaloids ibogaine and voacangine on the viability of *E. histolytica* cultures.

Alkaloid	Molar mass (g/mol)	IC_{50} (µg/mL)	IC_{50} (µM)	CC_{50}* (µg/mL)	SI
Alkaloid fraction	ND	3.5	ND	35.5	10.14
Ibogaine	310.44	1.4	4.5	354	252.8
Voacangine	368.48	3.0	8.1	40.8	13.6
Metronidazole	171.16	1.17	6.8	> 1000	> 854.7

* Cytotoxic Concentration 50 previously reported in [21], except for metronidazole, which was previously reported by us in [30]; SI: Selectivity Index (CC_{50}/IC_{50}); ND: Not determine

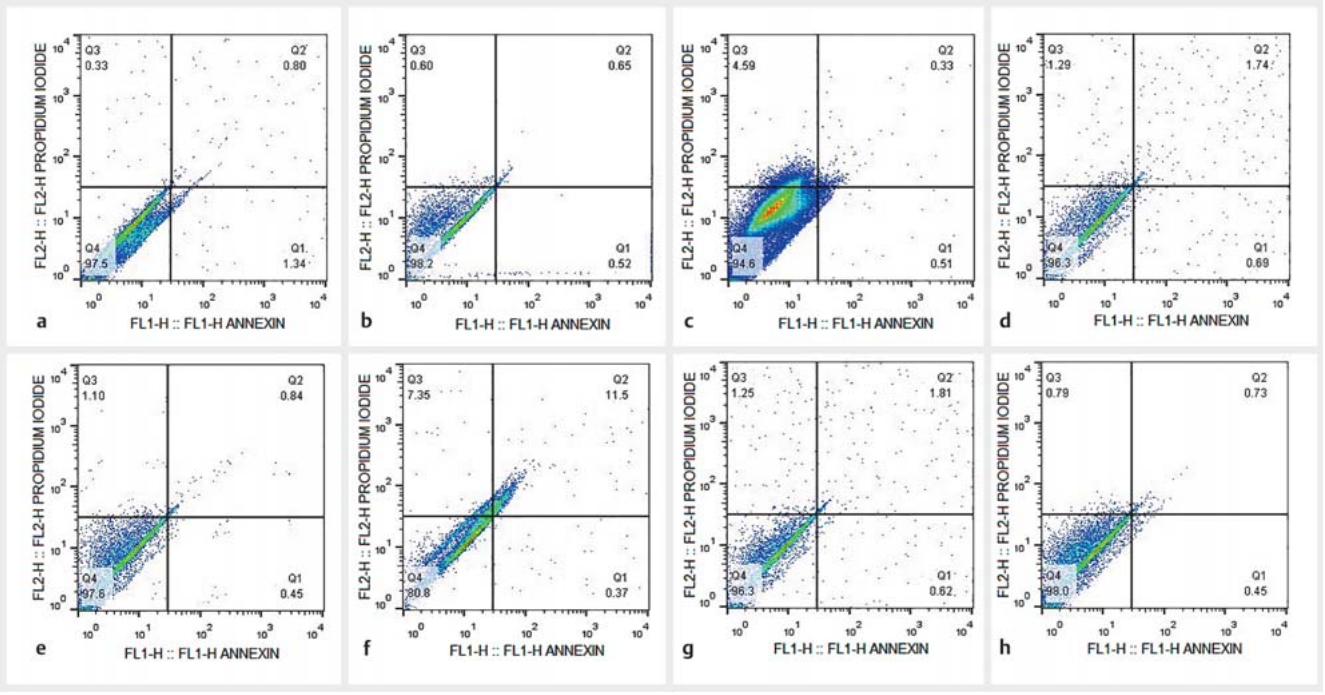


▶ **Fig. 3** Changes on amoebic trophozoites morphology induced by *T. arborea* alkaloid fraction and isolated ibogaine and voacangine evaluated at 24 h post-exposure. Trophozoites (10^5/mL) were cultured in the presence of previously determined IC_{50} values for 24 h at 37 °C and then analyzed by flow cytometry in a FACSCalibur. **a** and **b** untreated amoebae stained with Annexin or propidium iodide, respectively. **c** and **d** amoebae exposed to heat or metronidazole, respectively. **e** amoebae exposed to DMSO as vehicle control. **f–h** amoebae treated with *T. arborea* alkaloid fraction or the pure alkaloids ibogaine and voacangine, respectively. The results shown are representative of three independent experiments.

Discussion

Amoebiasis continues to be one of the most frequent parasitic diseases in the world and a public health problem in developing countries. Although treatment with metronidazole is usually highly efficient, it has the disadvantage of causing many secondary effects that promote its abandonment [5]. Furthermore, the appearance of drug-resistant strains of amoebae is a latent reality supported by the ease with which resistant cultures are obtained by continuous exposure to increasing concentrations of the drug [9]. As such, amoebiasis requires more efficient control mechanisms, mainly based on the development of new drugs of natural origin.

In this paper, we show that both the alkaloid fraction and its alkaloids ibogaine and voacangine, from the plant *T. arborea,* exhibit a potent *in vitro* anti-amoebic activity, almost comparable with metronidazole, the drug of choice for treatment of intestinal amoebiasis. Anti-parasitic properties of plant extracts of several species of the genus *Tabernaemontana* have previously been reported as mentioned in the introduction. However, few studies on the anti-amoebic activity of *Tabernaemontana* extracts are available. In a survey of herbalists in a central Kenyan town, it was reported that based on their empirical knowledge, extracts of *Tabernaemontana pachysiphon* are effective against amoebiasis [32]. Studies in India [33] demonstrated that *Tabernaemontana spp.* roots have also been reported to be anti-amoebic, although



▶ **Fig. 4** Evaluation of apoptosis/necrosis death induced by *T. arborea* alkaloid fraction and isolated ibogaine and voacangine evaluated at 24 h post-exposure. Type of cell death was determined using the FITC Annexin V-Sytox green assay. Trophozoites (10^5/mL) were cultured in the presence of previously determined IC_{50} values for 24 h at 37 °C and then analyzed by flow cytometry in a FACSCalibur. **a** and **b** untreated amoebae stained with Annexin or Sytox-green for basal autofluorescence and necrosis, respectively. **c** and **d** amoebae exposed to heat or metronidazole, respectively. **e** amoebae exposed to DMSO as vehicle control. **f–h**, amoebae treated with *T. arborea* alkaloid fraction or the pure alkaloids ibogaine and voacangine, respectively. The results shown are representative of three independent experiments.

no information was provided on effective concentrations for the extracts. In a unique experimental antique report on the effect of ethanolic extracts from 15 *Tabernaemontana* species from Africa, Asia, and Guyana, on *E. histolytica* DU strain, it was found that extracts from leaves and root and stem barks of all species showed significant activity killing most amoebae at 4.5 mg/mL, whereas extracts from only three species, stem barks of *T. contorta*, stem barks of *T. penduliflora,* and leaves of *T. psorocarpa*, showed some activity at 0.45 mg/mL [34]. They showed that the active substance from leaves, which does not contain alkaloids, was the seco-iridoid sweroside. In contrast, the compounds responsible for pharmacological activity in the extracts of stem barks were not identified; however, they suggested these could be the alkaloids [34].

To our knowledge, our work is the first report regarding the anti-parasitic activity of *T. arborea*. Here we show that the alkaloid fraction and two MIAS from the root bark have potent anti-amoebic activity, inhibiting the growth of cultures after 24 h post-exposure with a striking IC_{50} lower than 4 µg/mL (4.5 and 8.1 µM for ibogaine and voacangine, respectively). In addition, the alkaloid ibogaine showed a selectivity index (SI) of 252.8, which was very high in comparison to the SI of the alkaloid fraction and voacangine, suggesting that ibogaine is of low toxicity to mammalian cells. Although our study is not completely comparable with those mentioned above, since different strains of amoebae and extracts of different plant species were used, alkaloids of *T. arborea* seem to be much more active in killing amoebae at concentrations that were 2 to 3 orders of magnitude lower. It is worth emphasizing that both the alkaloid fraction and the isolated MIAs ibogaine and voacangine exerted anti-amoebic effects almost comparable to that of metronidazole (IC_{50} 6.8 µM), the drug of choice for the treatment of amoebiasis. However, the SI of metronidazole was much higher than that of the compounds tested, suggesting that it is even less toxic to VERO cells. There are few cases in the literature of compounds with amoebicidal activity equal to or close to metronidazole, with auranofin being perhaps the most recently known compound [35], which has even passed phase I clinical trial [36].

In a previous report, our group identified MIAs ibogaine and voacangine in *T. arborea* root bark methanol extract [21]. In contrast to the moderate antimycobacterial activity shown by these alkaloids (IC_{50} between 16 and 40 µg/mL), both showed potent amoebicidal activity, causing the death of all parasites in 24 h at a concentration of 10 µg/mL and yielding an IC_{50} between 1.4 and 3.5 µg/mL, as mentioned above. It is worth mentioning that the anti-amoebic effect was lost between 48 and 72 h post-exposure at concentrations below 10 µg/mL, suggesting their processing to inactive metabolites. Anti-bacterial and anti-fungal activity of ibogaine and voacangine has been widely reported [15]. In contrast, anti-parasite activity has been scarcely studied. As with the amoebae, voacangine and ibogaine have been shown to be highly effective against microfilariae and male adults of *Oncho-*

cerca ochengi [37]. Moreover, it has been shown that a *Tabernaemontana catharinensis* extract, rich in voacangine (53 %) exhibited potent antileishmanial activity against *L. amazonensis* [38]. Other alkaloids of natural origin, such as those related to emetine, have been shown to have a powerful anti-amoebic effect, but most of their studies have focused only on their *in vitro* effects [39]. Of them, for instance, 3-isocorynantheol has been recorded in PubChem as anti-amoebic with the AID 1 097 036 (https://pubchem.ncbi.nlm.nih.gov/bioassay/1097036).

Our results also showed that ibogaine and voacangine from *T. arborea* can cause the death of *E. histolytica* trophozoites by rapid or late necrosis (apoptonecrosis), suggesting that they could cause damage to the cell membrane. However, the cell targets through which they exert their anti-amoebic effect are unknown and further studies are needed to identify the possible receptor(s) for the alkaloids, as well as the intracellular changes they induce. Similar alkaloids, like cinchona, has been reported to possess anti-malarial activity acting by a mechanism alike to chloroquine, inhibiting the crystallization of the heme group in the digestive vacuole of the parasite [40]. Taken together, these findings point to the possibility of using alkaloids as anti-parasitic agents, particularly as anti-amoebics.

Since the effect of *T. arborea* alkaloid fraction, ibogaine, and voacangine was comparable to that of metronidazole, we decided to evaluate the ability of these compounds to inhibit the development of amoebic liver abscesses in hamsters. The results showed that although ibogaine and voacangine appear to reduce the number of parasites seen in histological sections of livers, abscess formation from the infection was not affected. This suggests that the remaining amoebic trophozoites were enough to promote abscesses by triggering and inflammatory responses that usually contribute to the pathology, as previously reported [41]. The reason for the lack of protection is unknown but could be related to the speed at which the alkaloids are catabolized in the blood of animals before they can reach the liver from the peritoneal cavity, where they were inoculated. In this regard, it has been reported that the half-life of ibogaine in rat plasma is only 2 h, rapidly o-demethylated to form the metabolite 12-hydroxyibogamine (noribogaine) [42]. Conversely, a low bioavailability of voacangine on rats has also been reported [43]. With this possibility in mind, we conducted trials administering the alkaloid fraction of *T. arborea* root bark, ibogaine, and voacangine via the vena cava using an indwelling catheter. However, the results were like those obtained when the compounds were administered by intraperitoneal route (data not shown). Since ischemia is characteristic of amoebic liver abscesses [44], studies on the bioavailability of these compounds to the liver and the possible processing of them by amoebae are warranted to understand the cause of their lack of protective effect. Furthermore, trials on the effect of these compounds on intestinal amoebiasis and *in vivo* cytotoxicity studies on human cells are also encouraging, to determine the potential of *T. arborea* MIAs ibogaine and voacangine as a new anti-amoebic drug.

Material and Methods

Plant material and preparation of alkaloid fraction

Tabernaemontana arborea Mill. was collected from the roads leading to the "Estación de Biología Tropical Los Tuxtlas" in the state of Veracruz, México. A voucher specimen was deposited at the herbarium of the Facultad de Ciencias (FCME), UNAM (voucher number 133 359) [29].

Crude extracts from the root bark were obtained by extraction with methanol, from which the alkaloid fraction was obtained by acid-base extraction and analyzed by GC-MS, and HPLC as described in [21], and where the chromatograms can be consulted. Pure ibogaine and voacangine kindly donated by Phytostan Enterprises, Inc. were used as external standards in these analyses.

Stock solutions

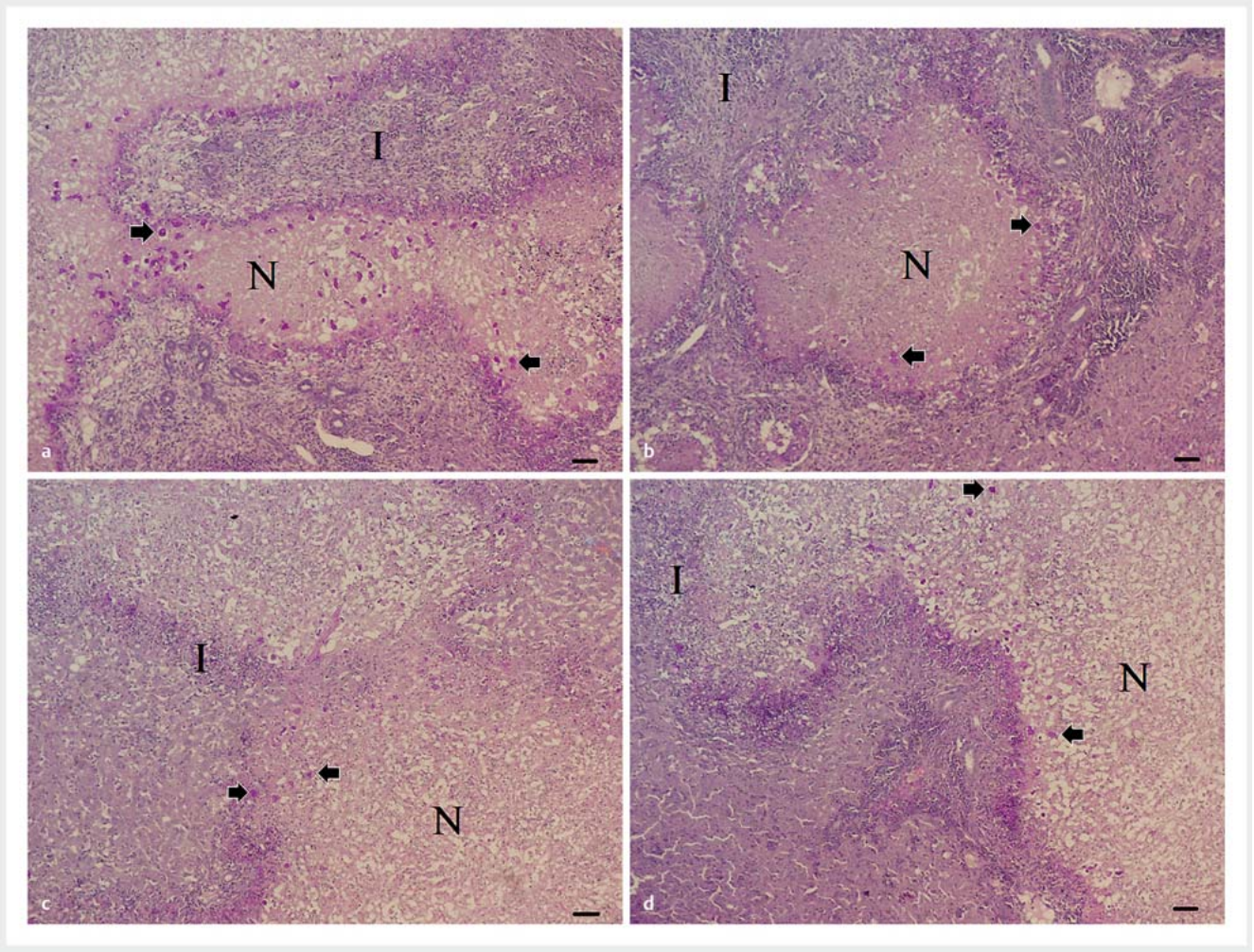
The stock solutions used in this work were prepared by solubilizing 10 mg of alkaloid fraction, ibogaine or voacangine in 1 mL of DMSO. All subsequent dilutions were also made in DMSO, which never exceeded 0.2 % in the tests, a concentration previously shown that does not affect the growth of *E. histolytica* trophozoites (data not shown).

Cultures of Entamoeba histolytica trophozoites

Axenic *E. histolytica* trophozoites of HM1:IMSS strain (originally isolated from a patient in México) were maintained in TYI-S-33 medium supplemented with 15 % adult bovine serum (Microlab) and 3 % Diamond vitamins (SAFC, Biosciences) at 37 °C in anaerobic conditions. Trophozoites grown for 72 h (log-phase) were harvested by ice-chilling for 5 min and centrifugation at 150 × g for 5 min at 4 °C. Trophozoite' virulence was maintained through successive passages in golden hamster livers and then recovering parasites from the induced amoebic abscesses [31].

Evaluation of activity on trophozoite cultures

Viability of *E. histolytica* trophozoites was performed, as previously described [30]. In brief, trophozoites were seeded in 96-well culture plates at 10^4 cells/100 µL of fresh TYI-S-33 medium. *Tabernaemontana arborea* alkaloid fraction, ibogaine, or voacangine were added at concentrations between 0.1–10 µg/mL. Once added, the plates were incubated for 72 h at 37 °C and the viability determined each 24 h by taken an aliquot and performing an MTT assay. In brief, 80 µL of MTT/well (1 mg/mL) was added and the plates were incubated for 1 h at 37 °C in darkness. Thereafter, the plates were centrifuged at 400 × g for 5 min and the supernatant removed to preserve a volume of 100 µL per well. Each well was added with 100 µL of 60 °C heated sodium dodecyl sulphate : hydrochloric acid (15 % SDS, 0.01 N HCl) and then homogenized to dissolve formazan salt. Finally, the absorbance was measured within 15 min at 595 nm using a Synergy HTX (BioTek) plate reader. The IC_{50} was obtained using the web IC_{50} calculator AAT Bioquest, at https://www.aatbio.com/tools/ic50-calculator (version 2021). The SI was obtained by dividing the CC_{50} of the alkaloid fraction of *T. arborea* root bark and the isolated alkaloids ibogaine and voacangine on VERO cells at 24 h over the respective IC_{50}.



▶ **Fig. 5** Effect of *T. arborea* alkaloids on the infectivity of amoebae. Golden hamsters were intraperitoneally treated with *T. arborea* alkaloid fraction (20 mg/Kg) or isolated alkaloids ibogaine and voacangine (10 mg/Kg) every other day, for 3 days, starting 1 day prior to infection. Animals were infected by intraporal route with *E. histolytica* trophozoites (10^6 parasites) and the development of amoebic liver abscesses evaluated 7 days later. Tissue sections from livers were obtained and stained with haematoxylin-eosin. Representative images of livers from DMSO (**a**), *T. arborea* alkaloid fraction (**b**), ibogaine (**c**), and voacangine (**d**) treated hamsters are shown. Arrows show amoebic trophozoites; N: necrotic areas; I: inflammatory infiltrates. All images were taken at 10 × magnification. Bars: 100 µm.

Apoptosis/necrosis analysis by flow cytometry

Flow cytometry studies were performed to determine whether *T. arborea* alkaloid fraction, ibogaine, and voacangine alkaloids lead to amoebic death by apoptosis or necrosis. In brief, *E. histolytica* trophozoites were cultured at 10^4 cells per 100 µL of fresh TYI-S-33 medium in 96-well culture plates. Amoebae were treated with the IC_{50} for the alkaloid fraction, ibogaine, or voacangine determined in the viability assays and cultured during 24 h at 37 °C. Trophozoites cultured with metronidazole (10 µg/mL) for 24 h at 37 °C or incubated for 30 min at 56 °C were used as positive controls of death. Treated amoebae were harvested by chilling on ice for 5 min and centrifugation (150 × g for 5 min) followed by two washes with PBS. The parasites were resuspended in 50 µL of 1 × Annexin V binding buffer (BD Pharmingen), and 5 µL of rh Annexin V-FITC (Enzo) and 500 nM Sytox Green (Thermo Fisher) were added. After 20 min at room temperature in darkness, 450 µL of 1 × Annexin V binding buffer was added and the samples analyzed using a FACSCalibur (Beckton Dickinson) flow cytometer. At least 10^4 gated events of each sample were considered. Data were analyzed using software package FlowJo v10 (BD Biosciences) to determine changes in size, granularity, and fluorescence intensity.

Evaluation of activity on amoebic liver abscesses

Male hamsters (*Mesocricetus aureus*) of 6 weeks old and weighing about 100 g were divided into four groups of five animals each (provided by the animal facility of the Faculty of Medicine, UNAM). One group was intraperitoneally injected with 100 µL *T. arborea* alkaloid fraction (20 mg/Kg) and the other two groups with 100 µL ibogaine (10 mg/Kg) or 100 µL voacangine (10 mg/Kg), every other day, for 3 days, starting 1 day prior to infection (− 1, 1 and 3 day). The vehicle in which the samples were prepared (DMSO) was administered to the fourth group as control.

Hamsters were then infected with *E. histolytica* trophozoites as described below. In brief, animals were anesthetized (Anestesal, 60 mg/kg) and the peritoneal cavity opened by surgical laparotomy. The portal vein was exposed and 10^6 axenic trophozoites resuspended in 100 µL PBS were injected into the portal vein bloodstream. The inoculation site was immediately occluded with sterile gel foam, the intestines returned to the peritoneum and the abdominal walls sutured (Vicryl, 4–0). Hamsters were sacrificed seven days post-infection using excess of anaesthesia, and the livers weighed. Fragments of the liver containing abscesses were fixed in 4% paraformaldehyde in PBS and stored in 30% sucrose for histology. Tissue sections of 5 µm were obtained in a microtome and haematoxylin-eosin stained for microscope analysis.

All animals were maintained in a common room under controlled temperature and a 14 h dark/10 h light cycle, and were inspected by the Internal Committee for the Care and Use of Laboratory Animals (CICUAL), Biomédicas, UNAM, ID 273 (date of approval May 2th, 2017). and Governmental agencies to ensure compliance with institutional and federal regulations and international guidelines.

Statistical analysis

Differences between the controls and experimental groups were determined using the software PAST 3.05 (http://www.toyen.uio.no/~ohammer/) with Tukey's and Dunett's tests, whereas the differences between the tested substances and the reference drug were examined by Tukey's test. The IC_{50} and confidence intervals were calculated using the software GraphPad Prism 8.

Contributors' Statement

Data collection: J. C. Carrero, V. Curay-Herrera, L. Chacón-Niño, F. Krengel, S. L. Guzmán-Gutiérrez, M. Silva-Miranda; design of the study: J. C. Carrero, C. Espitia, R. Reyes-Chilpa, J. P. Laclette; statistical analysis: L. C. González-Ramírez, R. Bobes-Ruíz; analysis and interpretation of the data: J. C. Carrero, V. Curay-Herrera, R. Reyes-Chilpa; drafting the manuscript: J. C. Carrero, C. Espitia, R. Reyes-Chilpa; critical revision of the manuscript: J. C. Carrero, R. Reyes-Chilpa, J. P. Laclette.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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