Inhibitory Effect of *Quassia amara* Linn. Crude Bark Extract on *Entamoeba histolytica* in vitro

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ABSTRACT

Background. *Entamoeba histolytica* is an important etiologic agent of diarrhea. Globally, it is estimated to infect 40 to 50 million people and cause 40,000 to 100,000 deaths per year. <local studies> Metronidazole is effective but can cause adverse reactions in certain individuals. In search of alternatives, traditional medicinal plants are being studied. Several plants in Family *Simaroubaceae* have shown anti-amoebic activity. *Quassia amara*, a member of this family, has not been tested.

Objective. To determine the effect of *Q. amara* crude extract on *Entamoeba histolytica* in vitro.

Methods. Initial testing of 10^4 µg/ml ethanolic bark extract was performed. Counts were made after 72 hours. Three trials in triplicates were performed.

Nine (9) dilutions of the extract were then tested (18.8 to 5,000 µg/ml). Test tubes were checked for viable amoeba after 24-hour and 72-hour incubation. Minimum inhibitory concentrations (MIC) were determined for the two incubation periods. At least two trials in triplicates for each dilution were performed. Metronidazole served as positive control.

Results. At 10^4 µg/ml incubated for 72 hours, no viable amoeba was obtained and counted. The MIC after 24 hours was 5,000 µg/ml, while the MIC at 72 hours was 37.5 µg/ml.

Conclusion. *Q. amara* crude extract has inhibitory effects on *E. histolytica* in vitro.

Key Words: Quassia amara, antiprotozoal agents, anti-amebic

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Background

Amoebiasis is an important cause of parasite-related diarrhea especially in countries where living conditions and sanitary systems are inadequate.[1] It is estimated to cause 40,000 to 100,000 deaths a year making it the third most important parasitic disease after malaria and schistosomiasis.[2] The disease is caused by the parasite *Entamoeba histolytica* through its invasion of the intestinal wall. The parasite is frequently transmitted by way of the fecal-oral route and releases parasite-derived hyaluronidases and proteases upon reaching the intestines. These enzymes create ulcerating mucosal lesions that often result in amoebic dysentery and amoebic colitis.[3] The parasite also affects other extraintestinal organs, mainly the liver such as in amoebic liver abscess.[4]

Globally, it is estimated to infect 10% of the population.[5] At one point, it was estimated to kill 700,000 people.[6] The global estimate dates back to 1986 and to date we have not found newer studies on the global burden of the disease. More recent local studies are however consistent with this estimate. In a study conducted by Sevilla and Cross[7] among fecal and serum samples collected from Luzon, Marinduque, Mindoro, Samar, Panay, and Palawan, 5%-6% tested positive for *E. histolytica* by direct fecal, concentration techniques, and indirect hemagglutination tests. Another local study by Rivera W et al. among households in an area of Northern Luzon showed that 8.119% having *E. histolytica* or *E. dispar* cysts. The same study utilized PCR techniques and showed found most cysts where *E. dispar* and *E. histolytica* are actually present in just 0.961% of that population.[8]

Saniel et al.[9] recovered the parasite from 18 out of 339 (5%) children and adults with acute diarrhea consulting at the Research Institute for Tropical Medicine (RITM) hospital from 1985 to 86.5. It is also implicated in amebic liver abscess patients of the Philippine General Hospital and Jose R. Reyes Memorial Medical Center especially in male patients aged 21-50 years old.[10]

Treatment of *E. histolytica* infection can be done through emetine or a nitroimidazole such as the usual choice, metronidazole. Though potent, they are not perfect drugs. Emetine exerts serious side effects by allowing high concentrations to build up in the liver, heart, and other viscera.[6] Metronidazole, meanwhile, is noted to have various side effects such as nausea, bitter taste, epigastric discomfort, diarrhea, vomiting, fatigue, reversible neutropenia, and cutaneous hypersensitivity reactions.[11] There are also case reports of peripheral neuropathy.[12,13] In addition, nitroimidazoles as a class are genotoxic and cytotoxic to human lymphocytes as tested in vitro.[14] Its use in pregnant and lactating women is also contraindicated due to some possibility of acting as a teratogen.[15]

With limited drug choices, drug resistance is an ever-present threat. Upcroft P and Upcroft J[6] cited an unpublished report indicating an increasing number of isolates that are less sensitive to metronidazole in Chandigarh, India. They have also successfully induced resistance in laboratory grown amoeba. These warrant a continuous search for a drug that would balance safety and efficacy against *E. histolytica*.

Traditional medicinal plants are often used in search of lead compounds for new drugs. Several local studies have tested plants for anti-protozoal in general and anti-amebic properties in particular. *Chromolaena odorata* and *Uncaria perrottetii* were shown to have effects on the growth of two protozoans, *Blastocystis hominis* and *Trichomonas vaginalis*.[16] *Ficus septica*, *Sterculia foetida*[17] and *Voacanga globosa*[18] have been shown to affect the growth of *T. vaginalis* and *E. histolytica*.

In this study, we look into the Family *Simaroubaceae*, members of which have been traditionally used in Mexico, China, and Ethiopia for the treatment of dysentery.[19] *Brucea antidysenterica*, *Brucea javanica* fruits and seeds, and *Simarouba amara* stem[20] were previously tested and were found to be effective against *E. histolytica*. Plants from this family also contain the quassinoid compounds ailanthone and glaucarbine which showed potent activity against the dysentery amoeba, *Entamoeba histolytica*, both in vitro and in vivo.[19]

Quassia amara (also known as "korales") is a locally available member of *Simaroubaceae*. It is a perennial tree or shrub with small red-tinged flowers and red oval fruits.[21] It is traditionally used as antipyretic, insect repellant, lice treatment, and anti-malarial.[22] It has also been found to have insecticidal[23] properties and antiviral effects on the human immunodeficiency virus.[24] Quassinoids from the plant and its relatives have been shown to have in vitro antimalarial activities.[25] Searching databases like PubMed and Herdin.ph showed that this plant has yet to be tested for its anti-amoebic activity. The anti-amoebic activity shown by the other family-related species may be a lead to finding other compounds found in *Q. amara* that could show the same activity against *Entamoeba histolytica*. The crude bark extract of *Q. amara* will be tested for anti-amoebic activity.

The life cycle of *E. histolytica* features two main morphologic forms: the lumen-dwelling trophozoite and the infective cyst. Notably, only trophozoite forms are observed in vitro. This phenomenon is useful in screening for the effects of substances against *E. histolytica* as it allows the use of simple light microscopy to differentiate viable from nonviable amoeba by considering only trophozoites as the viable amoeba. Inducing the cystlike structures in vitro requires special conditions and has been the subject of research. This state has been successfully induced in a recent study by Aguilar-Diaz et al.[26] Their group also cited other alternative methods required the use of CO2, intestinal enzymes or enteric bacteria.

Materials and Methods

Extract preparation

The bark of *Quassia amara* Linn. was collected and identified by the botanist from the Jose Vera Santos Herbarium, Institute of Biology, UP Diliman. A voucher sample was also sent to the herbarium of the National Museum.

The bark was air-dried for two weeks, homogenized using an osterizer and then soaked in 95% ethanol for 5 days. The mixture was filtered and the ethanolic filtrate was sent to the Department of Biochemistry and Molecular Biology, College of Medicine, University of the Philippines (UP) Manila for evaporation via rotary evaporator. A 5% (g/mL) stock mixture of the extract prepared by dissolving the extract in TYI-S-33 medium. This mixture was then sterilized by passage through a 3.5 μm microfilter. The crude extract and stock mixtures were stored at 4^0C.

Microorganism

E. histolytica HK-9 strain was obtained from the Molecular Protozoology Laboratory, Natural Science Research Institute, UP Diliman. The trophozoites were cultured axenically in complete medium or TYI-S-33 medium supplemented with 10% bovine serum, 40x Vit mixture and antibiotic (Pen-Strep) at 34.5 $^{\circ}$C

Pilot Study

A pilot study based on the procedure of Vital et al.[17] was performed to screen for the effect of *Q. amara* crude extract on the viability of *E. histolytica*. The amoebae were subjected to three treatments: (1) 1% (10^4 μg/ml) *Q. amara* crude extract (2) positive control (metronidazole at 17.1 μg/ml), (3) negative control or blank containing complete medium described in the previous section. Amoebae, at a concentration of 1.0 x 10^3 cells/ml, were incubated at 34.5^0C for 72 hours.

After 24 hours of incubation, tubes were viewed under scanning view of light microscope to check for viable amoebae. After 72 hours, the concentration of viable amoebae was determined using a Neubauer counting chamber. Counters were blinded to the treatment groups. Each treatment was done in triplicate. Three trials on three separate occasions were performed.

Anti-amebic assay

To determine the minimum inhibitory concentration (MIC), amoebae (10^3 cells/mL) were cultured with various concentrations (5,000, 2,500, 1,300, 600, 300, 150, 75, 37.5, 18.8 μg/ml) of *Q. amara* extract. After 24-hour and 72-hour incubation, the tubes were scanned for living amoebae. In similar studies, peak number of viable amoebae or growth was noted at 72 hours of incubation and the point where the effect of any substance on growth and viability would probably be most apparent. Results were reported as positive or negative for viable amoebae. Viewing and assessment of tubes were performed under the supervision of the research staff of Department of Parasitology of the College of Public Health.

Viable amoebae (Figure 1a) were distinguished from nonviable amoebae (Figure 1b) by the three criteria: shape, attachment, and motility. Amoebae need to meet the three criteria in order to be labeled viable. Viable amoebae are generally ovoid but can be pleomorphic. They are motile and have a finer granular texture (as if filled with sand). In general, nonviable amoebae resemble cyst-like structures being spherical with a coarse granular texture but do not contain nucleoli. They are often found floating in the medium. During assessment, only viable amoebae were considered (Table 1, Figure 1). Wong et al.[27] also described viable amoebae similarly in their study using *E. histolytica*.

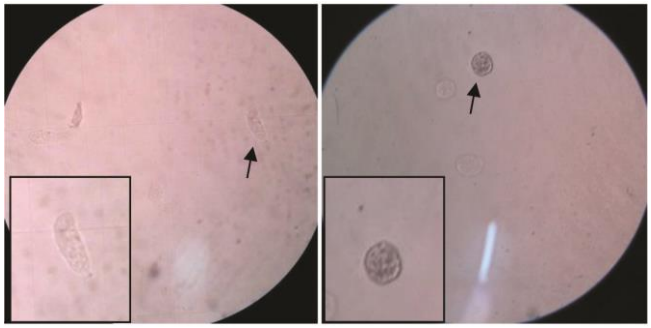
MIC is the lowest tested concentration wherein no viable amoebae was detected upon viewing of the tubes. Two MICs were determined, one for 24 hours (MIC24) and one for 72 hours (MIC72). At least two trials were performed for each concentration. Treatments per trial were performed in triplicate.

Determining the effect of ethanol on the viability of amoebae was necessary since it was the solvent used to obtain the crude extract. To check the effect of ethanol, amoebae were cultured with ethanol at a concentration equal to the determined MIC24.

Blank tubes are tubes where ameobae are grown in complete medium only. In tubes containing extract, ethanol or metronidazole, the amount of nutrient broth used was reduced by the volume of extract solution needed in order to maintain a maximum volume of 8.0 ml. Volume of nutrient broth was also adjusted according to the volume of amoeba inoculum needed. Maximum inoculum volume limit was set at 2.0 ml.

Table 1. Criteria for Differentiating Viable and Nonviable Amoeba

	Viable	Nonviable
Shape	Ovoid or pleomorphic	Round or Spherical
Motility	Motile	Non-motile
Attachment To Test tube wall	Attached	Usually floating in singles or clumps



A **B**

Figure 1. Morphology of viable and nonviable *E. histolytica* trophozoites (arrows) (100x). *E. histolytica* (arrows) loaded into counting chamber. Blown-up images of the amoeba are placed in the lower left corner. A is viable amoeba while B is nonviable. Viable amoeba are generally ovoid but can be pleomorphic. They are motile and have a finer granular texture. Nonviable amoeba are spherical with a coarse granular texture.

Disposal

After the experiment, Inoculated media, test tubes, and other materials were autoclaved. Media was then collected and disposed with other infectious waste of the Department of Medical Microbiology of the College of Public Health.

Results

One percent (1%) *Q. amara* extract inhibited the growth of *E. histolytica* after incubation for 72 hours. At 24 hours, no viable amoeba was detected in the 1% *Q. amara* and metronidazole on scanning of tubes under light microcope. Mean growth in the blank tube was 1,853 cells/ml (SD: 1821) after 72 hours. No amoeba was loaded into the counting chamber for both 1% *Q. amara* and Metronidazole.

Table 2 shows the results of incubation of amoebae with the extract after 24 hours and 72 hours. MIC24 is 5,000 ug/ml and MIC72 is 37.5 ug/ml. Viable amoebae were detected after 24-hour incubation at tested concentrations less than 5,000 μg/ml and after 72 hours at 18.8 μg/ml. The tubes at these concentrations were not highly colored by the extract (Figure 2).

Table 2. Growth and pH in different dilutions of *Q. amara* crude extract (in μg/ml)

	Blank	EtOH	Met	10^4	5 000	2 500	1 300	600	300	150	75	37.5	18.8
24h	+	+	–	–	–	+*	+*	+*	+	+	+	+	+
72h	+	+	–	–	–	–	–	–	–	–	–	–	+
pH	6.98	#	#	6.95	6.97	6.96	6.95	6.97	6.81	6.97	6.98	6.82	6.98

EtOH – ethanol, Met - Metronidazole
* - One tube in one of the trials, showed no growth of amoeba.
# - pH was not measured

Figure 2. Dilutions of *Q. amara* crude extract in complete medium. Tubes are arranged from least concentrated (blank, 0 μg/ml) to most concentrated (1,000 μg/ml). Marked tubes are those at MIC concentrations, the first marked tube from the left is MIC 72 (37.5 μg/ml) and the second is MIC 24 (5,000 μg/ml).

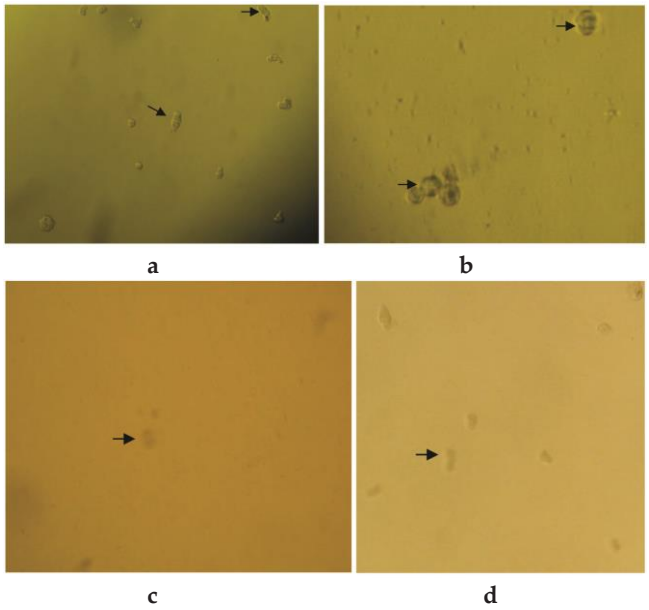


Figure 3. Amoeba grown under different conditions (100x). Arrows point to some of the amoeba in the picture. (a) Amoeba in blank (0 μg/ml) tubes after 72h Under scanning lens, amoeba are refractile oval or amorphous motile bodies attached to the test tube walls. Amoeba are found in the bottom part of the test tube due to preference for microaerophilic conditions. (b) Nonviable amoeba in 5,000 μg/ml after 72h. No change in shape was observed. (c) Viable Amoeba in 2,500 μg/ml after 24h. Growth was sluggish. (d) Viable amoeba in ethanol (600 μg/ml) after 72h.

It should be noted that in one replicate of the 5,000 μg/ml, there were traces of attached nonviable amoebae. They were assessed as nonviable because of the lack of motility and difference in the texture of the cells (Figure 3b). At 2 500 μg/ml, the amoebae were noted to be very sluggish and less ovoid in shape (Figure 3c) compared to blank tubes (Figure 3a). One replicate had no detectable amoeba. At 600 and 1,300 μg/ml, there seemed to be less viable amoebae compared to the negative control. There were also replicates that had no detectable amoeba. Based on the scan, less viable amoebae were also seen at 18.8 μg/ml compared to the negative control. The difference not confirmed via quantification.

The evaluation of the effect of ethanol on amoebic growth was done qualitatively. There were viable amoebae in ethanol (600 μg/ml) both after 24 hours and 72 hours of incubation. The morphology of amoebae incubated in ethanol was the similar to the blank tubes (Figure 3d). The level of growth in ethanol seemed lower than that in the blank tubes; however, the difference was not quantified using the counting chamber because there was difficulty loading the amoeba despite multiple attempts.

Percent yield using bark of *Q.amara* was 2.604%. Most of the pH of the dilutions lie in the range of 6.95–6.98. The dilutions 300 and 37.5 μg/ml, had a pH of 6.81 and 6.82, respectively.

Discussion

Results showed that *Q. amara* crude bark extract may have anti-amoebic activity specifically on motility, shape and attachment. This activity may be due to several quassinoid compounds such as simaliklactone D, picrasin B, picrasin H, neoquassin, quassin, picrasin I, and picrasin J.[25] This finding strengthens the claim that members of *Simaroubaceae* have anti-amoebic activity. *Q,amara has* been found to be active in vivo against *Plasmodium berghei*.[28,29] This anti-malarial activity was attributed to simalikalactone D.[30] This supports the observation of Wright et al.[20] that anti-amoebic property parallels with anti-malarial property.

We hypothesize that the extract has effects on the motility of the amoebae and their ability to undergo division. This would explain the difference in growth in extract-containing media compared to blank tubes. The hypothesized effects also explain the sluggish movement observed at 24 hours after incubation with 600–1,300 μg/ml extract.

Changing the pH and osmotic pressure of the external environment can adversely affect the growth, motility, and survival of amoeba. These changes, though, are insufficient to explain continued inhibition of growth at lower concentrations tested. The pH is not related to extract concentration, making it an insignificant factor. Increased osmotic pressures and turbidities may cause cell lysis or other cellular changes, but concentrations that are not turbid or grossly indistinguishable with the negative control also show anti-amoebic activity.

Since the extract was not lyophilized, traces of ethanol might still be present. One study[20] showed that ethanol has no inhibitory effect on amoebic growth at 0.25% concentration. Similarly, our results show that ethanol at 5,000 μg/ml is not sufficient to kill amoeba. However, it still seems to exert some inhibitory effect in our case. Although the level of ethanol tested would not reflect what is actually in the extract, we established that ethanol can affect growth but is insufficient to kill all amoeba. Whether the extract without ethanol can inhibit amoebic growth cannot be conclusively shown through this study but the extract seems to be a necessary factor in inhibiting growth and causing amoebic death. The actual amount of ethanol contained in the crude extract is expected to be less than that in the ethanol only treatment. We observed that in the ethanol only set-up, there was still positive amoebic growth. Therefore, it is valid to attribute the death of amoeba in the crude extract treatment to the extract itself. Testing the lyophilized extract can also be done to conclusively prove that the extract alone has anti-amebic activity.

Limitations

We were unable to quantify the effect of the extract on the growth of amoeba. Similar studies such as those of Cedillo-Rivera et al.[31] and Vital and Rivera[17] often determine the IC50 for plant extracts and substances but this requires quantification of amoeba or plotting of growth curves through the period of incubation. However, the group found it difficult to grow sufficient amoeba that can allow reliable determination using counting chambers. We also had experiences that despite observing a number of living amoebae attached to the test tube walls, no amoebae was loaded into the counting chamber. If this quantification technique was used, amoeba concentrations of zero will be used to describe even replicates that have visually confirmed living amoebae. Due to this, we opted to use MIC since it relies only on the presence or absence of trophozoites in the test tubes.

Conclusion and Recommendations

Q. amara crude extract may have anti-amoebic properties against *Entamoeba histolytica* trophozoites in vitro. MIC24 is 5,000 μg/ml and MIC72 is 37.5 μg/ml.

As bark is not a sustainable source of the extract, it is recommended to test the leaves of *Q.amara* for anti-amoebic activity. Future studies can focus on determining the IC50 of the extract and its active fractions and molecules. It is also suggested to employ high throughput methods such as the microdilution method used by Wright et al.[20] for these future *in vitro* studies. Dye exclusion techniques such as the used by Radvin and Skilogiannis[30] can be used to aid in identification of viable from non-viable trophozoites. In other studies also, viability was confirmed not just by microscopy but by subculture method which assesses if the amoeba retained ability to multiply. These types of methods may be able to address the limitations in quantification we encountered. In vivo testing can also be performed using the crude extract. Studies on the possible mechanisms of action can also be performed.

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