Philippine Tsaang Gubat (*Ehretia microphylla* Lam) and Ampalaya (*Momordica charantia* L.) Leaf Extracts Lack Amoebicidal Activity *in vitro*

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ABSTRACT

Background. Amoebiasis is a global health problem affecting poor regions in the world. Few drugs such as metronidazole are available to treat this disease; unfortunately, it is associated with several serious side effects. Tsaang gubat and ampalaya have been used by traditional healers from different cultures to treat dysentery.

Objective. The aim of this research was to provide evidence to validate the use of tsaang gubat and ampalaya leaf extracts for dysentery by determining their anti-amoebic activity.

Methods. The tsaang gubat and ampalaya leaves were sourced from the University of the Philippines at Los Baños and processed into a lyophilized aqueous extract. Anti-amoebic activity was determined in an *in vitro* assay using *Entamoeba histolytica* HK-9 strain against 10 dose levels (18-10,000 µg/mL). The amoeba and leaf extracts were incubated for 24, 48, and 72 hours. The trophozoites were stained with Trypan blue and dispensed into chambers of a Neubauer hemocytometer. The live trophozoites (unstained) were counted under a binocular microscope. The MIC and IC_{50} were determined. Metronidazole and DMSO served as positive and negative controls, respectively.

Results. Tsaang gubat and ampalaya leaves failed to show anti-amoebic activity and even had increased growth of amoeba at all dose levels. The IC_{50} of tsaang gubat and ampalaya leaf extracts were >500 µg/mL at 24, 48, and 72 hours. Metronidazole was able to eradicate the amoeba parasite at 24 and 72 hours, while exposure to DMSO did not result in inhibition nor death of the parasite.

Conclusion. Tsaang gubat and ampalaya aqueous leaf extracts did not exhibit any anti-amoeba activity.

Key Words: Ehretia microphylla, Carmona retusa, tsaang gubat, Momordica charantia, ampalaya, anti-amoeba, antiparasitic

INTRODUCTION

Plants have been used as therapy for thousands of years by numerous cultures. Plant products could provide boundless prospects for new drugs or drug leads because of their chemical diversity.[1] Many modern day drugs are plant-based or plant-derived. These include treatments such as quinine and artemisinin for parasitic diseases. Knowledge and experiences of traditional healers are important clues to the potentials of these plants as was seen in the 2 earlier mentioned anti-malarial agents.[2] Numerous compounds from traditional herbal medicines may serve as scaffolds for rational drug design.[3] Research exploring plants which are used by traditional healers as therapy for infectious diseases are worth investing in. We are also more familiar with the safety and tolerance of traditional herbal products

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as compared to new chemical entities wherein the risks are usually higher. Traditional medicine-inspired reverse pharmacology research has already resulted in faster usable products and with lower financial costs compared to synthetic drugs.[4]

Amoebiasis affects millions of people worldwide by causing diarrhea, dysentery, and extraintestinal abscesses. This disease leads to approximately 100,000 deaths annually.[5] Available pharmacologic therapy for amoebic colitis, which is the most common form of the disease is metronidazole. This drug is decades old and numerous adverse effects including headache, nausea, vaginitis, metallic taste, and influenza-like symptoms in more than 5% of patients may be experienced.[6] The rare but serious side effects of metronidazole include encephalopathy, peripheral neuropathy, Stevens-Johnson syndrome, and aseptic meningitis.[7] Cheap, well-tolerated, and safe alternatives need to be sought for the treatment of amoebiasis. Unfortunately, since this infection affects poor regions of the world, it is not a priority disease for drug development by most pharmaceutical companies.[5]

Tsaang gubat (*Ehretia microphylla* Lam. family Boraginaceae [synonym *Carmona retusa* (Vahl) Masam.]) is an erect, branched shrub found from Batan Island, Northern Luzon and in most provinces in Mindanao. Other common names of *Ehretia microphylla* are "alangit," "buyo-buyo," Philippine tea tree, and Fukien tea tree.[8] It can grow to 1.5-4 meters high with its leaves growing in clusters, lobed near the apex and pointed at the base.[8] The leaves have been used by Filipino traditional healers for diarrhea, colic, stomach ache, and gas pain.[9] Other cultures have used this plant for cachexia, syphilis, dysentery, and fever.[8]

Ampalaya (*Momordica charantia* L. family Cucurbitaceae) is also called bitter melon and has different varieties grown in Asia, Africa, and the Caribbean. This annual vine has simple tendrils up to 20 cm long, with rounded leaves 2.5-10 cm in diameter with 5-7 oblong, ovate and heart-shaped at the base.[8] The leaves are used by Philippine traditional healers for diabetes, anemia, fever, coughs and colds, malaria, asthma, ringworm, and worms.[9] Other traditional medical systems use this plant for hemorrhoids, leprosy, headaches, and dysentery.[8] The leaves of the 'Makiling" variety have shown to have anti-hyperglycemic effects as well as decreasing the glycosylated hemoglobin in type 2 diabetes patients in clinical trials.[10]

Both ampalaya and tsaang gubat have been recommended for the treatment of bloody diarrhea, thus testing its activity against amoeba is a step towards validating these remedies for this particular indication. The objective of this study was to determine if tsaang gubat and ampalaya have anti-amoebic activity in order to produce scientific evidence for its folkloric and traditional use.

MATERIALS AND METHODS

Study design

An *in vitro* model was used to screen the anti-amoebic activity of tsaang gubat and ampalaya. A standard laboratory strain of HK-9 amoeba that has been maintained in culture at the UP Manila Department of Parasitology laboratory was used to test the anti-amoebic effect of the aqueous leaf extracts of tsaang gubat and ampalaya. The minimum inhibitory concentration (MIC) or the lowest concentration of extract to be tested when no living amoeba is detected was determined for each plant extract.

Plant materials

Tsaang gubat and ampalaya plants were grown and cultivated by the Agriculture team at UP Los Baños who also identified and authenticated the samples (Table 1). The plant parts needed (leaves) were selected and foreign materials were removed after which, it was transported to the Pharmacy Team at UP Manila. At the formulation area, garbling was performed followed by washing with clean tap water. On the same day, the leaves were air dried on drying beds with fans at room temperature for 2 days until all superficial water was eliminated. The leaves were oven dried for 72 hours at 60°C until the moisture content was <10%. The leaves underwent milling for 30 minutes using the Rotor Beater Mill Retz until a fine powder was achieved.

Plant extract preparation

Plant extracts were prepared by the Pharmacy team. The plant extracts were prepared following the procedure from the Philippine Pharmacopeia 2004.[11] The plant material was initially air dried. For aqueous extraction, distilled water was added at a ratio of 4:1 (water: powdered leaves). The solution was boiled for 15 minutes to produce a decoction, then cooled and filtered using previously boiled cheese cloth. The residue was washed with distilled water. All filtrates were combined and subjected to secondary filtration using Whatman filter paper No. 1. Final filtrate was lyophilized. Prior to the anti-amoeba screening, the extracts were rendered sterile by filtering it through a 0.22 μm filter disk.

Table 1. Philippine medicinal plants evaluated for anti-amoebic activity

Scientific name	Synonyms	Local names	Family	Type of extract
Ehretia microphylla Lam.	*Carmona retusa* (Vahl) Masam., *Ehretia heterophylla* Spreng.	Tsaang gubat Philippine tea	*Boraginaceae*	Aqueous
Momordica charantia L.	*Momordica cylindrica Blco*	Ampalaya Amargoso Bitter gourd	*Cucurbitaceae*	Aqueous

Axenic cultivation of E. histolytica (trophozoites)

E. histolytica HK-9 strain obtained from the Natural Science Research Institute, University of the Philippines Diliman and were axenically cultivated at 35.5°C in the BI-S-33 medium. *E histolytica* was cultured in 8 mL screw-capped glass tubes containing 6.06 ml BI-S broth (K_2HPO_4, KH_2PO_4, NaCl, Biosate Peptone, glucose, L-cysteine HCl, ascorbic acid, ferric ammonium citrate) 870 mL distilled water, maintained at a pH of 6.8. It was autoclaved for 15 minutes at 121°C and supplemented with heat-inactivated bovine serum, Diamond Vitamin Tween® 80 Solution 40x (Sigma-Aldrich 58980C), and Penicillin-Streptomycin (Gibco 15140-122). Sub-culturing was done every 3 to 4 days by placing the tube on ice for 20 minutes to detach the amoeba trophozoites.

Anti-amoeba assay

This assay was modified from the procedure of Upcroft.[12] Lyophilized extracts were dissolved in BI-S broth and sterilized using a 0.22 µm syringe-driven filter unit (Millex® SLHV033RS Merck Millipore). *E. histolytica* trophozoites, 2×10^3 cells/mL, were incubated with tsaang gubat or ampalaya leaf extract, using 10 dose levels with capped tubes containing TYI-S-33 medium. Three sets of each preparation (triplicates) were made. Ten dose levels (10,000 µg/mL, 5,000 µg/mL 2,500 µg/mL, 1250 µg/mL, 625 µg/mL, 312 µg/mL, 150 µg/mL, 75 µg/mL, 37 µg/mL, and 18 µg/mL) of each plant extract were tested for its activity against *Entamoeba histolytica*. Metronidazole (17 µg/mL) and complete medium with added DMSO were used as positive and negative controls, respectively. Tubes were incubated at 35.5 °C under anaerobic conditions. After 24, 48, and 72 hours of incubation, counting of living or viable trophozoites from each tube was performed using the Trypan blue exclusion assay.[13] Triplicate tubes per dose level were chilled on ice for 20 minutes and centrifuged for 10 minutes. From each tube, the supernatant was pipetted out until the remaining volume was 500 µL. From the remaining trophozoite solution, 50 µL was pipetted and mixed with 50 µL of 0.4% Trypan blue stain (Gibco 15250-061). After 30 to 60 seconds, 10 µL of the stained-trophozoite solution was dispensed into both chambers of a Neubauer hemocytometer and examined under a binocular microscope. Live trophozoites (unstained) were counted and recorded.

Data processing and analysis

The lowest concentration that would be able to inhibit the growth of the amoebae after 24 or 72 hours were determined (minimum inhibitory concentration). The plot of the number of live amoeba against the dose of the plant extract concentration was planned. The best straight line was to be determined by regression analysis and concentrations which cause 50% inhibition (IC_{50}) was calculated. The criteria used for determining the degree of the anti-amoebic effects was adopted from Sawangjaroen[14] and are as follows:

IC_{50} <20 µg/mL=highly active
20 <IC_{50} ≤100 µg/mL=active
100 <IC_{50} ≤250 µg/mL=moderately active
250 <IC_{50} ≤500 µg/mL=weakly active
IC_{50} >500 µg/mL=inactive

RESULTS

Two commonly used medicinal plants for dysentery were screened for their anti-amoebic activity. In the *in vitro* assay, tsaang gubat did not inhibit the growth of amoeba at any time period, or in any of the ten dose levels (18-10,000 µg/mL). There was even an increase in the number of viable amoebae over time after exposure to tsaang gubat even at the highest dose (Figure 1). The IC_{50} of tsaang gubat at 24, 48, and 72 hours ranged from 3,791 to 1,209,726 µg/mL (Table 2), far above the 500 µg/mL cut-off for anti-amoebic activity. The MIC of tsaang gubat against *E. histolytica* was also >10,000 µg/mL because all tubes had increased growth of cells at all time periods. Metronidazole was able to completely inhibit and eradicate amoeba at 24 and 48 hours at the dose tested while DMSO which served as negative control did not show any anti-amoebic activity.

Table 2. IC_{50} for the tsaang gubat and ampalaya aqueous leaf extracts

	IC_{50} µg/mL		
	24 hrs	48hrs	72hrs
Tsaang gubat	3,791.0	1,209,726.0	845,921.0
Ampalaya Trial 1	10,602.0	235,124.0	65,315.0
Ampalaya Trial 2	13,555.2	16,102.8	11,144.6

Two *in vitro* trials were performed using ampalaya. Ampalaya also did not show any anti-amoebic activity. There was an increase in live amoeba at all time points observed at all doses with the IC_{50} ranging from 10,602 to 235,124 µg/mL in both trials (Table 2). Again, no dose-response relationship in the inhibition of the growth of amoeba nor in the deaths of the protozoa was seen (Figures 2 and 3). The MIC of the ampalaya leaf extracts were >10,000 µg/mL against *E. histolytica.* Thus both plants were considered to be inactive against *Entamoeba histolytica.* Metronidazole completely eradicated the amoeba parasite at 24 and 72 hours of trial 2 while DMSO which served as negative control did not eradicate the amoeba and there was a further increase in the number of parasites.

DISCUSSION

In this study, it was found that tsaang gubat and ampalaya did not inhibit the growth nor cause the death of *Entamoeba histolytica*. This study does not validate the folkloric use of both these medicinal plants for dysentery caused by *Entamoeba histolytica*.

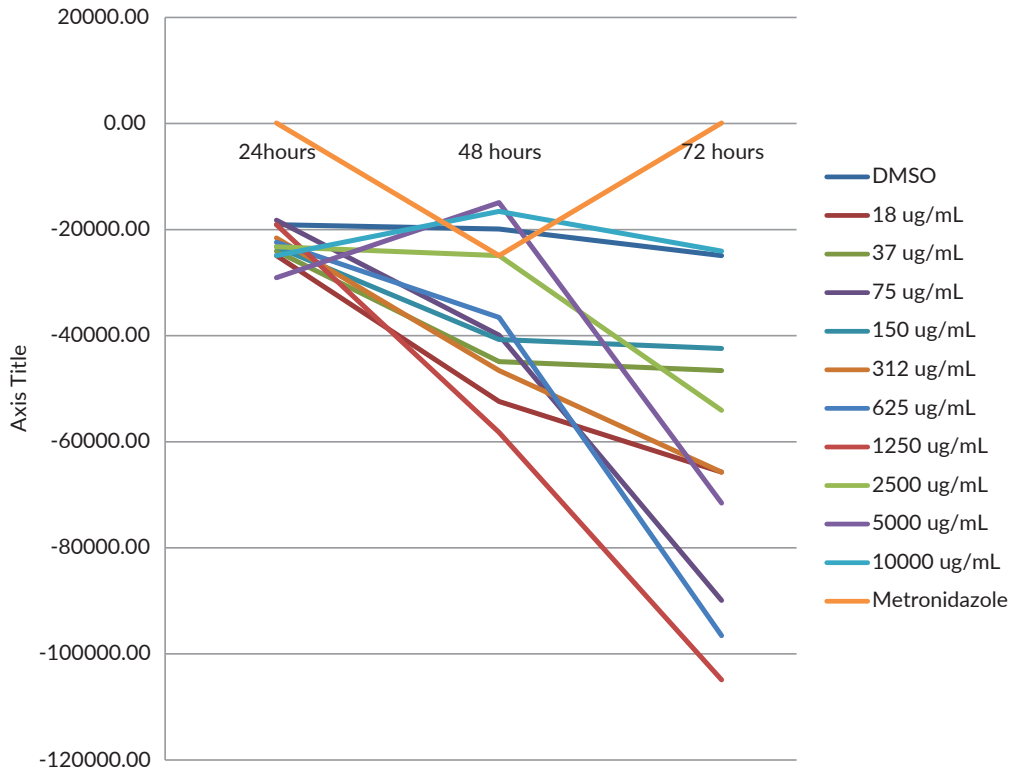

Figure 1. Mean percent inhibition of amoeba after treatment with different doses of tsaang gubat at 24, 48, and 72 hrs.

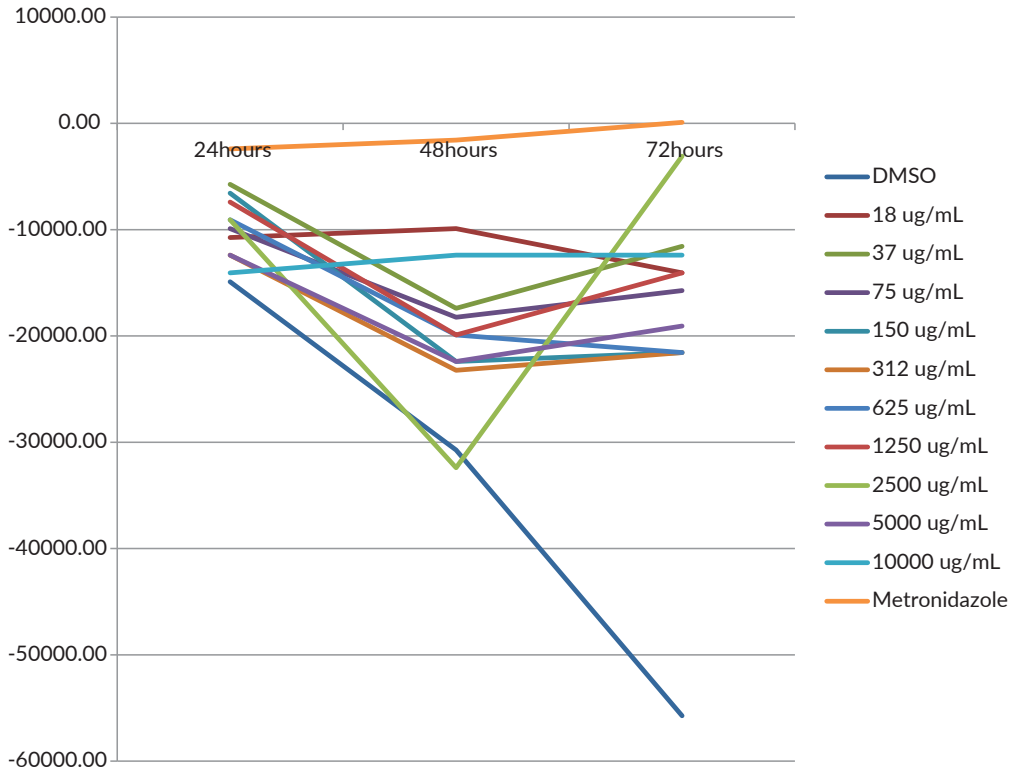

Figure 2. Mean percent inhibition of amoeba (Trial 1) after treatment with different doses of ampalaya leaf extract at 24, 48, and 72 hrs.

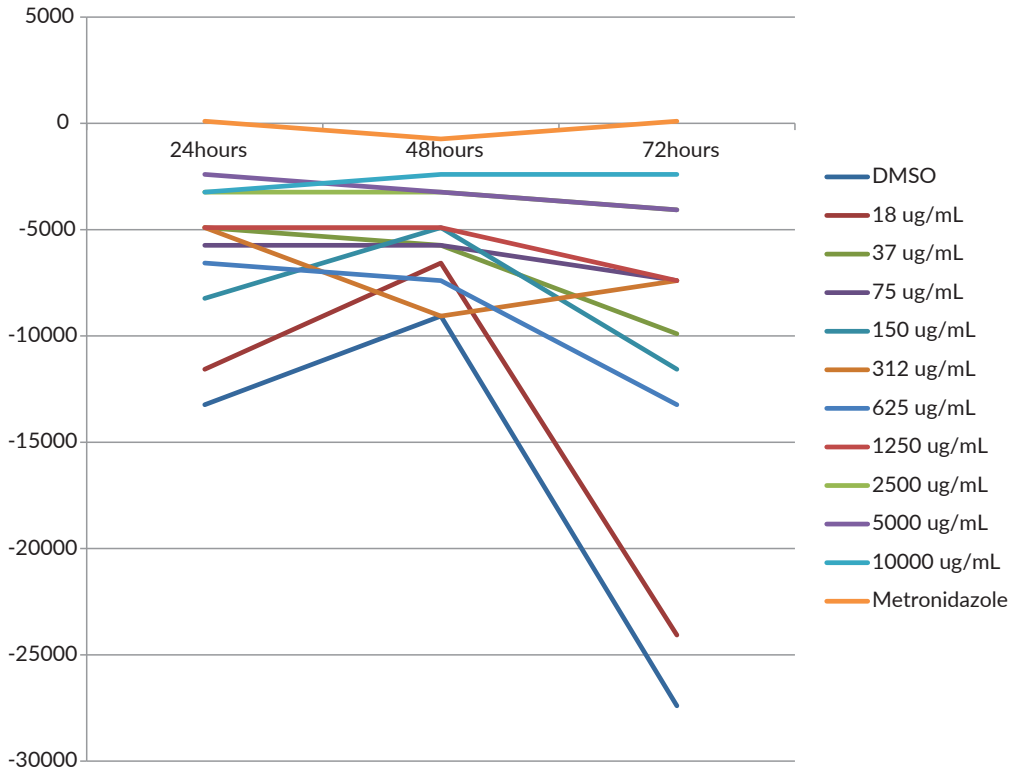

Figure 3. Mean percent inhibition of amoeba (Trial 2) after treatment with different doses of ampalaya leaf extract at 24, 48, and 72 hrs.

Tsaang gubat NIRPROMP formulations, both the tablet and syrup, have been proven in clinical studies to be safe and effective in patients with mild, moderate, or severe gastrointestinal colic, as well as mild, moderate, or severe biliary colic and were equivalent to dicycloverine.[15] Expanding its indication to infectious disease causes of gastroenteritis would be beneficial. There have been no previous published data on the antiparasitic activity of tsaang gubat. Unfortunately, this study showed that tsaang gubat did not have any activity against *E. histolytica.* Thus its use for treatment of bloody diarrhea has not been validated in this study.

Ampalaya has been previously studied against the free-living amoeba *Naegleria fowleri* and showed 100% inhibitory activity.[16] A pure compound from ampalaya, diosgenin, was found to have activity against *N. fowleri*.[17] Diosgenin has activity against the surface membrane and the *nf cysteine protease* of *N. fowleri* trophozoites. *Naegleria* and *Entamoeba* are very different parasites which differ in the life cycle, pathophysiology of disease, as well as treatments. For *Naegleria*, amphotericin, a polyene antifungal is used.[18] On the other hand, metronidazole and its analogs such as tinidazole are the drugs of choice for *Entamoeba* infections,[19] thus they have different mechanisms necessary to inhibit them. Thus, even if ampalaya is able to inhibit *Naegleria*, it may not have the same effect on *Entamoeba*. Unfortunately, the local species of ampalaya leaf extract, in this study, did not exhibit any activity against *E. histolytica.*

Another cause of dysentery or bloody diarrhea includes *Shigella*. Tsaang gubat from Kamataka, India was tested for its antibacterial activity using the disk diffusion method.[20] Different leaf and stem extracts showed some activity against *Shigella* with a zone of inhibition ranging from 13 to 20 mm, although the minimum inhibitory concentration was not determined. The Philippine variety was also tested against *Shigella* to further see if it will be effective against this etiology of dysentery. It also showed poor antibacterial activity for the *Shigella* clinical isolate.[21] *Momordica charantia* leaf extracts from India has also been tested for its activity against *Shigella flexneri* at 25-100% which showed inhibition of 15-19 mm although the minimum inhibitory concentration was not tested. Our local variety of ampalaya should also be tested against *Shigella*.[22]

CONCLUSION

Tsaang gubat and ampalaya had poor activity against *Entamoeba histolytica*, and even promoted the growth of the parasite. These results do not validate the use of these herbal extracts as therapy for dysentery, of which it has been used traditionally.

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Statement of Authorship

All authors participated in drafting the protocol. Rivera and Villacorte were involved in data collection. Maramba, Rivera and Villacorte were involved in data analysis. All authors were involved in writing the manuscript and approved the final version submitted.

Author Disclosure

All authors declared no conflict of interest.

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