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Avelina Espinosa

Guillermo Paz y Mino C

Yoly Santos

Hang Ma

Michael Nadeau

See next page for additional authors

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Authors

Avelina Espinosa, Guillermo Paz y Mino C, Yoly Santos, Hang Ma, Michael Nadeau, Navindra P. Seeram, and D C. Rowley



Research article

Anti-amebic effects of Chinese rhubarb (*Rheum palmatum*) leaves' extract, the anthraquinone rhein and related compoundsAvelina Espinosa^{a,b,*}, Guillermo Paz-y-Miño-C^b, Yoly Santos^a, Hang Ma^c, Michael Nadeau^c, Navindra P. Seeram^c, David C. Rowley^c^a Department of Biology, Roger Williams University, RI 02809, United States^b New England Center for the Public Understanding of Science, Roger Williams University, RI 02809, United States^c Department of Biomedical & Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI 02881, United States

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ABSTRACT

Entamoeba histolytica infects 50 million people worldwide and causes 55 thousand fatalities every year. Current anti-amebic drugs (e.g. paromomycin) work either at the level of the intestinal lumen (where trophozoites proliferate via cell divisions) or on the invasive trophozoites that have penetrated the gut or colonized internal organs (e.g. metronidazole). Some of these drugs are highly toxic to patients, have generated trophozoite resistance, or caused mutations and cancer in laboratory animals. Thus, alternative anti-amebic compounds need to be identified to minimize the side effects (on patients) or resistance (by amebas) to current treatments. The literature suggests that anthraquinones (chemicals found in medicinal plants) have antibacterial, antiparasitic, anti-inflammatory and antioxidant properties. Here we provide experimental evidence that Chinese rhubarb (*Rheum palmatum*) leaves' extract (rich in the anthraquinone rhein) inhibits *E. histolytica* trophozoite growth *in vitro*. In addition, from a set of ten isolated/synthetic anthraquinones (which we suspected to have anti-amebic properties), four analogs (rhein; AHHDAC = 1-amino-4-hydroxy-9, 10-dioxo-9, 10-dihydro-anthracene-2-carboxylic acid; unisol blue AS; and sennoside B) efficiently inhibited amebic growth at EIC₅₀ concentrations comparable to metronidazole. The mechanism of action of these compounds still needs to be determined, although anthraquinones might enhance the production of toxic oxygen metabolites as it has been suggested for various protists (e.g. *Leishmania*, *Plasmodium*, *Trypanosoma*). Our research is the first to explore anti-amebic effects of Chinese rhubarb leaves' extract and isolated/synthetic anthraquinones on pathogenic *Entamoeba*.

1. Introduction

Entamoeba histolytica infects 50 million people worldwide and causes 55 thousand fatalities every year [1, 2]. This protist's life cycle consists of the infectious trophozoite stage and the transmissible cyst stage. Cysts are ingested with contaminated food or water. Despite the epidemiological importance of *E. histolytica*, there are no ideal methods to prevent infection in areas without appropriate sanitation [1, 3]. The development of a vaccine for amebiasis remains in early stages, although some progress has been made at identifying potential immunogenic proteins and their effectiveness in animal models [4, 5, 6]. Current anti-amebic drugs seem to work either at the level of the intestinal lumen, where trophozoites proliferate via cell divisions (e.g. paromomycin, iodoquinol and diloxanide furoate [7, 8]) or at the level of the intestine's endothelium or tissue abscesses, on the invasive trophozoites that have already

penetrated the gut or colonized internal organs (e.g. metronidazole, tinidazole and ornidazole, broadly called nitroimidazoles), but not at both levels [7, 8, 9]. These drugs, however, are highly toxic to patients (causing nausea, vomiting, headaches, dizziness, vertigo, neuronal damage) [7], have generated resistance in *E. histolytica* [10], or triggered DNA mutations and cancer in laboratory rodents [11, 12, 13]. Thus, alternative anti-amebic compounds need to be identified to minimize the side effects (on patients) or resistance (by amebas) to current treatments.

More than 50% of FDA-approved drugs are natural products or their derivatives [14, 15]. Anthraquinones found in medicinal plants (e.g. *Rheum*, *Hemerocallis*, *Aloe*) have antibacterial, antiparasitic, anti-inflammatory, antioxidant and antitumorogenic properties [16, 17, 18, 19, 20]. Here we show that Chinese rhubarb (*Rheum palmatum*) leaves' extract and four isolated/synthetic anthraquinones inhibit *E. histolytica* trophozoite growth in the laboratory. We compare the

* Corresponding author.

E-mail address: aespinosa@rwu.edu (A. Espinosa).

Table 1. Anti-amebic effects of Chinese rhubarb (*Rheum palmatum*) leaves' extract (i.e. "rhein extract" in Figures 1 and 3).

Compound	at 24 h			at 48 h		
	No. amebas x10 ³	% inhibition	ED ₅₀	No. amebas x10 ³	% inhibition	ED ₅₀
"rhein extract" 100 µg/ml	0.97	78.52	42.96	0.63	90.31	19.39
metronidazole 20 µM	0.60	86.67	5.33	0.60	90.82	3.67
TYI-S-33 media	4.13	NA	NA	7.67	NA	NA
TYI-S-33 media + DMSO	4.50	NA	NA	6.53	NA	NA

µg/ml = micrograms per milliliter; µM = micromolar; DMSO = dimethyl sulfoxide. Initial trophozoite inoculum 900 cells at 0h. ED₅₀ = estimated concentration of plant extract required to inhibit at least 50% of trophozoites in comparison with the control.

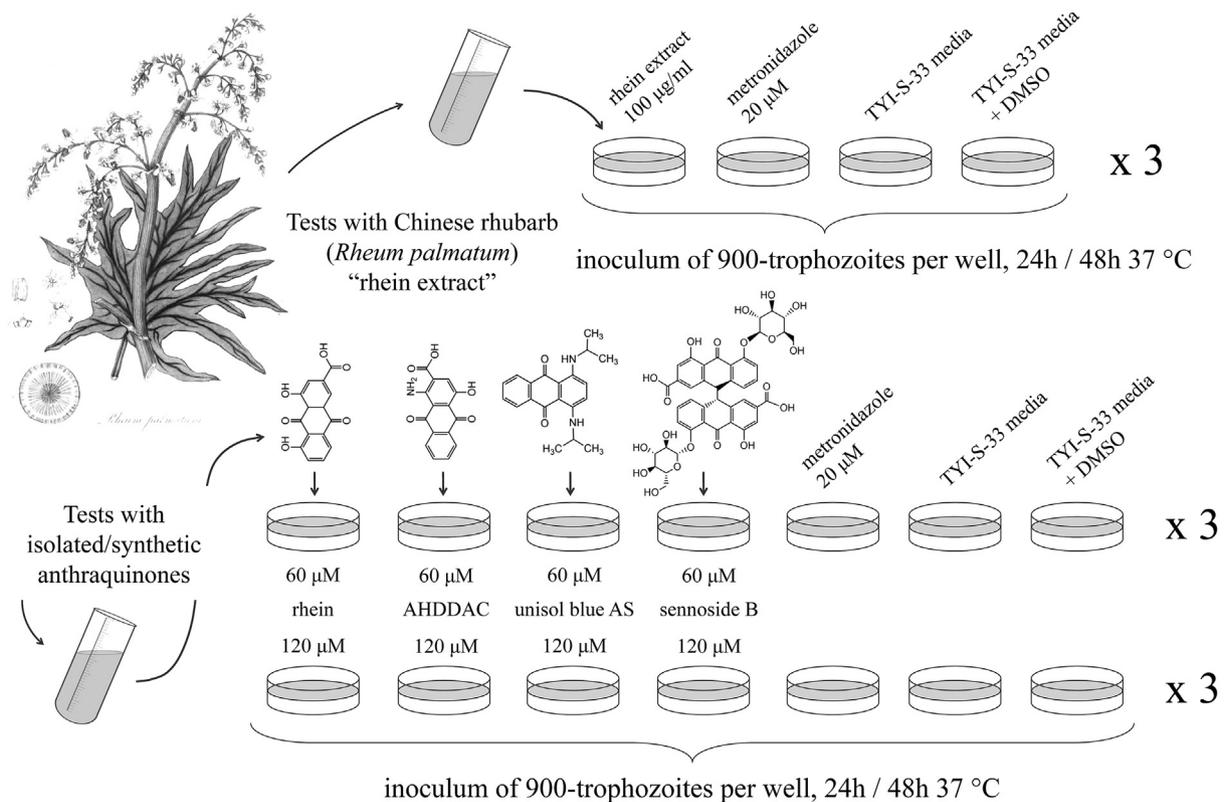


Figure 1. Experimental design: tests with Chinese rhubarb (*Rheum palmatum*) extract ("rhein extract") or with isolated/synthetic anthraquinones. Top: Extract obtained from Chinese rhubarb leaves was added to suspensions of *Entamoeba histolytica* trophozoites and incubated for 24h or 48 h at 37 °C; likewise, metronidazole (20 µM), TYI-S-33 media or TYI-S-33 media + DMSO (dimethyl sulfoxide) were added to separate suspensions (both controls); each series was done in triplicate (x3, total 24 wells). Bottom: From commercially available isolated/synthetic anthraquinones (rationale in Methods), four analogs (rhein; AHDDAC = 1-amino-4-hydroxy-9, 10-dioxo-9, 10-dihydro-anthracene-2-carboxylic acid; unisol blue AS; or sennoside B; structures in Figure 2) were added (concentrations 60 µM or 120 µM) to the suspensions of trophozoites and incubated for 24h or 48 h at 37 °C; metronidazole, TYI-S-33 media or TYI-S-33 media + DMSO were separately added as in above; each series was done in triplicate (x3, total 84 wells). Illustration of *R. palmatum* comes from Edward Hamilton 1853 [28].

inhibition levels of the leaves' extract and isolated/synthetic anthraquinone analogs to those of metronidazole.

2. Materials and methods

2.1. Selection of extracts and isolated/synthetic anthraquinone compounds for testing

Based on antimicrobial and antiparasitic properties reported in the literature for *Rheum* spp. [16, 17, 18, 19, 20], we tested the Chinese rhubarb "rhein extract" (from leaves) for potential anti-amebic effects. A commercial powder containing >90% rhein, "rhein extract", was purchased from Natural-Field Bio-Technique Co. (Xi'an, China). Relying on the chemical structure of rhein (i.e. the anthraquinone present in *R. palmatum* [17, 18] and main component of the commercial "rhein extract"), we selected ten isolated/synthetic anthraquinone analogs,

which we suspected could inhibit amebic growth, for additional testing, as follows: AHDDAC (1-amino-4-hydroxy-9, 10-dioxo-9, 10-dihydro-anthracene-2-carboxylic acid); unisol blue AS; anthraquinone-2-carboxylic acid; 2,3-bis-bromomethyl-1, 4-dihydroxy-anthraquinone; and quinalizarin (these five were purchased from Sigma Aldrich, St. Louis, MO); as well as rhein, sennoside B, diacetyl rhein, sennoside A, and carminic acid (these five were purchased from Cromadex, Irvine, CA). All compounds were stored in dimethyl sulfoxide (DMSO) at -20 °C prior to inhibition assays (below).

2.2. Amebas' cell-line cultures and inhibition assays

Entamoeba histolytica HM-1:1MSS trophozoites were cultured under axenic conditions in Diamond's TYI-S-33 medium plus 10% ABS at 37 °C as described previously [21, 22, 23, 24]. To assess inhibition of *E. histolytica* growth, by either "rhein extract" or the ten

Table 2. Anti-amebic effects of isolated/synthetic anthraquinones: rhein, AHDDAC, unisol blue AS, or sennoside B (as in Figure 2).

Compound	No. amebas x10 ³	% inhibition	No. amebas x10 ³	% inhibition	IC ₅₀
	at 24 h		at 48 h		
rhein 20 μM	5.5	15.82	4.20	48.78	–
rhein 40 μM	2.23	65.87	2.30	71.95	–
rhein 60 μM	0.90	86.22	1.7	86.99	–
rhein 120 μM	0.37	94.39	0.37	95.53	–
IC ₅₀ rhein μM	–	–	–	–	20.74
AHDDAC 20 μM	5.90	9.69	5.87	28.41	–
AHDDAC 40 μM	3.52	46.12	3.79	53.78	–
AHDDAC 60 μM	1.27	80.61	1.37	83.33	–
AHDDAC 120 μM	0.97	85.20	0.77	90.65	–
IC ₅₀ AHDDAC μM	–	–	–	–	36.07
unisol blue AS 20 μM	6.52	0.20	1.83	28.05	–
unisol blue AS 40 μM	3.80	41.84	1.27	57.39	–
unisol blue AS 60 μM	2.17	66.84	1.83	77.64	–
unisol blue AS 120 μM	1.67	74.49	1.27	84.55	–
IC ₅₀ unisol blue AS μM	–	–	–	–	33.59
sennoside B 20 μM	6.50	0.51	5.50	32.93	–
sennoside B 40 μM	3.20	51.02	3.10	62.20	–
sennoside B 60 μM	1.53	76.53	1.33	83.74	–
sennoside B 120 μM	0.63	90.31	0.63	92.28	–
IC ₅₀ sennoside B μM	–	–	–	–	29.96
metronidazole 1 μM	4.20	35.71	5.90	28.05	–
metronidazole 5 μM	1.90	70.92	2.30	71.95	–
metronidazole 10 μM	0.80	87.76	1.10	86.59	–
metronidazole 20 μM	0.50	92.35	0.30	96.34	–
IC ₅₀ metronidazole μM	–	–	–	–	3.16
TYI-S-33 media	6.17	NA	9.20	NA	NA
TYI-S-33 media + DMSO	6.53	NA	8.20	NA	NA

μM = micromolar; DMSO = dimethyl sulfoxide. Initial trophozoite inoculum 900 cells at 0h. IC₅₀ = concentration of compounds required to inhibit 50% of trophozoites after 48h. AHDDAC = 1-amino-4-hydroxy-9,10-dioxo-9,10-dihydro-anthracene-2-carboxylic acid.

isolated/synthetic anthraquinones (above), we designed two sets of experiments. First, flat-bottomed 24-well plates (Fisher Scientific, Pittsburgh, PA) containing an initial inoculation of 900 trophozoites per well (log phase) were grown for 24 h or 48 h in TYI-S-33 media (i.e. “amebas in TYI-S-33 media”), or supplemented with Chinese rhubarb leaves’ extract (“rhein extract”) at 100 μg/ml concentration dissolved in DMSO. A similar volume of DMSO was added to wells with ameba (i.e. “amebas in TYI-S-33 media + DMSO”) to control for potential solvent toxicity [22, 23, 24, 25, 26]. Metronidazole (market drug of choice to treat amebiasis) at 20 μM concentration was used as a positive control of inhibition. The 24-well plates were sealed with parafilm to minimize the exposure to oxygen. The estimated concentration of plant extract required to inhibit at least 50% of trophozoites in comparison with the control (Table 1) was determined by the following formula: $ED_{50} = [(A/B) * \text{drug concentration } \mu\text{g/ml}] / 0.5$; where A = mean value of trophozoites counted on plant extract treated wells at the end of each incubation period (e.g. 24 h/48 h) and B = mean value of trophozoites counted on media control wells at the end of each incubation period (24 h/48 h) [20, 22]. The experiments were done in triplicate (total 24 wells; experimental design for “rhein extract” is depicted in Figure 1 top) during three independent experiments. Counts of viable ameba cells were estimated with a Cellometer Vision HS RF-150 (Nexcelom BioScience Lawrence, MA) as detailed previously [22, 23, 24, 25, 26]. Second, all ten isolated/synthetic anthraquinones were dissolved in DMSO (as in above), added to the suspensions of trophozoites at concentrations of 60 μM or 120 μM, and incubated for 24 h or 48 h at 37 °C. Metronidazole, TYI-S-33 media or TYI-S-33 media + DMSO were separately added to the trophozoites as in

above (controls). Each series was done in triplicate (total 84 wells; experimental design for isolated/synthetic anthraquinones is depicted in Figure 1 bottom) during three independent experiments. To calculate the IC₅₀ (= the concentration of compound required to inhibit 50% of trophozoites after 48h, Table 2), we used the formula $IC_{50} = 10^{\log(A/B) \times (50-C)/(D-C) + \log(B)}$, where A is the concentration of test compound directly above 50% inhibition, B is the concentration of test compound directly below 50% inhibition, C is the percentage of inhibition directly below 50% inhibition, and D is the percentage of inhibition directly above 50% inhibition [27].

Out of the ten compounds, four inhibited amebic growth: rhein, AHDDAC, unisol blue AS, or sennoside B (structures in Figure 2). Below we only discuss these four compounds (all other data available from authors).

3. Results

Chinese rhubarb *R. palmatum* “rhein extract” (from leaves) inhibited *E. histolytica* HM-1:1MSS trophozoite growth at 24 h and 48 h at 100 μg/ml concentration, and differed from the controls TYI-S-33 media or TYI-S-33 media + DMSO (Figure 3). “Rhein extract” showed noticeably higher percentage of growth inhibition and lower ED₅₀ values at 48 h (90.31%, 19.39 μM; Table 1) than at 24 h (78.52%, 42.96 μM; Table 1). The rate of “rhein extract” inhibition was roughly comparable to that of metronidazole at 24 h or 48 h (Table 1). ED₅₀ was, of course, much lower for metronidazole than for “rhein extract” at both times (Table 1), which was expected for a purified commercial drug.

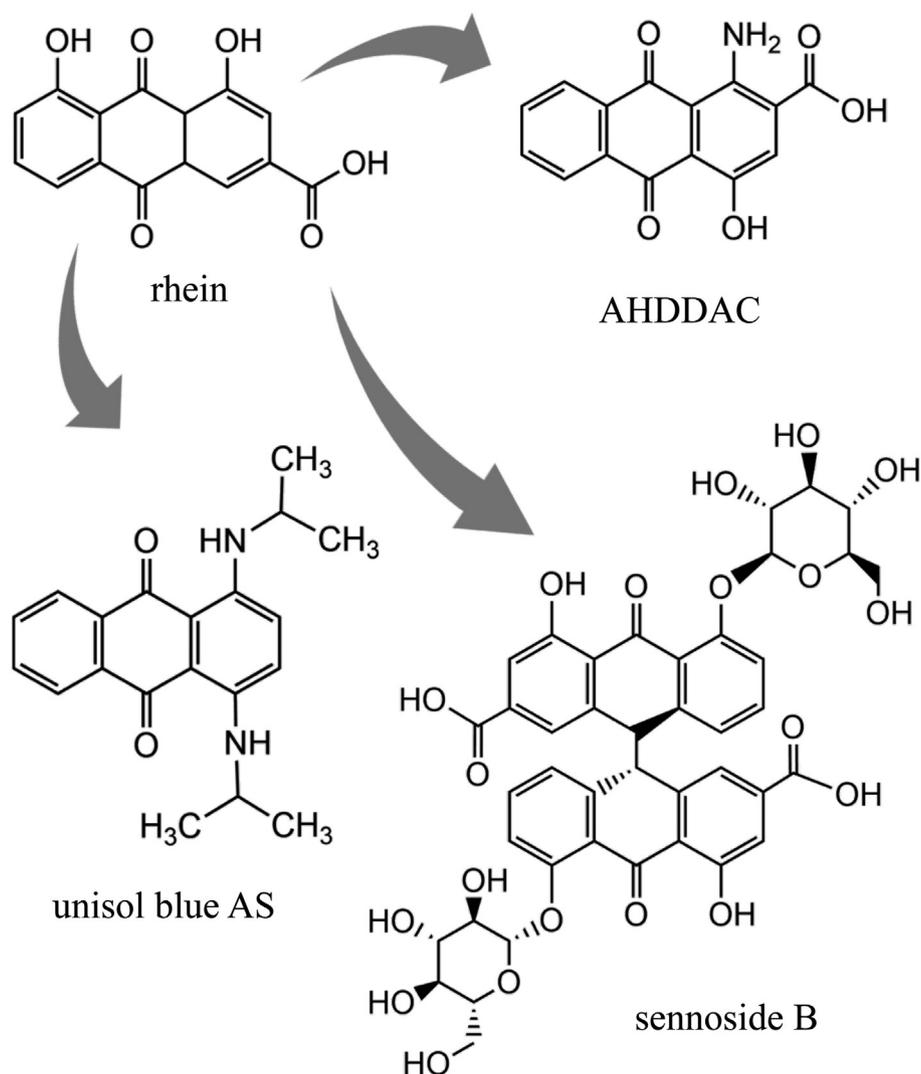


Figure 2. Isolated/synthetic anthraquinone analogs with anti-amebic properties. When *E. histolytica* trophozoites were exposed to these four anthraquinones (rhein or its derivatives AHDDAC = 1-amino-4-hydroxy-9,10-dioxo-9,10-dihydro-anthracene-2-carboxylic acid; unisol blue AS; or sennoside B), amebic growth was inhibited at IC_{50} concentrations comparable to the anti-amebic drug metronidazole, as shown in Figure 4 (structures generated by Hang Ma).

The isolated/synthetic anthraquinones rhein, AHDDAC, unisol blue AS, or sennoside B were effective at inhibiting amebic growth; they were all equivalent to metronidazole when compared to the controls (Figure 4). Rhein reached high inhibition values at 24 h, with little change at 48 h for 60 μ M or 120 μ M concentrations (60 μ M: 86.22%/86.99%; 120 μ M: 94.39%/95.53%; Table 2). Rhein IC_{50} value was 20.74 μ M, better than those of the other three compounds (AHDDAC IC_{50} = 36.07 μ M, unisol blue AS IC_{50} = 33.59 μ M, sennoside B IC_{50} = 29.96 μ M; Table 2). The three compounds AHDDAC, unisol blue AS and sennoside B showed noticeably higher inhibition at 120 μ M concentration at 48 h (90.65/84.55/92.28%; Table 2) than at 24 h (85.20/74.49/90.31%; Table 2). Again, the rates of inhibition of the four compounds were roughly comparable to those of metronidazole at 24 h or 48 h (Table 2). And the IC_{50} was much lower for metronidazole than for the four compounds (Table 2), rationale above.

4. Discussion

As shown in this study (*in vitro* experiments), “rhein extract” from Chinese rhubarb (*R. palmatum*) leaves and four isolated/synthetic anthraquinones (rhein, AHDDAC, unisol blue AS, and sennoside B) had anti-amebic properties. Their inhibition effects on trophozoite growth were comparable to the commercial drug metronidazole.

Although some anthraquinones can be toxic to humans, rhein and derivatives seem to have little to no toxicity, even at mg levels, when added to food [29]. A review published by the European Food Safety Authority (EFSA, 2018) states that after evaluating *in vitro* and *in vivo* studies on hydroxyanthracene derivatives, rhein and sennoside B showed no genotoxic effects at 78.8 mg- and 24.84 mg-/adult/day, respectively [29]. Baqui et al. 2009 showed that the anthraquinone scaffold (1-amino-4-anilino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate) showed preferential cytotoxicity for cancerous compared to non-cancerous cells [30]. Due to the low IC_{50} values reported in this study (20.74–36.07 μ M), we suggest that they could be less toxic than the current anti-amebic drugs, which have high toxicity in patients [7, 10, 11, 12, 13]. The low toxicity of these compounds in patients would need verification.

Several studies have reported antiparasitic effects of natural and isolated/synthetic anthraquinones on diverse pathogenic unicellular eukaryotes (*Leishmania donovani*, *L. major*, *Plasmodium falciparum*, *Trypanosoma brucei*), but the mechanism of action remains speculative [31, 32, 33, 34, 35, 36]. When interacting with the cellular chemistry of the pathogen, these compounds seem to generate free oxygen metabolites (which are toxic), alter DNA replication/repair enzymes, or cause direct damage on the DNA [31, 32, 33, 34, 35, 36].

In *Escherichia coli*, rhein inhibits growth by targeting the AlkB DNA repair enzyme; the inactive AlkB cannot fix methyl lesions on the

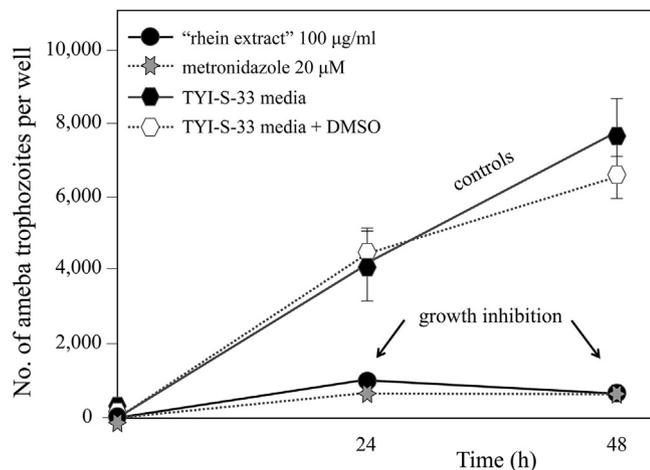


Figure 3. Growth inhibition of *E. histolytica* trophozoites by Chinese rhubarb (*Rheum palmatum*) extract (“rhein extract” as in Figure 1 top). A 100 µg/ml concentration of “rhein extract”, as well as the exposure to the anti-amebic metronidazole (20 µM), inhibited trophozoite growth at 24h and 48h (inoculum of 900-cells per plate well, 37 °C incubation). Experiments were done in triplicate (as shown in Figure 1 top). Controls contained TYI-S-33 media or TYI-S-33 media + DMSO (the latter used as solvent).

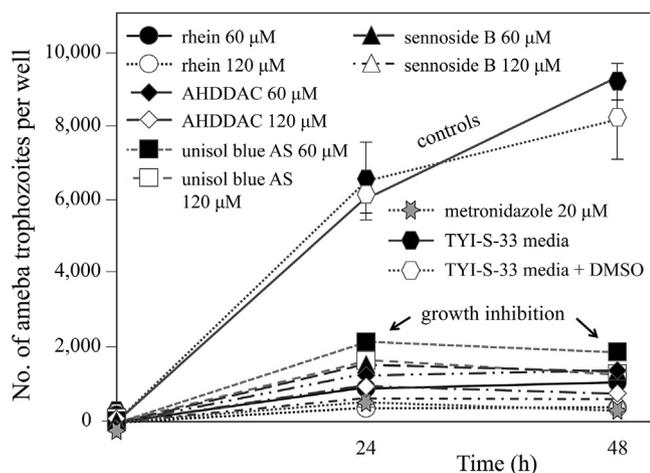


Figure 4. Growth inhibition of *E. histolytica* trophozoites by isolated/synthetic anthraquinones (rhein; AHDDAC = 1-amino-4-hydroxy-9, 10-dioxo-9, 10-dihydro-anthracene-2-carboxylic acid; unisol blue AS; or sennoside B; each at concentrations of 60 µM or 120 µM) or by the anti-amebic metronidazole (20 µM) at 24h and 48h (inoculum 900-cells per plate well, 37 °C incubation). Experiments were done in triplicate (as shown in Figure 1 bottom). Controls contained TYI-S-33 media or TYI-S-33 media + DMSO (the latter used as solvent).

bacterial DNA, which eventually leads to cell death [37]. There is no AlkB homolog in the *E. histolytica* genome [37], instead amebas rely on the Base Excision Repair (BER) pathway to restore DNA damage, which includes AlkD and other repair enzymes [38]. We think that rhein might target AlkD and/or other BER repair enzymes in its pathway. Our future research will focus on the bioinformatics and molecular analysis of these enzymes, and the effects that anthraquinones could have on them.

Recent studies [39] suggest that rhein's anti-inflammatory and immunomodulatory effects during kidney chronic malfunction (humans) results from restraining the expression of key proteins in the transcription factor NF-κB pathway (immune system), which remains active during organ malfunction. Because activation of the same pathway (NF-κB) is associated with amebic-liver-abscess formation, which can lead to organ malfunction and necrosis [40], we speculate that rhein could be used to treat both inflammation-related trophozoite invasion (as inferred from

our *in vitro* experiments) and/or prevent tissue/organ damage (based on the commonality of the NF-κB pathway activation during kidney or liver failure), but this needs further research.

We have previously shown that aggregations of *Entamoeba* spp. secrete into the milieu signals associated with cell proliferation, cell adhesion, cell movement, and stress-induced encystation (i.e. RasGap/Ankyrin, coronin-WD40, actin, protein kinases, heat shock 70, and ubiquitin [26, 41, 42]). Because encystation and abscess formation require cell aggregation (i.e. abscesses are bound to cell recruitment), we have postulated that disruption of *E. histolytica* capacity to group, via silencing of alleles yet to be discovered, or drugs that alter the chemistry of the recruitment signals secreted into the milieu, might be approaches to mitigating amebiasis [26]. Thus, by combining current or novel therapeutics (this paper) with the disruption of *E. histolytica* to aggregate, we might find new approaches to treatments [26, 41, 42].

5. Conclusion

Chinese rhubarb leaves' “rhein extract” and the isolated/synthetic anthraquinones rhein, AHDDAC, unisol blue AS and sennoside B inhibit *E. histolytica* trophozoite growth *in vitro*. Their level of inhibition is comparable to that of the commercial drug metronidazole. These compounds possibly act by generating toxic oxygen metabolites in the amebas, altering DNA replication/repair enzymes or directly damaging the DNA. Because current amebiasis drugs are toxic to patients, generate resistance in the pathogen, or can potentially induce cancer (as in has been documented in animal models), alternative anti-amebic compounds need to be discovered and tested to treat and manage amebiasis. Our study suggests that anthraquinones might be such alternative compounds.

Declarations

Author contribution statement

Avelina Espinosa, Guillermo Paz-y-Miño-C: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yoly Santos: Performed the experiments.

Hang Ma, Michael Nadeau, Navindra P. Seeram, David C. Rowley: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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