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Bioassay-Guided Fractionation of Extracts from *Codiaeum variegatum* against *Entamoeba histolytica*

Discovers Compounds That Modify Expression of Ceramide Biosynthesis Related Genes

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Abstract

Leaves of *Codiaeum variegatum* (“garden croton”) are used against bloody diarrhoea by local populations in Cameroon. This study aims to search for the active components from *C. variegatum* against *Entamoeba histolytica*, and thereby initiate the study of their mechanism of action. A bioassay-guided screening of the aqueous extracts from *C. variegatum* leaves and various fractions was carried out against trophozoites of *E. histolytica* axenic culture. We found that the anti-amoebic activity of extracts changed with respect to the collection criteria of leaves. Thereby, optimal conditions were defined for leaves’ collection to maximise the anti-amoebic activity of the extracts. A fractionation process was performed, and we identified several sub-fractions (or isolated compounds) with significantly higher anti-amoebic activity compared to the unfractionated aqueous extract. Anti-amoebic activity of the most potent fraction was confirmed with the morphological characteristics of induced death in trophozoites, including cell rounding and lysis. Differential gene expression analysis using high-throughput RNA sequencing implies the potential mechanism of its anti-amoebic activity by targeting ceramide, a bioactive lipid involved in disturbance of biochemical processes within the cell membrane including differentiation, proliferation, cell growth arrest and apoptosis. Regulation of ceramide biosynthesis pathway as a target for anti-amoebic compounds is a novel finding which could be an alternative for drug development against *E. histolytica*.

Introduction

Medicinal plants are recognized by the World Health Organization as alternatives in the treatment of various diseases and the interest of health professionals for medicinal plants is increasing everyday [1]. Medicinal plants contain a variety of secondary metabolites, which can be used to prevent or cure diseases, or to promote general health and well-being [2],[3]. During the last decades, scientific evidences of the medicinal values of plant products (through in *vivo* investigation) aroused public concerns about the conservation of such plants, in order to retain their economic and therapeutic significance [4]. Modern pharmaceutical industry relies mainly on the diversity of secondary metabolites in medicinal plants for the discovery of new compounds with novel biological properties. It is estimated that natural products and their derivatives and analogues represent over 50% of all drugs in clinical use [5],[6]. Therefore, further evaluation of drugs derived from plants requires the screening of large numbers of plant extracts, isolation and identification of the active compounds, the study of their mechanism of action, as well as the proof of its non-toxicity to human cells. In Cameroon, biodiversity is an important source of bioactive natural compounds and exploration of this biodiversity, based on ethnopharmacology approach from traditional healers, represents a promising strategy to fight against diseases such as intestinal infections. We have focused on amoebiasis, a human infectious disease caused by the amoebic parasite *Entamoeba histolytica*, which mainly targets intestine and liver. Humans are the only relevant host of this parasite and infection occurs upon ingestion of contaminated water or food containing cysts forms of *E. histolytica*. In rural areas of some developing countries, up to 20% of the population is infected with *E. histolytica*. Trophozoites (the vegetative form) are released in the intestine by excystation. Once the trophozoite has penetrated the intestinal mucosal layer, it induces intestinal amoebiasis responsible of colitis and bloody diarrhoea [7],[8]. An acute immune response is triggered during the early stages of the parasite infection. Amoebic liver abscesses are the most frequent with severe extra-intestinal clinical manifestations of...
Amoebiasis is a disease caused by a protozoan parasite, *Entamoeba histolytica*, with or without clinical symptoms. Humans are the only relevant host of this parasite, which mainly targets the large intestine and the liver. The current drug, metronidazole, has been successfully used against this parasite for several years. However, some reports have shown either parasite resistance or adverse effects due to its long term usage. Our study thereby pointed to alternative treatment of this infection by investigating the rational use of *Codiaeum variegatum* also referred as “garden croton” which is a medicinal plant used in Cameroon against bloody diarrhoea. We moved into the identification of the most efficient fraction of the aqueous extract of this plant, and initiated the characterization of the mechanism of action of this fraction. Upon treatment with the active fraction, parasite death occurs within two days through morphological changes such as cell membrane disorganization and cell destruction. More deeply, we found that components of the active fraction modify expression of genes involved in ceramide biosynthesis, a pathway responsible for cell death and growth inhibition. Our study therefore suggests a novel finding which could be further explored for screening of anti-amoeobic drugs.

Methods

Preparation of extracts

Leaves of *C. variegatum* were harvested in Yaoundé (Cameroon) according to several criteria: sites of cultivation (forest and garden), period of the day (morning, midday, afternoon and midnight) and stage of development (young and old leaves). It should be noted that young or old leaves as well as leaves from the forest and the garden were all collected in the morning. Leaves from more than 20 different plants were collected. The leaves were thoroughly washed with tap water, rinsed with distilled water, dried at room temperature and ground. The powder obtained, 200 grams (g) for each batch, was mixed with 2 litres of distilled water for the preparation of aqueous extracts by decoction for 1 hour. After filtration with Whatman No. 1 filter paper, the filtrate collected was dried by lyophilization.

Fractionation procedure and isolation of compounds

The sequential decoction of powdered leaves (start with 4100 g) yielded 992.34 g of aqueous extract which after washing with methanol led to 470.18 g extract. The methanol extract was then partitioned on silica gel by flash chromatography using a gradient of ethyl acetate (EtOAc) and methanol (MeOH). Eight stages of polarity were used: EtOAc (Fraction 1); EtOAc/MeOH 10% (Fraction 2); EtOAc/MeOH 20% (Fraction 3); EtOAc/MeOH 30% (Fraction 4); EtOAc/MeOH 40% (Fraction 5); EtOAc/MeOH 50% (Fraction 6); EtOAc/MeOH 80% (Fraction 7) and MeOH (Fraction 8). Fraction 1 (30.67 g) was further partitioned with a gradient polarity of ethylene chloride (CH₂Cl₂)/methanol (MeOH) solvent using a silica gel column chromatography. Four solvent systems (CH₂Cl₂; CH₂Cl₂/MeOH 2%; CH₂Cl₂/MeOH 5% and CH₂Cl₂/MeOH 10%) were used during the elution and more than 100 samples (80 ml each) were collected and grouped in 14 sub-fractions according to their chemical profiles analysed by thin layer chromatography. In all the fractionation, solvent was removed in collected samples by using a rotary evaporator. The action is still unclear. Fifty-five medicinal plants belonging to different families, selected on the basis of their traditional use against intestinal and liver disorders, were tested for their anti-amoebic activities on polyxenic cultures of *E. histolytica*. From these plants, the aqueous extract of leaves of *C. variegatum* exhibited a pronounced anti-amoebic activity [19]. Identification of its active compounds and characterization of their mechanism of action might provide new candidates for development of anti-amoebic drugs. The present study goes further in the characterization of *C. variegatum* amoebicide fraction. A bioassay-guided screening of various fractions of the aqueous extracts from *C. variegatum* leaves was carried out against trophozoites in axenic culture. The EC₅₀ values were determined for toxic fractions inducing parasite death. Then, an attempt to understand the mode of action of the compounds within active fraction was addressed by differential gene expression analysis using high-throughput RNA Sequencing. The data suggested a multi-target mechanism of action inducing cell death through the disturbance of lipid metabolism in the parasite. This cellular response is different when compared to results obtained by MTZ treatment. We therefore hypothesize that cell death might occur through the disturbance of certain biochemical processes within the cell membrane involving ceramide, a bioactive lipid known as cellular signal implicated in induction of apoptosis and cell growth inhibition. This study suggests a new mechanism of action for anti-amoebic compounds, which can be further explored as a strategy for drug development.
final fraction or powder was stored at 4°C. Figure 1 depicts the general scheme of fractionation.

In vitro culture of *E. histolytica*

The virulent strain of *E. histolytica* (HM1:IMSS) was grown in 15 ml screw cap glass tubes at 37°C on TYI-S-33 axenic medium supplemented with 15% (v/v) complement-inactivated bovine serum (PAA laboratories GmbH, Austria), 3% Diamond vitamin Tween 80 (Sigma-Aldrich, Saint Quentin Fallavier, France) and 1% Penicillin-Streptomycin (Sigma-Aldrich, Saint Quentin Fallavier, France) [20]. The culture medium was renewed twice in a week and trophozoites at the exponential phase of growth were used in all experiments.

In vitro anti-amoebic assay

Plant extracts, fractions, sub-fractions and isolated compounds were prepared using sterile DMSO (Sigma-Aldrich, Saint Quentin Fallavier, France) and culture medium leading to concentrations of 100, 50 or 10 mg/ml respectively. Each mixture was filtered with sterile syringe filters (Ø 22 μm) and aliquots of two-fold serial dilutions were prepared from these stock solutions. A fresh culture of $5 \times 10^3$ trophozoites per milliliter was introduced in each well of the 48 well microtiter plate and after allowing the parasite to adhere at the bottom of the well, 5 μl of the tested extract or compound was added. The concentration of DMSO did not exceed 0.5% in all assays performed. Each test included a blank (medium only) and two controls (one consist of trophozoites with medium only and the other consist of trophozoites with medium containing DMSO). Metronidazole (Sigma-Ultra, CA, USA) was used as the positive control in each assay (see tables and figures in the results section for the concentrations tested). For microarray experiments, MTZ was used at 8 μg/ml, which correspond to 50 μM. The plates were introduced in Genbag anaer (Biomerieux, Marcy l'Etoile, France) and incubated for 48 to 72 hours at 37°C and the cell viability was evaluated with a hemocytometer using the trypan blue exclusion technique. In some cases (as indicated in the tables) cells were incubated only for 24 hours. The mortality rate of trophozoites was calculated for each concentration tested according to the formula below. The 50% efficient concentrations (EC$_{50}$) were determined by plotting the graph of mortality rate versus the concentration tested and using a normalised sigmoidal function of the software Statgraphics, Plus Version 5.0.

\[
\text{Mortality rate (\%) = } \frac{\text{Number of viable cells (DMSO) - Number of viable cells (Assay)}}{\text{Number of viable cells (DMSO)}} \times 100
\]

Cytotoxicity assay on human enterocytes from the Caco-2 cell line

The human colon carcinoma cell line TG7 (Caco-2) was grown to 21 days confluence in Dulbecco’s modified Eagle’s medium (Life Technologies, Saint Aubin, France) supplemented with 15% fetal calf serum (Eurobio, Les Ulis, France) and 1% non-essentials amino acids (Life Technologies, Saint Aubin, France) at 37°C in a
10% CO₂ incubator. Differentiated Caco-2 cells were incubated for 48 hours with varying concentrations of ethyl acetate fraction (F1), sub-fractions (SF9, SF10, SF11 and SF9B) and the aqueous extract. The highest concentration tested on these cells was 1 mg/ml; over this concentration the aqueous extract was no longer dissolved in DMSO. Staurosporine 0.1 μM (a chemical which induces apoptosis) was used as a control for cell death and the solvent (DMSO) was the control for viability. After incubation, the culture medium was removed and the viability count was performed using the trypan blue exclusion technique.

Statistical analysis
The tests were performed in triplicate and all data are presented as mean ± SD (standard deviation) values. Statistical analysis was performed using GraphPad Instat and student's t-test was used to determine P-values for the differences observed between test compounds and control. Results were considered significantly different when *P*≤0.05.

Generation of transcriptome sequencing data
Trophozoites of *E. histolytica* (approximately 1×10⁶) grown in 15 ml glass tubes were treated with plant extract sub-fraction SF9B (at EC₅₀ discussed later) or DMSO (control) for 12 and 24 hours (in 3 biological replicates, n=4×3). Total RNA was extracted from these trophozoites using Trizol reagents (Invitrogen, Saint Aubin, France) and poly(A)+ RNA was purified from 10 μg of total RNA using oligo(dT) coated Sera-Mag Magnetic Particles according to manufacturer’s instructions (Thermo Scientific, Fremont, USA). PolyA enriched RNA is chemically fragmented to ~100 bp (Ambion, USA) and purified with RNeasy MiniElute Cleanup Kit (Qiagen, Venlo, Netherlands) according to manufacturer’s instructions. Strand-specific cDNA libraries (n=12) were prepared using an RNA ligation protocol based on Illumina TruSeq Small RNA Sample Preparation Kit [21]. Sequencing of these libraries was performed on a HiSeq 2000 (Illumina) in a multiplexed single-ended setting for 50 cycles using TruSeq SR Cluster kit v3 cBot HS and TruSeq SBS kit v3 HS (Illumina). After sequence files generation using CASAVA 1.7 (Illumina), 3’ adapter sequence was trimmed using Cutadapt [22]. These processed short reads data have been deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB3953).

Analysis of transcriptome sequencing data
Sequence reads were mapped to *E. histolytica* genome assembly (AmoebaDB v1.7, http://amoebadb.org/amoeba/) using Tophat version 2.0.6 [23] with default parameters. Coding genes differentially expressed in cells treated with SF9B versus control (in triplicates) at 12 or 24 hours were identified using Cuffdiff version 2.0.2 [23] and DESeq version 1.12.0 [24] with default parameters. Coding gene models were based on the bona fide gene models defined in previous work [25]. Differentially expressed genes were defined as genes at ≥5% false discovery rate with ≥2-fold change identified in either Cuffdiff or DESeq analyzes.

Microarray analysis of *E. histolytica* upon treatment with metronidazole
Trophozoites of *E. histolytica* (4×10⁶) grown in axenic culture and treated or not with metronidazole (at 50 μM for one hour), were lysed with Trizol reagent and total RNA isolated according to the manufacturer’s protocol. RNA was analyzed for integrity and the concentration determined by capillary electrophoresis using the Agilent Bioanalyzer 2100 RNA nanochip Assay (Agilent Technologies). Agilent microarrays EH-IP2008, scanning the entire amoebic genome, were used as previously described [26]. Dye swap hybridizations were performed for the three biological replicates leading to a total of 6 competitive hybridizations. The whole data set was submitted to the ArrayExpress database (Accession number: E-MTAB-1763). After pooling data from technical and biological replicates, differential analysis was carried out as published [26] and includes paired Student’s t-test. The raw P-values were adjusted by the Benjamini and Yekutieli method which controls the false discovery rate (FDR) [27]. We considered as being differentially expressed the genes with a Benjamini and Yekutieli P-value <0.05 and expression fold change ≥2.

Indirect immunofluorescence of treated trophozoites of *E. histolytica*
Trophozoites of *E. histolytica* (5×10⁵ ml⁻¹) were mixed with 5 μl of the active sub-fractions to obtain final concentrations of EC₅₀. The microtiter plate was introduced in Genban agar and then incubated at 37°C for 12, 24 and 48 hours and cell viability was evaluated as above described. The same assay was carried out on 8-wells Chamber Slide System (Brumath, France), the chamber was introduced in a Genban agar for 12 hours and the morphology of amoebae was examined using indirect immunofluorescence assay. Briefly, amoebae cells were fixed with 300 μl of formaldehyde 3.7% (Thermo scientific, Waltham-MA, USA) for 30 minutes, washed with 500 μl of 3% BSA in PBS and then incubated for 30 minutes at 37°C. The primary antibody Gal/GalNac lectin (1:100 diluted in 1% BSA-PBS) was added and the plates incubated for one hour at 37°C in humidified atmosphere. The plates were washed twice with 1% BSA-PBS. The secondary antibody Alexa Fluor 546 goat anti-rabbit IgG (1:200 diluted in 1% BSA-PBS) was added and incubated for 45 minutes at 37°C. At the end of the experiment, the plates are washed thrice with PBS and the mounting medium Vectorshield with nuclear stain DAPI (Vector-ABCYS) was used. For every assay, DMSO is used as negative control. The cells were observed by epifluorescence microscopy (Olympus).

Results

In vitro anti-amoebic activity of extracts
The anti-amoebic action of *C. variegatum* was assessed on axenic culture of trophozoites and the results are presented as follow: in the presence of the plant aqueous extract, the growth inhibition or mortality of *E. histolytica* increases in a concentration dependent manner and the collection criteria of leaves for extract preparation as well as the period of incubation significantly influenced the mortality rate. By plotting the mortality rate against concentration, EC₅₀ of the extracts were determined (Table 1). Despite the fact that no extract induced total mortality (100%) in the performed assay, we did not observe any stationary phase in amoebic growth, (Figure 2). We noticed that the anti-amoebic activity of the extracts depends on the leaves harvest criteria and it increases with the time of incubation. No significant difference was observed among the extracts from different sites of plant collection; extract from plants harvested at midnight (E6) showed significantly higher anti-amoebic activity compared to extracts from plants harvested at other period of the day. Extract obtained from old leaves and collected in the morning (E8) exhibited the highest significant anti-amoebic activity amongst all samples tested and displayed a EC₅₀ of 120.00 μg/ml after 48 hours of incubation and 60.54 μg/ml after 72 hours of incubation. However, when compared to pure MTZ (EC₅₀ = 0.73 μg/ml after 72 hours of incubation), extract E8 showed significantly lower anti-amoebic activity. Due to its
complex chemical composition, which may prevent activity of some compounds, we promptly initiated the identification and characterization of active compounds in E8.

Fractionation of aqueous extract enhances the anti-amoebic activity

After extraction with methanol (MeOH), we attempted to identify the active components in different fractions derived from chromatography (Figure 1). Three fractions (F1, F2 and F8) showed significant anti-amoebic activity, while F1 incubation for 48 hours was the most active amongst all conditions tested. Then, these three active fractions were further tested during 72 hours of incubation. The first observation was that the unfractionated MeOH extract achieved total mortality (100%) at the concentration of 500 μg/ml. Determination of EC_{50} was carried out for the unfractionated MeOH extract and three active fractions, which all exhibited at least 50% mortality (Table 2). The unfractionated MeOH extract was more potent than any of the three active fractions alone, with EC_{50} of 126.50 μg/ml and 53.00 μg/ml after 48 and 72 hours of incubation, respectively. Although the fraction F1 did not achieve total mortality, more than 90% of trophozoites were killed and this fraction was significantly more potent than other two active fractions, with EC_{50} of 202.00 μg/ml and 61.83 μg/ml after 48 and 72 hours of incubation, respectively. F1 was further explored owing to its relatively simpler chemical composition and its relatively higher efficacy compared to the other two active fractions.

Guided anti-amoebic paths to purify the ethyl acetate fraction

Silica-gel column chromatography was performed on F1, yielding 14 sub-fractions grouped according to their frontal ratio on thin layer chromatography profiles (Figure 1). Three of these sub-fractions (SF9, SF10 and SF11) exhibited significant anti-amoebic activity. Table 3 summarises EC_{50} of the active sub-fractions. Mortality due to these sub-fractions increased in a concentration dependent manner and 100% mortality was observed for sub-fractions SF9 at 125 μg/ml and for SF11 at 250 μg/ml after 72 hours of incubation. SF9 showed significantly higher anti-amoebic activity compared to other sub-fractions, with EC_{50} of 15.62 μg/ml after 72 hours of incubation. The chemical analysis of the 3 sub-fractions (SF9, SF10 and SF11) using thin layer chromatography and nuclear magnetic resonance revealed a common spot at the same frontal ratio and similarity between the three spectra meaning that these sub-fractions contain similar compounds, respectively. Interestingly, the presence of a small amount of observable crystals in these sub-fractions might imply the existence of certain compounds in the sub-fractions at a considerably high purity. In order to identify these compounds, the most active

| Table 1. EC_{50} of different aqueous extracts against E. histolytica after 48 and 72 hours of incubation. |
|-----------------------------------|----------------|----------------|
| **EC_{50} (Mean ± SD) [μg/ml]**   | 48 hours       | 72 hours       |
|-----------------------------------|----------------|----------------|
| E1 (Forest)                       | 204.33±12.02   | 102.00±4.33    |
| E2 (Garden)                       | 219.00±3.53    | 118.50±4.35    |
| E3 (Morning)                      | 191.16±5.00    | 119.33±3.61    |
| E4 (Midday)                       | 491.00±3.53    | 169.37±0.17    |
| E5 (Afternoon)                    | N              | 296.50±7.77    |
| E6 (Midnight)                     | 156.75±3.88    | 93.16±1.52     |
| E7 (Young)                        | N              | 182.50±2.82    |
| E8 (Old)                          | 120.00±7.77**  | 60.54±0.07**   |
| Metronidazole                     | 1.38±0.02**    | 0.73±0.03**    |

*significant difference between extracts;  
**significant difference of extracts compared to metronidazole (n = 3, p ≤ 0.05);  
Extracts E1, E2, E3, E4, E5, E6, E7, E8 were obtained from the forest. N means test non-performed.

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Figure 2. Anti-amoebic activity of different aqueous extracts according collection criteria (E1 = forest; E2 = garden; E3 = morning; E4 = midday; E5 = afternoon; E6 = midnight; E7 = young leaves and E8 = old leaves). Extracts were tested at increasing concentrations (31.25; 62.5; 125; 250; 500 μg/ml) for 48 hours (A) and 72 hours (B) of incubation. Data are obtained from three experiments and are presented as mean with bars as standard deviation (SD) compared to the control (DMSO).

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sub-fraction SF9 was further analysed chemically and the fractionation process is described in Figure 3. In fact, the sub-fraction SF9 was separated into a crystal fraction (SF9A) and a soluble fraction (SF9B). The crystal fraction was unfortunately less efficient than the soluble fraction. Chemical analysis of the soluble fraction suggested that SF9B is likely consisted of mainly 3 compounds (SF9B1, SF9B2 and SF9B3). The comparison of nuclear magnetic resonance (NMR) spectra of these sub-fractions suggests that they contain similar compounds with a common skeleton and some additional chemical groups which are important for their potency. Figure 4 described the superposition of different spectra of isolated sub-fractions or compounds. Further assays showed that SF9B1, SF9B2 and SF9B3 kill trophozoites with different potencies, while SF9B2 exhibited a pronounced activity against trophozoites comparable to the unfractionated soluble fraction (i.e. SF9B) and MTZ. EC50 of these active compounds were showed in Table 4. Based on the calculated EC50, the soluble fraction SF9B, which contained the mixture of at least 3 compounds, appeared as the most efficient killer of trophozoites compared to any of the isolated compounds alone (Figure 5). The high potency of SF9B can result from synergistic or additional action between isolated compounds and therefore SF9B was used in the following experiments.

Active components kill trophozoites of *E. histolytica* in axenic culture by inducing morphological changes and cell death

After incubating the trophozoites with SF9B for various durations (12, 24 and 48 hours) and concentrations (1.56–50 μg/ml), we determined an optimal condition which is sufficient to visualize cell morphology changes while keeping cell viability. The optimal condition is to expose trophozoites at EC50 with SF9B fraction and for a maximum period of 24 hours. The treatment of trophozoites with 3.78 μg/ml of SF9B2 and 2.75 μg/ml of SF9B caused different mortality rate according to the period of incubation (Figure 6). Death of trophozoites is firstly reflected by cell rounding and finally cell lysis. Dying cells detached from the bottom of the microtiter plate and attached viable cells could be counted.

Viability count of Caco-2 cells incubated with active components

Treatment of Caco-2 cells with the aqueous extract, fraction F1 and sub-fractions SF9, SF10, SF11 and SF9B at a wide range of concentrations did not show significant difference in cell death when compared to the negative control (DMSO). Therefore, at the tested concentrations, extracts and active fractions from *C. variegatum* had no observable cytotoxic effect on Caco-2 cells up to 1 mg/ml, while the positive control (Staurosporine 0.1 μM) induced substantial cytotoxicity between 30–40% of these cells.

**Table 2.** EC50 of methanol extract and active fractions against *E. histolytica* after 48 and 72 hours of incubation.

| EC50 (Mean ± SD) (μg/ml) | Incubation time | MeOH Extract | F1 | F2 | F8 |
|-------------------------|----------------|--------------|----|----|----|
|                         | 48 hours       | 53.00 ± 0.50*| 61.83 ± 0.57*| 202.25 ± 0.35| 234.25 ± 1.06|
|                         | 72 hours       | 126.50 ± 0.70*| 202.00 ± 3.50*| 398.00 ± 1.83| 479.50 ± 2.50|

| Metronidazole | 1.38 ± 0.02abc | 0.73 ± 0.03abc |

*Significant difference between methanol extract and fractions; 
*Significant difference between fractions; 
Significance difference of methanol extract and fractions compared with metronidazole (n = 3, p≤0.05).

doi:10.1371/journal.pntd.0002607.t002

**Table 3.** EC50 active sub-fractions against *E. histolytica* after 48 and 72 hours of incubation.

| EC50 (Mean ± SD) (μg/ml) | Incubation time | SF9 | SF10 | SF11 |
|-------------------------|----------------|-----|------|------|
|                         | 48 hours       | 18.87 ± 1.23* | 35.87 ± 2.69 | 26.50 ± 1.14 |
|                         | 72 hours       | ≤15.62* | 22.87 ± 1.59 | 17.91 ± 1.46 |
| Metronidazole           | 1.38 ± 0.02ab  | 0.73 ± 0.03ab |

*Significant difference between sub-fractions. 
Significance difference of sub-fractions compared to metronidazole (n = 3, p≤0.05).

doi:10.1371/journal.pntd.0002607.t003

**Table 2.** EC50 of methanol extract and active fractions against *E. histolytica* after 48 and 72 hours of incubation.

| EC50 (Mean ± SD) (μg/ml) | Incubation time | MeOH Extract | F1 | F2 | F8 |
|-------------------------|----------------|--------------|----|----|----|
|                         | 48 hours       | 53.00 ± 0.50*| 61.83 ± 0.57*| 202.25 ± 0.35| 234.25 ± 1.06|
|                         | 72 hours       | 126.50 ± 0.70*| 202.00 ± 3.50*| 398.00 ± 1.83| 479.50 ± 2.50|

| Metronidazole | 1.38 ± 0.02abc | 0.73 ± 0.03abc |

*Significant difference between methanol extract and fractions; 
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*Significant difference between sub-fractions. 
Significance difference of sub-fractions compared to metronidazole (n = 3, p≤0.05).

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Figure 3. Fractionation process of active sub-fraction (SF9). Activity-guided isolation of compounds using silica gel column chromatography. Active sub-fractions and compounds are marked in bold.
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Figure 4. Superposition of nuclear magnetic resonance (NMR) spectra of sub-fractions separated from SF9. (from top to bottom: SF9; SF9A; SF9B1; SF9B2 and SF9B3 respectively). Each product (1–10 mg) was dissolved in 0.7 ml of deuterated DMSO and the mixture was introduced into a tube adapted to the NMR engine and then was subjected to magnetic field to measure these resonances.
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Table 4. EC₅₀ of compounds and mixtures against *E. histolytica* after 24 and 48 hours of incubation.

| EC₅₀ (Mean ± SD) (μg/ml) | Incubation time | 24 hours | 48 hours |
|--------------------------|-----------------|----------|----------|
| SF9A                     | >50.00          | >50.00   |
| SF9B                     | 5.71±0.24a      | 2.75±0.10a|
| SF9B1                    | >50.00          | >50.00   |
| SF9B2                    | 10.80±0.35      | 3.78±0.31|
| SF9B3                    | >50.00          | >50.00   |
| Metronidazole            | 2.26±0.03ab     | 1.38±0.02ab|

*a*Significant difference between compounds and mixtures; 
*b*Significance difference of compounds or mixtures compared to metronidazole (n = 3, p<0.05).

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deficiency of ceramide in amoebae treated with SF9B. However, other suggested functions for TLC containing proteins are their role in protecting proteins from proteolysis through their binding to vacuolar ATPases [28] and their function as linkers of lipid transporters between the endoplasmic reticulum (ER)-to-Golgi traffic [29]. The categories of other downregulated genes include stress and oxysreduction, ATP binders, two cysteine proteinases (CP), cysteine synthase and a protein carrying myb transcription factor homology. Almost all of these genes are transcriptionally downregulated after 24 hours of compound treatment. The downregulation of heat shock proteins (Hsp 70 and Hsp 90), known to act as chaperones for signal transducers by blocking some steps of apoptotic pathways [30],[31], revealed the inhibition of any cell damage repair therefore impairing cell survival upon exposure to SF9B. Moreover, it is known that cysteine, a major thiol which replaces glutathione in *E. histolytica* and which plays a major role in growth and survival of *E. histolytica* [32],[33], is synthesized via a pathway consisting of two steps catalyzed by serine acetyltransferase and cysteine synthase [34],[35]. The reduced expression of cysteine synthase by treatment with SF9B implies low level of cysteine which is supposed to protect *E. histolytica* against oxidative stress and external environment that may cause cell death. Furthermore, down-regulation of two cysteine proteinases (CP-A5 and CP-A8) demonstrated loss or reduction of cytolytic activity of treated trophozoites. In fact, CP, especially CP-A5 was found to be associated with the trophozoite membrane and has been suggested to play a key role in host tissue invasion and destruction [36],[37].

Overall the findings of the RNAseq experiment were reinforced by transcriptome analysis of *E. histolytica* treated in the presence of MTZ (92% of cell survival determined by trypan blue assay). The expression of the entire genes set of *E. histolytica* was examined using EH-IP2008 microarray [26]. By MTZ treatment, we highlighted the downregulation of peroxiredoxin encoding gene but no changes in ceramide biosynthesis genes were found (Table S3). Notice that genes whose transcription was modified by the presence of MTZ were not significantly modulated by SF9B treatment.

SF9B treatment of *E. histolytica* modifies Gal/GalNAc lectin at the surface

A dual role of acid shingomyelinase (ASM) has been suggested. First, it has an essential housekeeping function within the lysosomes and late endosomes of virtually all cells, participating in membrane turnover. Second, ASM translocates from intracellular compartments to the cell membrane, where hydrolysis of sphingomyelin into ceramide initiates membrane reorganization and facilitates the formation and coalescence of lipid micro-domains, bringing inactive monomeric signalling proteins into active oligomers responsible for cell death. High levels of cholesterol and sphingolipid characterize these membrane domains. In *E. histolytica*, lipid rafts are enriched in Gal/GalNAc lectin, a surface protein complex involved in parasite adhesion to cells [38]. An immunofluorescence assay characterizing the Gal/GalNAc lectin, was thus used to examine the morphology changes and localization of the Gal/GalNAc lectin induced by active components from SF9B. In general, SF9B treatment caused significant changes on trophozoites surface after 12 hours of incubation. These changes were characterized by the accumulation of the cell surface Gal/GalNAc lectin in an
agglomerate patches at certain points of the parasite surface (Figure 7).

Discussion

In our previous study, fifty-five medicinal plants belonging to different families, selected on the basis of their traditional use against intestinal and liver disorders were tested for their anti-amoebic activities on polyxenic cultures of *E. histolytica* [19]. The aqueous extract from leaves of *C. variegatum* exhibited a clear anti-amoebic activity, therefore strengthening the local usage of this medicinal plant in the treatment of dysentery [19]. In the present research, the bioassay of extracts from different collection criteria aimed to search and adopt optimal conditions of phytochemical synthesis of secondary metabolites sometimes using the primary metabolites as precursors [43],[44]. While describing the fractionation process of compounds from the aqueous extract of *C. variegatum*, the anti-amoebic activity of each fraction or compound give a strong proof of the potency of this medicinal plant in the clearance of *in vitro* culture of *E. histolytica*.

Table 5. Changes in gene expression determined by RNASeq upon SF9B treatment of trophozoites.

| Gene ID* | Description | Fold Change** |
|----------|-------------|---------------|
|          | Cellular organization/Cell growth | 12 h | 24 h |
| EHI_040600 | Acid sphingomyelinase | nc | −2.16 |
| EHI_131880 | Acyl-CoA synthetase | nc | −4.28 |
| EHI_079300 | Long-chain-fatty-acid-CoA ligase | −5.88 | −9.90 |
| EHI_140740 | hypothetical protein (acyl-carrier) | nc | −3.31 |
| EHI_073450 | hypothetical protein (glycosyl transferase) | −6.71 | −6.13 |
| EHI_161070 | hypothetical protein (lipid binding) | nc | −2.51 |
| EHI_092490 | hypothetical protein (synthase, fatty acid) | −8.83 | −7.28 |
| EHI_009430 | hypothetical protein (synthase, fatty acid) | −18.81 | −6.15 |
| EHI_031640 | hypothetical protein (synthase, fatty acid) | −6.68 | −9.05 |
| EHI_139080 | Longevity-assurance family protein | nc | 8.15 |
| EHI_130860 | Longevity-assurance family protein | 5.5 | 4.53 |
|          | Stress and Oxyreduction | | |
| EHI_199590 | 70 kDa heat shock protein putative | nc | −4.38 |
| EHI_163480 | 90 kDa heat shock protein | nc | −3.36 |
| EHI_073480 | ADP-ribosylation factor | nc | −3.66 |
| EHI_189960 | ADP-ribosylation factor | nc | −3.05 |
| EHI_071590 | Protein disulfide isomerase | nc | −1.97 |
| EHI_021560 | Thioredoxin | nc | −3.17 |
| EHI_022950 | DNAJ family protein | nc | −2.94 |
| EHI_182520 | DNAJ homolog subfamily A member 1 | nc | −1.94 |
| EHI_019630 | hypothetical protein (Dipeptidyl-peptidase) | nc | −3.64 |
|          | Energy generation | | |
| EHI_095620 | ATP-binding cassette protein | −5.39 | −4.12 |
| EHI_178050 | ATP-binding cassette protein | −5.18 | −3.91 |
| EHI_131560 | hypothetical protein (ATP-binding cassette) | −7.76 | −4.18 |
| EHI_170940 | hypothetical protein | nc | −2.71 |
| EHI_119510 | hypothetical protein | nc | −3.45 |
| EHI_117580 | hypothetical protein | nc | −4.65 |
| EHI_013340 | hypothetical protein (Myb factor) | nc | −3.74 |
|          | Virulence | | |
| EHI_060340 | Cysteine synthase A | nc | −2.03 |
| EHI_010850 | Cysteine proteinase A5 | nc | −2.20 |
| EHI_039610 | Cysteine proteinase A8 | nc | −2.26 |

*Accession number from Amoeba DB data base, nc: non-significant changes. **Reads are the cDNA fragments sequenced and then mapped to the genome. Fold Change refers to the number of reads from cells in the tested condition divided by the number of reads from the non-treated control cells. doi:10.1371/journal.pntd.0002607.t005

The sub-fraction SF9B was identified for its strong anti-amoebic activity and when sub-fractionated, the resulting samples display reduced activity against *E. histolytica* suggesting that components of SF9B could act in synergy on single or multiple target sites associated with a physiological process or alternatively, the interaction between these compounds can improve their solubility and thereby enhance their bioavailability [45]. Synergic action has been noticed in several examples as a beneficial effect since it can help to eliminate eventual side effects associated with the predominance of a single xenobiotic
may modify the salvage pathway which utilizes long-chain sphingoid

agents and stress stimuli (such as chemotherapeutic agents) have

Ceramide accumulation most often occurs before any identifiable

that although phytotherapy do need isolation of active constituents, their identification is mostly required so that chemical profiling and establishment of phytoequivalence can be more precise in order to ensure herbal product approval and standardization [48]. The modifications of the parasite morphology when treated with active SF9B suggest that the mechanism of action passes through destabilization of the cell membrane constituents. Moreover, the transcriptome analysis of treated parasites revealed reduction in level of ceramide through downregulation of acid shingomyelinase and several enzymes implicated in fatty acids production which supports the disorganization of the cell membrane. The up regulation of Lag genes, encoding proteins recently renamed ceramide synthase [49] might overcome the reduction of ceramide in treated amoebae therefore leading to high level of ceramide which may modify lipid modifications of the parasite morphology when treated with active SF9B, thereby suggesting that SF9B may modify the salvage pathway which utilizes long-chain sphingoid bases to form ceramide; alternatively a possible reduction of ceramide release may occur due to reduction fatty acid metabolism and lipids trafficking in E. histolytica.

Conclusion

Figure 7. Localization of the Gal/GalNAc lectin on trophozoites surface after 12 hours treatment. DMSO is used as negative control and SF9B is tested at 2.75 µg/ml. The analysis was carried out with the epifluorescence microscope and photographs taken under 60 x objective lens.

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compound in the body [46],[47]. In addition, it has been suggested

modifications of the parasite morphology upon one hour treatment of trophozoites with 50 µM Metronidazole. Gene ID refers to the accession number of genes in AmoebaDB (http://amoebadb.org/amoeba/). FC: fold change; BY: False discovery rate by Benjamini and Yekutieli multiple testing; rawp: raw p-value.

Table S1 Modulation of gene expression in E. histolytica upon 12 hours treatment with SF9B fraction. Genes that are identified as differentially expressed in treated amoebae (i.e. FDR adjusted p-value <0.05) in either DESeq or Cuffdiff analyses were listed. Fold-changes were shown in linear scale. “Average normalized read count” refers to the upper-quartile normalized read count averaged over the triplicates. “Average abundance in RPKM” refers to Reads Per Kilobase per Million mapped reads averaged over the triplicates.

Table S2 Modulation of gene expression in E. histolytica upon 24 hours treatment with SF9B fraction. Genes that are identified as differentially expressed in treated amoebae (i.e. FDR adjusted p-value <0.05) in either DESeq or Cuffdiff analyses were listed. Fold-changes were shown in linear scale. “Average normalized read count” refers to the upper-quartile normalized read count averaged over the triplicates. “Average abundance in RPKM” refers to Reads Per Kilobase per Million mapped reads averaged over the triplicates.

Table S3 Genes modulated in E. histolytica upon metronidazole treatment. Differences in gene expression were evaluated by microarray upon one hour treatment of trophozoites with 50 µM Metronidazole. Gene ID refers to the accession number of genes in AmoebaDB (http://amoebadb.org/amoeba/). FC: fold change; BY: False discovery rate by Benjamini and Yekutieli multiple testing; rawp: raw p-value.

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Author Contributions

Conceived and designed the experiments: EMN CW CCH NAHC YJ MFGK PFM NG. Performed the experiments: EMN CW NAHC CCH YJ MFGK. Analyzed the data: EMN NAHC CCH NG. Contributed reagents/materials/analysis tools: EMN YJ CCH PFM. Wrote the paper: EMN CCH NG.

Supporting Information

compound and photographs taken under 60 x objective lens.
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