In vitro activity of the F-6 fraction of oregano against *Giardia intestinalis*

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SUMMARY

Giardiosis is a neglected parasitic disease that produces diarrhoea and different degrees of malabsorption in humans and animals. Its treatment is based on derivatives of 5-nitroimidazoles, benzimidazoles, nitrofuranes, acridine and nitrotiazoles. These drugs produce undesirable secondary effects, ranging from a metallic taste in the mouth to genetic damage and the selection of resistant strains; therefore, it is necessary to develop new therapeutic alternatives. We demonstrated that a 2-h treatment with 2·87 $\mu g\ ml^{-1}$ of fraction 6 of *Lippia graveolens* (F-6) was sufficient to kill half of an experimental *Giardia intestinalis* (Syn. *G. duodenalis, G. lamblia*) population, based on the reduction of MTT-tetrazolium salt levels. F-6 breaks the nuclear envelope and injures the ventral suckling disc. The major compounds of F-6 were characterized as naringenin, thymol, pinocembrin and traces of compounds not yet identified. The results suggest that *Lippia* is a potential source to obtain compounds with anti-*Giardia* activity. This knowledge is an important starting point to develop new anti-giardial drugs. Future studies will be required to establish the efficacy of F-6 *in vivo* using an animal model.

Key words: *Giardia intestinalis*, *Lippia* extracts, alternative treatments, naringenin, ultrastructure.

INTRODUCTION

Giardia intestinalis (syn. *G. lamblia, G. duodenalis*) is a mammal-infecting parasite (Adam, 2001). Since 1988, the World Health Organization has recognized that there are more than 280 million infections per year in Africa, Asia and America alone (Comité OMS d'Experts, 1988; Feng and Xiao, 2011). Giardiosis can be symptomatic or asymptomatic; however, independent of the clinical course, trophozoites harm enteric cells, hinder the absorption of nutriments and cause different degrees of malabsorption (Astiazarán-García *et al*. 2000), which can lead to malnutrition and delayed cognitive development (Berkman *et al*. 2002). The main treatments against *G. intestinalis* are based on derivatives of the following compounds: acridine, mepacrine (Mendelson, 1980) and quinacrine (Harris *et al*. 2001); nitroimidazoles, including metronidazole (Freeman *et al*. 1997), tinidazole (Jokipii and Jokipii, 1980), ornidazole (Jokipii and Jokipii, 1982), and other 5-nitroimidazoles (Upcroft *et al*. 1999); benzimidazoles, albendazole (Dutta *et al*. 1994), mebendazole (Bulut *et al*. 1996), nitrofuranes, and furoxone (Pickering, 1985); and more recently, nitazoxanide, a nitrothiazole (Romero *et al*. 1997; Ponce-Macotela *et al*. 2001). However, these drugs all produce undesirable secondary effects, ranging from nausea, (Davidson, 1984) and metallic taste in the mouth (Spellman, 1985) to psychosis (Upcroft *et al*. 1996), carcinogenesis (Gardner and Hill, 2001) and possible genetic damage (Legator *et al*. 1975; Mitelman *et al*. 1976). In addition, there is evidence suggesting the selection of resistant strains to anti-giardial drugs (Upcroft, 1994; Upcroft and Upcroft, 2001; Sangster *et al*. 2002; Dunn *et al*. 2010). Therefore, it is necessary to research and develop new alternatives for giardiosis treatment. Traditional indigenous medicine and ethnobotanical knowledge are commonly used as sources to find new drugs. In previous studies (Ponce-Macotela *et al*. 1994, 2006),

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whole *Lippia* extracts were found to be more potent than tinidazole; however, which substance(s) are responsible for the activity against *Giardia* is still unknown. In this study, we characterize the anti-*Giardia* activity of fraction 6 (F-6) from whole oregano ethanol extract.

MATERIALS AND METHODS

Giardia isolate

Giardia intestinalis INP020300B2 isolated from a lamb and identified as genotype A-I (not published) was cultured in TYI-S-33, harvested in log phase, washed 3 times with cold sterile phosphate-buffered saline (PBS, pH 7·0), counted in a Neubauer chamber, and used in bioassays.

Oregano plants

Oregano from Milpillas, Guanajuato was identified, and a sample was deposited at the Biology Institute with the identification code MEXU-IB-UNAM 1 022 934 as *Lippia graveolens* (HBK, 1818, syn. *Lantana origanoides, Lippia berliandieri*). *Lippia* leaves were frozen with dry ice and grounded in a mortar to a fine powder. The powder was then extracted in 75% ethanol/water (v/v) at 4 °C, with continuous stirring. The extractable substances were dried in a centrifuge (SpeedVac) at 1800 rpm for 8 h, then weighed and stored in amber bottles until experimental use.

Fractions

To identify the compound(s) responsible for the anti-giardial activity, the whole extract was fractionated on a chromatography column packed with silica gel and eluted with hexane/ethyl acetate (4:1), in fractions of 100 ml. We then analysed collected fractions using silica gel thin layer chromatography (TLC), and TLC spots were detected by spraying with 2% ceric sulphate ($Ce(SO_4)_2$) solution in 50% sulphuric acid and heating at 100 °C. Fractions with the same retardation factor (Rf) were pooled, vacuum-dried in a rotary evaporator TLC-re-analysed and stored in amber bottles at 4 °C until experimental use.

Pooled fractions that showed strong anti-giardial activity were further analysed for purity by HLPC. To characterize the major compounds within the fraction spectroscopy at 800 and 4000 nm, nuclear magnetic resonance (^{H}-NMR Variant Gemini) 200 MHz, mass spectrograph in a Joel RX 505 HA, and polarizer light rotation was performed.

Bioassays

To ascertain anti-*Giardia* activity, bioassays were performed as previously described (Ponce-Macotela *et al*. 2006). Briefly, 1·5 million G. *intestinalis* trophozoites were exposed to different *Lippia* fractions in PBS (pH 7·0), at concentrations ranging from 2 μg ml^{-1} to 50 μg ml^{-1} (fractions were dissolved in dimethyl sulphoxide (DMSO) before each single experiment, the final DMSO concentration was less than 0·05%) in 1.5 Eppendorf tubes, and incubated at 37 °C for 2 h. To eliminate traces of extract, the trophozoites were washed twice with PBS. Cellular viability was tested in 2 ways. First, the reduction of MTT-tetrazolium salts to MTT-formazan was measured. For this technique, exposed parasites were washed and subsequently incubated for 30 min at 37 °C in a solution containing 40 μl of MTT-tetrazolium salts (5·0 mg ml^{-1}) and catalysed with 20 μl of PMS (2·5 mg ml^{-1}). The synthesized dye was dissolved in isopropanol/hydrochloric acid and its concentration was measured in a spectrophotometer set at 570 nm (Ponce-Macotela *et al*. 1994). Second, cellular viability was tested by re-growth in fresh TYI-S-33 medium. The washed trophozoites, from experimental and control populations, were inoculated into culture tubes with fresh TYI-S-33 culture medium and incubated at 37 °C. To determine if there were changes in the *Giardia* populations, we looked for living cells attached to the tube walls every 24 h for 1 week. If there were trophozoites still alive, then the cells were harvested and counted in a Neubauer chamber.

Ultrastructure

To identify ultrastructural modifications, *Giardia* trophozoites from experimental and control groups were fixed in 2·5% glutaraldehyde buffered with phosphate buffer (0·1 M, pH 7·2), post-fixed in 1% osmium tetroxide, dehydrated in an ethanol series and embedded in LR white resin. Thin sections were mounted on formvar-coated copper grids and stained with lead citrate/uranyl acetate. The structural changes from 50 fields for each sample were recorded using a Carl Zeiss EM-109 transmission electron microscope.

Mimicking F-6 with commercial compounds

To reproduce F-6 mix we bought the 3 major compounds identified in *Lippia*-F-6 (LF-6). Bioassays were performed with every single compound to identify the substance with the main anti-giardial activity and with the artificial mix, because we hypothesize that these substances could have synergic activity. Assays to measure the *Giardia* sensitivity were the reduction of tetrazolium salts to MTT-formazan and re-growth, as we have described previously.

Every experiment was performed 4 times in a triplicate fashion and the controls were: unexposed trophozoites, trophozoites exposed to DMSO, tinidazole 250 μg ml^{-1}, and trophozoites treated with 3 freeze and thaw cycles in liquid N_2.

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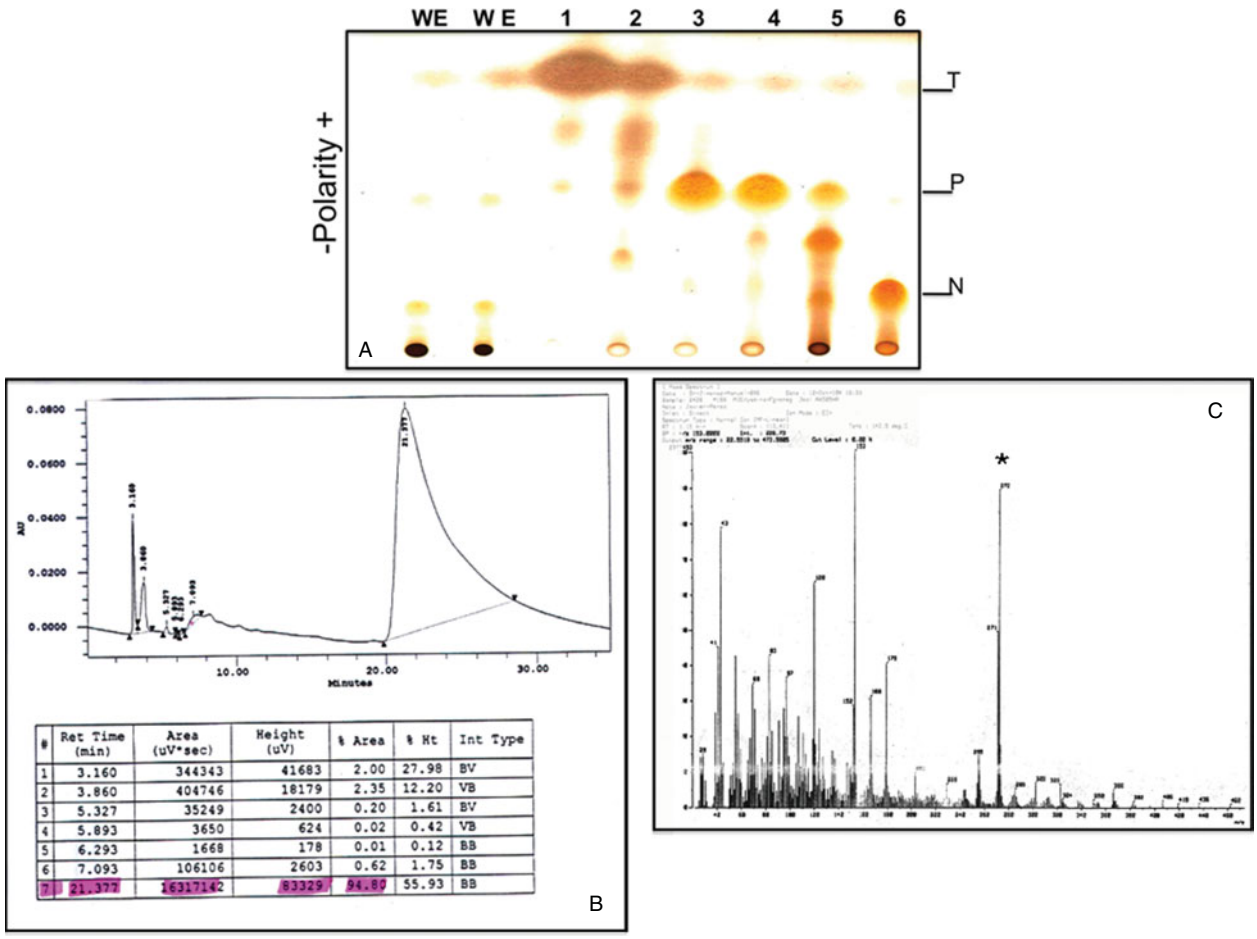

Fig. 1. TLC showing the pattern of 6 isolated fractions (1–6) and the whole extract (WE) from *Lippia graveolens*. (A) Relative positions of T: thymol; P: pinocembrin; N: naringenin. (B) Shows the F-6 relative concentration of compounds by HPLC, major component was naringenin. (C) The MS from F-6 shows a molecular ion (*) with 272 molecular weight, that corresponds to $C_{15}H_{12}O_5$.

Toxicity test on human lymphocytes

Lymphocytes were isolated from a whole blood sample obtained from a healthy donor using Ficoll-Paque gradient centrifugation. Approximately 300 000 lymphocytes were exposed to increasing concentrations of F-6 from 2–10 μg ml^{-1} in RPMI-1640 and incubated for 2 h at 37 °C in 5% CO_2. The cells were then washed with PBS and 1×10^5 lymphocytes were re-cultured in 100 μl of RPMI-1640 culture medium plus, 49 μl of XTT-tetrazolium salts (1 mg ml^{-1}), and 1 μl of PMS (0·383 mg ml^{-1}) in a 96-well plate and incubated at 37 °C for 24 h in 5% CO_2. Synthesized formazan was measured using a spectrophotometer set at 490 nm. As a negative control, unexposed lymphocytes were used, and as a positive control, cells treated with 3 freeze-thaw cycles in liquid N_2 were used.

Statistics

Statistical analysis was performed using the SPSS version 17 program. ANOVA and Tukey tests at a significance level of $P = 0{\cdot}05$ and Probit analyses were performed.

RESULTS

Fractions

Whole oregano extract was separated into 32 fractions, which were examined by TLC with ceric sulphate as the developing reagent. Fractions with the same Rf were mixed together, resulting in 6 fractions, the TLC-pattern of these fractions is shown in Fig. 1.

Bioassays

Lippia fractions were tested by measuring the reduction of MTT-tetrazolium salts to formazan and re-culturing in fresh medium. The performance of the 6 fractions was charted in Table 1. ANOVA analysis indicated significant differences between fractions at $P < 0{\cdot}05$. Probit analysis revealed that fractions F-5 and F-6 had the best levels of anti-*Giardia* activity in 2-h experiments, with LD_{50} values of 4·94 and 2·87 μg ml^{-1} (Table 2). Fraction F-6 had the highest level of anti-*Giardia* activity, killing more than the 80% of the population detected with tetrazolium salts assay and 100% of the population with the growth inhibition method at

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Table 1. Anti-giardial activity of fractions from *Lippia graveolens* from whole extract and control[a]

	F-1	F-2	F-3	F-4	F-5	F-6[c]
C μg/ml	% M ± S.D.[b]	% M ± S.D.	% M ± S.D.	% M ± S.D.	% M ± S.D.	% M ± S.D.
50	75·67 ± 35·24	88·81 ± 15·3	60·69 ± 32·49	87·18 ± 6·63	86·51 ± 5	87·42 ± 2·42
40	22·36 ± 19·33	67·62 ± 8	61·21 ± 12·5	76·23 ± 4·32	84·25 ± 3·68	85·67 ± 4·95
30	6·67 ± 6·43	34·63 ± 26·16	47·99 ± 8·31	74·51 ± 7·34	80·73 ± 1·27	84·35 ± 2·59
20	3·4 ± 5·9	4·48 ± 7·76	16·55 ± 2·97	26·12 ± 4·33	83·33 ± 3·19	84·55 ± 4·95
10	0	3·71 ± 6·43	9·8 ± 6·37	0·63 ± 1	76·61 ± 6·92	80·07 ± 4·36
8	0	0	0	0	76·6 ± 7·5	81·02 ± 3·89
6	0	0	0	0	69·49 ± 19·7	80·24 ± 5·12
4	0	0	0	0	13·52 ± 14·7	74·36 ± 6·97
2	0	0	0	0	5·99 ± 4·15	33·66 ± 29·21
0	0	0	0	0	0	0

[a] Tinidazol at 250 μg/ml produced a mortality of 44·52 ± 2·41, and inhibited 99·95% of *G. intestinalis* re-growth in fresh TYI-S-33.
[b] % mortality ± standard deviation.
[c] F-6 inhibited 99·95% of *G. intestinalis* re-growth in fresh TYI-S-33.

Table 2. Concentrations required to kill 50% of *Giardia intestinalis* populations and their significance values by Tukey tests for *Lippia graveolens* fractions, and commercial compounds

Fraction number	[μg/ml]*	Tukey test
F1	44·94	$P > 0·05$
F2	35·63	$P > 0·05$
F3	33·81	$P > 0·05$
F4	31·55	$P > 0·05$
F5	4·94	$P > 0·05$
F6	2·87	$P < 0·05$
Thymol	40·58	$P > 0·05$
Pinocembrin	38·88	$P > 0·05$

* Probit test, LD_{50}.

the lowest concentration 6 μg ml^{-1}. HPLC analysis of F-6 demonstrated that there were several compounds, the major one accounting for 94·80% of the area and traces in concentrations as low as 0·01% of the area (Fig. 1). Infrared spectroscopy analysis revealed absorption spectra consistent with a double bond of an aromatic ring from a phenolic or carbolic group and $^{H-}$NMR suggested the presence of a flavanone. Mass spectra yielded a molecular ion with a molecular weight of 272 (Fig. 1). These data are compatible with the compound 5,7,4′-trihidroxiflavanona, more commonly known as naringenin (Dominguez *et al*. 1989; Arcila-Lozano *et al*. 2004).

Re-culture and ultrastructure

In re-culture experiments, 2 μg ml^{-1} of F-6 was sufficient to produce profound physiological injuries that inhibited the growth of *Giardia* trophozoites in fresh TYI-S-33 medium. This result was substantiated by ultrastructural analysis, in which F-6 treatment at 2 μg ml^{-1} led to more evident endoplasmic reticulum cisterns (*) and irregularly shaped nuclei (Fig. 2B, C). Near the nuclei, large gaps of amorphous material and glycogen granules were found. In some cases, the double nuclear envelope was discontinuous (arrowheads Fig. 2B1, B2, C1 and C2). There was also destruction of the suckling (ventral) disc structure (VD arrows) and vesicles under the cytoplasm membrane. Trophozoites treated with concentrations of 4, 6, and 8 μg ml^{-1} presented a similar injury (data not shown). In contraposition with untreated trophozoites that present the classic structure, nucleus, axonemes, ventral suckling disc, ventrolateral flange and lateral crest (Fig. 2A).

Toxicity assays

Toxicity assays did not reveal any differences between *Lippia*-F-6 treated lymphocytes and untreated lymphocytes.

Mimicking the F-6 activity

The anti-giardial activities of pure thymol, naringenin and pinocembrin are summarized in the Table 3, it showed that neither pure compounds nor the artificial mix were able to mimic the anti-giardial activity of the Lippia-F-6. Values of LD_{50} were thymol 40·58 μg ml^{-1} and pinocembrin 38·88 μg ml^{-1}; data from the reduction of tetrazolium salts to formazan viability test are shown in Table 2.

DISCUSSION

Oregano is a plant used as a spice in many parts of the world. It is also a source of compound(s) with activity against *Giardia* and, as we previously demonstrated, it has a stronger anti-giardial effect than tinidazole

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Table 3. Performance of commercial compounds against *Giardia intestinalis* trophozoites[a]

	XTT Dead trophozoites % (±S.D.)			Re-growth Dead trophozoites % (±S.D.)		
C μg/ml	Naringenin	Thymol	Pinocembrine	Naringenin	Thymol	Pinocembrine
2	13·39 (135)	12·23 (4·87)	14·03 (7·8)	6·12 (10·61)	5·83 (10·10)	5·19 (8·99)
10	4·07 (5·38)	1·85 (1·62)	2·69 (2·76)	1·94 (3·36)	0 (0)	2·38 (4·12)
40	2·34 (1·93)	52·78 (49·11)	57·03 (37·51)	12·08 (9·03)	62·10 (54·20)	36·90 (54·90)
100	7·95 (5·09)	98·25 (2·33)	99·57 (0·53)	15·69 (27·19)	99·98 (0·02)	100 (0)

[a] Artificial F-6 with 3 compounds has a poor effectiveness against *G. intestinalis*, less than naringenin.

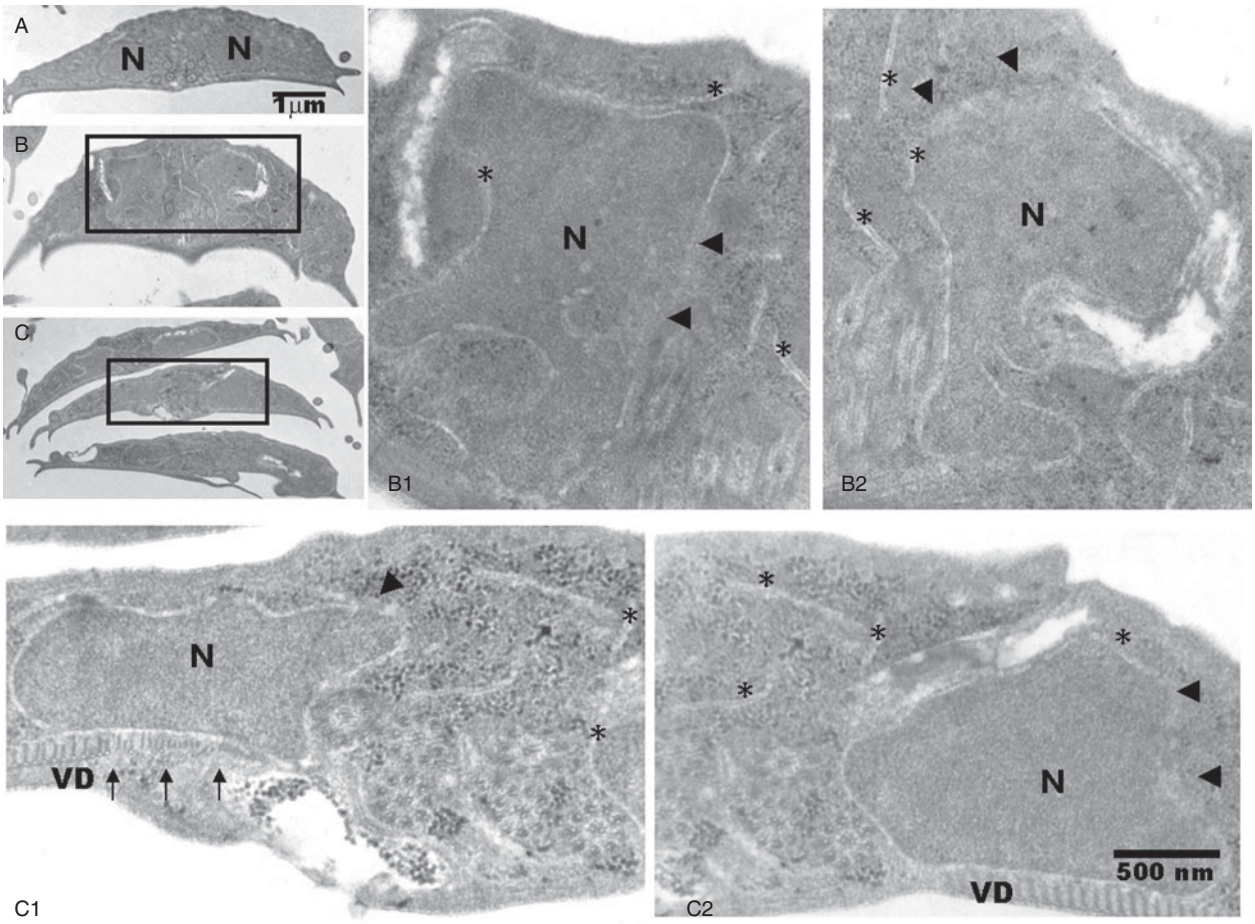

Fig. 2. (A) Untreated *Giardia intestinalis* trophozoites showing the classic morphology (N) nucleus. (B and C) Trophozoites treated with F-6 from *Lippia graveolens* at 2 μg ml^{-1} Scale bar = 1 μm. B1, B2, C1 and C2 highlight nuclear envelope discontinuities (arrowheads) membrane systems emphasized with asterisk, there are electron-lucid places filled with an amorphous proteinaceous substance. C1, arrows show damage in ventral suckling disc (VD) Scale bar = 500 nm.

(Ponce-Macotela *et al*. 1994, 2006). In those studies, however, the total extract was used at very high concentrations, ranging from 58·88 to 300 μg ml^{-1}. Here we analysed the anti-giardial activity of fractions isolated by column chromatography on *Giardia* trophozoites. The damage was evaluated using the reduction of MTT-tetrazolium salts to MTT-formazan and re-culturing in fresh TYI-S-33. Although the MTT-tetrazolium salt assay has low sensitivity, it allowed us to find a gross concentration that killed nearly 80% of the *Giardia* population and to discard concentrations that were below the efficacy of tinidazole.

We found that whole extract of *Lippia* contained several substances that were able to kill *Giardia* trophozoites. Fraction F-1 was mainly comprised of essential oils and had an LD_{50} of 44·94 μg ml^{-1}. This value is lower than that recorded by Machado *et al*. (2010). Probit analysis, indicated that fractions F5 and F6 had good activity against *Giardia* in experiments as short as 2 h. These experiments were performed with the B2 *Giardia* isolate, which is

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more resistant to tinidazole and nitazoxanida treatment than the MM or WB isolates (Ponce-Macotela *et al*. 2001).

In re-culture experiments, we found that a concentration of $6\,\mu g\,ml^{-1}$ produced irreversible damage on 100% of the B2-*Giardia* isolate population, as even treated trophozoites were unable to grow in fresh culture medium. From these results we inferred that the lethal dose at 100% was $6\,\mu g\,ml^{-1}$; ($LD_{100} = 6\,\mu g\,ml^{-1}$). This concentration was 10 times lower than values previously reported for the MM-isolate (Ponce- Macotela *et al*. 2006).

To further understand the failure of the trophozoites to re-populate tubes in re-culture experiments, we looked for ultrastructural damage on *Giardia* trophozoites. The analysis revealed that the lowest concentration of fraction F-6 tested, $2\,\mu g\,ml^{-1}$, was enough to damage the nuclear envelope, giving rise to perforations on the nuclear membrane (Fig. 2, arrowheads). In previous studies, we demonstrated that total *Lippia* ethanol extract produced changes in permeability, causing trophozoites to appear swollen. In addition, we observed a lack of glucocalix, gaps on the nuclear envelope and a loss of the nucleoskeleton structure (Ponce-Macotela *et al*. 2006). Similar gaps on the *Giardia* nuclear envelope treated with *Lippia* essential oils were observed by Machado *et al*. (2010), suggesting that oils reduce *Giardia* adherence. We also found that the ventral (suckling) disk showed protein pattern destruction, which could explain the findings of Machado *et al*. (2010). The fraction F-6 is mainly comprised of naringenin, thymol, pinocembrin and traces of undetermined compounds. Naringenin is a flavonoid found in many plants; in a previous study, anti-giardial activity with an $IC_{50} = 47{\cdot}84\,\mu g\,ml^{-1}$ was demonstrated (Calzada *et al*. 1999). In addition, it has anti-bacterial activity as tested in *Staphylococcus aureus*, *Salmonella enterica*, and *Listeria monocytogenes* (Mandalari *et al*. 2010). Pinocembrin from *Teloxys graveolens* has an activity with an $IC_{50} = 57{\cdot}4\,\mu g\,ml^{-1}$ (Calzada *et al*. 1999, 2003). The F-6 mix from *Lippia* had activity against *G. intestinalis* after 2 h of treatment, with a LD_{100} of $6\,\mu g\,ml^{-1}$.

At this moment, we are yet unable to establish the identity of the compound responsible of the anti-giardial activity because the LD_{50} found for thymol and pinocembrin were higher than those observed for F-3. On the other hand, we failed to mimic the *Lippia* F-6 because artificial F-6 had a very low performance when we compared between them. These facts can only be explained if we assume that some of the components at the trace level had the main anti-giardial activity, or that all compounds are necessary because they have synergic activity. This is an ongoing study with a successful mix of compounds, which is capable of killing 50% of a *Giardia* trophozoite population at concentrations as low as $2{\cdot}87\,\mu g\,ml^{-1}$ in experiments as short as 2 h.

In addition, toxicity experiments did not show differences between experimental and control groups ($P > 0{\cdot}5$), suggesting that F-6 (at $10\,\mu g\,ml^{-1}$) did not damage the defensive cells. Additional studies should be performed to confirm the lack of toxicity of F-6.

Many important follow-up experiments are possible, including identification of the F-6 molecular targets on *Giardia* trophozoites and performing experiments *in vivo*.

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