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Antiprotozoal Activity of a *Thymus vulgaris* Methanol Extract and Its Fractions

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Abstract

Introduction: *Thymus vulgaris* is used in traditional medicine to treat gastrointestinal diseases because of its antifungal, antibacterial, and antispasmodic activity. **Objective:** To verify whether *Thymus vulgaris* also has antiprotozoal activity against *Trichomonas vaginalis*, *Giardia lamblia* and *Entamoeba histolytica* trophozoites. **Materials and methods:** Conventional cultures of parasites were measured on the third day during the logarithmic growth phase. The antiprotozoal activity of the methanol extract and its fractions were evaluated comparing growth in cultures with and without extracts. Next, the extract was fractionated by polarity-based partitioning. Then, the purity of each fraction was determined by thin layer chromatography (TLC). The percentage of growth inhibition was calculated with respect to untreated controls. The 50% inhibitory concentration (IC_{50}) of each extract was calculated by PROBIT analysis. **Results:** We found that a methanol extract of the aerial parts of *Thymus vulgaris*, at 300 μg/mL, inhibited the *in vitro* growth of *G. lamblia* and *T. vaginalis*, while *E. histolytica* growth was poorly inhibited. The methanol extract was further separated into mixtures of ursolic, oleanolic, and betulinic acids. The IC_{50} values of ursolic acid against *G. lamblia* and *T. vaginalis* were 8.12 μg/mL and 5.51 μg/mL, respectively. **Conclusions:** The methanol extract fraction containing ursolic acid obtained from *Thymus vulgaris* has antiprotozoal activity against *G. lamblia* and *T. vaginalis* trophozoites.

Keywords

Thymus vulgaris, *Trichomonas vaginalis*, *Giardia lamblia*, *Entamoeba histolytica*, Antiprotozoal Agents

1. Introduction

Gastrointestinal diseases are one of the most frequent causes of medical consultation in Mexico and in the world. For this reason they are regarded as public health problems. These diseases affect people of any age or gender, but the most vulnerable are children and the elderly. Globally, these diseases are the leading causes of illness and death in these age groups. The most common agents associated with gastrointestinal infections are the bacteria *Escherichia coli*, *Salmonella typhi*, and *Shigella* spp., parasites such as *Giardia lamblia* and *Entamoeba histolytica*, and viruses such as rotavirus and Norwalk virus. Oral-fecal transmission, *i.e.* ingesting feces-contaminated water or food, is the most common route of infection [1].

G. lamblia, the causative agent of giardiasis, is a water-borne intestinal protozoan that infects human and other mammals worldwide [2] [3]. Approximately 200 million people are affected by giardiasis in tropical and subtropical countries [4]. Every year, 500,000 new cases of giardiasis are reported [5]. Over 55% of the Mexican population is seropositive for this parasite [6].

Another common gastrointestinal infection is amoebiasis, which is caused by *E. histolytica*. Around the world, 50 million people per year are infected by *E. histolytica*, but only 5 million develop the disease, resulting in 100,000 deaths each year [7]. In this disease, the most common clinical forms are dysentery and amebic liver abscess (ALA) [8] [9]. In Mexico, 8.4% of the population is seropositive for *E. histolytica* [7] [9]. It is estimated that just over one million cases are treated and 1216 deaths per year are caused by this disease [10].

Sexually transmitted diseases are the leading cause of acute illness worldwide. Trichomoniasis, caused by the protozoan parasite *T. vaginalis*, is the most common, curable, sexually transmitted disease, generating more than 170 million cases each year around the world [11].

The most common drugs to treat of amoebiasis, giardiasis and trichomoniasis are 5-nitroimidazoles derivatives, such as metronidazole or tinidazole [12] [13] [14]. A recently reported increase in the resistance to metronidazole and 5-nitroimidazoles of *E. histolytica*, *G. lamblia* and *T. vaginalis* strains poses a serious problem [15], and has caused a heightened interest in the discovery and development of new compounds to treat these diseases [16].

Thymus vulgaris is a plant of European origin and belongs to the Lamiaceae family. In the chemical composition of *Thymus vulgaris*, essential oils are predominant, especially the monocyclic monoterpenes, (thymol and carvacol), as well as other monoterpenes (p-cymene, camphene, limonene, borneol, among others). Besides, *Thymus vulgaris* contains flavonoids (apigenin and luteonin),

methoxylated flavones (cirsilineol, cirsimartina), and some other minor components, such as phenolic acids (caffeic acid and rosmarinic acid), tannins, and triterpenes (ursolic acid and oleanolic acid) [17]. The latter have been reported in methanol and hexane extracts.

The antiseptic and anti-inflammatory activity of *Thymus vulgaris* is well-known. *In vitro* studies have proven high antibiotic activity against *Mycobacterum tuberculosis* [18], cutaneous leishmaniasis [19], *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* [20]. Furthermore, *Thymus vulgaris* has fungicide activity and was able to protect germinating crop seeds from fungal attack [21].

The purpose of this study was to investigate the *in vitro* antiparasitic activity of the *Thymus vulgaris* methanol extract and of some of its components by verifying their capacity to inhibit the growth of *G. lamblia*, *E. histolytica*, and *T. vaginalis* trophozoites.

2. Materials and Methods

2.1. Chemicals and Stock Solutions

All chemicals were reagent grade. DMSO and the standard drugs were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals used in this study were purchased from J. T. Baker (Xalostoc, Edo. de Mexico, Mexico). Sterile bovine serum and PEHPS medium were prepared in our laboratory as described elsewhere [22]. Metronidazole (Sigma Chemical Co.), a nitroimidazole antibiotic and antiprotozoal agent was dissolved in DMSO to get 500 μg/mL concentration and further diluted adding 500 μL in 10 mL of dimethyl sulfoxide (DMSO), finally this solution was added to media 1:10 v:v to give 12.5 μg/mL final concentration. Ursolic acid and betulinic acid solutions (Sigma Chemical Co.), antiprotozoal pentacylclic triterpenoids, were prepared adding 2 mg of the pure compound (Sigma Chemical Co.) in 2 mL of DMSO and further diluted to 25 μg/mL. Stock solutions were stored at −20˚C until use.

2.2. Plant

Thymus vulgaris was obtained from local markets and identified at the herbarium of the Faculty of Forestry of the Autonomous University of Nuevo Leon, Mexico (Boucher number 21,344). The aerial parts of the plant were dried in electric oven at 40˚C for 72 h (J.M. Ortiz. Aparatos Eléctricos S.A. de C.V., Monterrey, N.L. México) and ground with the use of an electric mill (Molino del Rey, S.A. de C.V., San Nicolas de los Garza, NL, Mexico).

2.3. Protozoa

G. lamblia 0989-IMSS strain (5×10^3 trophozoites) was cultivated in 5.5 mL TYI-S33 medium in 13 × 100 culture tubes (Pyrex, Corning life Sciences, Corning, New York, NY, USA) at 36.5˚C. The duplication time for *G. lamblia* trophozoites was 13.1 h (**Figure 1**).

E. histolytica HM1-IMSS strain (5×10^3 trophozoites) and *T. vaginalis* GT-15

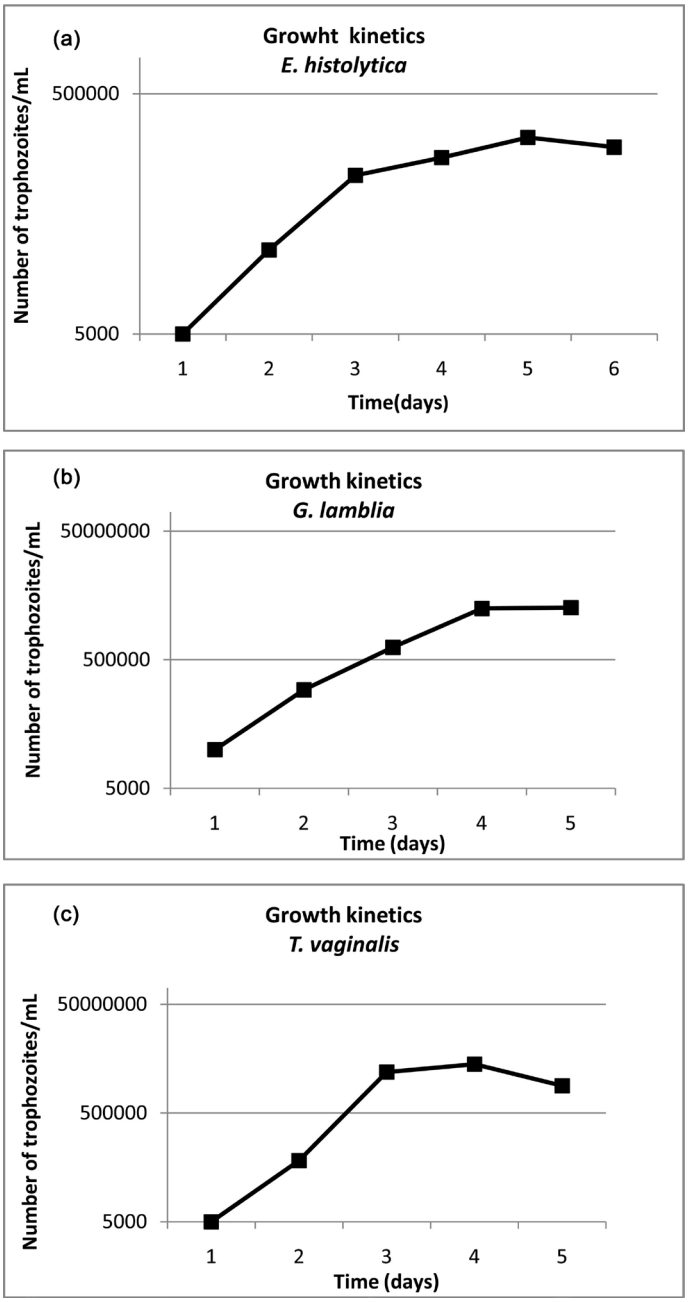

Figure 1. Growth curve of *E. histolytica* (a) *G. lamlia* (b) and *T. vaginalis* trophozoites (c).

strain (5×10^3 trophozoites) were cultivated in 5.5 mL PEHPS medium [22] at 36.5˚C. The duplication time for *E. histolytica* was 21.3 h and for *T. vaginalis* 11.7 h (Figure 1).

2.4. Preparation of the Methanol Extract

The crude extract was obtained by maceration and mixing with methanol. Briefly, 150 g of the dried, ground, aerial *Thymus vulgaris* parts were stirred with 500 mL methanol for 72 h on a rotary shaker (PC 622 Corning, New York, NY, USA). The supernatant of crude methanol extract was filtered using Whatman #

1 papers (Whatman International, LTD, Maidstone, England) and air-dried. This extraction was repeated two more times. The pooled filtrates were stored in the dark at 4˚C in the dark until use. Thirty mg of the extract was dissolved in 250 µL dimethylsulfoxide (DMSO), which was further diluted in distilled water to create a stock of 6 mg/mL.

2.5. Fractionation of the Extract

Once the anti-parasitic activity of the methanol extract had been verified (Figure 2), the extract was fractionated by polarity-based partitioning: after a single

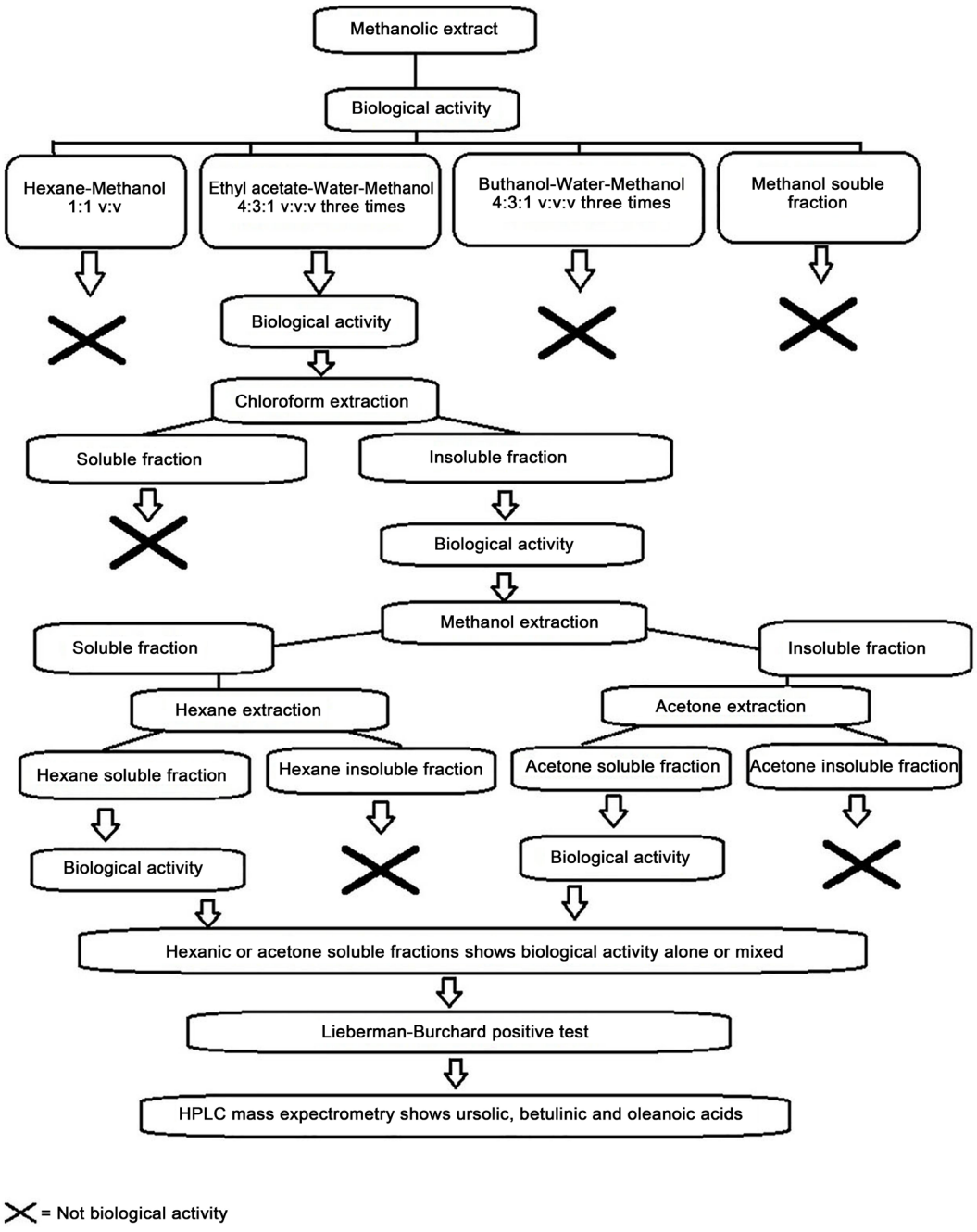

Figure 2. Work flows of bio-guided extraction and fractionation process.

hexane:methanol (1:1, v/v) fractionation, and triple ethyl acetate:water:methanol (4:3:1, v/v) and n-butanol:water:methanol (4:3:1, v/v) fractionations, the soluble methanol fraction was recovered. All fractions were allowed to dry completely before being tested for antiprotozoal activity (see below). The best-performing fraction was extracted with chloroform, filtered, and air-dried completely. Both the chloroform-soluble and insoluble fractions were tested for anti-parasitic activity. As only the chloroform-insoluble fraction had significant anti-parasitic activity, it was re-suspended in methanol and filtered. Next, the methanol-soluble portion was three times extracted with n-hexane:methanol (3:1, v/v), whereas the methanol-insoluble part was extracted with acetone, so that four fractions were obtained: 1) a hexane-soluble fraction, 2) a methanol-soluble fraction, 3) an acetone-soluble fraction, and 4) a fraction insoluble in any of the above solvents. The purity of each fraction was determined by thin layer chromatography (TLC), (**Figure 3**). As the hexane and acetone-soluble fractions produced the same TLC pattern, they were combined before the various fractions were tested again for antiprotozoal activity. Only the mixed hexane/acetone-soluble fraction

Figure 3. Outcomes of thin layer chromatograpy spots represents methanolic extract in the lane a, mixture hexane-acetone extracts in the lane b, and commercial ursolic acid in the lane c.

had antiprotozoal activity. This fraction was characterized by high-performance liquid chromatography (HPLC) coupled to orthogonal acceleration time-of-flight mass spectrometry, mass Spectrometer and the results of data obtained from the database were compared Mascot-Blast Spectrophotometric [23].

2.6. Antiprotozoal Assay

The antiprotozoal activity of the methanol extract and its fractions were evaluated as previously described [24] [25] [26]. Trophozoite suspensions (2×10^4/mL for *E. histolytica*, 1×10^5/mL for *T. vaginalis*, and 2×10^5/mL for *G. lamblia*) in their respective media supplemented with 10% bovine serum were incubated with the various extracts and control solutions at 36.5˚C for 72 h for *E. histolytica* and 24 h *G. lamblia* or *T. vaginalis*. These incubation periods were chosen because of their differential growth kinetic characteristics under standard conditions (**Figure 1**). Test samples included the crude methanol extract and the pooled hexane/acetone-soluble fraction at 0 - 300 μg/mL. Positive antiprotozoal solutions included two-fold serial dilution series (0 - 25 μg/mL) in appropriate media of ursolic acid and betulinic acid, and metronidazole at 1.25 μg/mL, whereas 5% DMSO served as a negative control. Incubations were stopped by placing the suspensions in ice-cold water for no less than 20 min. The number of trophozoites/mL was determined using a hemocytometer.

The percentage of growth inhibition was calculated with respect to untreated controls. The 50% inhibitory concentration (IC_{50}) of each drug was calculated by PROBIT analysis [27]. Each drug was assayed in triplicate in three independent experiments for each protozoan species, and the mean and 95% confidence limits were calculated.

2.7. Vero Cell Cytotoxicity

Vero cells (African green monkey epithelial kidney cells, ATCC CCL-81) were maintained in complete RPMI media/1 mM pyruvate/10% fetal bovine serum at 37˚C and 5% CO_2. To evaluate cytotoxicity, 1×10^5 cells/well were seeded in 96-well plates and incubated for 24 h. Next, the supernatant was replaced with 100 μL of test or control solutions and incubated for another 24 h. Test samples included crude methanol extracts and the hexane/acetone-soluble fractions (9.37 - 300 μg/mL); the positive control was 10% DMSO and the negative control was fresh medium. After the incubation time, the viable cells/well were counted with the use of the trypan blue exclusion method and a hemocytometer. The IC_{50} was determined by PROBIT [27] [28]. All assays were performed in triplicate [29].

2.8. TLC

The plant extracts with the most promising antiprotozoal activity were analyzed by TLC on pre-coated silica gel plates with benzene:acetone 9:1 v/v as the mobile phase. The air-dried TLC plates were observed at both 254 nm and 366 nm, and developed by iodine vapors and also exposed to different spraying reagents, in order to determine the Rf values of the observed spots.

2.9. Liebermann-Burchard Test

A drop of the extract was mixed with 1 drop of chloroform and three drops Liebermann-Burchard reagent (sulfuric acid/anhydrous acetic acid/chloroform, 20:1:1 v/v) on a porcelain plate. A change of color to orange, red, blue or green color is indicative of the presence of cholesterol-like compounds.

2.10. Statistical Analysis

For each experiment the mean and standard deviation of three independent experiments performed in triplicate were determined. The IC_{50} for each protozoa-antiprotozoal extract combination was calculated with the PROBIT trial [27]. The *t* student test was performed to compare the means of IC_{50} of each antiprotozoal extract combination using the statistical software SPSS version 15.0. A p-value < 0.05 was considered as statistically significant.

3. Results

As 18.22 g dry-weight was obtained from the methanol extract of the aerial parts of 150 g *Thymus vulgaris*, the production efficiency was 12.14%.

3.1. Antiprotozoal Activity of Thymus Vulgaris Extracts

The 300 µg/mL crude methanol extract of *Thymus vulgaris* had antiprotozoal activity against *G. lamblia* (IC_{50} 86.41 ± 2.9 µg/mL) *and T. vaginalis* (IC_{50} 115.41 ± 2.29 µg/mL), but not against *E. histolytica* trophozoites (Table 1 and Table 2).

The subsequent fractionation steps were bio-guided by the antiprotozoal assay. Only fractions with antiprotozoal activity underwent further work-up as in-

Table 1. Anti-parasitic activity of *Thymus vulgaris* extracts.

Thymus vulgaris extract/fraction	Growth inhibition (%)		
	E. histolytica	*G. lamblia*	*T. vaginalis*
crude methanol extract	14.12	95.86	96.42
1st fractionation step, ethyl acetate- methanol	ND	97.0	97.3
2nd fractionation step, Chloroform-insoluble	ND	97.4	96.8
3rd fractionation step, mixed hexane-acetone fraction	ND	96.1	99.0

Table 2. IC_{50} antiprotozoal activity and cytotoxicity of the extracts, mixture of fractions and commercial compounds.

Parasite/ Cell line	*IC_{50} of Compounds* (µg/mL, mean ± SD)		
	Crude methanol extract	*Mixture of hexane- and acetone-soluble fraction*	*Commercial ursolic acid*
T. vaginalis	115.41 ± 2.23	75.93 ± 1.17	5.51 ± 0.16
G. lamblia	86.41 ± 1.86	41.68 ± 1.23	8.12 ± 0.43
Vero cells	260.46 ± 5.34	260.74 ± 3.16	318.77 ± 8.51
ΔIC_{50} Vero/parasite	2.2 - 3.1	3.4 - 6.3	39.2 - 57.9

dicated in Figure 2. After the first step, the fraction with highest antiprotozoal activity was methanol extract from the ethyl acetate/water/methanol fractionation, causing a ≥ 97% growth inhibition of *T. vaginalis* and *G. lamblia* cultures. Next, it was the chloroform-insoluble fraction that caused growth inhibition of about 97% in aforementioned cultures. After the third step, both the hexane and acetone fractions from the chloroform-insoluble, methanol-recovered extract yielded antiprotozoal activity against both species. The mixed hexane-acetone fraction induced had excellent antiprotozoal activity, inducing 96% - 99% growth inhibition (Table 1).

3.2. Identification

The color change to red in the Liebermann-Burchard test indicated the presence of triterpenes in the mixed hexane-acetone fraction. Further characterization by mass spectrometry chromatography allowed the identification of oleanolic acid, betulinic acid and ursolic acid. These compounds were commercially obtained and their antiprotozoal activity was compared with the mixed hexane-acetone fraction. Only ursolic acid had antiprotozoal activity against both *T. vaginalis* and *G. lamblia*. The antiprotozoal activity of ursolic acid was 5 - 14 times stronger than that of the mixed hexane-acetone fraction, which was 1.5 - 2 times stronger than that of the crude methanol extract (Table 2).

3.3. Citotoxicity

Although the crude methanol extract, the hexane-acetone fraction, and commercial ursolic acid were all toxic for Vero cells, the concentration required to achieve an IC_{50} on Vero cells was 2.2 - 57.8 times higher than the IC_{50} for the protozoa (Table 2).

4. Discussion

Previous studies have reported the antiparasitic and nutritional activities of *Thymus vulgaris* [17] [30]. Thymol and carbachol are *Thymus vulgaris*-derived essential oils that have been recognized as active agents with analgesic, antifungal, antibiotic, antioxidant, antispasmodic, and insect repellent activities [17] [30] [31].

We aimed to extract an antiprotozoal agent from *Thymus vulgaris* with methanol, because methanol is recognized to be among the most effective solvents to extract compounds from plants [31] [32] [33], especially to obtain antibiotic and antiparasitic agents [25] [31] [34] [35].

Most antiprotozoal plant extracts are functional at 2 - 100 micrograms/mL [35], while extracts that require ≥ 1000 μg/mL to sort effect are considered inactive [31]. Some researchers encourage that crude extracts with IC_{50} of about 100 μg/mL should be further investigated as they may contain new, active agents, that could be discovered by bio-guided fractionation using polar solvents that favor compounds with hydroxyl and amine groups [36] [37]. Applying the principles of this idea, we decided to work up a crude methanol extract with anti-

protozoal activity at ≤300 μg/mL that was further fractionated in a multi-step partition process in which a variety of solvent (mixtures) were used and the obtained fractions were tested for antiprotozoal activity. In this way we were able to isolate a mixture of oleanolic acid, ursolic acid, and betulinic acids in the final hexane-acetone fraction. The characterization of these compounds was realized by chromatographic mass spectrometry. Remarkably, there was a similarity between the obtained mass spectrum of the hexane-acetone fraction obtained from *Thymus vulgaris* and a spectrum obtained from a previously-reported *Carya illinoensis* extract *[38]*. Others have reported that oleanolic acid and ursolic acid, obtained after column chromatography fractionation of a *Thymus vulgaris*-derived methanol extract, had anti-tuberculosis activity [18]. Antiprotozoal activity against *G. lamblia* trophozoites has been reported for *Thymus zigis* subsp. sy*lvestris*-derived essential oil. This essential oil had an IC_{50} of 185 μg/mL and mainly contained thymol and carvacrol [39]. Here we present a *Thymus* vulgaris-derived methanol extract that contains oleanolic acid, ursolic acid, and betulinic acids and has anti-protozoal activity against *G. lamblia* and *T. viginalis* trophozoites. The antiparasitic activity of ursolic acid had been reported before [40], and was confirmed to be the best-performing when commercially obtained oleanolic acid, ursolic acid and betulinic acid were tested in our antiprotozoal assay against *T. vaginalis* and *G. lamblia.* As our crude methanol extract (at 300 μg/mL) could not inhibit convincingly inhibit the growth *E. histolytica* trophozoites, it can be inferred that ursolic acid is not active against this parasite. Behnia *et al.* have reported positive biological activity against *E. histolytica* in a water-ethanol extract of *Thymus vulgaris* with an IC_{50} of 3 mg/mL [41]. The different extraction technique and very high concentration of the compound may explain their result. However, bioactivity testing of plant-derived extracts at a concentration beyond a 100 μg/mL is uncommon [35]. Furthermore, the study by Behnia *et al.* did not include a cytotoxicity study on mammalian cells [41]. In our study, the therapeutic index was studied by comparing the IC_{50} cytotoxicity on Vero cells [42] and the IC_{50} for growth inhibition of protozoa species. In our study design, protozoal growth inhibition and Vero cell cytotoxicity were evaluated in separate bioassays, although in antiprotozoal studies co-cultures are common practice. The reasons for our alternative approach were: 1) the protozoa and Vero cells require different culture conditions, and 2) *Trichomonas* sp. are known to be cytotoxic to Vero cells and other human monolayer cell lines and would interfere in the interpretation of the cytotoxicity results [43]. The performance of therapeutic indices we found were: commercial ursolic acid ≫ hexane-acetone fraction > methanol extract. In this assay we did not find any cytotoxic activity on the Vero cells for any of the compounds tested.

Thus, in this paper we report the anti-parasitic activity of the methanol extract of *Thymus vulgaris.* Anti-parasitic activity was contundent against *G. lamblia y T. vaginalis*, but insignificant against *E. histolytica* trophozoites. The mixture of hexane-soluble and acetone-soluble fractions of this methanol extract, which contained ursolic, betulinic, and oleanic acids, had the strongest antiparasitic ac-

tivity. We provide evidence that ursolic acid was the compound that was mainly responsible for antiprotozoal activity in the methanol extract of *Thymus vulgaris*.

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Conflict of Interest

There are not conflicts of interest to develop its research or elaborate this manuscript.

References

[1] Hernández-Cortéz, C., Aguilera, A.M.G. and Castro, E.G. (2011) Situación de las enfermedades gastrointestinales en México. *Enf Infect and Microb,* **31**, 137-151.

[2] Adam, R.D. (2001) Biology of *Giardia lamblia*. *Clinical Microbiology Reviews*, **14**, 447-475.

[3] Lane, S. and Lloyd, D. (2002) Current Trends in Research into the Waterborne Parasite Giardia. *Critical Reviews in Microbiology*, **28**, 123-147. https://doi.org/10.1080/1040-840291046713

[4] Thompson, R.C. and Monis, P.T. (2004) Variation in Giardia: Implications for Taxonomy and Epidemiology. *Advances in Parasitology*, **58**, 69-137. https://doi.org/10.1016/S0065-308X(04)58002-8

[5] (1996) The World Health Report. World Health Organization, Geneva.

[6] Cedillo-Rivera, R., Leal, Y.A., Yépez-Mulia, L., Gómez-Delgado, A., Ortega-Pierres, G., Tapia-Conyer, R. and Muñoz, O. (2009) Seroepidemiology of Giardiasis in Mexico. *American Journal of Tropical Medicine and Hygiene*, **80**, 6-10.

[7] Chacín-Bonilla, L. (2013) An Update on Amebiasis. *Revista Médica de Chile,* **41**, 609-615. https://doi.org/10.4067/S0034-98872013000500009

[8] Petri, W.A.Jr. (1996) Recent Advances in Amebiasis. *Critical Reviews in Clinical Laboratory Sciences*, **33**, 1-37. https://doi.org/10.3109/10408369609101485

[9] Caballero-Salcedo, A., Viveros-Rogel, M., Salvatierra, B., Tapia-Conyer, R., Sepúlveda-Amor, J. and Gutiérrez, G. (1994) Sero-Epidemiology of Amebiasis in México. *American Journal of Tropical Medicine and Hygiene*, **50**, 412-419. https://doi.org/10.4269/ajtmh.1994.50.412

[10] Stanley, S.L.Jr. (2003) Amoebiasis. *The Lancet*, **361**, 1025-1034. https://doi.org/10.1016/S0140-6736(03)12830-9

[11] World Health Organization (2001) Global Prevalence and Incidence of Selected Curable Sexually Transmitted Infections.

[12] Petri, W.A. (2003) Therapy of Intestinal Protozoa. *Trends in Parasitology*, **19**, 523-526.

[13] Busatti, H.G., Santos, J.F. and Gomes, M.A. (2009) The Old and New Therapeutic Approaches to the Treatment of Giardiasis: Where Are We? *Biologics*, **3**, 273-287.

[14] Sobel, J.D., Nyirjesy, P. and Brown, W. (2001) Tinidazole Therapy for Metronida-

zole-Resistant Vaginal Trichomoniasis. *Clinical Infectious Diseases*, **33**, 1341-1346. https://doi.org/10.1086/323034

[15] Löfmark, S., Edlund, C. and Nord, C.E. (2010) Metronidazole Is Still the Drug of Choice for Treatment of Anaerobic Infections. *Clinical Infectious Diseases*, **50**, S16-S23. https://doi.org/10.1086/647939

[16] Upcroft, J.A., Campbell, R.W., Benakli, K., Upcroft, P. and Vanelle, P. (1999) The Efficacy of New 5-Nitroimidazoles against Metronidazole-Susceptible and -Resistant Anaerobic Protozoa. *Antimicrobial Agents and Chemotherapy*, **43**, 73-76.

[17] Cañigueral, S. and Vanaclocha, B. (2000) Usos terapéuticos del tomillo. *Fitoterapia*, **1**, 5-13.

[18] Jiménez-Arellanes, A., Martínez, R., García, R., León-Díaz, R., Luna-Herrea, J., Molina-Salinas, G. and Said-Fernandez, S. (2006) *Thymus vulgaris* as Potential Source of Antituberculous Compounds. *Pharmacologyonline*, **3**, 569-574.

[19] Nilforoushzadeh, M.A., Shirani-Bidabadi, L., Zolfaghari-Baghbaderani, A., Saberi, S., Siadat, A.H. and Mahmoudi, M. (2008) Comparison of *Thymus vulgaris* (Thyme), *Achilleamillefolium* (Yarrow) and Propolishydroalcoholic Extracts versus Systemic Glucantime in Treatment of *Cutaneous leishmaniasis* in Balb/C Mice. *Journal of Vector Borne Diseases*, **45**, 301-306.

[20] Al Maqtari, M.A., Alghalibi, S.M. and Alhamzy, E.H. (2011) Chemical Composition and Antimicrobial Activity of Essential Oil of *Thymus vulgaris* from Yemen. *Turkish Journal of Biochemistry*, **36**, 342-349.

[21] Valizadegan, O. (2013) Study on the Influence of Thyme (*Thymus vulgaris*) Extract on Fungal Control of Some Crop Seeds during Germination Stage. *Advances in Environmental Biology*, **7**, 109-112.

[22] Said-Fernández, S., Vargas-Villarreal, J., Castro-Garza, J., Mata-Cárdenas, B.D., Navarro-Marmolejo, L., Lozano-Garza, G. and Martínez-Rodríguez, H. (1988) PEHPS Medium: An Alternative for Axenic Cultivation of *Entamoeba histolytica* and *E. invadens*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **82**, 249-253.

[23] Molina-Salinas, G.M., Peña-Rodríguez, L.M., Mata-Cárdenas, B.D., Escalante-Erosa, F., González-Hernández, S., Torres de la Cruz, V.M., Martínez-Rodríguez, H.G. and Said-Fernández, S. (2011) *Flourensia cernua*: Hexane Extracts a Very Active Mycobactericidal Fraction from an Inactive Leaf Decoction against Pan-Sensitive and Pan-Resistant *Mycobacterium tuberculosis*. *Evidence-Based Complementary and Alternative Medicine*, **2011**, Article ID: 782503. https://doi.org/10.1155/2011/782503

[24] Mata-Cárdenas, B.D., Vargas-Villarreal, J., González-Salazar, F., Palacios-Corona, R. and Said-Fernández, S. (2008) A New Vial Microassay to Screen Antiprotozoal Drugs. *Pharmacologyonline*, **1**, 529-537.

[25] Calzada, F., Yépez-Mulia, L. and Aguilar, A. (2006) *In Vitro* Susceptibility of *Entamoeba histolytica* and *Giardia lamblia* to Plants Used in Mexican Traditional Medicine for the Treatment of Gastrointestinal Disorders. *Journal of Ethnopharmacology*, **108**, 367-370.

[26] Cedillo-Rivera, R. and Muñoz, O. (1992) *In Vitro* Susceptibility of *Giardia lamblia* to Albendazole, Mebendazole and Other Chemotherapeutic Agents. *Journal of Medical Microbiology*, **37**, 221-224. https://doi.org/10.1099/00222615-37-3-221

[27] Finney, D.J. (1971) Probit Analysis. 3rd Edition, Cambridge University Press, London.

[28] Reynolds, C.P., Black, A.T. and Woody, J.N. (1986) Sensitive Method for Detecting

Viable Cells Seeded into Bone Marrow. *Cancer Research*, **46**, 5878-5881.

[29] González-Garza, M.T., Mata-Cárdenas, B.D. and Said-Fernández, S. (1989) High Susceptibility of Five Axenic Entamoeba Histolytica Strains to Gossypol. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **83**, 522-524.

[30] Oluwatosin, K.S., Habibat, U. and Usman, Y.U. (2013) Effect of Methanolic Leaf Extract of *Thymus vulgaris* on Some Biomarker Enzymes in *Trypanosoma brucei* Infected Rats. *International Journal of Pharmaceutical and Biomedical Research*, **4**, 83-87.

[31] Ríos, J.L. and Recio, M.C. (2005) Medicinal Plants and Antimicrobial Activity. *Journal of Ethnopharmacology*, **100**, 80-84.

[32] Chun, H., Shin, D.H., Hong, B.S., Cho, H.Y. and Yang, H.C. (2001) Purification and Biological Activity of Acidic Polysaccharide from Leaves of *Thymus vulgaris L. Biological and Pharmaceutical Bulletin*, **24**, 941-946. https://doi.org/10.1248/bpb.24.941

[33] Morimitsu, Y., Yoshida, K., Esaki, S. and Hirota, A. (1995) Protein Glycation Inhibitors from Thyme (*Thymus vulgaris*). *Bioscience, Biotechnology, and Biochemistry*, **59**, 2018-2021. https://doi.org/10.1271/bbb.59.2018

[34] Mahboubi, A., Kamalinejad, M., Ayatollahi, A.M. and Babaeian, M. (2014) Total Phenolic Content and Antibacterial Activity of Five Plants of Labiatae against Four Foodborne and Some Other Bacteria. *Iranian Journal of Pharmaceutical Research*, **13**, 559-566.

[35] Calzada, F., Yépez-Mulia, L. and Tapia-Contreras, A. (2007) Effect of Mexican Medicinal Plant Used to Treat Trichomoniasis on *Trichomonas vaginalis* Trophozoites. *Journal of Ethnopharmacology*, **113**, 248-251.

[36] Touchstone, J.C. (1992) Practice of Thin Layer Chromatography. 3rd Edition, John Wiley & Sons, INC., Hoboken.

[37] Cannell, R.J.P. (1998) Natural Products Isolation. Humana Press Inc., Clifton, 165-208. https://doi.org/10.1007/978-1-59259-256-2

[38] Sáenz-Esqueda, M.A., Álvarez-Roman, R., Castro-Ríos, R., Gómez-Flores, R., Nuñez-Rodríguez, M.A., Galindo-Rodríguez, S.A. and Chávez-Montes, A. (2012) Actividad Antituberculosa del extracto de *Carya illinoensis*. *Revista Mexicana de Ciencias Farmacéuticas*, **43**, 12-21.

[39] Machado, M., Dinis, A.M., Salgueiro, L., Cavaleiro, C., Custódio, J.B.A. and Sousa, M.C. (2010) Anti-Giardia Activity of Phenolic-Rich Essential Oils: Effects of *Thymbra capitata*, *Origanum virens*, *Thymus zigis* subsp. *Sylvestris* and *Lippia graveolens* on Trophozoites Growth, Viability, Adherence and Ultrastructure. *Parasitology Research*, **106**, 1205-1215. https://doi.org/10.1007/s00436-010-1800-7

[40] Al Musayeib, N.M., Mothana, R.A., Gamal, A.A., Al-Massarani, S.M. and Maes, L. (2013) *In Vitro* Antiprotozoal Activity of Triterpenoid Constituents of *Kleinia odora* Growing in Saudi Arabia. *Molecules*, **18**, 9207-9218. https://doi.org/10.3390/molecules18089207

[41] Behnia, M., Haghighi, A., Komeylizadeh, H., Javad, S., Tabael, S. and Abadi, A. (2008) Inhibitory Effects of Iranian *Thymus vulgaris* Extracts on *in Vitro* Growth of *Entamoeba histolytica*. *The Korean Journal of Parasitology*, **46**, 153-156. https://doi.org/10.3347/kjp.2008.46.3.153

[42] Sonboli, A., Mirjalili, M.H., Hadian, J. and Yousefzadi, M. (2014) The Biological Activity and Composition of the Essential Oil of *Sclerorhachis leptoclada* (*Asteraceae-Anthemideae*) from Iran. *Iranian Journal of Pharmaceutical Research*, **13**, 1097-1104.

[43] Alderete, J.F. and Pearlman, E. (1984) Pathogenic *Trichomonas vaginalis* Cytotox-

icity to Cell Culture Monolayers. *The British Journal of Venereal Diseases*, **60**, 99-105.