Roca-mezquita et al., Afr J Tradit Complement Altern Med. (2016) 13(4):162-165
doi: 10.21010/ajtcam.v13i4.21

ANTIPROTOZOAL ACTIVITY OF EXTRACTS OF *ELAEODENDRON TRICHOTOMUM* (CELASTRACEAE)

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Abstract

Background: Chagas disease, amebiasis, giardiasis and trichomoniasis represent a serious health problem in Latin America. The drugs employed to treat these illnesses produce important side effects and resistant strains have appeared. The present study was aimed to evaluate the antiprotozoal activity of leaves, stem bark and root bark of *Elaeodendron trichotomum*, a celastraceus, that is used in Mexico as an anti-infective in febrile-type diseases.
Materials and methods: Dichloromethane and methanol extracts of leaves, bark and roots of *Elaeodendron trichotomum* were tested against *Entamoeba histolytica*, *Giardia lamblia*, *Trichomonas vaginalis*, and *Trypanosoma cruzi*. A quantitative HPLC analysis of pristimerin and tingenone was performed.
Results: The dichloromethane extract of roots was active against *E. histolytica*, *G. lamblia*, *T. vaginalis*, and *T. cruzi*, at IC_{50}'s of 0.80, 0.44, 0.46, and 2.68 µg/mL, respectively. The HPLC analysis revealed the presence of tingenone (3.84%) and pristimerin (0.14%).
Conclusions: The dichloromethane extract of the roots bark showed significant activity against all screened protozoa.

Keywords: *Elaeodendron trichotomum*, antiprotozoal activity, Celastraceae, tingenone, pristimerin, root bark.

Introduction

Infections caused by protozoan parasites affect more than billion people worldwide causing a serious health problem, mainly in developing countries (Schmidt et al., 2012). Chagas disease, amebiasis, giardiasis and trichomoniasis are among the main neglected parasitic diseases in Latin America (Nava-Zuazo et al., 2014; Peter et al., 2014). The impact of Chagas disease on annual productivity in Latin America has been estimated in US$ 1.2 billion. The acute phase of this disease may not shows clinical manifestations but may progresses to a chronic phase, in which the patients may suffer cardiomyopathy, digestive megasyndrome or both (Zingales et al., 2014).

The infections caused by the intestinal protozoans *Entamoeba histolytica* and *Giardia lamblia*, frequently give rise diarrhea, and are considered one of the most common causes of death worldwide (Camacho-Corona et al., 2015).

Amoebiasis affects more than 10% of the world's population, and untreated infection may leads to severe complications including hepatic amoebiasis and intestinal tissue destruction. *E. histolytica* is considered the second leading cause of death from a protozoan parasite (Mortimer et al., 2010). Giardiasis is another major diarrheal disease found throughout the world. Symptomatic infection is characterized besides diarrhea, by epigastric pain, nausea, vomiting, cramps, and weight loss; chronic giardiasis causes malabsorption syndrome and, thus, malnutrition, especially in children. WHO has estimated that 280 million people are infected each year (Pasupuleti et al., 2014). *Trichomonas vaginalis* causes trichomoniasis, a common sexually transmitted human infection, which is associated with the predisposition to cervical cancer, pelvic inflammatory disease, infertility, increasing infections by human papillomavirus (HPV), birth outcomes, and human immunodeficiency virus (HIV). According to the WHO, approximately 180 million cases are reported annually worldwide (Hotez, 2014).

Despite the significant mortality and devastating social and economic consequences of parasitic diseases, these have been largely neglected for drug development. Presently there is a limited number of drugs commercially available for the treatment of these illnesses. In addition, the efficacy of some of them is low and all have side effects, including toxicity. This fact is aggravated by the recently appearance of several resistant clinical strains (Renslo et al., 2006).

Thus, there is an evident continuing need for effective and safe antiprotozoal agents, and natural products are promising sources of such compounds (Pink et al., 2004). In this context, plants of the Celastraceus family are known to produce several bioactive

doi: 10.21010/ajtcam.v13i4.21
compounds, including antiprotozoal compounds (González et al., 2000). Continuing our search for potential sources of antiprotozoal compounds from Celastraceae species belonging to Yucatan peninsula (México) flora, we have screened *Elaeodendron trichotomum*, against *Giardia lamblia*, *Entamoeba histolytica*, *Trichomonas vaginalis* and *Trypanosoma cruzi*. *E. trichotomum* is a celastraceus species distributed in the south and southeast of Mexico, and Central America, growing at coastal dunes, margins of mangrove areas or semi deciduous medium forest. The species is a branched large shrub or small tree that grows up to 12 ft tall. The leaves are yellowish green, leathery, from oval to elliptical shaped (1-5 in) with serrated margins. The fruit is a globular drupe (0.5-1.5 in) (CICY, 2015). Regarding its ethnobotanical uses, *E. trichotomum* has been used in Mexico as an anti-infective in febrile-type diseases and also as antitumor agent (Ulubelen et al, 1965).
This paper reports the results of the evaluation of the antiprotozoal activity of leaf, stem bark and root bark of *Elaeodendron trichotomum*.

Materials and Methods

Sample collection

Leaves, stem and roots of *Elaeodendron trichotomum* (Turcz.) Lundell were collected in February 2012 at semi deciduous medium forest in Celestún, Yucatán, México (N 20° 51.0', W 90° 11.5'); and authenticated by Dr. Juan Tun. A voucher specimen (J. T. 2328) was deposited in the herbarium ¨Alfredo Barrera Marín¨ of the Universidad Autónoma de Yucatán.

Plant extract preparation

Plant materials were dried at room temperature and powdered. Fifty grams of leaf stem bark and root bark of *E. trichotomum* each one were extracted successively with CH_2Cl_2 and methanol in a Soxhlet apparatus. The extract solutions were concentrated in vacuum, to obtain six crude extracts. The dry extracts were then stored at 4 ºC until use. The yields are shown in Table 1.

Biological assays

In vitro giardicidal, amebicidal and trichomonicidal assay

G. lamblia strain IMSS: 0696:1 was cultured in TYI-S-33 modified medium, supplemented with 10% calf serum and bovine bile. *T. vaginalis* strain GT3 and *E. histolytica* HM1-IMSS were cultured in TYI-S-33 medium, supplemented with 10% bovine serum. A sensitive and stringent method for assessing the anti-protozoal effects ('gold standard') was used for *in vitro* susceptibility assays (Cedillo-Rivera et al., 2002). For the bioassay, the extracts were dissolved in 1 mL of dimethylsulfoxide (DMSO) and added to microtubes containing 1.5 mL of medium in order to reach concentrations of 0.5, 1.0, 5.0 y 10.0 µg/mL. The solutions were inoculated with *G. lamblia* or *T. vaginalis* or *E. histolytica* trophozoites to achieve an inoculum of 4×10^4 trophozoites/mL and then were incubated for 24 h (*T. vaginalis*) or 48 h (*G. lamblia* or *E. histolytica*) at 37 ºC. Each test included metronidazole as positive control and trophozoites incubated in culture medium with DMSO used in the experiments as the negative control. After the incubation, trophozoites were washed and subcultured for another 48 h in fresh medium alone. At the end of this period, trophozoites were counted and the 50% inhibitory concentration (IC_{50}) was calculated by Probit analysis. Experiments were carried out in triplicate and repeated at least twice.

In vitro antitrypanosomal bioassay

Trypanosoma cruzi strain H1 was isolated from a human patient in the Yucatan Peninsula, Mexico. The bioassay was performed according to a method previously described (Leon-Deniz et al., 2009). Parasites were cultured at 28°C in liver infusion tryptose (LIT) broth medium supplemented with 10% of fetal bovine serum (FBS), hemine (50 mg/mL), penicillin (100 IU/mL), and streptomycin (100 µg/mL). The trypomastigotes were harvested at day 7 in the "log" phase, counted in a Neubauer's chamber, and adjusted to a concentration of 1×10^5 trypomastigotes/100 µL with fresh medium. For the bioassay, extracts were dissolved in 1 mL of DMSO aqueous solution (1%) and diluted with broth medium to obtain serial dilutions at concentrations of 5, 10, 50, and 100 µg/mL. Then, 100 µL of inoculum (1×10^5 trypomastigotes) was dispensed in 96-well plates containing 100 µL of serial dilutions and incubated at 28°C. Live mobile trypomastigotes were counted with a Neubauer's chamber. The growth inhibition percentage was determined after 48 h of incubation, by comparison with controls. Violet crystal was used as a positive control, whereas untreated *T. cruzi* trypomastigotes were used as negative controls. All assays were performed in triplicate. IC_{50} was determined by nonlinear regression analysis, by plotting the number of viable trypomastigotes versus log EC (effective concentration) values by use of GraphPad Prism 4 software.

High Performance Liquid Chromatography (HPLC) Analysis

The HPLC system consisted of a Beckman system gold high performance liquid chromatograph equipped with a binary pump model 126 and a diode array detector model 168. The samples were introduced using an injection valve fitted with a 20 µL loop. The mobile phase consisted of HPLC grade acetonitrile:water mixture (75:25, isocratic mode) at a flow rate of 1.5 mL/min for 25 min. A C-18 column (4.6 x 250 mm, 5 µm, Zorbax, Agilent) was utilized at 25°C. UV detection was performed at 245 nm (spectral acquisition in the range 200–400 nm). *Elaeodendron trichotomum* root bark dichlorometane extract (10 mg) was dissolved in the solvent mixture used as mobile phase (10 mL), and then 1 mL was filtered through a 0.45 µm membrane filter before being injected into the HPLC system for

doi: 10.21010/ajtcam.v13i4.21

analysis. Pristimerin and tingenone used as standard were previously isolated from *Hippocratea excelsa* root bark (Mena-Rejón et al., 2007). Its identity and purity were confirmed by NMR spectroscopy.

The quantification of pristimerin and tingenone was done by the external standard method, under the above described chromatographic conditions. Calibration curves were constructed using five points dilutions of each compound: 2, 4, 6, 12, and 20 ppm in acetonitrile : water mixture (75:25). Each determination was carried out in triplicate. The detection (LOD) and quantification (LOQ) data obtained were determined using signal-to-noise (S/N) ratios of 3.3 and 10, respectively.

Results and Discussion

Six organic extracts obtained from leaves, stem bark and root bark of *E. trichotomum* were screened against the pathogenic protozoa *E. histolytica*, *G. lamblia*, *T. vaginalis*, and *T. cruzi*. The IC_{50} values are shown in Table 1.

Table 1: *In vitro* antiprotozoal activity of *Elaeodendron trichotomum* extracts

Plant material	Extract	Yield (%)	Protozoa IC_{50} µg/mL±DS			
			E. histolytica	***G. lamblia***	***T. vaginalis***	***T. cruzi***
Leaves	CH_2Cl_2	5.03	> 10	> 10	> 10	31.22 ± 2.11
	MeOH	25.86	> 10	> 10	> 10	> 100
Stem Bark	CH_2Cl_2	7.03	> 10	> 10	> 10	27.21±2.33
	MeOH	22.30	> 10	> 10	> 10	> 100
Root Bark	CH_2Cl_2	6.10	0.80	0.44	0.46	2.68 ± 1.25
	MeOH	36.91	> 10	> 10	> 10	20.76 ± 1.63
MZT[a]			0.030	0.16	0.12	
VC[b]						0.60

[a]Metronidazole; [b]Violet crystal

Based on previous reports, an extract with an $IC_{50} \leq 10$ µg/mL was considered active (Moo-Puc et al, 2008; Leon-Deniz et al., 2009). Thus, only the dichloromethane extract of root bark of *E. trichotomum* was considered active against *T. cruzi* ($IC_{50} = 2.68$ µg/mL) and the three amitochondriate protozoa, *E. hystolytica* ($IC_{50} = 0.80$ µg/mL), *G. lamblia* ($IC_{50} = 0.44$ µg/mL), and *T. vaginalis* ($IC_{50} = 0.46$ µg/mL).

This results showed that the dichloromethane extract of roots bark of *E. trichotomum* possess a potent antiprotozoal activity against *G. lamblia*, *T. vaginalis* and *T. cruzi*. In contrast, this extract exhibited an IC_{50} against *E. hystolytica* 27 times higher than metronidazole (MZT). This fact was unexpected because *E. hystolytica* exhibited high sensitivity to this antiprotozoal drug (Table 1); besides the dichloromethane extract was active against the other two amitochondriate protozoa only at a concentration 2.6-3.8 times higher than MZT.

It has been stablished that if a pure compound shows antiprotozoal activity at $IC_{50} < 1$ µg/mL, it is considered as a hit (Pink, R. et al., 2005); therefore it is remarkably that the dichloromethane extract showed inhibitory activity against the amitochondriate protozoan at concentrations below to above mentioned threshold. On the other hand, the IC_{50} of the same extract against *T. cruzi* was higher than 1 µg/mL; however, according to Calderon et al. (2006), it can be classified as an extract with high antitrypanosomal activity ($IC_{50} < 10$ µg/mL).

Some species of Celastraceae family have been studied in order to isolate antiprotozoal compounds. In this context, it has been determined that pristimerin and tingenone, the main isolated celastroloids from roots of celastraceous species, possess activity against trophozoites of *G. lamblia* (Mena-Rejon et al., 2007) and trypomastigotes of *T. cruzi* (Liao et al., 2008). It is well-known that many species of Celastracea family contain pristimerin and tingenone (Gunatilaka, 1996); then, in order to confirm the presence of either celastroloid above mentioned in the dichloromethane extract of roots bark of *E. trichotomum*, a quantitative HPLC analysis was performed.

As was expected, both celastroloids were present in the analyzed extract. Tingenone eluted at 2.11 min, while the retention time of pristimerin was 5.18 min.

The regression equations for pristimerin and tingenone were $y = 0.1123x - 0.1344$ ($r = 0.996$) and $y = 0.0694x - 0.0626$ ($r = 0.993$), respectively. LOD and LOQ were 0.4079 and 1.2361 for tingenone, and 0.4280 and 1.297 for pristimerin. Based on these calibration curves, it was determinate that dichloromethane extract of roots bark of *E. trichotomum* contains 3.84 % of tingenone, and very low concentration of pristimerin (0.14%). This result was unexpected, since pristimerin is known as the major celastroloid of the roots of Celastraceae species (Gunatilaka, 1996).

IC_{50}'s of 0.05 and 0.31 µg/mL against *Giardia lamblia*, of pristimerin and tingenone, respectively have been previously reported, (Mena-Rejón et al., 2007); while the dichloromethane extract exhibited antigiardial activity at IC_{50} 0.44 µg/mL. It is noteworthy that at this concentration the solution contains 6.16×10^{-4} µg of pristimerin and 0.017 µg of tingenone; therefore the amount of each celastroloid in the extract could be not enough to produce the observed significant activity.

The antitrypanosomal assay using extracellular trypomastigotes, has been reported as an excellent model for *in vitro* antitrypanosomal screening. In the present study, the dichloromethane extract using this model exhibited an IC_{50} of 2.68 µg/mL against *T. cruzi* trypomastigotes, and at this concentration the bioactive solution contains 0.10 µg of tingenone and 3.8×10^{-3} µg of pristimerin. It

is important to point out that tingenone has been assayed against *T. cruzi* trypomastigotes showing an IC_{50} of 204.4 µg/mL. (Liao et al., 2008) Then, in the same fashion of the antigiardial assay result, tingenona could not be responsible for the antitrypanosomal activity of the dichloromethane extract.

Regarding antytripanosomal activity of pristimerin, this celastroloid has been evaluated using epimastigotes (extracellular form), but the reported results (IC_{50} = 1.4 x 10^{-3} µg/mL) cannot be compared with observed activity by the difference between the composition of the plasmatic membrane of the epimastigote and trypomastigote forms (dos Santos et al., 2013).

In conclusion, the extracts from leaves, root bark and stem bark of *Elaeodendron trichotomum* were screened against *Giardia lamblia*, *Entamoeba histolityca*, *Trichomonas vaginalis* and *Trypanosoma cruzi* for first time. Only the dichloromethane extract of the roots bark showed significant activity against the four screened protozoa. The celastroloids pristimerin and tingenona are present in the bioactive extract (0.14 and 3.8 %, respectively). But, very likely they are not the main active compounds for the observed antiprotozoal activity of the extract, since their concentrations in the active solution are very low (Pristimerin = 6.16 x 10^{-4} µg and tingenone = 0.017 µg). Finally, the obtained results confirm the traditional use of *E. trichotomum* and encourage us to perform further research conducted to isolate and characterize the antiprotozoal compounds present in the root bark of this celastraceous species.

Acknowledgments

In memory of Dr. José Antonio Manzanilla Cano (1964-2015). This work was funded by the grant 101265 provided by Consejo Nacional de Ciencia y Tecnología (CONACYT). This paper was taken in part from the doctoral thesis of C. Roca-Mézquita. L. León-Déniz and C. Roca-Mézquita are grateful to CONACYT for the postdoctoral (No. 19875) and doctoral (No. 326013 and No. 21560) fellowships, respectively. The authors are grateful to Dr. Leovigildo Quijano for his critical review of the manuscript.

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