ANTITRYPANOSOMAL ACTIVITY OF *SENNA VILLOSA* IN INFECTED BALB/C MICE WITH *TRYPANOSOMA CRUZI* DURING THE SUB ACUTE PHASE OF INFECTION.

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Abstract

Antitrypanosomal activity of chloroform extract of *Senna villosa* leaves was evaluated in the sub acute phase of mice infected with *Trypanosoma cruzi*. Oral doses of 3.3, 6.6 and 13.2 µg/g were tested during 15 days on infected mice BALB/c, beginning treatment 40 days after infection to evaluate specifically the antitrypanosomal activity over the amastigote form of the parasite. Two different amount of parasites (100 and 500) were inoculated to 25 mice for each doses tested. At the end of the assay the animals were sacrificed and cardiac and skeletal tissue sections were stained with hematoxylin-eosin (HE) for identification and quantification of amastigote nest. In mice infected with 100 parasites, a significant reduction in the number of amastigote nest was observed in cardiac tissue of treated animals at all doses evaluated (p<0.05). An important reduction of amastigote nest was also observed in treated animals and infected with 500 parasites in comparison with no treated mice or treated with allopurinol.

Résumé

Activité antitrypanosomal de chloroforme extrait de feuilles *Senna villosa* a été évaluée dans la sous phase aiguë de souris infectées par *Trypanosoma cruzi*. Des doses orales de 3,3, 6,6 et 13,2 µg / g ont été testés pendant 15 jours sur des souris infectées BALB /c, le début du traitement 40 jours après l'infection d'évaluer précisément l'activité antitrypanosomal sur la forme amastigote du parasite. Deux montant différent de parasites (100 et 500) ont été inoculés à 25 souris pour chaque doses testées. À la fin de l'essai les animaux ont été sacrifiés et des coupes de tissus cardiaques et squelettiques ont été colorées à l'hématoxyline-éosine (HE) pour l'identification et la quantification du nid amastigote. Chez les souris infectées avec 100 parasites, une réduction significative du nombre de nids amastigote a été observée dans le tissu cardiaque des animaux traités à toutes les doses évaluées (p <0,05). Une réduction importante du nid amastigote a également été observée chez les animaux traités et infectés avec 500 parasites par rapport à pas de souris traitées ou traitées avec l'allopurinol.

Keywords*:* Antiprotozoal, *Senna villosa*, *Trypanosoma cruzi*, amastigote.

Introduction

Chagas disease is a systemic chronic parasitic infection caused by *Trypanosoma cruzi* and continues to represent a health threat for an estimated in 28 million people, living mostly in Latin America. While Chagas disease occurs throughout Mexico, central and southern America, the clinical manifestations and the epidemiological characteristics are different in the diverse endemic zones and actually at least 55,585 persons per year would require treatment against *Trypanosoma cruzi* (OMS, 2007). There are limitations of existing drugs for treatment. For more than 20 years, two drugs have been mainly used to treat Chagas’ disease: benznidazole (Rochagan ®, Rodanil®, Roche) and nifurtimox (Lampit®, Bayer, recently discontinued. While the efficacy of drug treatment has been validated in infected people less than 16 years of age, it is not known if the same drugs can indeed halt disease progression in adult patients or if their use can be recommended to those at the indeterminate or chronic phase of the disease (OMS, 2007). Many clinical trials with nifurtimox and benznidazole have shown that these compounds have very low activity in preventing the development of chronic Chagas disease. Moreover, the drugs had been associated with numerous toxic effects (Guedes *et al*. 2006; Bern et al. 2007).

However, natural products have proved to be an important source of lead compounds in the development of new drugs (Sülzen et al. 2006). In the last decade several research groups have reported the effect of plant derived compounds against *T. cruzi* (Boza and Cassels, 1996; Bastos *et al*. 1999; Grael *et al*. 2000); more recently it has been demonstrated the presence of some compounds isolated from medicinal plants with antiprotozoal activity from *in vivo* experimental conditions (Cuhna *et al.* 2006; Dantas *et al*. 2006; Saraiva et al. 2007).

There is an urgent need to develop of alternative drugs to replace nifurtimox and benznidazole, currently used for the treatment of chagasic patients (Coura and De Castro, 2002). Screening natural products provides the chance to discover new molecules of unique structure with high activity and selectivity (Kayser *et al.* 2003). The discovery of new active, non-toxic

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compounds from a natural source as plants would probably expand treatment alternatives, especially in individuals in the sub-acute and chronic phase of infection.

Mayan culture, as other historical cultures, developed an important cumulus of information about the approach of their environmental plants and used them as empirical medicine. The ancestral information about traditional medicine from the Mayan medicine culture has survived, still stays and is used in Yucatan Peninsula until actual date (Heinrich et al. 2005), mainly in the rural areas. *Senna villosa* (*S. villosa*) is a leguminous plant from the southern of Mexico and the Caribbean; it grows at an altitude range of 10 to 1600 meters above sea level. The antimicrobial and antifungal properties of *Senna villosa* (*S. villosa*) had been previously reported (Flores, 2001). In Mayan language *S. villosa* known as "Booxsaal che'" or "Saal che', meaning "black bean", has been used traditionally in herbal medicine to treat stomach disorders (specially as a laxative) dysmenorrheal or the topical application of the leaves as cicatrizing agent in skin lesions associated with mycosis. Considering the medicinal use of the plant in traditional medicine, other studies had been performed. The phytochemical analysis has shown the existence of alkaloids, sterols, flavonoids, and two 9, 10 anthraquinones (Mena et al. 1997). Crude extracts (methanol, aqueous and chloroform) from *S. villosa* leaves has been previously tested and a strong antiprotozoal activity *in vitro* against epimastigote forms of *T. cruzi* was found in chloroform extract at concentration of 1.6 µg/ mL (Guzmán *et al.* 2004). From the chloroformic extract, the compound (8-hydroxymethylen)-trieicosanyl acetate was isolated which demonstrated antiprotozoal activity against epimastigotes and trypomastigotes of *T. cruzi* under *in vitro* conditions (at concentrations of 1.65, 3.3 and 6.6 µg/mL) (Guzman et al. 2008) and antitrypanosomal activity *in vivo* at 33.6 µg/g in mice which were treated 24 hours after inoculation (Jimenez-Coello et al. 2010). However, it is not easy to obtain enough amount of the compound because the yield is very low (2.7%) and is not practical to be used during longer periods of time. Otherwise, the chloroform extract is a good option, because it contains an active compound with a demonstrated antitrypanosomal activity (Guzman et al. 2008; Jimenez-Coello *et al.* 2010), also contains elements with anti-inflammatory properties (Unpublished data) and can be easily obtained in the area. For that reason, the aim of this study was to determinate the antiprotozoal activity *in vivo* of the chloroform extract of *Senna villosa* leaves against amastigote form of *Trypanosoma cruzi* during the sub acute phase of the infection.

Methods and Materials

Plant material and extract preparation

Senna villosa leaves were collected from the rural community Komchem town, some 17 km from Merida, Yucatan state, Mexico, from July through September of 2005, 2006 and 2007. The plant was authenticated by Dr. Salvador Flores-Guido and a voucher (10284) was deposited at the herbarium of Universidad Autonoma de Yucatan (UADY). Leaves were separated and dried at room temperature under shadow conditions. From the dried powdered leaves, chloroform extracts were obtained in accordance with the previously methodology described by Guzman et al. (2004)

Parasites and experimental animals

Trypomastigotes of *T. cruzi* H4 strain were used. The selected strain produces a mortality rate of 50% in mice after 30 days of inoculation and has mainly tropism to invade cardiac tissue and a minor tropism to skeletal muscle (Barrera *et al*, 2001). It has been previously classified as a highly virulent strain (Dumonteil et al. 2004). Eight weeks old BALB/c mice were used to assay the antitrypanosomal activity. Animals were maintained on a 12:12 light-dark cycle and had access to food and water *ad libitum* during the entire assay (60 days).

In vivo assay

Fifty BALB/c mice were randomly divided into 10 groups (n=5 each). Five groups were infected with 100 parasites and the remaining five groups with 500 trypomastigotes through intraperitoneal injection (IP) (A Positive control group, a negative control group and three more mice groups for the different doses were evaluated respectively).

After 40 days post-infection, during the sub acute phase of the infection, the mice in the experimental groups commenced a daily oral dose of the chloroform extract of *S. villosa* for 15 days. The chloroform extract of *S. villosa* was evaluated at 3 doses: 3.3, 6.6 and dose 13.2 µg/g. The chloroform extract was resuspended in phosphate buffer saline (PBS, NaCl 13.7 mM, KCl 2.7 mM, Na_2HPO_4 4.3 mM y KH_2PO_4 1.4 mM pH 7.4), and administered orally (adjusted to 50 µL per animal) every 24 hours during 15 days. For negative controls, un-infected mice groups (n=5) received only 50 µL of the vehicle (PBS) orally. Positive controls included infected mice (n=5) treated orally with allopurinol (8.5 µg/g) (Nakajima-Shimada et al. 1996) diluted in 50 µL of PBS, every day with the same scheme that animals treated with the chloroform crude extract. To determine the activity of the chloroform extract of *S. villosa* over the intracellular *T. cruzi* amastigote forms, cardiac tissue samples from treated and untreated mice were collected and fixed in formaldehyde (10%) for further processing. Paraffin embedded tissue sections were stained with hematoxylin-eosin (HE) and examined under a light microscope (40X). Four nonconsecutive slides from the heart of each mouse were examined in a blinded fashion. The number of amastigote nests was quantified in 100 fields for each heart. All procedures were conducted in accordance with the internationally accepted principles for laboratory animal use and care.

Statistical analysis

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Data are expressed as mean ± S.D. statistical analysis was performed using ANOVA ($P < 0.05$), followed by Duncan multiple comparison test, in order to compare more than two groups. The Statistical Package for the Social Sciences (SPSS) v.16.0 was used to perform the statistical analysis.

Results

The two control negative groups (100 and 500 inoculated parasites respectively) showed a bad clinical appearance at the end of the assay in comparison with all groups of mice treated with the extract). Regarding to the number of amastigote nests observed in cardiac tissue, they were more frequent and of apparently bigger size in mice none treated (negative control groups) in contrast with the number of nest observed in mice from experimental treated groups. The aspect of the amastigote nests were predominating rounded forms in treated mice groups and extended forms in none treated groups (Figure 1).

The antitrypanosomal activity from the chloroform extract of *S. villosa* leaves were demonstrated in the assay developed with mice infected with 100 parasites. The amount of amastigote nests observed in cardiac tissue from infected and treated mice were minor in comparison with none treated mice (p <0.043). The antiprotozoal response was observed with the three doses evaluated (3.3, 6.6 and 13.2 µg/g) (Figure 3).

The results obtained from the *in vivo* assays in mice infected with 500 parasites were reasonable, however even there was observed an important reduction from the amount of amastigote nests as well in cardiac as in skeletal muscle (specially at higher doses), there were not found statistical differences between treated and none treated mice (Figure 2). Comparing the activity of the chloroform extract with none treated mice, it was observed a reduction of 95% of the amastigote nest (p<0.059), and a stronger activity of the chloroform extract of *S. villosa* than allopurinol was showed (Figure 3).

Discussion

The observed differences in the appearance between infected mice treated and untreated coincided with those reported previously by Diaz Limay et al. (2004), who point out that these clinical signs are observable in BALB/c mice infected with *T. cruzi* and none treated. Particularly, mice from the control groups, was observed alopecia in neck and chest as well as adynamia and emaciation. In contrast, treated mice showed less deterioration and the clinical signs were almost barely visible.

It has been described that the drug allopurinol has an important antitrypanosomal activity against amastigotes in Vero cell line (Nakajima-Shimada et al. 1996; Paulino et al. 2005). Results observed in these assays under *in vivo* conditions, during the sub acute phase of infection by *T. cruzi*, showed only a slight reduction of amastigote nest in mice treated with that drug, in comparison with none treated mice (p=<0.05). Previously, it had been reported the effectiveness of allopurinol when were used in murine models infected with *T. cruzi* (Stopanni, 1999). However, the results has been controversial in humans (Rodriguez-Morales, 2005; Apt et al. 2005) probably because under *in vivo* condition, drug cannot efficiently penetrate the tissues where the amastigote nest are located, as described by Urbina (1999).

In this study, the best results were observed as a reduction in the number of cardiac tissue nests in mice infected with 100 parasites. However, the number of amastigote nests did not reduced from skeletal muscle probably because of the poor crude extract penetration in muscular tissue. In mice infected with 500 parasites, although a reduction in the number of amastigote nests in cardiac and muscular tissue was observed, the difference was not statistically different from control groups (Figure 2). The *in vivo* activity from the chloroform extract of *S. villosa* against the amastigote form of *T. cruzi* determinate in this work, is similar at the previously described by Guzman et al. (2004) when it was reported the antiprotozoal *in vitro* activity from the chloroformic extract obtained besides the leaves of *S. villosa* against epimastigotes and tripomastigotes of *T. cruzi* at the concentrations 3.3 and 6.6 µg/mL.

The presence of amastigote nests showed a direct relation with the number of parasites inoculated. Which could be indicating that parasites after infection and tissue recognition, (depending their specific tropism), has an intensively replication in an intracellular level proportional to the amount of parasites inoculated and the damage they produce in the infected host (Diaz-Limay *et al.* 2004). On the other hand, the amount of amastigote nest can be associated with the strain heterogeneity, and the parasitemia and the presence of cardiac lesions may depend of the strain virulence. The intrinsic immune response of the host may also be determinant to the development and degree of damage, susceptibility or resistance to *T. cruzi* infection in the different experimental animal models (Postman et al. 1999). Also, it is important consider the *T. cruzi* infection in mammals as in vectors is used to be multiclonal, meaning the constellation of clones present in an infected host could behave different between the infected individuals and produce diversity on the intensity of clinical signs and tissue lesions, depending several times of the particular situation of the infected host (age, immunological condition etc.) (Macedo et al. 2002)

The behavior of *T. cruzi* H4 strain in this study, showed a mainly tropism to cardiac tissue as it has been previously described by Barrera et al. (2001). The activity of the chloroform extract of *S. villosa* leaves in mice treated during the sub acute phase of the infection, probably is due to the compounds present in the crude extract evaluated, maybe could be interfering the cellular cycle of the parasite, particularly in the amastigote form which is the replicate form in the mammal host, as well has been described previously that the presence of secondary metabolites as flavonoids, sterols, terpens and others contents in the crude extract of *S. villosa* leaves (Mena et al. 1997; Guzman et al. 2004) could be implicated with the antiprotozoal activity under *in vivo* conditions.

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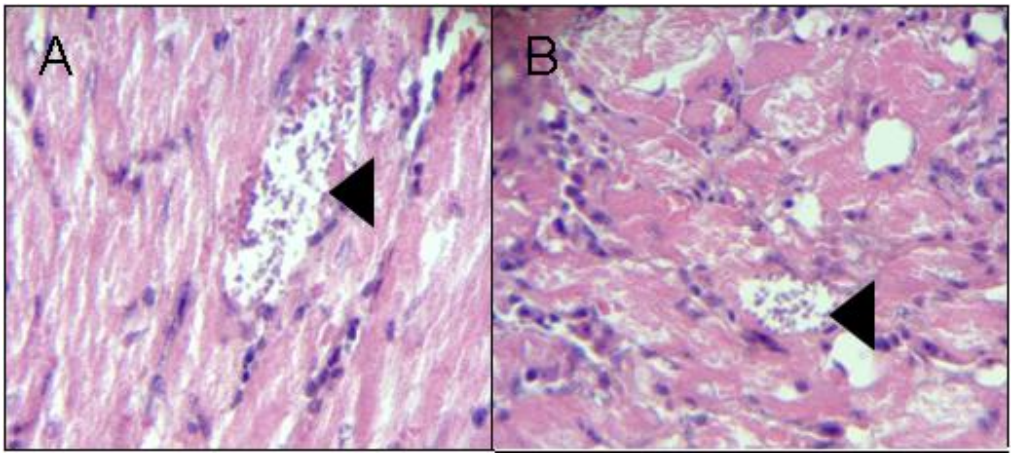

Figure 1: Representative haematoxylin-eosin-stained slides (magnification ×40) obtained from the heart of non treated (A) and chloroform crude extract of *S. villosa* leaves treated mice (B). The amastigote nests were different in size and form in treated and none treated mice (arrow).

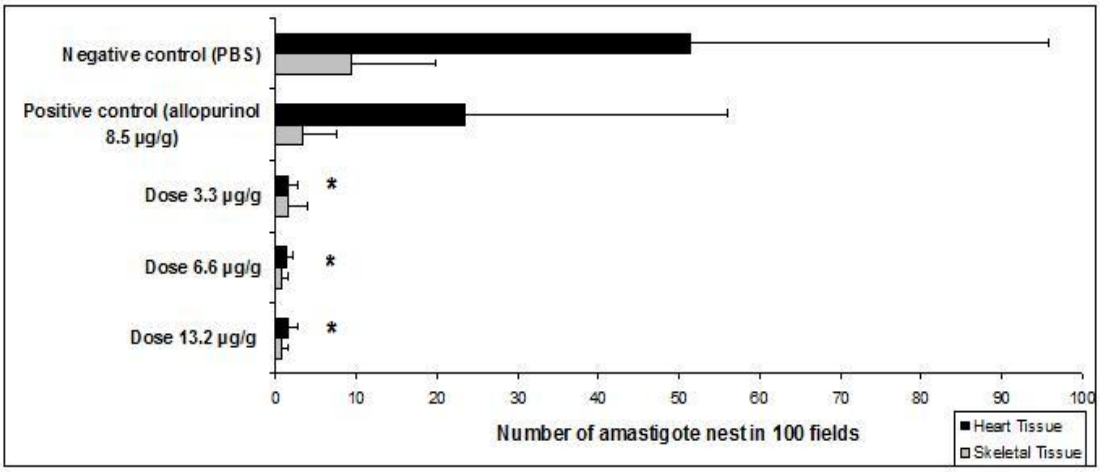

Figure 2: Effect of chloroform extract of *S. villosa* over the number amastigote nests observed in cardiac and skeletal tissue from mice BALB/c infected with 100 trypomastigotes of *T. cruzi* and treated *at* doses 3.3, 6.6 and 13.2 µg/g (* p <0.05)

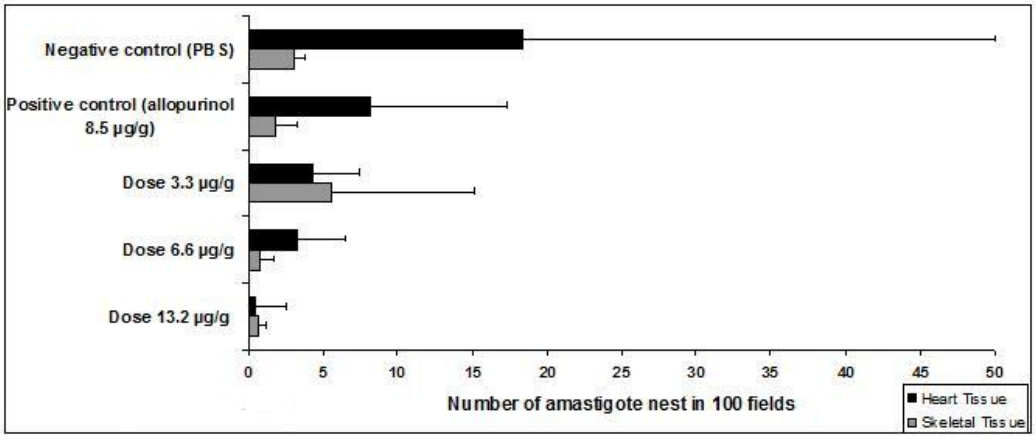

Figure 3: Effect of chloroform extract of *S. villosa* over the number amastigote nest observed in cardiac and skeletal tissue from mice BALB/c infected with 500 trypomastigotes of *T. cruzi* and treated *at* doses 3.3, 6.6 and 13.2 µg/g .

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The antiprotozoal activity of another plant from the Mayan traditional medicine, *Senna racemosa* has been also described. In this plant, it has been identified the presence of the piperidine alkaloid cassine, chrysophanol and an anthracenone, all this compounds has been relational with antiprotozoal activity against *E. hystolitica* and *G. lamblia* (Moo-Puc et al. 2007).

In contrast, in the chloroform crude extract of *S. villosa* it has been also described the presence of (8-hydroxymethylen)-tricosanyl acetate, which has demonstrated antiprotozoal activity *in vitro* and *in vivo* against epimastigotes, trypomastigotes and amastigotes of *T. cruzi*, being apparently not cytotoxic when tested in a Vero cell line (Guzman et al. 2008) and in BALB/c mice treated during 28 days (Jimenez-Coello et al. 2010). Indicating the possibility that the presence of (8-hydroxymethylen)-tricosanyl acetate in the chloroform crude extract of *S. villosa* it is concerned in the antiprotozoal activity against amastigote forms described in this study. However, the administration of the crude extract appears to show a better antiprotozoal effect *in vivo* against amastigotes of *T. cruzi*, probably due to anti-inflammatory properties of the other components of the extract as even *S. villosa* has been used empirically as an anti-inflammatory in Mexican traditional medicine. Treatment with the crude extract can directly show a better response in the inhibition of parasite replication and perhaps allow a better prognosis in chemotherapy of individuals infected by *T. cruzi*.

Conclusion

The chloroform crude extract from *S. villosa* leaves showed antiprotozoal activity under *in vivo* conditions. Mice infected with 100 parasites and treated during the chronic phase of the infection with doses 3.3, 6.6 and 13.2 µg/g, showed a reduction in the amount of amastigote nests of *T. cruzi* in cardiac tissue ($p<0.043$) in comparison with untreated mice.
Biotransformation of the chloroform crude extract from *S. villosa* leaves is apparently not toxic, but it is necessary the pharmacodynamic determination of chloroform crude extract from *S. villosa* leaves in an animal model, as well as assay the antiprotozoal activity of the crude extract treating infected mice during long time periods, in the chronic phase of the infection and maybe higher doses be investigated.

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