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Original article

5-Nitro-2-furyl derivative actives against *Trypanosoma cruzi*: Preliminary *in vivo* studies

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ABSTRACT

Ten 5-nitro-2-furyl derivatives, with good to excellent *in vitro* anti-*Trypanosoma cruzi* activity, and nifurtimox were tested oral and intraperitoneally on healthy animals for its acute toxicity on murine models. According to animals' survival percentage, organ histological results, biochemical and haematological findings, three new derivatives, with toxicity like nifurtimox, were selected to test *in vivo* as antichagasic agents. Clearly, dependences between chemical structure and both acute toxicity and *in vivo* anti-*T. cruzi* activity were observed. 4-Hexyl-1-[3-(5-nitro-2-furyl)-2-propenylidene]semicarbazide displayed good profile as anti-*T. cruzi* agent and better acute toxicity profile than nifurtimox.

1. Introduction

Chagas' disease is a major health problem in South and Central America, affecting 16–18 million people [1]. In spite of such high prevalence, only two synthetic compounds, nitroheterocycles nifurtimox (**Nfx**, Lampit®) and benznidazole (**Bnz**, Rochagan®), have been used [2]. Both are effective in the early stages of trypanosomiasis, but are practically useless in the chronic disease. Only 50% of patients are parasitologically healed after treatment [3]. Furthermore, in Latin America the commercial situation with these two drugs is very terrible. On the one hand, **Nfx**'s production has been discontinued and on the other hand, Roche's right patent of **Bnz** production has been got to the Brazilian government covering pharmacologically the population of this country.

The limited efficacies as well as their toxic side effects and the commercial problems justify the continue research for new trypanocidal substances [4,5]. In this way, new 5-nitro-2-furyl derivatives have been synthesized [6–10]. Previously, were reported the anti-*Trypanosoma cruzi* properties *in vitro* and *in vivo* of some of these derivatives [11,12]. It was also demonstrated that the presence of a nitro moiety, as a source of free radicals, seems to be a feature that increases the anti-*T. cruzi* activity [13]. *In vitro* anti-*T. cruzi* activity of these new 5-nitro-2-furyl derivatives was evaluated on epimastigotes. Almost all of them were effective at 5 μM. The active derivatives belong to two categories, namely 5-nitrofurfurylidene derivatives ($n = 0$, see Table 1) and (5-nitro-2-furyl)propene derivatives ($n = 1$, see Table 1). In the present study we selected representative examples of each group of compounds (from the first category derivatives **1**, **2**, **4**, and **5** and from the second category derivatives **3**, and **6**–**10**, Table 1) to *in vivo* screening studies, analyzing the potential use of these compounds as antichagasic drugs.

2. Chemistry

The 5-nitro-2-furyl derivatives **1**–**2** [10], **3** [7], **4**–**5** [8], **6**–**9** [7], and **10** [8] were obtained as previously described. **Nfx** (Lampit®, Bay 2502) was obtained from Bayer. Identity of the studied compounds was confirmed by MS, ^{1}H NMR, and ^{13}C NMR, and their purity established by microanalysis.

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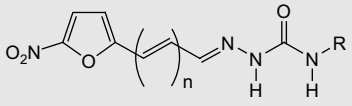
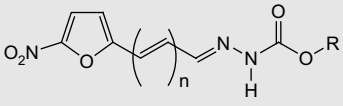
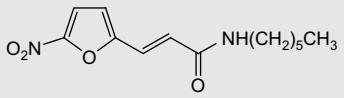
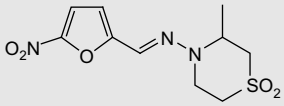
Table 1
Chemical structures of 5-nitro-2-furyl derivatives assayed and *in vitro* trypanocidal activities on epimastigote forms (Tulahuen 2 strain).

Compound					PGI[a] (%)
	n	–R	*n*	–R	
1	0	$-(CH_2)_3CH_3$	–	–	30
2	0	$-(CH_2)_2OCH_3$	–	–	20
3	1	$-(CH_2)_5CH_3$			82
4	–	–	0	$-(CH_2)_5CH_3$	93
5			0	$-(CH_2)_7CH_3$	84
6	–	–	1	$-(CH_2)_3CH_3$	87
7	–	–	1	$-(CH_2)_5CH_3$	86
8	–	–	1	$-(CH_2)_6CH_3$	90
9	–	–	1	$-(CH_2)_7CH_3$	92
10					82
Nfx					46

[a] PGI: percentage of growth inhibition at 5 μM at 5th day of incubation.

3. Pharmacology

3.1. Preliminary acute toxicity study

Firstly, in order to select the compounds for the pharmacological studies we established the adverse effects in the whole population of derivatives including **Nfx**. For that, healthy animals were treated with a single oral or a single intraperitoneal (i.p.) dose of compounds higher than the pre-established therapeutic dose (60 mg/kg/day). At the third day post-administration the animal was weighted and sacrificed [14,15]. Differences in the weights, animal-survival percentages, clinical biochemistry findings, haematological and organ histological results respect to healthy non-treated animal (NTA) were analyzed. Tables 2–4 show these results. According to animal survival percentages when the compounds were administered intraperitoneally (single doses of 300 mg/kg, Table 2) 5-nitrofurfurilydene derivatives, **1**, **2**, **4**, and **5**, resulted the most toxic of the evaluated drugs and compound **5** also caused animal death when it was orally administrated in a single doses of 450 mg/kg (Table 2). On the other hand,(5-nitro-2-furyl)propene derivatives, **3**, and **6**–**10**, resulted in this study less toxic than the 5-nitrofurfurilydene ones without animals' death and specially derivatives **3**, **7** and **9** showed weight's increase as the behaviour of NTA. When observing the clinical biochemistry and haematological findings (Table 3) of living animals, derivatives **7**–**9** belonging to (5-nitro-2-furyl)propene family, showed the best results.(5-Nitro-2-furyl)propene derivatives **3**, **6**, and **10** presented the highest levels of leukocytes while 5-nitrofurfurilydene derivative **4** possessed lower levels than normal animals [16]. Compound **4** also presented the highest level of GOT [17,18] when it was orally administered while derivative **7** and **Nfx** presented high levels of GOT in both administrations via, however for the three compounds these values fall in the normal range. When the animals' organs were histologically examined (Table 4) compounds **1**, **2**, **9** and **10** presented the worst results especially on kidneys, heart and lungs.(5-Nitro-2-furyl)propenes **3**, **6**–**8** results were similar to that of **Nfx**.

These results allowed us to select for *in vivo* antichagasic activity studies five different 5-nitro-2-furyl derivatives. On the one hand, derivatives belonging to the (5-nitro-2-furyl)propene family, semicarbazone **3** and carbazate **7**, with toxic effects in healthy animals similar to **Nfx**, and amide **10**, more toxic than **Nfx**. On the other hand, it was included derivatives **1** and **2**, as toxic derivatives in healthy animals, belonging to 5-nitrofurfurilydene family. These derivatives, **1** and **2**, were previously analyzed *in vivo* [12] in different experimental conditions.

3.2. In vivo activity against T. cruzi

In the *in vivo* studies wild strain isolated from Uruguayan patients was employed [19]. *In vivo* anti-Chagasic activity was

Table 2
Change in weight (PDW) and survival of healthy animals treated with compounds **1**–**10** and **Nfx**.

Compound	Via oral			Via intraperitoneal		
	Dose (mg/kg)	PWD[a] (%)	Animals survival	Dose (mg/kg)	PWD[a] (%)	Animals survival
1	450	1	5/5	300	–	0/5
	300	4	5/5	150	6	5/5
2	450	0	5/5	300	–	0/5
	300	0	5/5	150	1	5/5
3	450	(21)[b]	5/5	300	(8)	5/5
4	450	(3)	5/5	300	0	2/5
5	450	11	4/5	300	(4)	1/5
6	450	2	5/5	300	(2)	5/5
7	450	(2)	5/5	300	(2)	5/5
8	450	0	5/5	300	(4)	5/5
9	450	(2)	5/5	300	(1)	4/5
10	450	0	5/5	300	(2)	5/5
Nfx	450	6	5/5	300	3	5/5
	300	0	5/5			
NTA[c]	Vehicle	(6)	5/5	Vehicle	(8)	5/5

[a] PWD: percentage of weight diminution respect to day zero.
[b] Numbers in parentheses show increase in the weight respect to day zero.
[c] NTA: non-treated animals. For experimental conditions see Experimental Section.

Table 3
Mean values of the biochemical and the haematological findings in healthy animals treated orally and i.p. with single doses of compounds **3**–**10** and **Nfx**.

Animal treated with[a]	via	Leukocyte[b,c] (/μL)	Haemoglobin[c,d] (g/L)	Hematocrite[c,e] (%)	GPT[c,f] (UI/L)	GOT[c,g] (UI/L)
3	Oral	32,400	16.3	ND[h]	29.4	94.1
	i.p.	31,700	12.8	ND	76.2	109.0
4	Oral	2700	10.6	34.4	34.0	192.0
	i.p.	4900	12.3	38.7	32.0	140.0
5	Oral	6600	11.9	36.4	30.0	119.0
	i.p.	5300	13.8	43.6	ND	ND
6	Oral	26,600	13.5	ND	22.6	72.0
	i.p.	31,800	11.8	37.2	17.0	76.5
7	Oral	6600	13.5	42.1	42.0	164.0
	i.p.	5300	12.0	22.9	33.0	172.0
8	Oral	9010	13.4	39.4	62.7	139.0
	i.p.	11,100	14.2	ND	46.1	77.7
9	Oral	7750	13.9	ND	70.2	84.0
	i.p.	10,600	13.4	ND	50.7	57.0
10	Oral	86,000	11.5	30.4	22.7	69.8
	i.p.	12,100	11.3	ND	52.8	129.0
Nfx	Oral	6700	13.5	42.4	49.0	162.0
	i.p.	5100	13.2	39.3	32.5	177.0
NTA	Oral	6130	13.3	37.0	47.0	130.0
	i.p.	8410	13.7	38.0	68.0	119.0

[a] Oral doses = 450 mg/kg, intraperitoneal doses = 300 mg/kg.
[b] Normal value: 5000–13,700/μL.
[c] Normal values from Ofert et al. 1993.
[d] Normal value: 11.0–14.5 g/L.
[e] Normal value: 35.0–45.0%.
[f] GPT: glutamic-pyruvate transaminase (alanine aminotransferase); normal value: 28.0–184.0.
[g] GOT: glutamic-oxalacetate transaminase (aspartate ketoglutarate aminotransferase); normal value: 55.0–251.0.
[h] ND: not determined.

Table 4
Histological results for selected organs in healthy animals treated with compounds **1**–**10** and **Nfx**.

Animal treated with[a]	via	Liver[b]	Intestine	Kidney	Heart	Lung	Brain
1	Oral	+++	++	++	−	−	−
	i.p.	+++	−	++	−	−	−
2	Oral	+++	−	++	−	+	−
	i.p.	+++	−	−	+	−	−
3	Oral	+++	−	−	−	−	−
	i.p.	+++	−	++	−	−	−
4	Oral	+++	−	−	−	−	−
	i.p.	+++	−	−	−	−	−
5	Oral	++	−	−	−	−	−
	i.p.	+++	−	−	−	+	−
6	Oral	+++	−	−	−	−	−
	i.p.	+++	−	−	−	−	−
7	Oral	+++	−	++	−	−	−
	i.p.	+++	−	−	−	−	−
8	Oral	+++	−	−	−	−	+
	i.p.	+++	−	−	−	−	−
9	Oral	+++	−	++	−	−	−
	i.p.	+++	−	−	−	−	+
10	Oral	++	−	−	++	++	−
	i.p.	++	−	−	+++	++	−
Nfx	Oral	+++	−	++	−	−	−
	i.p.	+++	−	−	−	−	−
NTA	Oral	++	−	−	+	+	−
	i.p.	+++	−	−	+	+	−

[a] Oral doses = 450 mg/kg, intraperitoneal doses = 300 mg/kg.
[b] The histological results were summarized as, −:without changes respect to normal tissue; +: the organ not present relevant changes respect to normal tissue; ++: the organ present moderate kind of changes respect to normal tissue; +++: the organ present a great number of changes respect to normal tissue.

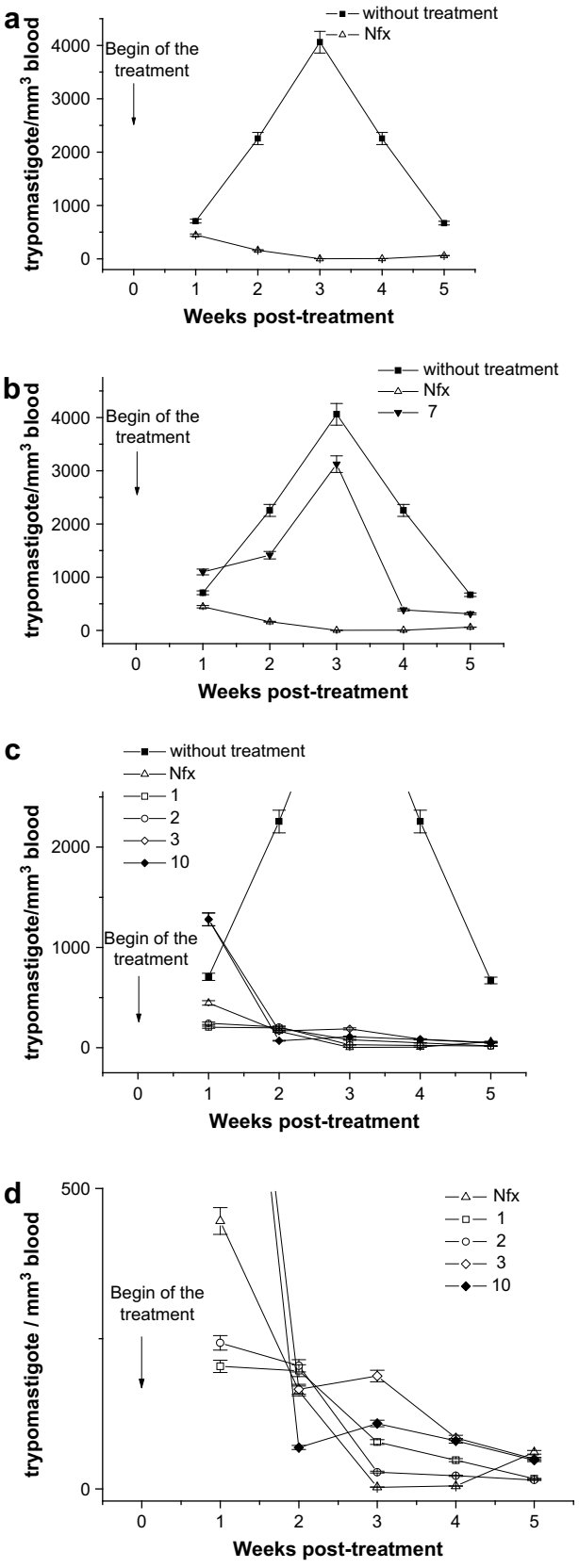


Fig. 1. Different parasitaemia treatments.(a) NTA, vehicle- and **Nfx**-treated animals.(b) *In vivo* behaviour of derivative **7**.(c) *In vivo* behaviours of derivatives **1**, **2**, **3**, and **10**.(d) Increased view of (c).

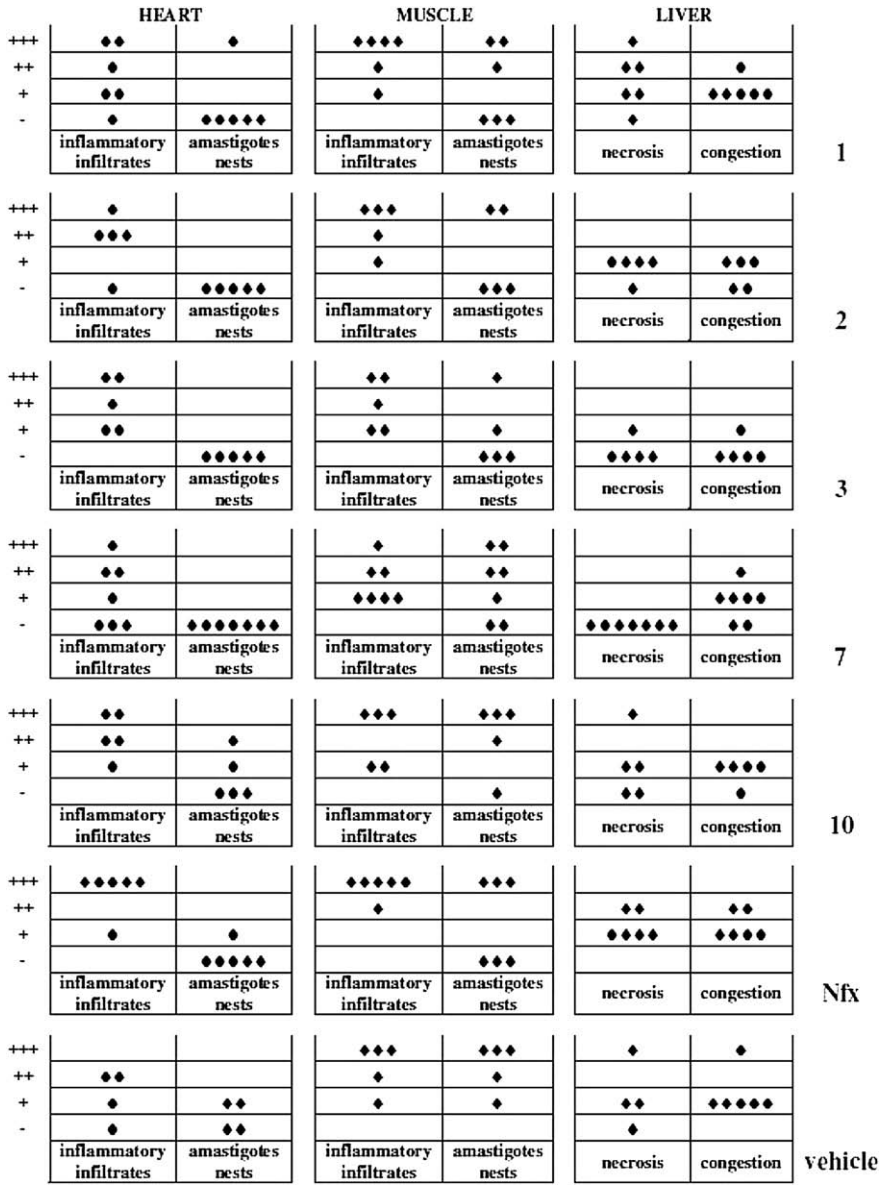


Fig. 2. Dispersion diagrams indicating the intensities of inflammatory infiltrates, amastigotes nests, necrosis or congestion in the infected animals treated with derivatives **1**, **2**, **3**, **7**, **10**, **Nfx** and without treatment. "− to +++" denotes increase of degree of histopathological alterations or amastigotes presence.

evaluated by means of parasitaemia and selected-organs' histopathology. In the histological studies were also included some organs that indicate compounds toxicity during the biological assay. Fig. 1 shows the means of the parasitaemia for the different treatments. According to these results, derivatives **1**, **2**, **3**, and **10** had similar *in vivo* behaviour of **Nfx** being compound **7** the least active of the studied compounds. Compounds **1**, **2**, **3**, and **10** reduced and maintained constant the trypomastigote levels in blood on the end of treatment (second week, Fig. 1). On the other hand, not all treated animals with derivatives **1** and **2** survived, after fifteen days of treatment one animal of seven died in each group.

In the histopathological studies of Chagasic animals, as signs of cure, the presence of amastigotes and inflammatory infiltrates in heart and muscle was determined by two independents observers (Fig. 2). The liver was studied searching for necrosis and congestion. According to these data, derivatives **1**, **2**, **3** and **7** were good anti-*T. cruzi* agents for myocardial muscle (compare degrees of inflammatory infiltrates and amastigotes nests with NTA and **Nfx**-treated animals, Fig. 2). Derivative **7** possessed the best inflammatory infiltrates profile and absence of amastigotes nests. All the new 5-nitro-2-furyl derivatives presented lower inflammatory infiltrates than the reference compound, **Nfx**. On the other hand, derivative **3** showed the best behaviour as anti-*T. cruzi* agent on smooth muscle being more efficient than **Nfx** (compare degrees of inflammatory infiltrates and amastigotes nests, Fig. 2).

From the histological studies other findings could be extracted. Derivative **1** and **Nfx**, and in lower order derivatives **2** and **10**, produced adverse effects on liver at the studied dose. Furthermore, in these studies some level of toxicity was observed in kidneys when Chagasic animals were treated with compounds **1**, **2**, **7**, **10**, and **Nfx** producing cortical congestion. Furthermore, it was evidenced, in these animals, some degree of spleenic congestion produced by compound **10** during the treatment.

4. Conclusions

In the present studies clear dependences between chemical structure and both acute toxicity and *in vivo* anti-Chagasic activity

were observed. On the one hand, 5-nitrofurfurilydene derivatives (**1**, **2**, **4**, and **5**) were more toxic, in the assayed conditions, than the (5-nitro-2-furyl)propene-analogues (**3**, **6**, and **9**). On the other hand, a carbazate, derivative **7**, was lesser anti-trypomastigote agent than the semicarbazones, derivatives **1**, **2**, and **3**, or the amide, **10**. It also shows a possible chemical structural dependence with the *in vivo* activity. Besides, in this *in vivo* study we confirmed that *in vitro*-active 5-nitro-2-furyl derivatives, especially (5-nitro-2-furyl)propene-semicarbazone derivatives, could be employed as therapeutic alternative in the treatment of Chagas' disease after further studies. Based on the *in vivo* trypanocidal activities compound **3** posses an excellent profile for the smooth muscle Chagasic disease with adequate profile in the cardiac one.

Compound **3**'s behaviour in the *in vivo* toxicity experiments promotes a deepest studies related to mutagenic and genotoxic properties of this compound. We are currently performing this kind of studies. Further work designed to examine the ability of compound **3** to provide a cure for *T. cruzi*-infected mice, changing dose, posology, and combination with other drugs is currently in progress.

5. Experimental

5.1. Chemistry

All starting materials were commercially available research-grade chemicals and used without further purification. The 5-nitro-2-furyl derivatives **1**–**10** were obtained as previously described [7,8,10]. **Nfx** (Lampit®, Bay 2502) was obtained from Bayer.

5.2. Pharmacology

5.2.1. Formulation of drugs for in vivo trials

Derivatives **1**–**3**, **7**, **10** and **Nfx** were suspended in sterile physiological saline:Tween 80 (4:1) solution (vehicle solution) immediately prior to injection. These preparations were made under aseptic conditions and in all cases homogeneous suspensions were obtained by shaking under ultrasound conditions.

5.2.2. Animals

The experiments were carried out on two month-old CD1 female mice (20–22 g) bred under specific pathogen-free conditions. Animals were housed in wire mesh cages at 20 ± 2 °C with natural light–dark cycles. The animals were allowed to feed "ad libitum" to standard pellet diet and water and were used after a minimum of 3 days acclimation to the housing conditions [20]. Control and experimental group consisted of 5–7 animals. The experimental protocols with animals were evaluated and supervised by the local Ethics Committee and the research adhered to the Principles of Laboratory Animal Care [21]. Animals were evaluated by supervision of international protocols and they were sacrificed in a humane way in accordance with recognized guidelines on experimentation. At the end of experiments they were anaesthetised with ethyl ether and sacrificed by cervical dislocation.

5.2.3. Biological samples

For the *in vivo* studies two kinds of biological samples were obtained: (1) Blood for parasitaemia, biochemical and haematological studies was drawn by sectioning the subclavian artery and studied immediately or maintained in EDTA or heparin anticoagulant at 0 °C. The biochemical and haematological determinations were carried out no more than 24 h post extraction.(2) Organs (lung, kidney, liver, spleen, heart, and intestine) were obtained by autopsy and maintained in aqueous formalin solution (10%) for further histological studies.

5.2.4. In vivo generation of Chagas disease

T. cruzi was isolated from Uruguayan patient and was inoculated in the mice by intradermal inoculation of $10–150 \times 10^6$ cells. Pharmacological studies were initiated on day 15 after observed parasitaemia.

5.2.5. Treatment of healthy animals with higher doses than the established posological dose

The animals were treated orally with a unique dose of 450 mg/kg, and for derivatives **1**, **2** and **Nfx** also a dose of 300 mg/kg was used. Intraperitoneally (i.p.) with one dose of 300 mg/kg, and for derivatives **1** and **2** also a dose of 150 mg/kg was assayed. The oral administration was done via intragastric syringe (1.0 mL) and the i.p. administration was done via injection (0.5 mL). Also, negative control, animals treated with vehicle, was included. The experiments lasted 3 days during these the animals were daily weighted and observed for alterations in skin, physical aspect, activity and faeces aspect, also the microenvironment was examined. At the end of the experiments the animals were sacrificed and dissected and the organs and blood were submitted for further studies.

5.2.6. In vivo anti-Chagasic trials

Derivatives were administered orally using intragastric syringe (1.0 mL) daily during 14 days. Suitable controls of NTA and **Nfx**-treated animals were also included. The effect of each drug was weekly evaluated by determining the trypomastigote levels. Parasitemia determinations were carried out as described previously [12,19]. On day 45th after the beginning of the treatment the animals were sacrificed and the dissected organs histologicaly studied. The presence of amastigotes and inflammatory infiltrates in heart and muscle and liver necrosis and congestion was determined by two independents observers [19].

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