

Antioxidant effect of *Morus nigra* on Chagas disease progression

Michelly Cristina Montenote¹, Vithor Zuccaro Wajzman¹, Yoichi Takaki Konno¹, Paulo César Ferreira², Regildo Márcio Gonçalves Silva², Altino Luiz Silva Therezo³, Luciana Pereira Silva⁴, Luciamáre Perinetti Alves Martins¹

ABSTRACT

Considering the widespread popular use of *Morus nigra* and the amount of scientific information on its antioxidant and anti-inflammatory activity, the effectiveness of this phytotherapeutic compound in the parasitemia progression during the acute phase of Chagas disease and its role in the development of the inflammatory process as well as its effects on the oxidative damage in the chronic phase of infection were evaluated. Thus, 96 male Swiss mice were randomly divided into eight groups, four groups were uninfected controls, and four groups were intraperitoneally infected with 5.0×10^4 blood trypomastigotes forms of *T. cruzi* QM2 strain. Four batches composed of one uninfected and one infected group were respectively treated with 70% alcohol solution and 25 μ L, 50 μ L and 75 μ L of the phytotherapeutic compound. Levels of antioxidant elements (TBARS, FRAP, GSH and Sulfhydryl groups) were measured in plasma samples. The phytotherapeutic compound's antioxidant activity was measured by polyphenol and total flavonoid quantification, DPPH, NO, and FRAP method. Our results showed that the vehicle influenced some of the results that may have physiological relevance in Chagas disease. However, an important action of *M. nigra* tincture was observed in the progression of Chagas disease, since our results demonstrated a reduction in parasitemia of treated groups when compared to controls, especially in the group receiving 25 μ L. However, in the chronic phase, the 50- μ L dosage presented a better activity on some antioxidant defenses and minimized the tissue inflammatory process. Results indicated an important action of *M. nigra* tincture on the Chagas disease progression.

KEYWORDS: Phytotherapy. Herbal medicine. Herbal compounds. Phytotherapeutic compound. Plant extracts. Parasitic diseases. Blackberry. Oxidative stress.

INTRODUCTION

Chagas disease is a parasitic and inflammatory infection caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*) discovered in 1909. It is estimated that, from 2000 to 2011, more than 1,200 cases were recorded, 70% of infections were acquired by oral transmission, 7% by vectorial transmission and 22% did not have the transmission mode identified¹.

The vectorial transmission occurs with penetration of metacyclic trypomastigote forms in the host leading to the development of the disease's acute phase. This initial phase can last from thirty to ninety days, with presence of high levels of parasites in bloodstream (parasitemia) and tissue, which may go unnoticed in most infected individuals due to insufficient clinical signs and symptoms². Subsequently, the chronic phase of disease may be asymptomatic or symptomatic when cardiac and

⁽¹⁾Faculdade de Medicina de Marília, Departamento de Parasitologia, Marília, São Paulo, Brazil

⁽²⁾Universidade Estadual Paulista "Júlio de Mesquita Filho", Laboratório de Fitoterápicos e Produtos Naturais (FitoLab), Assis, São Paulo, Brazil

⁽³⁾Faculdade de Medicina de Marília, Departamento de Anatomia Patológica, Marília, São Paulo, Brazil

⁽⁴⁾Fundação Educacional do Município de Assis, Departamento de Imunologia, Assis, São Paulo, Brazil

Correspondence to: Michelly Cristina Montenote
Faculdade de Medicina de Marília, Departamento de Parasitologia, Rua Dona Maria Feres, 165, CEP 17519-070, Marília, SP, Brazil

E-mail: mi_montenoti@hotmail.com

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digestive clinical manifestations are observed. Symptomatic chronic phase of Chagas disease is characterized by low parasitemia and high level of antibodies, which makes the laboratory diagnosis possible through several different techniques^{3,4}.

During the life cycle of *T. cruzi*, free radicals resulting from oxidative stress caused by both host immune response and parasite's aerobic metabolism and, occasionally, by drugs used to treat the disease, are associated with the appearance of tissue damage⁵.

In this sense, the excessive production of free radicals can trigger oxidative stress and, consequently, the loss of primordial cellular functions, which can lead to apoptosis and / or cell necrosis⁶. Studies have shown that increased oxidative stress is associated with disease progression and the use of antioxidant elements has been effective in attenuating the oxidative damage caused by disease and may influence its course^{7,8}.

In this context, several studies have demonstrated the efficacy of different phytotherapeutic compounds as effective antioxidant agents against many diseases, such as Alzheimer's, thyroid disorders, hypertension and obesity⁹⁻¹¹.

Morus nigra (*M. nigra*), popularly known as mulberry or blackberry, stands out for its edible fruits with bittersweet flavor and also for the wide popular use of its leaves as an hypoglycemic agent in the complementary treatment of diabetes mellitus and in menopause by attenuating climacteric symptoms¹². Other studies have also highlighted its use as an analgesic, anti-inflammatory, antipyretic, antiseptic, healing, depurative and diuretic, anthelmintic and emetic¹³⁻¹⁵. Several chemical compounds such as alkaloids, coumarins, flavonoids, triterpenes and steroids have been investigated in the *Morus* genus and researches have been carried out to prove these compounds' action on specific receptors, especially polyphenolic ones present in leaves and fruits of *M. nigra*, due to the presence of a wide spectrum of biochemical activities, such as antioxidant, antimicrobial, antimutagenic and immunoregulatory properties^{16,17}.

Although the role of free radicals in inflammatory processes is widely discussed, their interactions in the body deserve extensive investigations¹⁸. In this context, the activity of *M. nigra* in the parasitemia progression during the acute phase of Chagas disease, as well the influence of this phytotherapeutic compound in the development of the inflammatory process in the chronic phase of infection and its effects on the oxidative damage originated from infection in a murine model were evaluated, considering its widespread popular use and the amount of scientific information on its antioxidant, anti-inflammatory and anti-aging activities.

MATERIALS AND METHODS

Phytotherapy compound's antioxidant activity

The phytotherapy compound used in this research was a 20% *M. nigra* (blackberry) leaf dye purchased from the Panizza laboratory (batch N° 16013B124, reference MAP012), produced in May/2012 with expiration date in May/2016.

This phytotherapy compound was diluted in different concentrations, which were later used in *in vitro* antioxidant activity tests, as well as in the characterization of polyphenols and flavonoids. As for the animal's treatment, the phytotherapy compound was administered in different dosages according to the distribution of study groups. The Ethics Committee on the Use of Animals of the Medical School of Marília/CEUA-FAMEMA has approved the experimental study under the protocol N° 178/14.

Antioxidant activity test (DPPH)

The phytotherapy compound's antioxidant activity was determined by the H + donor ability to the stable radical 1,1-diphenyl-2-picrylhydrazyl, according to the *in vitro* methodology proposed by Blois¹⁹. The phytotherapy compound reacted with DPPH radical for a period of thirty minutes under low light and then subjected to a UV-vis Femto® spectrophotometer at a 517-nm wavelength. The antioxidant activity of the phytotherapy compound can be seen by the reagent's degree of discoloration required for the reaction to attain a plateau, and also by the low IC₅₀ value translating the phytotherapy compound's ability to inhibit radical oxidation by 50%. Gallic acid was used as the standard.

Ferric Reducing the Antioxidant Power (FRAP)

The FRAP assay was performed as previously described by Benzie and Strain²⁰. Sample or Trolox standard was mixed with distilled water to prepare the FRAP reagent extemporaneously, which was subsequently incubated at 37 °C for 30 min. Maximum absorbance values were read at 595 nm UV-vis Femto® spectrophotometer. Results were expressed as micromoles of Trolox Equivalents (TE) per mL of extract.

Antioxidant ability assessment through the nitric oxide (NO) scavenging assay

The method used to measure this phytotherapy compound's antioxidant ability was based on the Griess

*et al.*²¹ methodology with adaptations. The experiment was performed by transferring a 320- μ L aliquot of each extract into test tubes, adding 360 μ L of 25 mM NPS solution and 215 μ L of Griess reagent. They were kept in a water bath for 150 min at 37 °C and were then allowed to stand for an additional 60 min at room temperature. For the preparation of the standard curve, solutions ranging in concentrations from 5 to 60 μ M/mL were prepared from the standard sodium nitrite solution of 500 μ M/mL. Absorbance readings were performed using UV-vis Femto[®] spectrophotometer at 540 nm and the mean and nitrite concentration in μ M/mL were calculated from the absorbances obtained.

Total phenol content

The Folin-Ciocalteu method was used to determine the total phenol content in the extracts, with gallic acid as the comparative standard²². Phytotherapy compound's samples at different concentrations, distilled water and the Folin-Ciocalteu reagent were added. Then, the absorbance was measured at 725 nm using a UV-vis Femto[®] spectrophotometer. All measurements were performed in triplicate and results were expressed in μ g of gallic acid/mL of the phytotherapy compound.

Flavonoid content

The total flavonoid content of the phytotherapy compound was determined by a UV-vis spectrophotometer, and samples were prepared according to the methodology proposed by Yao *et al.*²³, based on the flavonoid complexation with AlCl_3 , which dislocates the absorption bands to higher wavelengths. Samples were shaken in a vortex shaker and the absorbance was measured at 510 nm using UV-vis Femto[®] spectrophotometer. All the tests were performed in triplicate and the results were expressed in μ g of rutin/mL of the phytotherapy compound.

Animal setting and infection methodology

A total of 96 male Swiss mice with 20 days old were randomly divided into eight groups of 12 animals, consisting of 4 (-) groups of uninfected animals and 4 (+) groups of infected animals. The infected groups (+) received 5.0×10^4 blood trypomastigotes forms of *T. cruzi* QM2 strain intraperitoneally, characterized by Martins *et al.*²⁴, from another infected mouse. One (C (+)) and one (C (-)) groups were used as controls and received 50 μ l of 70% alcohol solution because this solution was the basis of the *M. nigra* phytotherapy compound.

The other 6 groups were treated with the phytotherapy compound and received 25 μ L, 50 μ L and 75 μ L, these groups were named 25(+), 25(-), 50(+), 50(-), 75(+) and 75(-). All treatments were carried out every day in the morning and the mice received the alcohol solution or the phytotherapy compound orally (in their mouth) through a Gilson[®] automatic pipette tip for a period of 180 days. Treatment, care and euthanasia of the mice followed CEUA/FAMEMA standards (protocol: 178/14).

Parasitemia analysis

Parasitemia was investigated during the acute phase of infection (60 days), according to Brener's method²⁵. Twice a week we collected 5 μ l of blood from the tails of five animals from each infected group, starting on the 7th day after infection, totaling 17 counts to each animal. These animals were randomly chosen and kept in isolated cages until the end of the experiments and treatment was administered normally until the end of the chronic phase.

Euthanasia, samples collection and preparation

For the histopathological and biochemical study, animals were euthanized with 100% CO_2 on the 180th day after infection. The blood sample of each mouse was collected by cardiac puncture in tubes containing heparin, and the hemolysate was immediately performed to determine glutathione reduction according to the methodology used. The remainder of the blood sample was centrifuged at 1,110 g for 5 min at 4 °C and the collected plasma was stored at -20 °C for use in other biochemical tests.

Histopathological study

For histopathological analysis, fragments of heart, colon and skeletal muscle tissue were collected from all mice that survived until the 180th post-infection day. The tissues were embedded in paraffin and 5 μ m sections, were stained with hematoxylin-eosin and examined under a light microscope with 400x magnification. For each fragment, five sequential histological sections were performed, which were analyzed and graded according to the intensity of the inflammation process and the amount of amastigotes nests. A total of 10 high magnification fields were analyzed for each type of tissue. A semiquantitative scale varying from zero to three was used to grade the inflammatory process, the amastigote nests and the necrosis. "Zero" was considered as the absence of inflammation, necrosis and amastigote nests, "+" - mild inflammation, and rare amastigote nests and necrosis, "++" - moderate inflammation, moderate number of amastigote

nests and necrosis, and “+++” - intense inflammation, frequent amastigote nests and necrosis.

Antioxidant activity and oxidative stress assessment *in vitro*

FRAP

The antioxidant ability was determined using the technique described by Benzie and Strain²⁰. It is based on the ability of the plasma to reduce the Fe^{III} to Fe^{II} ions in the presence of 2,4,6-tripyridyltriazine at low pH, which results in the formation of a blue color read in a spectrophotometer at 593 nm. Plasma antioxidant ability was determined for each sample by comparing the absorbance test with the absorbance of Fe^{II} solutions of known concentrations (100-1000 mmol/L) tested in parallel.

TBARS

The thiobarbituric acid reactive species (TBARS), used as biomarkers of lipid peroxidation, was measured in plasma using a method adopted from Yagi²⁶. TBARS samples concentrations were determined by comparing their absorbance with a standard malondialdehyde solution.

GSH

This method relies on the reduction of 5,5'-dithiobis acid (2-nitrobenzoic acid) (DTNB). The concentration of erythrocyte glutathione (GSH) was determined by the colorimetric method developed by Beutler²⁷. The DTNB in the presence of glutathione produces a yellow compound, the optical density of which is measured at 412 nm. The concentration of GSH was expressed in mmol/g of Hb.

Total sulfhydryl groups (Ellman's test protocol)

The methodology proposed by Sedlak and Lindsay²⁸ using the Ellman reagent DTNB (5,5'-dithio-bis 2-nitrobenzoic acid) was employed. The thiol groups of the sample react with DTNB, forming a complex that absorbs light at 412 nm. The concentration of the total sulfhydryl groups was calculated by the equation $\{[(\text{Absorbance}_2 - \text{Absorbance}_1) - \text{White}] / \epsilon * \text{DTNB}\} \times \text{dilution} \times 1000$ with the molar absorption coefficient used for DTNB $\epsilon = 13,600 \text{ cm}^{-1} \text{ M}^{-1}$.

Statistical Analysis

Data normality was verified using the Shapiro-Wilk test (W). The ANOVA test was used for the comparisons between three or more groups complemented by the Tukey test. The Games-Howell test was used when the homogeneity of the

variances was unidentified. Kruskal-Wallis non-parametric analysis of variance (KW), complemented by Dunn's test for multiple comparisons, was used when data normality was not identified in at least one of the groups in comparison²⁹. The level of significance was set at 5%.

RESULTS

Phytotherapy compound's antioxidant activity: polyphenol and total flavonoid quantification, DPPH, NO and FRAP method

The assessment of the phytotherapy compound's polyphenol and total flavonoid concentrations showed 261.66 µg of gallic acid equivalent/mL and 361.83 µg of equivalent routine/mL of the phytotherapy compound, respectively.

To determine the antioxidant activity by the FRAP method, 32.09 µM of trolox/g equivalent of the phytotherapy compound was used. Similarly to the DPPH test, **Figure 1** shows that once different dilutions and pure phytotherapy compound were used, the antioxidant activity was progressively increased with increasing concentrations and the highest antioxidant activity was observed at 250 µL/mL of concentration presenting 81% of reduction, as shown in **Table 1**, and EC₅₀= 72.28 µL/mL (**Figure 1**). The potential for NO sequestration has also been tested. For this assessment, it was possible to verify a concentration of 13.64 µM/mL of nitrite formed, values close to those obtained with the gallic acid control that presented 10.11 µM/mL.

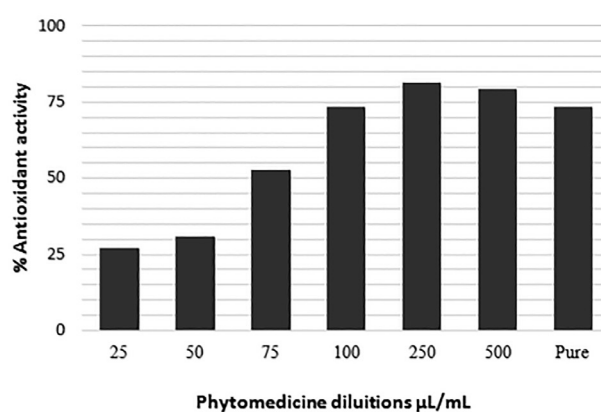


Figure 1 - Free radical scavenging activity DPPH in *M. nigra* - phytotherapy compound

Assessment of parasitemia in the acute phase

It is possible to observe that, in **Figure 2**, since the first count, the group C(+) presented with higher parasitemia when compared to the groups treated with the phytotherapy

Table 1 - Free radical DPPH and nitric oxide (NO) scavenging activity, polyphenols, total flavonoid and FRAP test in *M. nigra* - phytotherapy compound

	Total Polyphenols ^a	Total Flavonoids ^b	FRAP ^c	DPPH EC50% (µl/mL)	Nitric Oxide ^d Control: 10.11 ^e
Phytomedicine	261.66	361.83	32.09	72.2	13.64

^aµg of gallic acid equivalent/mL of the phytomedicine, ^bµg of rutine equivalent/mL of phytomedicine. ^cµM trolox equivalent/g of phytomedicine. ^dµM/mL of nitrite concentration formed by the antioxidant capacity of NO capture. ^e µM/mL of gallic acid.

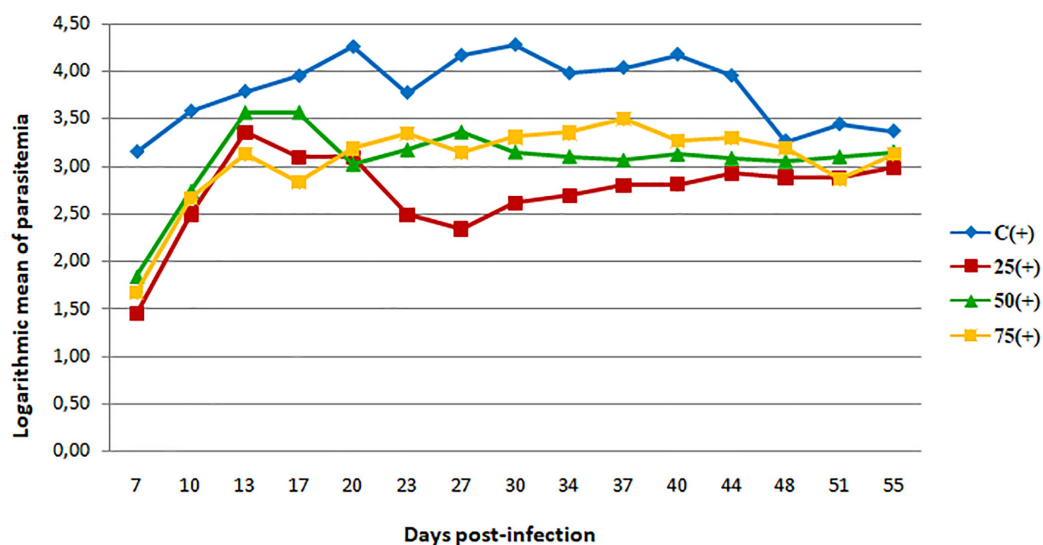


Figure 2 - Parasitemia curve by logarithmic mean of the number of trypomastigotes/5 µL of blood in the different experimental groups during the acute phase from the 7th to the 60th day of infection

compound, remaining superior throughout the analyzed period.

It was observed that the different concentrations were statistically different when compared to control groups, which exhibited the highest levels of parasitemia. It was verified that the 25(+) group exhibited the lowest parasitemia with $p < 0.05$, when compared to 50(+), 75(+) and C(+). The groups that received 50(+) and 75(+) showed $p < 0.05$ when compared to the C(+)

Assessment of antioxidant ability in plasma

The antioxidant capacity of the phytotherapy compound was performed in the animals that survived the studied period, as can be observed in [Table 2](#).

FRAP

Taking into account the absence of infection, it is possible to observe that in [Figure 3](#) the phytotherapy compound administration increased the concentrations of FRAP at the doses of 50(-) and 75 (-) in a similar way ($p=0.05$), but these results differed from the one obtained

Table 2 - Number of animals that survived in each group during the chronic phase of infection

	Group	Number of animals
Uninfected	Control(-)	11
	25µL(-)	11
	50µL(-)	11
	75µL(-)	9
Infected	Control(+)	8
	25µL(+)	10
	50µL(+)	8
	75µL(+)	12

for 25(-) ($p < 0.05$). These values did not differ statistically from group C(-).

When comparing FRAP concentrations in the different dosages with C(-), although increments can be observed, these differences did not reach statistical significance ($p > 0.05$). This may suggest an effect of the alcoholic vehicle acting in a dose dependent manner, with a maximum effect in the 50(-) group.

To evaluate the animals, compared with the C (+) group,

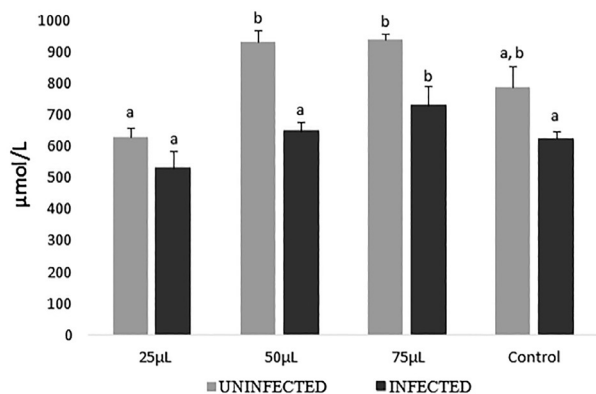


Figure 3 - FRAP results in different concentrations of the phytotherapy compound of *M. nigra* expressed in mean and standard deviation. Averages followed by the same designation do not differ from one another by the Dunn test at the 5% probability level

only the group 75 (+) showed a statistically significant difference ($p < 0.05$) with increasing FRAP concentrations.

When the effect of different dosages of each uninfected group was compared with its infected counterpart, differences ($p < 0.05$) were observed in the group that ingested 50 µL, which, when under infection, had their FRAP values decreased.

When comparing the controls with their infected counterparts, the 50 µL group showed a greater reduction of approximately 30% when under infection condition ($p < 0.05$). The effects of the different dosages of each uninfected group were compared with –the infected counterparts and significant differences ($p < 0.05$) were observed in the group that ingested 50 µL, which, when under infection, had their FRAP values decreased in approximately 30% ($p < 0.05$).

TBARS

It was observed that, in **Figure 4**, the C(-) group produced TBARS levels which were relatively higher than those obtained in the other groups with or without infection, not differing only from the 25(-) group, and this dosage showed the highest level of peroxidation in the absence of infection.

However, in relation to the 50(-) and 75(-) groups, when compared to C(-), there was a statistically significant reduction in TBARS levels of approximately 80%. This may indicate that the presence of the phytotherapy compound at these concentrations minimizes the possible alcohol-induced lipid peroxidation.

The C(+) group did not differ statistically from the other infected groups. The animals of 75(+) presented higher levels of TBARS when compared to C(+), but without

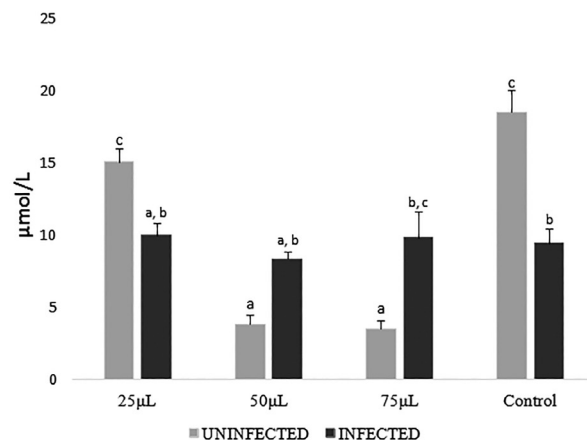


Figure 4 - TBARS results in different concentrations of the phytotherapy compound of *M. nigra* expressed in mean and standard deviation. Averages followed by the same designation do not differ from one another by the Dunn test at the 5% probability level

statistical significance. Between the 25(+) and 50(+) groups, there were also no statistically significant differences.

GSH

Regarding GSH concentrations, as seen in **Figure 5**, the group's 25(-), 50(-) and 75(-) presented higher concentrations of GSH when compared to C(-) group receiving the alcoholic solution, indicating that the phytotherapy compound induced effects mainly among animals receiving 50 µL and 75 µL of tincture.

When comparing C(+) and C(-), there was no statistically significant difference, evidencing that, in the chronic phase, the infection is not acting as an inducer of GSH synthesis.

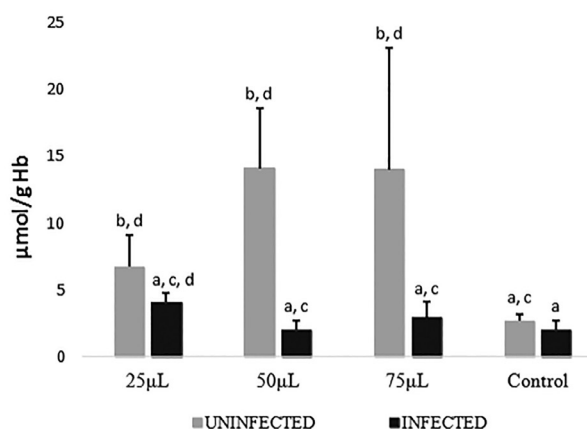


Figure 5 - GSH results in different concentrations of the phytotherapy compound of *M. nigra* expressed in mean and standard deviation. Averages followed by the same designation do not differ from one another by the Dunn test at the 5% probability level

It is also possible to observe that, among the infected groups, differences were not statistically significant among them, nor when they were compared to C(+), suggesting that the phytotherapy compound does not increase glutathione in the chronic phase infection.

However, for the groups that received 50 μL and 75 μL , the values are relevant when compared to the respective uninfected groups, because there is a statistically significant decrease, suggesting that the glutathione decrement is inherent to its consumption during the infection process. When comparing 50(-) and 75(-) with each other, they were not statistically different, inferring a maximal inducing effect at 50 μL .

Total Sulfhydryl Groups

Figure 6 shows that the 25(-) dosage decreased the total sulfhydryl groups in relation to 50(-) ($p < 0.05$). This dosage, however, was not different from the concentration observed in 75(-), differing, however, from C(-) ($p < 0.05$). This may evidence a possible phytotherapeutic effect at this concentration in increasing this type of defense mechanism.

Among the animals in the infected groups, no statistically significant difference was observed, a fact that has also occurred when they were compared to C(+). When comparing the uninfected groups with their respective infected counterparts, the differences were also not statistically significant.

Histopathological study

Considering uninfected animals that received tincture, no inflammation, necrosis or amastigote nests were observed in the histopathological analysis. Table 3 demonstrates exclusively the animals that presented any change in one or more of the analyzed parameters.

It was observed that the highest levels of inflammation were found in the skeletal muscle, heart and colon (Figure 7A), respectively, in all study groups.

In skeletal muscle, all animals in the 25 (+) group presented inflammatory process, being 90% discrete and 10% moderate; however, 37.5% of the animals in group C (+) presented moderate inflammation. Regarding the heart, although C (+) presented a greater number of animals (87.5%) with mild inflammatory process in the heart, in the group 25 (+) 40% of the animals presented moderate inflammation. As for the colon, the group C (+) had a higher number of animals with inflammatory process.

However, as shown in Figure 7B, the 25 (+) group had a higher number of animals with amastigote nests. In the

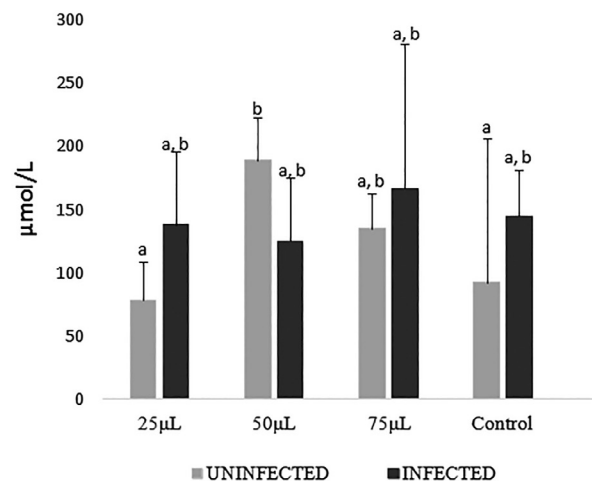


Figure 6 - Sulfhydryl groups results in different concentrations of the phytotherapy compound of *M. nigra* expressed in mean and standard deviation. Averages followed by the same designation do not differ from one another by the Dunn test at the 5% probability level

case of necrosis, all groups had necrotic processes in skeletal tissues in 2 animals from each group, as shown in Figure 7C.

DISCUSSION

Several studies have demonstrated the relationship between oxidative stress and antioxidant defenses during the course of infection and progression of Chagas disease in humans and experimental models^{7,30}.

The intense pharmacological potential of phenolic compounds and the presence of flavonoids can result in the ability to act on inflammation and on the immune system³¹. In addition, the antioxidant activity of these compounds is predominantly due to their ability to act as reducing agents of reactive oxygen species (ROS), in addition of reducing or chelating ferric ions that catalyze lipid peroxidation³².

Thus, the *in vitro* results obtained in this research (Table 1) corroborated several studies demonstrating that *M. nigra* leaves constitute a rich source of phenolic compounds capable of positively impacting human health, in addition to presenting a higher content of phenolic compounds and antioxidant activity when compared to its fruits^{33,34}.

Our results showed that the 25 (+) group exhibited a lower parasitemia when compared to the other groups (Figure 2). This decrease could be related to the anti-inflammatory activity of *M. nigra* leaves that contain germanicol among other compounds, which is described as an important natural anti-inflammatory, since flavonoids act modulating cells involved with inflammation¹⁶.

In addition, this decrease may be related to the availability of NO, since the ability to inhibit NO synthesis

Table 3 - Histopathological analysis performed in skeletal muscle, cardiac muscle and colon during the chronic phase of infection, in mice experimentally infected by *Trypanosoma cruzi* QM2 strain and treated with alcoholic solution (control), 25, 50 and 75 µL of *Morus nigra* phytotherapy compound. "%" indicates the percentage of mice with inflammation or necrosis in each group, and () * indicates the absolute number of animals in each group

Groups	Histological Analysis		Skeletal muscle	Cardiac muscle	Colon
C(+)	Inflammation %(8)*	+	50.0 (4)	87.5 (7)	62.5 (5)
		++	37.5 (3)	---	---
		+++	---	---	---
	Amastigote %(8)*	+	---	25.0 (2)	12.5 (1)
		++	---	---	---
		+++	---	---	---
	Necrosis %(8)*	+	25.0 (2)	---	---
		++	---	---	---
		+++	---	---	---
25(+)	Inflammation %(10)*	+	90.0 (9)	40.0 (4)	10.0 (1)
		++	10.0 (1)	40.0 (4)	---
		+++	---	---	---
	Amastigote %(10)*	+	8.3 (1)	---	30.0 (3)
		++	---	---	---
		+++	---	---	---
	Necrosis %(10)*	+	20.0 (2)	---	---
		++	---	---	---
		+++	---	---	---
50(+)	Inflammation %(8)*	+	62.5 (5)	25.0 (2)	37.5 (3)
		++	12.5 (1)	12.5 (1)	---
		+++	---	---	---
	Amastigote %(8)*	+	12.5 (1)	---	---
		++	---	---	---
		+++	---	---	---
	Necrosis %(8)*	+	12.5 (1)	---	---
		++	12.5 (1)	---	---
		+++	---	---	---
75(+)	Inflammation %(12)*	+	83.3 (10)	58.3 (7)	24.9 (3)
		++	---	---	---
		+++	---	---	---
	Amastigote %(12)*	+	8.3 (1)	---	---
		++	---	---	---
		+++	---	---	---
	Necrosis %(12)*	+	16.6 (2)	---	---
		++	---	---	---
		+++	---	---	---

by leaf extracts of the *Morus* genus is described in a dose-dependent manner³⁵. Thus, results could indicate that the lower dose of tincture used in the treatment induced a greater control of parasitemia (Figure 2) by allowing a

greater availability of nitric oxide, which plays an important role in the defense system to combat the parasite.

However, animals from the C (+) group showed patent parasitemia for a longer period of time probably due to

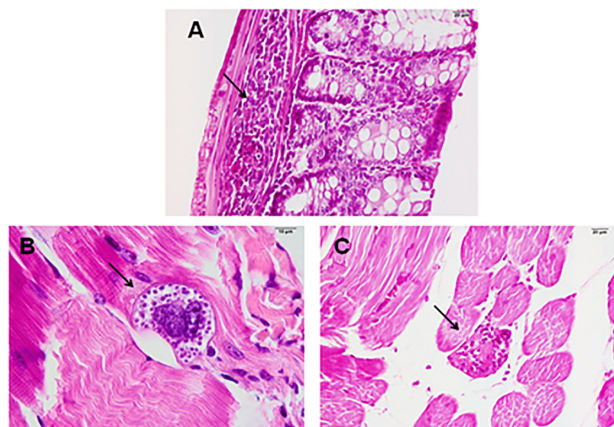


Figure 7 – Histological preparations of infected animals stained with hematoxylin-eosin: A) presence of inflammation in the control animal's colon; B) presence of amastigote nests in the skeletal musculature of animals from the 50 µL group; C) myocardial necrosis in a sample from a 25 µL group animal

the chronic use of ethanol, which according to Gomes and Pereira³⁶ can induce alterations in the nonspecific defense mechanisms or in immune responses. Thus, the chronic use of alcohol could explain the results disagreements reported by Gusmão *et al.*³⁷ when studying the parasitemia infected with the QM2 strain of *T. cruzi* receiving only water.

Although some studies have shown that the decrease of parasites influences the reduction of cardiac damage and the increase of animals' survival, our results showed that although the 25(+) group presented lower parasitemia in the acute phase, the reduction of the inflammatory process in histopathological studies was observed in the 50(+) group (Table 3). These differences may be related to the levels of active compounds present in the different dosages administered, since they may inhibit oxidation processes in certain systems, but this does not mean that they can protect cells and tissues from all types of oxidative damages, since they may present pro-oxidant activity under certain conditions^{38,39}.

Our results evidenced complex interactions in the defense systems according to the infection status, as well as the possible effect of the phytotherapy compound's vehicle itself on the evaluated parameters.

Thus, administration of the phytotherapy compound showed a maximal effect on FRAP at the 50 µL dosage (Figure 3) that could translate a beneficial protection effect for cells and tissues from the oxidative damage, since this method mainly reflects the reducing action of these phenolic compounds⁴⁰.

This dosage was still effective in increasing another defense mechanism, such as total sulfhydryl groups reaction (Figure 6) that represent the first line of defense, and the most ready source of antioxidants in plasma that can act in

situations in which vitamins C and E are not effective, acting also as savers of these vitamins⁴¹. This parameter relates to all thiols found in GSH and plasma proteins, such as albumin, and low molecular weight compounds consisting of free sulfhydryl, reduced cysteine residues in particular, cystenyl glycine and others which are targeted during the oxidative attack^{38,42}.

The reduction of sulfhydryl groups concentration could reflect the oxidative alteration of proteins resulting in a decrease in the arsenal of antioxidant defenses, which could be observed in the FRAP result of the infected groups which would be used to neutralize the free radicals generated, minimizing their damage on cellular structures.

In the present study, it is possible that *T. cruzi* infection has induced a minimized oxidative stress by mobilizing a defense arsenal that also combated the damage produced by alcohol. Unexpectedly, the presence of infection also appears to decrease TBARS levels (Figure 4), as can be observed in the C(+) group which had a 50% reduction when compared to C(-) ($p < 0.05$).

However, when comparing the uninfected groups with their infected counterparts, it was evidenced that the 25µL dosage resulted in a statistically significant reduction of parasitemia. Considering that this group presented, in absence of infection, a high concentration of TBARS, this expressive reduction in the presence of infection may suggest, once again, that the mobilized antioxidant defenses during infection has also acted in this situation.

It was observed that the phytotherapy compound was able to increase glutathione concentrations (Figure 5) in the absence of infection, which provided a possible defense against the oxidative stress caused by infection, as can be observed by the reduction of glutathione concentration in infected groups.

The possible regulatory role of some flavonoids on the level of glutathione γ -SGC (γ -glutamylcysteine synthetase) was investigated in incubated cells that received different treatments. Flavonoids usually limit the rate of protein synthesis and regulate intracellular GSH levels, capable of increasing the intracellular concentration of glutathione γ -GCS by approximately 50%⁴³.

The metabolism of glutathione and the interaction with the chronic use of ethanol is not well defined and may be related to the time of exposure. Studies have reported that chronic ethanol administration may increase the activity of glutathione peroxidase enzymes by 45% as a consequence of their action on peroxidation and glutathione reductase activities leading to a 15% reduction as a result of increased GSH demand for their oxidized form^{44,45}.

Molina *et al.*⁴⁶ reported that ethanol and its metabolites facilitate biomolecules' oxidation, acting directly as

oxidants reducing antioxidant levels or by combining both phenomena, increasing the levels of molecules with antioxidant properties such as flavonoids. These molecules have been increasingly investigated as possible therapeutic agents for the processes of tissue injury resulting from the excessive ethanol intake, demonstrating the protective effect of these substances against the oxidative stress caused by the excess consumption of ethanol. In our study, the increase of glutathione observed in the groups treated with the phytotherapy compound, has demonstrated a beneficial effect in the process of *T. cruzi* infection, more specifically regarding the level of tissue inflammation observed in the 50(+) group (Table 3). Apparently, the phytotherapy compound could induce a response and this response may have been relevant in the context of infection.

The presence of this response to the phytotherapy compound administration may be important, since previous research has reported that, in the chronic phase of disease, there is a depletion of the antioxidant defense system involving glutathione, which no longer responds to up regulation mechanisms as occurs in the acute phase^{47,48}.

Thus, the results of this study corroborate other researches, making it evident that natural products may be a source of new drugs with high activity and low toxicity of their secondary metabolites and that the *M. nigra* leaves are a promising source of natural compounds that should be considered for future studies, which could provide alternatives for Chagas disease treatment.

However, more detailed studies are needed mainly regarding the mechanisms of action and bioavailability, as well as the isolation and characterization of the phytotherapy compounds detected or not in this work, in order to collaborate with possible pharmacological applications of this *M. nigra* phytotherapy compound. herbal medicine.

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