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ANTHELMINTIC ACTIVITY OF LAPACHOL, β-LAPACHONE AND ITS DERIVATIVES AGAINST *Toxocara canis* LARVAE

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SUMMARY

Anthelmintics used for intestinal helminthiasis treatment are generally effective; however, their effectiveness in tissue parasitosis (i.e. visceral toxocariasis) is moderate. The aim of this study was to evaluate the *in vitro* activity of lapachol, β-lapachone and phenazines in relation to the viability of *Toxocara canis* larvae. A concentration of 2 mg/mL (in duplicate) of the compounds was tested using microculture plates containing *Toxocara canis* larvae in an RPMI-1640 environment, incubated at 37 °C in 5% CO_2 tension for 48 hours. In the 2 mg/mL concentration, four phenazines, lapachol and three of its derivatives presented a larvicide/larvistatic activity of 100%. Then, the minimum larvicide/larvistatic concentration (MLC) test was conducted. The compounds that presented the best results were nor-lapachol (MLC, 1 mg/mL), lapachol (MLC 0.5 mg/mL), β-lapachone, and β-C-allyl-lawsone (MLC, 0.25 mg/mL). The larvae exposed to the compounds, at best MLC with 100% *in vitro* activity larvicide, were inoculated into healthy BALB/c mice and were not capable of causing infection, confirming the larvicide potential *in vitro* of these compounds.

KEYWORDS: *Toxocara canis;* Quinones; Chemotherapy; Anthelmintics.

INTRODUCTION

Human visceral toxocariasis is a neglected zoonotic infection caused by the larvae of *Toxocara canis* and, less frequently, *Toxocara cati*[31]. According to recent reports, their prevalence seems to be underestimated mainly because of the difficulties of diagnosis and non-specific symptomatology[36]. The symptoms of this parasitic disease are characterized by cutaneous reactions, extensive eosinophilia, hepatomegaly, myocarditis, pulmonary infiltrates, and nodules accompanied by cough and fever[13,18]. The severity of symptoms depends on the location of the larvae and the number of larvae housed in tissues, which induces mechanical damage and, in turn, results in an immune-mediated inflammatory response[26]. Therefore, death is frequently associated with inflammatory granulomatous reactions around the larvae[15], which may persist for a long time and, with it, reactivated larval migration into the eye or the brain may occur at any time[40]. The long-term survival of *T. canis* larvae has been attributed to molecular strategies evolved by the parasite[26].

Generally, the drugs used to treat this disease have limited effectiveness, such as diethylcarbamazine and thiabendazole faced with poor tolerability and the need for prolonged use[30]. The low water solubility of benzimidazole compounds appears to collaborate with the low bioavailability of compounds in this group, such as albendazole[38], the drug of choice in the treatment of visceral toxocariasis[9]. Nevertheless, albendazole is the drug that crosses the blood brain barrier[34] and shows results superior to thiabendazole[37] and diethylcarbamazine, because it does not reduce the levels of specific IgE and produces side effects in treated patients[25]. Therefore, an effective drug for treating human infections caused by *T. canis* is still needed[28].

Among the possibilities of assisting in the treatment of visceral toxocariasis, natural and synthetic products[33] stand out. Plant extracts are important sources of biologically active natural products and may be a model for the development of new drugs[12,32].

Lapachol, an important representative of the quinone group, is isolated from plants of the Bignoniaceae family[19]. It performs biological activities against several pathogens, especially anti-parasitic activities against *Trypanosoma cruzi*, *Schistosoma mansoni, Leishmania amazonensis* and *L. braziliensis*[7,23,24].

β-lapachone is an ortho-naphthoquinone, a natural derivative of lapachol, present in small quantities in the woods of *Tabebuia* spp (Bignoniaceae). β-lapachone is easily synthesized by sulfuric acid treatment of lapachol[16] and has a wide range of biological activities, including trypanocidal, antibacterial, anti-inflammatory, and anticancer activity[2,3,4,7,29].

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Several heterocyclic compounds were synthesized from β-lapachone (i.e., phenazines) and have attracted considerable attention due to their biological activities, including antimalarial[5], antimycobacterial[2], antitumor, and antiparasitic[21] ones. Therefore, the use of this group of compounds as pharmacophores for the development of new drugs has consequently been investigated.

In this study, lapachol, β-lapachone and three of its derivatives, and 17 phenazines synthesized from β-lapachone analogues were tested against *T. canis* larvae.

MATERIALS AND METHODS

Synthesis: Lapachol was extracted from the heartwood of *Tabebuia* spp (*Tecoma*) and purified by recrystallization from ethanol, following a previously described procedure[11]. Nor-lapachol was synthesized from lapachol through Hooker oxidation[14].

β-lapachone, nor-β-lapachone, and β-C-allyl-lawsone were obtained through the cyclisation of the prenyl side chain of lapachol, nor-lapachol and C-allyl-lawsone, respectively. 10 mmol of the naphthoquinone were solubilized in 15mL of sulfuric acid and mixed for several minutes. The reaction was poured over cold water. The red solid was filtered, washed with cold water (3 × 100 mL) and purified by recrystallization using a mixture of acetone/hexane[17].

The phenazines were prepared by the reaction of the naphthoquinone (1.00 mmol), *o*-phenylenediamine (1.10 mmol) and sodium acetate (1.30 mmol) in glacial acetic acid (50 mL). The reaction was maintained under reflux for two hours and monitored by TLC. After the reaction, the mixture was poured over ice and left to incubate overnight. The yellow precipitate was filtered through a Buchner funnel, washed with cold water (3 × 100 mL), and the phenazine was isolated. All phenazines were synthesized with > 95% yield[35].

Test compounds: All synthesized compounds were solubilized in DMSO at 2.5% (Sigma®) and in sterile distilled water to obtain a concentration of 2 mg/mL[33].

Preparation of *T. canis* larvae: *T. canis* eggs were initially collected directly from the uterine tubes of female adult parasites following the treatment of young dogs with pyrantel pamoate (15 mg/kg). Afterwards, the eggs were incubated in a 2% formalin solution at 28 °C for 30 days in a humidity of > 90%[27]. By using a 5% sodium hypochlorite solution (Vetec), the eggs' protein cover was dissolved and the hatched *T. canis* larvae were collected in sterile tubes for cultivation with a (Gibco) RPMI-1640 medium supplemented with (Sigma) 25mM HEPES, 1% glucose, (Gibco) PSF antibiotic-antimycotic solution, and 0.4 μg/mL ofloxacin. Samples were maintained at 37 °C strain with 5% CO_2.

Larvicidal/larvistatic activity test: A microplate was used to measure the activity of substances at a concentration of 2 mg/mL. The tests were conducted in duplicate. 100 *T. canis* larvae, 200 μL of RPMI-1640 medium, and 100 μL of the test substances were added in each well. The larvae were then maintained at 37 °C for 48 hours with 5% CO_2.

The activity was tested *in vitro* and after exposure to the test compound the larval mobility was tested by the state of the larvae (i.e., motile, immobile but not dead, or dead). Cell viability was tested by using a 0.4% trypan blue indicator.

The substances that showed larvicidal activity in 100% of larvae with the *in vitro* test at concentrations of 2 mg/mL were re-tested at lower concentrations (MLC) (i.e., 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.05 mg/mL). Afterwards, the substances with larvicidal/larvistatic activity at the lowest concentrations were assessed for their viability of infection in mice. In order to assess their viability, the content of each microplate well was inoculated into 5-week-old BALB/c female mice by intraperitoneal injection. All mice were given food without antibiotics and had free access to water. The mice were kept on a 12 hour light to 12 hour dark cycle at a 22 °C (± 2 °C) room temperature.

Furthermore, a control group of live larvae (100 larvae/well) in mice was used to confirm the viability of larvae that were not exposed to the substances. A single mouse was used for each compound and each control. Mice were euthanized after 30 days of inoculation. The animals were examined for larvae by having their carcass, brain, liver, lungs, kidneys, heart, eyes, and spleen digested in a solution of 1% hydrochloric acid and 1% pepsin[39].

RESULTS

Lapachol, β-lapachone and three of its derivatives, and 17 phenazines were tested against *T. canis* larvae.

β-lapachone and β-C-allyl-lawsone showed the highest activity (MLC = 0.25 mg/mL), followed by lapachol (MLC = 0.5 mg/mL) and nor-lapachol (MLC = 1 mg/mL) (Table 1).

Out of the 17 phenazines tested on *T. canis* larvae, four compounds (i.e., compounds 1, 2, 3, and 4) showed 100% activity at a concentration of 2 mg/mL. Additionally, three compounds (i.e., compounds 5, 16, and 17) showed a larvicidal activity of 78.6-98.4% at the same concentration. The other phenazines showed < 14% activity (Table 2).

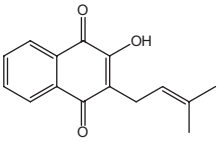
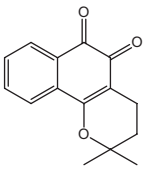
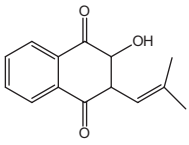
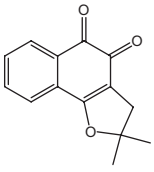
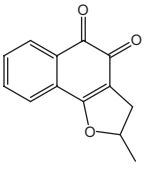
The larvae exposed to the compounds with 100% activity *in vitro* were not viable and, therefore, were not able to infect the mice. The control group consisted of live larvae and caused infection when inoculated into the mice, which validates the *in vitro* evaluation criteria used in this study.

DISCUSSION

The search for new therapeutic prototypes with effectiveness against *T. canis* larvae housed in human tissues is relevant for the efficacy of visceral toxocariasis treatment. The new drugs should eradicate all larvae housed in the tissues, not only decrease the intensity of infection as it was noted in the administration of albendazole[1,6,32,33], ivermectin, mebendazole, and thiabendazole[22] in mice.

In this study, the possible effect of lapachol and β-lapachone and its derivatives against *T. canis* larvae was tested. Among all the synthetic compounds tested, β-lapachone and β-C-allyl-lawsone showed the best anthelmintic activity *in vitro*. Although these results are relevant, the quinones present significant toxicity, possibly due to the redox potential. This toxicity may cause cell damage due to oxidative stress, which could result in undesirable side effects[10].

Table 1
Larvicide/larvistatic activity, MLC and *in vivo* viability of the *T. canis* larvae treated with lapachol and derivatives (n = 5)

Nº	Chemical structure	Chemical compound	Activity	Standart deviation	MLC	Larvae viability in mice
1		Lapachol $C_{15}H_{14}O_3$	100%	Zero	≤ 500 µg/mL	Negative
2		β- lapachone $C_{15}H_{14}O_3$	100%	Zero	≤ 250 µg/mL	Negative
3		Nor-lapachol $C_{14}H_{12}O_3$	100%	Zero	≤ 1,000 µg/mL	Negative
4		Nor-β-lapachone $C_{14}H_{12}O_3$	11.9%	0.8	-	-
5		β-C-allyl-lawsone $C_{13}H_{10}O_3$	100%	Zero	≤ 250 µg/mL	Negative
Control	Live larvae (no compound)		4.2%	0.4	-	Positive

Negative to detection of *T. canis* larvae in mice tissues; Positive to detection of *T. canis* larvae in mice tissues.

Nevertheless, due to the presence of larvicidal activity and by the easy access of quinones to natural sources from Brazilian flora[7], justify the utilization of these compounds as a pharmacophore to develop heterocyclic derivatives more active and less toxic.

This approach was previously used to synthesize trypanocidal naphthoimidazoles from β-lapachone and to demonstrate that naphthoimidazoles were more active and less toxic than β-lapachone[8].

The larvicidal potential of *in vitro* tests and the capacity to inhibit viability of infection in the mice, demonstrated by quinones, indicated the relevance of studies in this area. Furthermore, motivates realize cytotoxicity studies, for further evidence of the biological activity of these compounds, in preclinical trials in experimental models, aiming the development of prototype compound with anthelmintic activity which could be used in the treatment of visceral toxocariasis.

Four phenazines (i.e., compounds 1, 2, 3, and 4) out of the 17, showed 100% activity at a concentration of 2 mg/mL. However, these phenazines did not present satisfactory results when exposed to low concentrations; similar results were obtained with the same phenazines against *Plasmodium falciparum*, *P. berghei*[5], and *Mycobacterium tuberculosis*[2]. In these studies, the compounds showed 50% antimalarial activity *in vitro*, and only one-fourth of the phenazines tested against *M. tuberculosis* demonstrated strong antimycobacterial activity (minimum inhibitory concentration = 0.78 µg/mL). A significant antimalarial activity *in vitro* was also shown in the other phenazines synthesized from naphthols that were assayed against *P. falciparum* strains resistant to chloroquine. However, they are not able to promote an effective cure when tested against *P. berghei in vivo*[20].

Table 2
Larvicide/larvistatic activity, MLC and *in vivo* viability of the *T. canis* larvae treated with phenazines (n = 17)

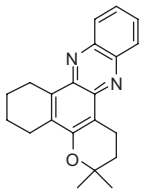
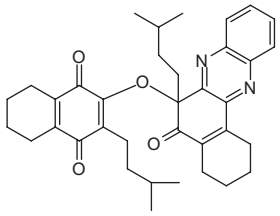
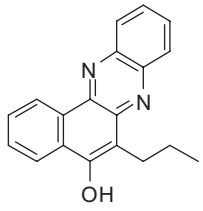
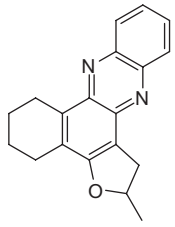
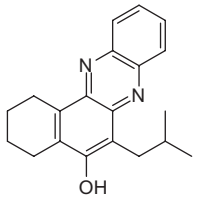
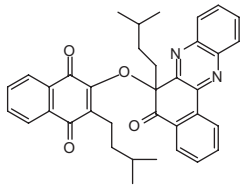
No.	Chemical structure	Chemical compound	Activity	Standart deviation	MLC	Larvae viability in mice
1		$C_{21}H_{22}N_2O$	100%	Zero	2,000 µg/mL	Negative
2		$C_{36}H_{42}N_2O_4$	100%	Zero	2,000 µg/mL	Negative
3		$C_{19}H_{16}N_2O$	100%	Zero	2,000 µg/mL	Negative
4		$C_{19}H_{18}N_2O$	100%	Zero	2,000 µg/mL	Negative
5		$C_{20}H_{22}N_2O$	78.6%	7.1	-	-
6		$C_{36}H_{34}N_2O_4$	1.76%	0.03	-	-

Table 2
Larvicide/larvistatic activity, MLC and *in vivo* viability of the *T. canis* larvae treated with phenazines (n = 17) (cont.)

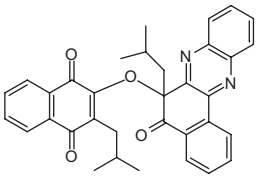
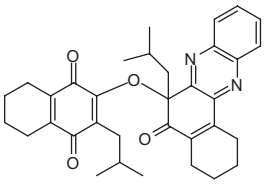
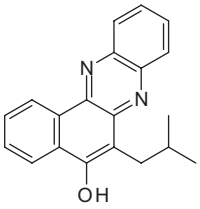
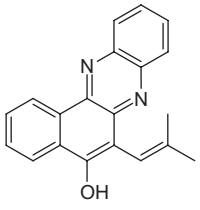
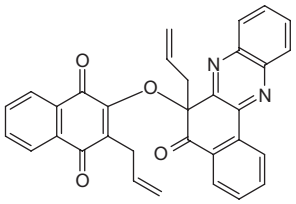
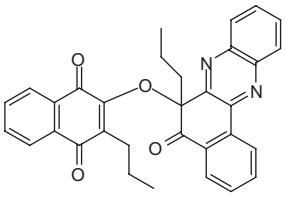
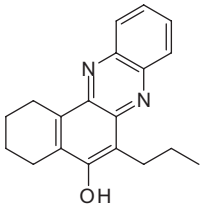
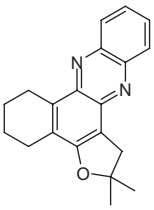
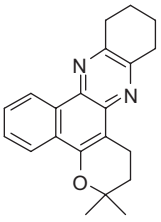
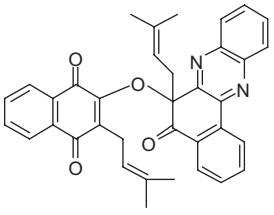
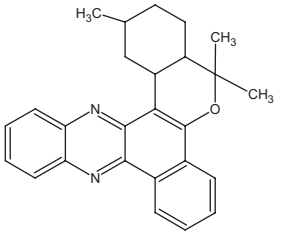
No.	Chemical structure	Chemical compound	Activity	Standart deviation	MLC	Larvae viability in mice
7		$C_{34}H_{30}N_2O_4$	1.0%	0.1	-	-
8		$C_{34}H_{38}N_2O_4$	3.8%	18.0	-	-
9		$C_{20}H_{18}N_2O$	4.2%	8.8	-	-
10		$C_{20}H_{16}N_2O$	1.3%	4.5	-	-
11		$C_{32}H_{22}N_2O_4$	6.5%	2.2		
12		$C_{32}H_{26}N_2O_4$	1.5%	1.1	-	-

Table 2
Larvicide/larvistatic activity, MLC and *in vivo* viability of the *T. canis* larvae treated with phenazines (n = 17) (cont.)

No.	Chemical structure	Chemical compound	Activity	Standart deviation	MLC	Larvae viability in mice
13		$C_{19}H_{20}N_2O$	14.0%	47.4	-	-
14		$C_{20}H_{20}N_2O$	2.5%	1.4	-	-
15		$C_{21}H_{22}N_2O$	4.0%	0.7	-	-
16		$C_{36}H_{30}N_2O_4$	94.5%	3.5	-	-
17		-	98.4%	0.4	-	-
CT		No compound	1.3%	0.4	-	Positive

CT: Control; Negative to detection of *T. canis* larvae in mice tissues; Positive to detection of *T. canis* larvae in mice tissues.

Structural changes that arose in other phenazines (i.e., compound 5-17) tested in this study did not increase the specific activity of the molecules. The lower activity of compounds 5-17, compared to compounds 1-4, indicates that new modifications to these molecules are necessary to promote effective action against *T. canis* larvae.

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RESUMO

Atividade anti-helmíntica do lapachol, β-lapachona e derivados contra larvas de *Toxocara canis*

Os anti-helmínticos empregados no tratamento das helmintoses intestinais, de modo geral, são eficazes, porém nas parasitoses teciduais, como é o caso da toxocaríase visceral, a eficácia é moderada. Este estudo teve como objetivo avaliar *in vitro* a atividade do lapachol, β-lapachona e fenazinas derivadas da β-lapachona sobre a viabilidade de larvas de *Toxocara canis.* Os compostos foram testados na concentração de 2 mg/mL (em duplicata) em placas de microcultivo, contendo larvas de *T. canis* em meio RPMI-1640, sendo incubados, a 37 °C, em tensão de CO2 de 5%, por 48 horas. Na concentração de 2 mg/mL, quatro fenazinas, o lapachol e três derivados, apresentaram atividade larvicida/larvostática de 100%. A seguir, foi realizado o teste de concentração larvicida/larvostártica mínima (CLM). Os compostos que apresentaram os melhores resultados foram o nor-lapachol (CLM, 1 mg/mL), lapachol (CLM, 0,5 mg/mL), a β-lapachona e a β-C-alil-lausona (CLM, 0,25 mg/mL). As larvas expostas aos compostos, na melhor CLM 100% *in vitro* foram inoculadas em camundongos BALB/c saudáveis não sendo capazes de causar infecção, confirmando o potencial larvicida *in vitro* desses compostos.

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