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ANTHELMINTIC ACTIVITY OF EUGENOL, TANNIN AND THYMOL AGAINST NEOECHINORHYNCHUS BUTTNERAE

(Atividade anti-helmíntica de eugenol, tanino e timol frente à *Neoechinorhynchus buttnerae*)

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ABSTRACT - This study assessed the *in vitro* anthelmintic activity of eugenol, thymol and tannic acid against the acanthocephalan endoparasite *Neoechinorhynchus buttnerae*. Tambaqui fish *Colossoma macropomum* intestines were collected and dissected for parasite removal. The anthelmintic activity of the compounds studied was tested *in vitro* using minimum essential medium (MEM) to maintain parasite viability. One control group and from 8 to 10 treatments were carried out in triplicate. Ten *N. buttnerae* specimens were introduced to each MEM plate, and after exposure to the compounds, parasite motility was observed every 15 min. The LC_{50} value of eugenol after 2, 6 and 24 h exposure was 1.01, 0.79 and 0.41 $mg.mL^{-1}$, respectively; to thymol the values were 1.97, 0.96 and 0.92 $mg.mL^{-1}$, and to tannic acid were 4.68, 1.79 and 1.30 $mg.mL^{-1}$. Among the compounds tested, eugenol was the most efficient against *N. buttnerae*, eliminating 100% of the parasites after 15 min exposure to the three highest concentrations tested (1.56, 3.125 and 6.25 $mg.mL^{-1}$). Thymol eliminated over 90% of the parasites after 2h exposure to the three highest levels tested (0.78, 1.56 and 3.125 $mg.mL^{-1}$), and tannic acid showed the lowest activity, eliminating 100% of parasites after 6h exposure to doses between 6.25 and 50 $mg.mL^{-1}$.

Key words: parasite; tambaqui; acanthocephalan; anthelmintic activity.

RESUMO - Este estudo avaliou a atividade anti-helmíntica in vitro do eugenol, timol e ácido tânico contra o endoparasito acantocéfalo *Neoechinorhynchus buttnerae*. Intestinos do peixe tambaqui *Colossoma macropomum* foram coletados e dissecados para remoção do parasito. A atividade anti-helmíntica dos compostos estudados foi testada *in vitro* usando meio mínimo essencial (MEM) para manter a viabilidade do parasita. Um grupo controle e de 8 a 10 tratamentos foram realizados em triplicata. Dez espécimes de *N. buttnerae* foram introduzidos em cada placa MEM e, após exposição aos compostos, a motilidade dos parasitos foi observada a cada 15 minutos. O valor de CL50 do eugenol após 2, 6 e 24 h de exposição foi 1,01, 0,79 e 0,41 $mg.mL^{-1}$, respectivamente; para o timol os valores foram 1,97; 0,96 e 0,92 mg.mL-1 e para o ácido tânico foram 4,68, 1,79 e 1,30 $mg.mL^{-1}$. Dentre os compostos testados, o eugenol foi o mais eficiente contra *N. buttnerae*, eliminando 100% dos parasitos após 15 min de exposição às três maiores concentrações testadas (1,56; 3,125 e 6,25 $mg.mL^{-1}$). O timol eliminou mais de 90% dos parasitos após 2h de exposição aos três maiores níveis

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testados (0,78; 1,56 e 3,125 mg.mL^{-1}), e o ácido tânico apresentou a menor atividade, eliminando 100% dos parasitas após 6h de exposição a doses entre 6,25 e 50 mg.mL^{-1}.
Palavras-chave - parasito; tambaqui; acantocéfalo; atividade anti-helmíntica.

INTRODUCTION

Parasite infestation is a common problem in fish farming. Worms are one of the most common fish parasites and are distributed among three phyla: Platyhelminthes, Nematoda and Acanthocephala (Kime, 1995). Based on phylogenetic studies and reviews on molecular taxonomy, Amin (2013) reports that acanthocephalans (thorny-headed worms) include 26 families, 157 genera and 1,298 species. Found throughout the world, adult acanthocephalans attach themselves to the intestinal walls of marine and freshwater fish by their hooked proboscides (Meyer and Olsen, 1966). In Brazil, the main acanthocephalan groups are *Polyacanthorhynchus, Echinorhynchus* and *Neoechinorhynchus* (Eiras et al., 2010). Currently, *Neoechinorhynchus buttnerae* is the only acanthocephalan described as a parasite of tambaqui *Colossoma macropomum* and its hybrids, namely tambacu *C. macropomum* x *Piaractus mesopotamicus* and tambatinga *C. macropomum* x *Piaractus brachypomus* (Malta et al., 2001; Dias et al., 2015). The tambaqui is of considerable economic interest and is the most important native fish species produced in fish farming in Brazil, yielding 136,990 tonnes in 2016 (IBGE, 2016). Although the damage caused by *N. buttnerae* to tambaqui is mostly slight to moderate, proboscis penetration into the intestinal walls can inflict a more pronouced inflammatory response, metaplasia and ireversible focal necrosis in muscle tissue in the intestinal wall. Taking into acount the role of the organ in absorption of nutrientes, these injuries can compromise the quality of *C. macropomum* juveniles in the stage of life in which the fish are obtained by fish farmers in the region for growth and fattening. (Aguiar et al., 2018; de Matos et al., 2017). There is evidence that acanthocephalosis can compromise fish zootechnical performance (Silva-Gomes et al., 2017) and production yields (Jerônimo et al., 2017). Therefore, the search for alternative strategies to control and prevent acanthocephalan infection in fish farms is of vital importance. In the aquaculture industry, antiparasite treatment is limited by the absence of effective and useable medications (Zuskova et al, 2018) and antibiotics are frequently employed indiscriminately to ensure high productivity (Luis et al, 2017). Synthetic drugs can be used to treat parasitic infection, but because of their toxicity, they can cause adverse side effects in fish (Srivastava et al, 2004), to the environment (Gürkan and Özdilek, 2018; Rico et al., 2012) and consumers (Zhao et al, 2020). A less harmful alternative treatment is the use of natural compounds such as tannins, eugenol and thymol, which display anthelmintic activity in lambs, pigs and fish (Katiki et al., 2014; Sutili et al., 2014; Williams et al., 2014; de Lima Boijink et al., 2015). These compounds are major components of essential oils and extracts from medicinal plants and are less toxic than synthetic drugs. Against this background, the present study evaluated the *in vitro* anthelmintic activity of eugenol, thymol and tannic acid against the acanthocephalan *N. buttnerae* infecting tambaqui (*Colossoma macropomum)*.

MATERIAL AND METHODS

Tambaqui *C. macropomum* juveniles weighing around 115g and naturally infected with *N. buttnerae* were acquired from commercial fish farms and transported to an experimental field at Embrapa Amazônia Ocidental (Manaus, Amazonas state). Fish were anesthetized with 10% benzocaine and euthanized by through cranial drilling with the aid of a pointed instrument, as recommended by CONCEA (National Council for Control of Animal Experimentation) (2018). Their intestines were removed at necropsy and dissected for parasite removal using tweezers.

The parasites used for the *in vitro* tests, identified according to the morphological characteristics described by Golvan (1956) and de Matos et al. (2017), were adult males and females of size 1,77±0,63 cm.

Eugenol (Isofar, Rio de Janeiro, RJ, BR, purity:>=99,0%) was tested at 9 concentrations (0.024, 0.049, 0.098, 0.19, 0.39, 0.78, 1.56, 3.125 and 6.25 $mg.mL^{-1}$) and Thymol (Neon, Suzano, SP, BR, purity:>=99,0%) was tested at 8 concentrations (0.024, 0.049, 0.098, 0.19, 0.39, 0.78, 1.56, 3.125 $mg.mL^{-1}$), and tannic acid (Sigma-Aldrich, St. Louis UM, USA) at 10 concentrations (0.098, 0.19, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 and 50 $mg.mL^{-1}$). All tested was made in triplicate. Each plate was filled with 4 mL of minimum essential medium (MEM) (Inlab, Diadema, SP, BR) to maintain parasite viability. Ten *N. buttnerae* specimens were added to each plate, totaling 30 specimens per concentration.

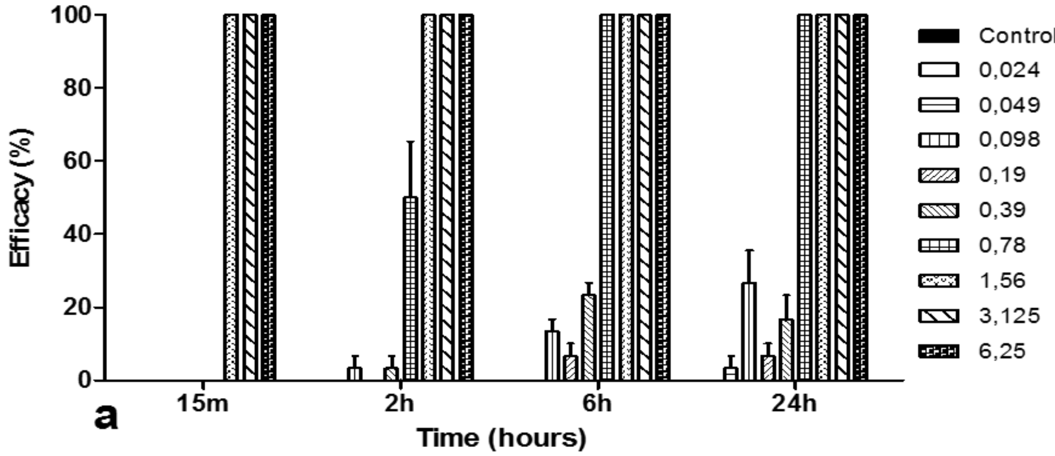
Thymol and eugenol were diluted in 3% Tween 80 (Synth, Diadema, SP, BR) aqueous solution and tannic acid in distilled water. A negative control used 3% Tween 80 aqueous solution was also tested. Each plate containing MEM received 1 mL of the test solution.

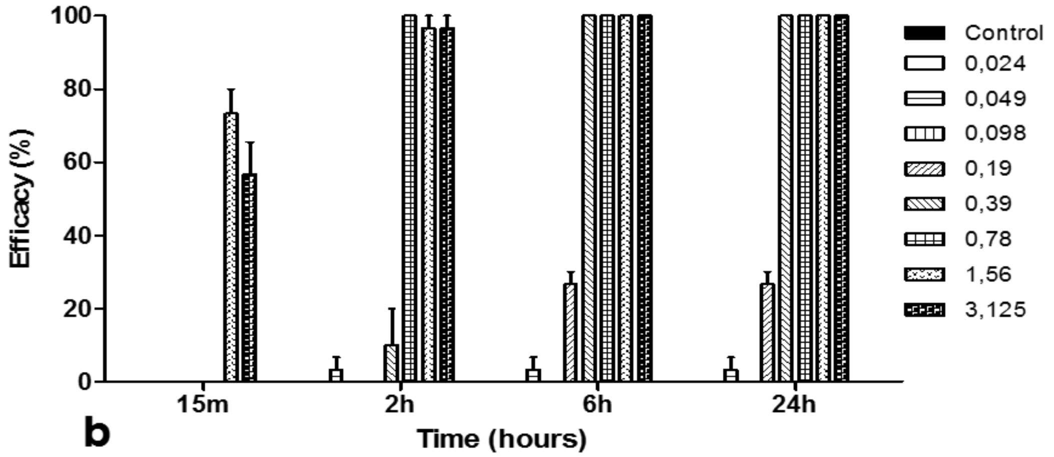
After introducing the test solutions, the plates were examined under a stereo microscope (ambiental conditions, temperature: 20-25°C) every 15 min, for 7 h, and parasite viability assessed by motility detection. Mortality rate was determined after 24 h exposure to the components tested. Parasites were considered dead when they were motionless, even after mechanical stimulation (Sutili, Gressler, Baldisserotto, 2014).

Mortality data were used to calculate the mean lethal concentration (LC_{50}) and treatment efficacy. Treatment efficacy was calculated using a modified version of Wang et al. (2008) equation: AE=[C - T] × 100%/B, where AE is the anthelmintic efficacy, C the mean live *N. buttnerae* specimen count in the control group (3% Tween 80 diluted in MEM) and T the mean live parasite count in the treatment. LC_{50}, which corresponds to the concentration capable of killing 50% of the parasite population, was determined 2, 6 and 24 h after exposure of each compound tested, applying the Trimmed Spearman Karber method (Hamilton, Russo, Thurston, 1977).

RESULTS

The compounds tested showed *in vitro* anthelmintic efficacy, and their activity was enhanced with an increase in concentration and exposure time (Figure 1). Eugenol was the most efficient against *N. buttnerae*, eliminating 100% of the parasites after 15 min exposure to the 3 highest concentrations tested (1.56, 3.125 and 6.25 mg.mL^{-1}) (Figure 1a). Thymol, in turn, required 2 h exposure to the 3 highest concentrations (0.78, 1.56 and 3.125 mg.mL^{-1}) to kill at least 90% of the parasites (Figure 1b), but after 6 h exposure, even at a low concentration of 0.39 mg.mL^{-1},it killed 100% of the parasites. Tannic acid exhibited the lowest efficacy, killing 100% of the parasites after 6h exposure to concentrations of 6.25 to 50 mg.mL^{-1} and after 24 h using concentrations of 3.125 to 50 mg.mL^{-1} (Figure 1c). There were no deaths in the controls tested.





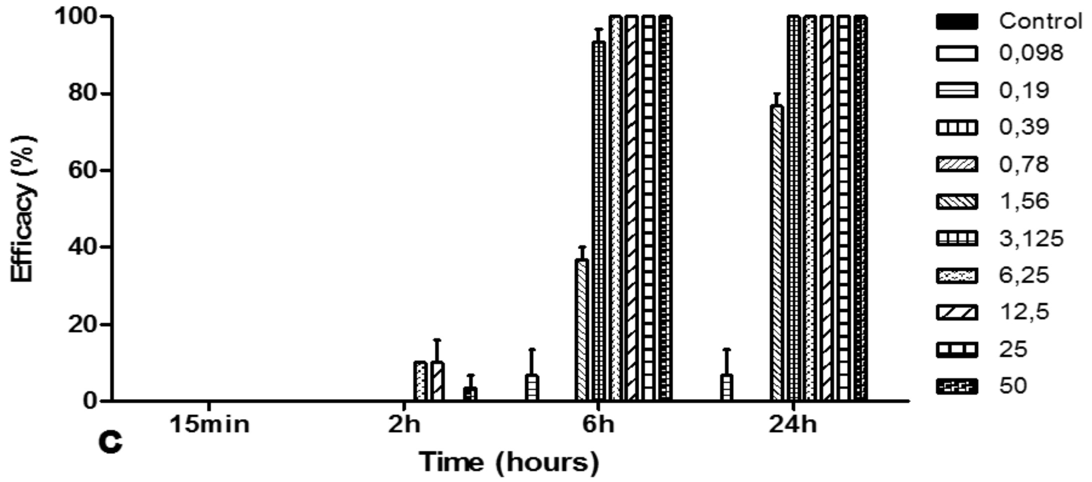


Figure 1 - *In vitro* anthelmintic efficacy of a) eugenol, b) thymol and c) tannic acid at different concentrations (in mg.ml^{-1}) and exposure times.

The LC_{50} value of eugenol after 2, 6 and 24 h exposure was 1.01, 0.79 and 0.41 mg.mL-^{1}, respectively; to thymol the values were 1.97, 0.96 and 0.92 mg.mL^{-1}, and to tannic acid were 4.68, 1.79 and 1.30 mg.mL^{-1} (Table I).

Table I: Anthelmintic activity of eugenol, thymol and tannic acid against the parasite *Neoechinorhynchus buttnerae*

Compound	Exposure time	LC_{50} mg.mL^{-1} (confidence interval)
	2 h	1.01 (0,92 – 1,10)
Eugenol	6 h	0.79 (0,67 – 0,94)
	24h	0.41 (0,35 – 0,48)
	2 h	1,97 (1,79 – 2,16)
Thymol	6 h	0,96 (0,87 – 1,06)
	24h	0,92 (0,82 – 1,03)
	2 h	4,68 (3,80 – 5,77)
Tannic acid	6 h	1,79 (1,56 – 2,06)
	24h	1,30 (1,17 – 1,44)

LC_{50}: Lethal concentration (concentration capable of killing 50% of the parasite population)

DISCUSSION

Meneses et al. (2018) carried out *in vitro* assays with the essential oil of *Ocimum gratissimum*, which is rich in eugenol, and found anthelmintic activity against the monogenoid *Cichlidogyrus tilapiae*, a Nile-tilapia parasite. They achieved LC_{50} with 0.04 mg.ml^{-1} and 96 h exposure to the oil, which is lower than the 0.41 mg.ml^{-1} we found using eugenol for 24 h. The higher efficacy of the essential oil may be explained by a number of factors, such as increased toxicity promoted by the synergism between eugenol and other components of *O. gratissimum*, or to differences between exposure time and parasite size.

Katiki et al. (2017) evaluated the effects of 10 compounds isolated from medicinal plants on the hatching of *Haemonchus contortus* eggs, and found an LC_{50} of 0.57 mg.ml^{-1} after exposure to eugenol for 24 h. Similarly, in the present study, LC_{50} was obtained after 24 h exposure to eugenol at 0.41 mg.ml^{-1}.

The anthelmintic activity of eugenol has been reported by numerous studies *(*Asha et al., 2001; Pessoa et al., 2002; de Lima Boijink et al., 2015; Sutili, Gressler, Baldisserotto, 2014) and corroborated here against *N. buttnerae*. Eugenol was more efficient at killing

N. buttnerae than reported in other studies for parasites such as *Gyrodactylus sp.*, which required 5 and 10 $mg.ml^{-1}$ of eugenol to eliminate 80 and 90% of the population, respectively (Sutili et al., 2014).

With respect to thymol, LC_{50} at 2, 6 and 24 h was 1.97, 0.96 and 0.92 $mg.ml^{-1}$, respectively (Table 1). A higher LC_{50} (2.49 $mg.ml^{-1}$ after 2-h exposure) against third-stage (L3) *H. contortus* larvae was found for thymol in a study on *Lippia sidoides* essential oil, which contains 59.65% of this compound (Camurça-Vasconcelos et al., 2007). A morphological trait of adult nematodes is a three-layered cuticle (Fetterer and Rhoads, 1993), but in the L3 stage, their cuticle has only 2 layers, separated by a glycoprotein-rich fluid (Bird, 1990). Thus, third-stage *H. contortus* larvae may resist the activity of essential oils and drugs, explaining the higher thymol doses required to achieve LC_{50}. On the other hand, the body walls of acanthocephalans contain pores and channels, and although these structures are used for nutrient absorption (Lee, 1966), they make parasites more susceptible to the action of essential oils and isolated compounds. André et al. (2017) report that 0.8 $mg.ml^{-1}$ thymol inhibits the motility of *H. contortus* larvae (70% in 6 h and 100% in 24 h). They also found that thymol damages the cuticle of adult nematodes.

With regard to tannic acid, its LC_{50} against *N. buttnerae* at 2, 6 and 24h was 4.68, 1.79 and 1.30 $mg.ml^{-1}$, respectively (Table 1). Plant-derived condensed tannins have been shown to be a promising option for treating pigs infected with gastrointestinal helminth parasites such as *Oesophagostomum dentatum* (Williams et al., 2014). Rich in condensed tannins, the fiber of green coconut shell was shown to have 40.56 mg ml^{-1} half maximal effective concentration (EC50) against the development of *H. Contortus* larvae and to cause 99.77% inhibition in a 6-day incubation period (Oliveira et al., 2009). By contrast, the efficacy of tannins in treating acanthocephalans in the present study was significantly higher, likely because the parasites lack the cuticles that increase resistance to the treatment.

Most studies on the anthelmintic activity of tannin-rich plants are carried out with species containing tannins in the condensed form. However, as observed here, hydrolyzed tannins such as tannic acid exhibit high anthelmintic activity. A similar result was reported by Katiki et al. (2013), who studied the leaf extracts of plants from the Appalachian Mountains, which are rich in condensed and hydrolyzed tannins. In tests against *Caenorhabditis elegans*, they found an LC_{50} of 1.03 $mg.ml^{-1}$ in 24 h using *Acer rubrum* extract, which is rich in hydrolyzed tannins of the same class as tannic acid.

CONCLUSION

The findings of the present study indicate that eugenol, thymol and tannic acid are promising compounds in the control of acanthocephalans isolated from tambaqui. The results encourage future *in vivo* tests as well as determination of the toxicity and palatability of feed containing these compounds.

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ETHICAL APPROVAL

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors. The experiment was previously approved by the Ethics Committee for Animal Experimentation (CEUA) of Embrapa Amazônia Ocidental, protocol 02/2017, and authorized by the Council for Genetic Heritage Management (CGen), protocol AE2F272.

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