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In Vitro Determination of Ovicidal and Larvicidal Activity of Curcumin on *Toxocara canis* Eggs

Selma Kocademir[1,2]✉, Kader Yıldız[3]

[1]*Kırıkkale University, Institution of Health and Science, Kırıkkale, Türkiye.*
[2]*Ministry of Agriculture and Forestry, Veterinary Control Central Research Institute, Laboratory of Parasitology, Ankara, Türkiye*
[3]*Kırıkkale University, Faculty of Veterinary Medicine, Department of Parasitology, Kırıkkale, Türkiye.*

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✉**Corresponding Author:** selmavetkocademir@hotmail.com

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ABSTRACT

Curcuma longa rhizome is the source of turmeric. Curcumin exhibits some encouraging antiparasitic properties in helminths. The purpose of this investigation was to determine the ovicidal and larvicidal activities of curcumin in *Toxocara canis* eggs in vitro. Curcumin dilutions (36.8 mg/ml, 18.4 mg/ml and 3.6 mg/ml) were prepared by adding RPMI-1640. The eggs and hatched infective-stage larvae were incubated with curcumin dilutions for 6, 12 or 24 hours. The ovicidal activity was evaluated after 28 days. Larvicidal activity was assessed after completing each incubation time. In the present study, no changes in the eggshell structure were observed in all curcumin groups. The lowest embryogenesis rate (75%) was observed only at the highest curcumin dilution (36.8 mg/ml) at the 12- and 24-hour incubations, but the difference was not found statistically significant. No significant larvicidal effect of curcumin was detected. The percentage of moving larvae was 80% at 12 hours and 76% at 24 hours in the highest curcumin dilution (36.8 mg/ml). *T. canis* larvae survived in RPMI-1640 for four days after being incubated with 36.8 mg/ml curcumin for 24 hours. However, the untreated larvae were still active at this time. Further studies focusing on the migration of *T. canis* infective larvae in animal models may shed light on the effect of curcumin, which is rapidly metabolized in the body and absorbed at low levels from the intestine, on the migrating larva.

INTRODUCTION

Turmeric is obtained from the rhizome of *Curcuma longa*, Zingiberaceae family. It has been used in complementary and alternative medicine for a long time (Bigford and Del Rossi, 2014). Curcumin (60-70%), dethoxycurcumin (20-27%), and bisdemethoxycurcumin (10-15%) are the three main curcuminoid chemicals found in turmeric (Nelson et al., 2017). Curcumin is known antioxidant, anti-inflammatory, antibacterial, immune system regulatory, anticarcinogenic, antidiabetic, neuroprotective, and protective agent for cardiovascular system and liver (Wang et al., 2018; Cao et al., 2018).

Curcumin has promising antiparasitic effects on helminths, including *Ascaridia galli* (Bazh and Bahy, 2013), *Trichinella spiralis* (Hamed et al., 2022), *Fasciola gigantica* (Ullah et al., 2017), *Schistosoma mansoni* and *Schistosoma haematobium* (Abou El Dehab et al., 2019), *Raillietina cesticillus* (El-Bahy and Bazh, 2015), *Taenia crassiceps* cysticercus (Martínez-González et al., 2022). Curcumin acts in different ways such as reducing the detoxification ability, increasing the amount of reactive oxygen species (Rehman et al., 2020), and altering the tegument of helminths (Ullah et al., 2017). Due to the tegumental changes, Na+ –K+ transport is impaired in the parasite, possibly resulting in a significant decrease in motility (Abou El Dehab et al., 2019; Faixová et al., 2021).

Toxocara canis, nematode, lives in the small intestine of dogs and other canids (Oge, 2018). There is no intermediate host in the biology of the parasite; however, some animal species and humans play a role as the paratenic host (Kocademir and Yildiz, 2022). *Toxocara canis* eggs are spherical-oval, 74-80 µm in diameter, brown in color and covered with thick shell (Oge, 2018). Embryogenesis begins in the eggs at air temperature above 15°C, and infective larvae (L3) develop about 3-4 weeks in nature (Abou-El-Naga, 2018). The eggs, including L3, cause infection in dogs and paratenic hosts. The dog is also infected by eating the L3-containing tissues of the paratenic hosts (Oge, 2018). The infective larvae are released in the small intestine and migrate to different tissues, including the liver, heart and lungs in the dog (Kocademir and Yildiz, 2022). The migration route of the larvae varies according to the age and gender of the dogs (Oge, 2018).

Infective larvae maintain their viability in the tissues of their paratenic hosts, and they are also transmitted between paratenic hosts. Infective larvae do not become adults in the paratenic host, but they cause a pathology called visceral larva migrans (VLM) during migration in the paratenic host tissues (Chen et al., 2018). Drugs have limited efficacy in treating VLM in humans. Albendazole is used as the first option for the treatment of VLM. However, the treatment regime has not yet been standardized (Hombu et al., 2019). In addition to drug treatment, the effectiveness of some plant extracts on VLM is being investigated. Several plant extracts have been shown to have ovicidal and larvicidal effects on *T. canis* in both in vitro and in vivo experiments (Mata-Santos et al., 2015; Orengo et al., 2016; El-Sayed, 2017).

This study aimed to determine the effect of curcumin on ovicidal activity in *T. canis* eggs in vitro. In addition, it was also aimed to determine larvicidal activity on *T. canis* infective stage larvae which are responsible for infection in both dogs and paratenic hosts including humans.

MATERIALS AND METHODS

Toxocara canis eggs

Adult *T. canis* were obtained from veterinary clinics in Ankara, Türkiye. The parasites were brought by owners after being excreted in the faeces of dogs naturally infected with toxocariasis. The samples were brought to the Parasitology Laboratory of the Central Veterinary Control Institute in Ankara. The parasites were carefully washed in distilled water and diagnosed as *T. canis* under a stereo microscope based on their morphological features. Female *T. canis* were washed three times with distilled water in a Petri dish. The uterine part was dissected with a sterile scalpel, and the eggs were collected in distilled water. After sieving the egg suspension through a sieve (200 µm pore), it was centrifuged three times using sterile distilled water (3 mins, 500 x g). Then the number of eggs were counted and adjusted to 1000 eggs per ml with sterile distilled water.

Preparation of the curcumin dilutions

Curcumin powder (Sigma C1386) was stored at -18°C and protected from light until used in the experiments. The curcumin dilutions were prepared by adding RPMI-1640 (Sigma) (36.8 mg/ml, 18.4 mg/ml and 3.6 mg/ml). They were prepared just before the experiments and protected from light until use.

Experimental design

A total 5 groups were consisted (3 different curcumin dilutions, the positive and negative controls) for each incubation time of this study. These groups were created separately for three different incubation times (6, 12 and 24 hours).

Determination of the ovicidal activity

In this assay, the experimental groups were consisted with *T. canis* eggs obtained from female parasites. The egg suspension (100 µl) was added to the microcentrifuge tubes. Three curcumin dilutions (36.8 mg/ml, 18.4 mg/ml and 3.6 mg/ml) were added (100 µl) separately to the tubes and gently dispersed by pipetting. The positive control was prepared from a commercial drug containing pyrantel pamoate at 725 µg/ml in RPMI-1640 and it was added (100 µl) to the eggs and gently dispersed. The untreated eggs were used as the negative control in the experiments. The tubes protected from light were incubated for 6, 12, and 24 hours in the incubator (28°C). After completing each incubation step, the supernatant was removed from the tubes and they were centrifuged with sterile distilled water three times (3 mins, 500 x g). Final centrifugation was performed with formalin solution (0.5%). The eggs were placed into the wells of sterile polystyrene microplates with a lid. The plates were put in the incubator (28°C) and mixed daily. Larvae development was evaluated using a light microscope (Leica ICC50) for 28 days.

Determination of the larvicidal activity

In this assay, the experimental groups were consisted with *T. canis* larvae after hatchled from the eggs after complated 28-day incubation. To obtain hatched larvae, *T. canis* eggs were incubated for 28 days (28°C). The eggs were washed with sterile distilled water at the end of the incubation period. To remove the protein cover on the eggshell, the hypochlorite solution was added to the eggs. Then the eggs were centrifuged twice with sterile distilled water, the final centrifugation was performed with RPMI-1640 (3 minutes at 500 xg). The hatched larvae number was counted and adjusted as 1000 larvae per ml in the larvae suspension.

To determine the larvicidal effect, the larvae suspension (100 ml) was placed in the microcentrifuge tubes. 100 µl of each curcumin dilutions (36.8 mg/ml, 18.4 mg/ml and 3.6 mg/ml) were added to the tubes and mixed gently. The larvae in RPMI-1640 and pyrantel pamoate served as the negative and the positive controls, respectively. The tubes protected from light were incubated at 37°C for 6, 12, and 24 hours. After completing each incubation period, the larvae were washed three times with RPMI-1640. The trypan blue dye test was used to determine the larvae viability (Sena Lopes et al., 2020). Larvae that did not move were considered dead (Reis et al., 2010).

To assess the impact of curcumin on the larvae's life span, the larvae were washed with RPMI-1640 after being incubated with the highest curcumin dilutions (36.8 mg/ml) for 24 hours. The larvae left untreated served as the negative control. They were incubated with daily replacement of RPMI-1640 (28°C). Larval viability was examined daily by a light microscope.

Statistical analysis

Data were analyzed using the Chi-square test. $P<0.05$ were considered as statistically significant.

RESULTS

The ovicidal activity of curcumin

At the 6-hour incubation, the ovicidal activity was not detected in all curcumin dilutions in this study ($P>0.05$).

The lowest embryogenesis rate (75%) was observed only in the highest curcumin dilution (36.8 mg/ml) at the 12 and 24 hours-incubations (Table 1). Only one or two damaged blastomers were observed in the undeveloped eggs of curcumin groups. The embryogenesis rate was lower in the curcumin dilutions than in the positive controls at the 12 and 24 hours-incubations, but the difference was not found statistically significant ($P>0.05$). Concerning the larval development, there was no difference in other curcumin dilutions at these incubation steps (12 and 24 hours). No changes in shell structure were observed in the eggs in all groups. Depending on the curcumin concentration and the length of incubation, the eggshell's color ranged from gray to yellow (Figure 1). The eggshell's color was not changed in the positive control (Figure 2). It was observed that the larvae were moved inside the eggs in all groups.

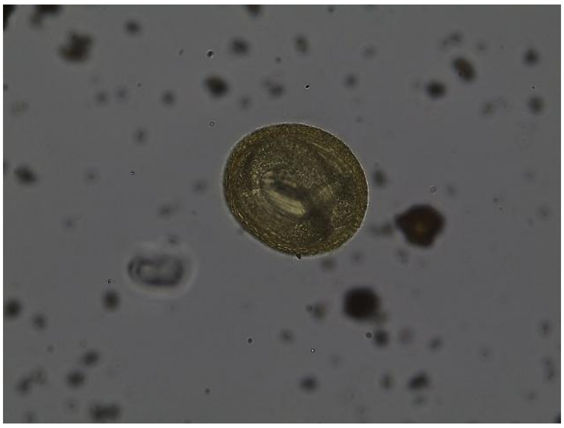

Figure 1. The larvae developed in *Toxocara canis* eggs after being incubated with the curcumin dilution (36.8 mg/ml) for 6 hours (x 40)

Figure 2. The larvae developed in *Toxocara canis* eggs after being incubated with pyrantel pamoate (725 µg/ml) for 6 hours (x40)

The larvicidal activity of curcumin

At the six-hour incubation, the motile larvae rate was similar (89-92%) in all curcumin dilutions, while motile larvae were not observed in the positive control at this time (Table 1). The motile larvae rates were detected as 80% and 76% in the highest curcumin dilution (36.8 mg/ml) at 12 and 24 hours incubations, respectively (Figure 3). Dead larvae were observed immobile and straight in shape in the curcumin groups (Figure 4). All larvae were observed as motionless and flat in the positive control at 12 and 24 hours incubations, and stained with trypan blue dye solution.

After incubating with 36.8 mg/ml curcumin for 24 hours, the larvae can live only for four days in RPMI-1640. But the untreated larvae were more motile at this incubation step.

Figure 3. Dead *Toxocara canis* larva after being incubated with the curcumin dilution (36.8 mg/ml) for 24 hours (x10)

Figure 4. Live *Toxocara canis* larva after being incubated with the curcumin dilution (36.8 mg/ml) for 12 hours (x40)

Table 1. Larval development rate in *Toxocara canis* eggs and the viability rate of the larvae incubated with different doses of curcumin for different times

Incubation time (hours)	Curcumin dilutions (mg/kg)	The larvae development rate in the eggs (%)	The viability rate of the larvae (%)
	36.8	90[a]	89[x]
	18.4	85[a]	92[x]
6	3.6	93[a]	90[x]
	PC	92	0
	NC	95	90
	36.8	75[b]	80[y]
	18.4	91[b]	93[y]
12	3.6	88[b]	90[y]
	PC	93	0
	NC	98	85
	36.8	75[c]	76[z]
	18.4	91[c]	84[z]
24	3.6	89[c]	93[z]
	PC	97	0
	NC	92	84

PC: Positive control
NC: Negative control
[a,b,c,x,y,z] The difference was not found statistically significant (P>0.05)

DISCUSSION AND CONCLUSION

Curcumin reduces egg production by the female parasite (Magalhaes et al., 2009). It alters the activity in many genes in the parasite, including significant signaling pathways that affect embryogenesis and oogenesis (Morais et al., 2013). Curcumin disrupts the shell structure of Schistosoma spp. eggs and adversely affects the larvae inside (Abou El Dehab et al., 2019). In this study, disruption of the eggshell was not detected depending on curcumin incubation. *T. canis* egg possesses a strong eggshell consisting of five layers (Bouched et al., 1986). The shell protects the eggs from the detrimental effects of disinfectants and chemicals besides strong environmental conditions (Aycicek et al., 2001). Curcumin dilutions in the current investigation had some negative effects on the development of *T. canis* eggs. The highest ovicidal activity (75%) was observed in the eggs at 12-hour incubation with 36.8 mg/ml curcumin dilutions. A similar result was observed in the 24-hour incubation. The larva development rate was lower in all curcumin dilutions than in the positive controls at the 12- and 24 hours-incubations.

Flavonoids, including curcumin, are low molecular-weight polyphenols and they have therapeutic potential for some diseases. The potential anthelmintic effects of curcumin are reported on mainly trematodes and cestodes (El-Bahy and Bazh, 2015; Ullah et al., 2017; Abou El Dehab et al., 2019; Martínez-González et al., 2022). Few studies have examined the effectiveness of curcumin on nematodes (Bazh and Bahy, 2013; Hamed et al., 2022). The efficacy of curcumin is dependent on in vitro concentration and incubation time, with the highest effect on adult *A. galli* reported after 48 hours of incubation at a dilution of 100 mg/ml curcumin (Bazh and Bahy, 2013). Caroccia et al. (2013) reported that *T. canis* larvae mobility decreased after incubation with curcumin dilutions (0.01, 0.05 and 0.1 mg/ml) for 48 and 72 hours. In the present study, the larvicidal activity of the curcumin dilutions (36.8 mg/ml, 18.4 mg/ml and 3.6 mg/ml) was limited to the infective stage *T. canis* larvae. No significant difference was found between the viability rates of infective stage *T. canis* larvae at all incubations with all curcumin dilutions, and the viable larvae rate was still higher (75%) even after 24 hours of incubation at the highest curcumin dilution (36.8 mg/ml).

Curcumin can regulate certain parasites' ion channels, receptors, and enzyme structures and functions, thus leading to deterioration in the physiology and death of parasites in vitro. In addition, it penetrates the tegument of platyhelminths, affects glycogen stores, and leading disruption of energy metabolism. It is also reported to interfere with enzymatic systems involved in muscle coordination (Faixova et al., 2021). After being incubated with curcumin at 60 µM concentration in vitro, adult *F. gigantica* were alive: however, a significant decrease in motility, tegumental distortions in the anterior and posterior regions, and erosion of the tegumentary spines of the parasite have been reported (Ullah et al., 2017). At doses of 50 and 100 µM, curcumin kills *S. mansoni*, and some morphological abnormalities have been observed on the parasite's surface (Magalhaes et al., 2019). Curcumin decreased the movement of *R. cestillus* depending on the concentration (25, 50 and 100 mg/ml) (El-Bahy and Bazh, 2015). In the present study, the viability rate was observed as 74% in the infective stage *T. canis* larvae after being treated with 36.8 mg/ml curcumin for 24 hours: however, the shortened life span was detected in the treated larvae compared to the untreated group. Faixová et al. (2021) reported that decreasing parasite motility triggered possibly impaired Na+ –K+ transport due to the tegumental changes caused by curcumin. Mostly *R. cesticillus* (65-80%) disappeared after 48 hours of exposure to curcumin at concentrations of 25 or 100 mg/ml in vitro. The detrimental effect of curcumin is primarily seen in the tegument of *R. cesticillus*, to cause death by affecting the metabolism of glucose absorption/penetration in the parasite (El-Bahy and Bahzy, 2013).

Tegumental damage affects excretory/secretory processes, alters signalling pathways, and affects metabolic pathways in the parasite (Ullah et al., 2017; Abou El Dehab et al., 2019; Rehman et al., 2020; Faixová et al., 2021). *Toxocara canis* infective stage larvae require minimal support to survive in vitro (Bowman, 2020). Cell culture mediums like RPMI-1640 contain some chemicals which important to surviving *T. canis* larvae in vitro. *Toxocara canis* larvae can survive in the cell culture media for a long time (Bowman, 2020). In the current investigation, infective stage *T. canis* larvae only survived in RPMI-1640 for 4 days after being exposed to 36.8 mg/ml curcumin dilution for 24 hours. The untreated larvae were still motile at this point. It was assumed that curcumin could interfere with the energy metabolism of *T. canis* L3, which would cause the larvae to lose all of their glycogen reserves and eventually die.

Curcumin has potential anthelmintic properties against *A. galli* in experimentally infected chickens (Bazh et al., 2013). The antiparasitic activity of curcumin (1000 mg) is relatively low in chickens infected with *R. cesticillus* contrary to in vitro experiments (El-Bahy and Bazh, 2015). Curcumin reduces the parasite load in mice experimentally infected with *T. spiralis*, moreover, the anti-inflammatory, antioxidant, and anti-angiogenic properties of curcumin help to reduce the trichinellosis-related pathology (Hamed et al., 2022). Triggered apoptotic-like activities and increased oxidative stress caused by curcumin, reduce the parasite's ability to survive in the host (De Paula Agular et al., 2016; Rehman et al., 2020; Faixová et al., 2021). In the present study, curcumin shortened the life span of the *T. canis* infective stage larvae in vitro. Shortening the life span of the larvae could affect the migration of the infected larvae of *T. canis* in the host tissues. Most of the infective stage *T. canis* larvae reach the host liver within 24 hours of post-infection and then they migrate to the lungs (Oge, 2018). The shortening life span of infective stage *T. canis* larva depending on curcumin needs to be tested in animal models experimentally infected.

The bioavailability of curcumin is relatively low because of inadequate intestinal absorption and quick metabolism in the liver (Shehzad et al., 2017). Curcumin is mostly converted to conjugated curcumin form in the digestive system after oral ingestion, but it is reduced to dihydrocurcumin, tetrahydrocurcumin and hexahydrocurcumin when administered intraperitoneally or intravenously (Prasad et al., 2014). Data on curcumin bioavailability is very limited in dogs. Liposomal curcumin is metabolised to tetrahydrocurcumin after being administered intravenously in Beagle dogs (total dose of 10 mg/kg for 2 hours) and the plasma half-life is 0.4-0.7 hours (Helson et al., 2012). After feeding with curcumin-rich food, red blood cells, neutrophils and lymphocytes numbers have been increased in Beagle dogs naturally infected with some pathogens, which is reported as the result of the anti-inflammatory effect of curcumin (Campigotto et al., 2020). According to the Author's opinion, a curcumin-rich diet may have negative effects on both the adult *T. canis* residing in the intestine and the infective stage *T. canis* larvae in dogs.

In the present study, the low ovicidal activity was detected in the highest curcumin dilution at 12 hours-incubations. The effect of curcumin on the larval viability was not found statistically significant at the doses and incubation stages determined in this study. The infective stage *T. canis* larvae can only survive for four days after 24 hours-incubation with 36.8 mg/ml curcumin. The effect of curcumin, which is absorbed at low levels from the intestine and metabolized rapidly in the body, on the migration of *T. canis* infective stage larvae in animal models can be revealed by future studies.

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Conflict of Interest

The authors declare that they have no competing interests.

Authorship contributions

Concept: S.K., K.Y., Design: S.K., K.Y., Data Collection or Processing: S.K., K.Y., Analysis or Interpretation: S.K., K.Y., Literature Search: S.K., K.Y., Writing: S.K., K.Y.

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