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In vitro and *in vivo* study of the antiparasitic effect of thymol on poultry drinking water

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ABSTRACT. The current study evaluates the antiparasitic effect of thymol on reducing parasite burden, especially *Cryptosporidium* load, in poultry drinking water and in improving zootechnical performances of chicks. The first experiment assessed in vitro the anti-*cryptosporidium* activity of NP (thymol-based product) on drinking water samples using microscopic counting. Samples were treated by increasing concentrations of thymol (1, 2 and 4 g L^{-1} of NP). In vivo, chicken were randomly assigned to three groups (control and chicks consuming treated water with two concentration of thymol (1 and 2 g L^{-1} of NP). Water treatment efficiency was evaluated on the intestinal parasitic load and zootechnical performances of animals (Body weight, body weight gain, food intake and the consumption index). In vitro the anti-cryptosporidium effect was dose dependent ($p < 0.05$; $p < 0.01$; $p < 0.001$). The in vivo test showed that the intestinal parasitic load was significantly lower ($p < 0.05$; $p < 0.01$; $p < 0.001$) in the group treated with 2 g L^{-1} of NP. Additionally, results showed a significant increase ($p < 0.05$; $p < 0.1$; $p < 0.001$) in the body weight and the body weight gain of treated groups during the whole rearing period compared to the control. Furthermore, treated groups represent a lower consumption index compared to the control.

Keywords: chlorination; *Cryptosporidium*; essential oils; intestinal flora; performance parameters.

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Introduction

Microbiological drinking water quality deterioration is a causal factor of disorders in farmed animals. Generally, poultry farms are located in rural areas without supply of clean water. It's loaded with microbiological pollutants such as bacteria, fungi, virus and parasite.

Waterborne diseases may involve bacteria or viruses but the public health spotlight is currently on protozoans, such as *Cryptosporidium* and *Giardia* (Angelici & Karanis, 2019).

For this reason, some active substances such as biocides, acids and agents neutralizers are commonly used to improve the quality of water. The most common disinfection treatment is chlorination using chlorine bleach. However, it has several disadvantages such as chlorine resistance especially for protozoa (Angelici & Karanis, 2019). In addition, disinfection byproducts can be harmful to human health (Li & Mitch, 2018).

The antiparasitic activity of essential oils and their components has long been recognized, and has continued to receive research interest in recent years (Tasdemir, Kaiser, Demirci, Demirci, & Baser, 2019; Azadbakht, Saeedi Akbarabadi, Motazedian, Monadi, & Akbari, 2020).

Our laboratory, which has extensively worked on essential oils and their major compounds, demonstrated the antiparasitic activity of these components (Remmal, Achahbar, Bouddine, Chami, & Chami, 2011; Tanghort et al., 2019). These studies suggested a potential use of these substances as alternatives to antiparasitic treatments used for treating poultry drinking water.

The aim of this present study was therefore to comprehensively evaluate the anti-*cryptosporidium* activity of thymol in vitro on drinking water samples and in vivo on intestinal *Cryptosporidium* load of chicks, as well as the improvement of their zootechnical parameters.

Material and methods

In vitro test

The anti-*cryptosporidium* activity of thymol was tested *in vitro* in different water samples used as drinking water in poultry farms. Samples were obtained from Tank (T) and water tower (WT) coming from a river and

four different groundwater points (W1, W2, W3, and W4). They were collected in sterile bottles, stored in a cooler at 4°C and transported directly to the laboratory. They were analyzed within 24 hours of arrival.

The antiparasitic agent used was thymol. It's the active principle of NP (15% of thymol), produced by the Industrial Laboratory of Veterinary Alternatives (LIAV, LLC) in Morocco. Thymol was obtained from *Origanum compactum*. In addition to thymol, other excipients have been added to provide stability and solubility. Different concentrations of the thymol (1, 2 and 4 g L^{-1} of NP) were added to each water sample. A negative control was also prepared.

The anti-*cryptosporidium* test was carried out using microscopy counting. 10 µL of each sample was transfered to a Malassez chamber. Number of parasites was counted in 10 fields of view using standard techniques (Ryley, Meade, Hazelhurst, & Robinson, 1976), and the mean number of parasites per milliliter of sample was calculated.

In vivo test

The main goal of the in vivo test was to assess the efficiency of the consumption of the treated water with thymol on the parasitic intestinal load of chicken and their performances. One-day-old chicks in a weight range of 37 g were used in this study. The present work was approved by the Committee on Ethics and guidelines for the use of animals, University of Sidi Mohamed ben Abdelah. The photoperiod was adjusted on a daily basis to 12 hours of light and 12 hours of darkness. A fan was used to ensure the aeration. At the beginning of the experiment, the ambient temperature was 32°C. It was reduced by 2 to 3°C each week to reach 23°C at the end of the experiment. Water and feed were provided for ad libitum consumption. They were fed with maize-based food, free of antibiotics and antiparasitics. For sanitation, drinkers were cleaned daily.

The drinking water was obtained from the water supply system on a poultry farm (sample T). Before any treatment, the parasitic load of the water (sample T) was 1.7 10^4Cells mL^{-1}. After treatment with thymol, it was reduced to 1.8 10^3 Cells mL^{-1} with 1 g L^{-1} and 10^2 Cells mL^{-1} with 2 g L^{-1} of NP.

Animals were divided by random selection into groups of twenty and housed separately:

Group 1 (control group): Animals that consumed untreated drinking water.

Group 2: Animals that consumed treated drinking water with thymol (1 g L^{-1} of NP).

Group 3: Animals that consumed treated drinking water with thymol (2 g L^{-1} of NP).

The effect of the consumption of untreated water compared with treated water with thymol on the assessment of the parasitic intestinal load of animals was tested during the whole rearing period. One day a week (Day 1, Day 7, Day 14 and Day 21), 1 g of fresh fecal specimens from each group were collected and placed into sterile tubes containing 9 ml of physiological saline. From this stock solution, dilutions were performed. The intestinal load was calculated using microscopy counting. 10 µL of each sample was transferred to a Malassez chamber. Number of parasites was counted in 10 fields of view using standard techniques (Ryley et al., 1976), and the mean number of parasites per milliliter of sample was calculated.

In that same experiment, the thymol effect on drinking water was evaluated by body weight, body weight gain, food intake and consumption index of animals.

Statistical analyses

All values were expressed as means and standard error. SigmaStat 4.0 was used to analyze the data. The significance was verified for parasitic analysis of drinking water, intestinal load of animals and zootechnical parameters. p values less than 0.05 were considered as significant.

Results

In vitro test

The variation of the *Cryptosporidium* load of the different tested samples is shown in Table 1. Before any treatment, the parasitic tests showed an important burden for all tested samples. After treatment with thymol, a significant reduction was observed for samples W2, W3, W4 and T ($p < 0.05$; $p < 0.01$; $p < 0.001$) with the concentration 1 g L^{-1} of NP. For all samples, a significant reduction ($p < 0.05$; $p < 0.01$; $p < 0.001$) of the *Cryptosporidium* burden was obtained thanks to the increase of the treatment concentration.

Table 1. Variation of the *Cryptosporidium* load in different samples depending on the thymol concentration.

	Parasitic load (log_{10} Cells mL^{-1})					
	GP N° 1	GP N°2	GP N°3	GP N°4	Tank	Water tower
Control	5.72 ± 0.07	4.11 ± 0.00	5.18 ± 0.04	5.26 ± 0.01	4.12 ± 0.04	4.21 ± 0.1
1 g L^{-1} of NP	5.54 ± 0.08	4.04 ± 0.02*	4.97 ± 0.02**	4.9 ± 0.03***	3.25 ± 0.03***	4.04 ± 0.01
2 g L^{-1} of NP	2.98 ± 0.08**	2.81 ± 0.09***	2.71 ± 0.01**	2.64 ± 0***	2.05 ± 0.11***	2.9 ± 0.04*
4 g L^{-1} of NP	1.91 ± 0.05**	1.84 ± 0.06***	1.43 ± 0**	1.31 ± 0.01***	1.69 ± 0.14***	1.44 ± 0.01**

GP (Groundwater point); Values are means (n=6) ± SEM (Standard error of the mean); Comparison with control: * p <0.05; ** p <0.01; *** p <0.001.

In vivo test

The variation of the *Cryptosporidium* intestinal load for different groups of animals is shown in Table 2. At the beginning of the experiment (Day 1), the *Cryptosporidium* burden of all groups was 4.15 10^{12} Cells g^{-1}. Throughout the experience, the group 3 treated with thymol (2 g L^{-1} of NP) was distinguished by a higher reduction load compared to the group treated with 1 g L^{-1} and to the control.

Table 2. Evolution of the *Cryptosporidium* intestinal load.

	Cryptosporidium intestinal load (log 10 Cells g^{-1})		
	Group 1	Group 2	Group 3
Day 1	12.61 ± 0.06		
Day 7	13.2 ± 0.37	13.14 ± 0.01	11.21 ±0.11
Day 14	12.94 ± 0.16	12.86 ± 0.16	10.33 ± 0.03*
Day 21	13.09 ± 0.13	12.73 ± 0.05	10.44 ± 0.16**

Group 1 (untreated water); Group 2 (treated water with thymol (1 g L^{-1} of NP)); Group 3 (treated water with thymol (2 g L^{-1} of NP)). Values are means (n=6) ± SEM (Standard error of the mean); Comparison with control: * p <0.05; ** p <0.01.

Temporal evolution of body weights, body weights gain, food intake and consumption index for each group is shown in Table 3. During the whole rearing period, results show a significant increase (p <0.05; p <0.01) in the weight of the group 2 compared to the control. This increase is more significant (p <0.05; p <0.001) after treatment with the concentration 2 g L^{-1}. The body weight gain of animals in both treated groups was significantly (p <0.05; p <0.01; p <0.001) higher than the control. Concerning the consumption index, the control represents a higher consumption index compared to treated groups.

Table 3. The effect of water treatment on body weight, body weight gain, food intake and consumption Index.

		Group 1	Group 2	Group 3
Body weight (g)	Day 1	37.16 ± 1.64	36.58 ± 3.01	36.83 ± 3.03
	Day 7	70.83 ± 6.33	80.41 ± 6.85*	86.63 ± 11.23*
	Day 21	214 ± 6.8	246 ± 25.12**	317 ± 7.6 ***
Body weight gain (g)	Day 1- Day 7	33.6 ± 7.04	43.8 ± 5.04**	49.8 ± 13.50**
	Day 7- Day 21	143 ± 3.43	166 ± 26.40*	230 ± 7.30***
Total body weight gain (g)		406	643	688
Food intake (g)	Day 1- Day 7	75	61.6	69
	Day 7 - Day 21	270	245	270
Consumption index	Day 1- Day 7	2.23	1.41	1.39
	Day 7 - Day 21	1.89	1.48	1.17

Group 1 (untreated water); Group 2 (treated water with thymol (1 g L^{-1} of NP)); Group 3 (treated water with thymol (2 g L^{-1} of NP)). Values are means (n=20) ± SEM (Standard error of the mean); Comparison with control: * p <0.05; ** p <0.01; *** p <0.001.

Discussion

In vitro test

Protozoa are difficult to treat and control because of their biological characteristics as cysts/oocysts resistant stage, very thick wall and small size of cellular body .They can survive in cold water for several months (Omarova, Tussupova, Berndtsson, Kalishev, & Sharapatova, 2018). They are responsible for a number of diseases such as severe gastro enteritis, chronic damages and high morbidity (Fletcher, Stark, Harkness, & Ellis, 2012).

Parasitic analysis revealed the contamination of both groundwater and surface water samples by parasites with dominance of *Cryptosporidium*. The *Cryptosporidium* load varies between 10^4 and 10^5 Cells mL^{-1}. Samples were treated with increasing concentrations of thymol, which are 1, 2 and 4 g L^{-1} of NP. The treatment with thymol at 1 g L^{-1} significantly reduced this load, and with the concentration 2 g L^{-1} of NP, reduction was more

important. This inhibitory effect is explained by the fact that thymol is one of the most effective antiparasitic agent. Tanghort et al. (2019) tested the oocysticidic efficiency of thymol and found that it decreases the number of *Cryptosporidium baileyi* and *Cryptosporidium galli* oocysts in a significant proportion (88%) at a concentration 0.5 mg mL-1 after only 3 hours of treatment, while no oocysts were visible at the concentration of 1 mg mL^{-1}. After 24 hours of treatment, all oocysts of both *Cryptosporidium* species are destroyed at the concentration of 0.5 mg mL^{-1}.

In vivo test

The intestinal *Cryptosporidium* load was significantly lower in thymol-treated groups (group 2 and group 3). The effect is more important after treatment with 2 g L^{-1}. Most researchers have reported the efficiency of essential oils, against intestinal parasites. Studies conducted by Silva et al. (2009); Tsinas, Giannenas, Voidarou, Tzora, & Skoufos (2011) and Alp et al. (2012) investigated that the supplementation of broilers with oregano essential oils had a protective effect against avian coccidiosis. Oocyst shedding in excreta was reduced compared to control birds.

In the same experiment, even if animals of the control group consumed the same amount of food as treated groups, they showed worse zootechnical performances. This difference in growth between animals groups could be due to thymol which reduces the pathogen parasitic load and allows an intestinal microflora balance. This reduction affects intestinal integrity, therefore food may be more easily absorbed. A study conducted by Greathead and Kamel (2006) reported a positive effect of thymol and carvacrol supplementation to experimentally infected birds with sporulated oocysts of *E. acervuline*. It was concluded that this supplementation enhanced intestinal integrity by reducing expression of coccidiosis. To this end, thymol could represent a natural alternative to chemical biocides frequently used in poultry farming.

Conclusion

Results of these experiments prove that thymol exercises a significant anti-*cryptosporidium* action on poultry drinking water. This action affect chicks by reducing the load of pathogen intestinal parasite and has a significant positive effect on zootechnical performance of animals.

In addition to demonstrating a high antiparasitic activity, this work offers an alternative solution to chemical biocides which are commonly used in poultry farming.

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