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Research Article
Oocysticidal Effect of Essential Oils (EOs) and their Major Components on *Cryptosporidium baileyi* and *Cryptosporidium galli*

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Abstract

Background and objective: Cryptosporidiosis is a common gastrointestinal disorder in humans and animals caused by various *Cryptosporidium* species. At present, many antiparasitic drugs have been tested but only a few have been shown to be partially effective in treating cryptosporidiosis. Therefore, the aim of this study was to evaluate the ability of some essential oils and their major compounds (MCs) to destroy both species of *Cryptosporidium*: *C. baileyi* and *C. galli*. **Materials and methods:** A screening of the oocysticidal activity of five EOs and three MCs was carried using the direct contact method in a liquid medium. The release of substances absorbing at 273 nm was measured after treatment of *Cryptosporidium* oocysts with thymol and carvacrol. **Results:** Among the EOs tested those of thyme, oregano and clove as well as their MCs; thymol, carvacrol and eugenol were the most effective, with low LC50 (<0.4 mg mL^{-1}). The release of substances absorbing at 273 nm after treatment of *Cryptosporidium* oocysts with thymol and carvacrol show that the treatment of oocysts with these components led to their lysis in a dose and time-dependent manner. **Conclusion:** We were able to conclude that these EOs and their MCs are of particular interest in fighting cryptosporidiosis since they have a destructive effect on oocysts at very low concentrations. They could also help in the formulation of radical and safe solutions to cryptosporidiosis.

Key words: *C. baileyi*, *C. galli*, cryptosporidiosis, Essential oils, oocysticidal activity, oocysts

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cryptosporidiosis is a cosmopolitan zoonotic disease caused by the protozoa of the genus *Cryptosporidium*[1]. Protozoan biological cycles take place in the epithelial cells in the gastrointestinal and respiratory tracts, in the bursa of Fabricius and, less frequently, in other organs causing clinical and subclinical infections[2-4]. The economic losses associated with this disease result from poor flock performance (growth retardation and increased consumption index), morbidity and high mortality[5-7]. To date, three *Cryptosporidium* species have been classified in birds: *C. meleagridis*[8], *C. galli*[9] and *C. baileyi*[10]. The first two species *(C. meleagridis* and *C. galli*) affect the gastrointestinal tract and show in different degrees of enteritis[11,12], whereas *C. baileyi* infects many sites, including, nasopharynx, trachea, bronchi, air sac, bursa of Fabricius, kidneys and urinary tract and displays in three clinical forms: respiratory disease, enteritis and renal disease[13,14].

A major issue with cryptosporidiosis is the lack of an effective means of prevention and therapy of this disease. Because oocysts are highly resistant to environmental stress and to many disinfectants, hygienic measures are in general not sufficient to reliably eliminate oocysts from animal housing facilities and thus to avoid infection[15,16]. Until now, many chemotherapeutic agents have been evaluated against *Cryptosporidium* sp., none of them has demonstrated a clear efficacy for a curative use and when given in a prophylactic way, results were better although not totally satisfying[17,18]. The resistance of *Cryptosporidium* to drugs agents results from the fact that it has an intracellular, rather than an extra-cytoplasmic localization[19].

With growing awareness of the dangers of *Cryptosporidium*, continuous efforts are invested in order to develop new potent drugs and experimental tools. Interest has shifted towards plant extracts used in traditional medicine as sources for new treatments[20-23].

The present study was designed to test *in vitro* the ability of some essential oils (EOs) and their MCs to destroy oocysts of both *C. baileyi* and *C. galli* species isolated from infected chicken faeces.

MATERIALS AND METHODS

***Cryptosporidium* oocysts isolation and purification:** The *Cryptosporidium* oocysts used in this study were originally isolated from fresh faeces of broilers, naturally infested in Morocco. The oocysts were purified and sieved by flotation in a saturated solution as described by Tumova *et al.*[24]. Species identification was achieved by evaluation of morphometric characteristics of the oocysts (size), using the microscope and micrometer[25]. The average measurements are of 6.3×5.2 µm and 8.3×6.3 µm for *C. baileyi* and *C. galli* respectively.

Counting of *Cryptosporidium* oocysts: The number of oocysts was determined by transferring 20 µL of the sample suspension of *Cryptosporidium* oocysts to a Malassez chamber for microscopic examination and counting. *Cryptosporidium* oocysts were counted in 10 fields of view by using standard techniques[26] and the mean number of oocysts per milliliter of the sample was calculated. The identification of *Cryptosporidium* species was done based on the microscopic observation thereby allowing the identification of the *Cryptosporidium* species according to the method described by Ng *et al.*[25]. The percentage of each species in the mixed suspension was approximately 43.44% for *C. galli* and 56.56% for *C. baileyi.*

Effect of EOs and MCs on the number of *Cryptosporidium* oocysts: The EOs used in this study were: oregano oil (*Oreganum compactum*), thyme oil (*Thymus vulgaris*), Clove oil (*Eugenia caryophylata*), rosemary oil (*Rosmarinus officinalis*) and Artemisia oil (Artemisia absinthium). These EOs were purchased from Seema International (India). Carvacrol, thymol and eugenol used in this study were purchased from Sigma Aldrich (France).

All EOs and their MCs were dispersed in a liquid medium containing 0.2% agar in distilled water. This dispersion method was improved by Mzabi *et al.*[27]. Each EO or EO component was tested in increasing concentrations (0, 0.25, 0.33, 0.5, 1 and 2 mg mL^{-1}). The activity of each EO or MC was determined in triplicate in Eppendorf tubes by incubation at room temperature (25°C) on rotary shaker for 24 h of an inoculum of 25 µL containing 1.22×10^7 oocysts mL^{-1} which was put in direct contact with the EO or MC at various concentrations for a final volume of 200 µL[28,29]. The different curves were drawn, expressing the number of oocysts with respect to the concentration of EOs or MCs, after 3 and 24 h for each species of *Cryptosporidium*. The LC_{50} was then inferred from this curve by identifying the concentration in which the number of oocysts was equal to half of the initial number.

Effect of anticoccidial agents on the decrease of the number of *Cryptosporidium* oocysts: The action of anticoccidials such as monensin, salinomycin and robenidine was tested using the previously described method on EOs with the following concentrations 0, 0.25, 0.33, 0.5, 1 and 2 mg mL^{-1}. These

concentrations were prepared from a concentration of 100 mg mL^{-1} of each of the three anticoccidial agents. All anticoccidials were purchased from Sigma Aldrich (France).

Decrease of the oocyst number in parallel with the release of substances absorbing at 273 nm after treatment with increasing concentrations of thymol and carvacrol: The release of cellular material absorbing at 273 nm from oocysts cells treated with increasing concentrations of carvacrol and thymol was determined. This experiment was performed on aliquots incubated for 24 h at ambient temperature with one milliliter suspension containing:

- 100 µL of washed suspension of *Cryptosporidium* oocysts at $1.22x10^7$ oocysts mL^{-1}
- 700 µL of PBS Phosphate Buffer Saline (PBS) (containing 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.13 g L^{-1} Na_2HPO_4, $2H_2O$ and 0.2 g L^{-1} KH_2PO_4)
- 200 µL increasing concentrations of thymol or carvacrol (0, 0.3, 0.5, 1 and 2 mg mL^{-1})

After incubation, the samples were centrifuged at 320 g for 5 min at 4°C. 500 µL of the supernatant was used to measure the UV absorption by a Beckman spectrophotometer[28,29]. For each concentration, the oocyst number was counted as previously described by Ding *et al.*[26].

Statistical analysis: The results were expressed as mean values ±S.E.M (standard error of mean). Statistical analysis of the data was performed with one-way analysis of variance followed by Tukey's Multiple Comparison Test (one way ANOVA followed by Tukey's test) (Graph Pad Prism, version 5.03). Significant differences were indicated by values inferior to 0.05.

RESULTS

Effect of EOs concentrations on *Cryptosporidium* oocysts number: Figure 1 and 2 show that the number of *C. baileyi* (Fig. 1: a-b) and *C. galli* (Fig. 2a-b) oocysts decreases with the majority of EOs tested in a dose-dependent manner at a concentration ranging between 0 and 2 mg mL^{-1}, after the 3 and 24 h of treatment.

After the 3 h of exposure of *Crypposptoridium* oocysts to various concentrations of EOs, the number of oocysts of both species of *Cryptosporidium* noticeably decreased by very low concentrations (0.25-0.5 mg mL^{-1}) of thyme oil, oregano oil and clove oil. EOs of artemisia and rosemary require a high concentration (2 mg mL^{-1}) for a significant reduction of more than 50% in the number of oocysts.

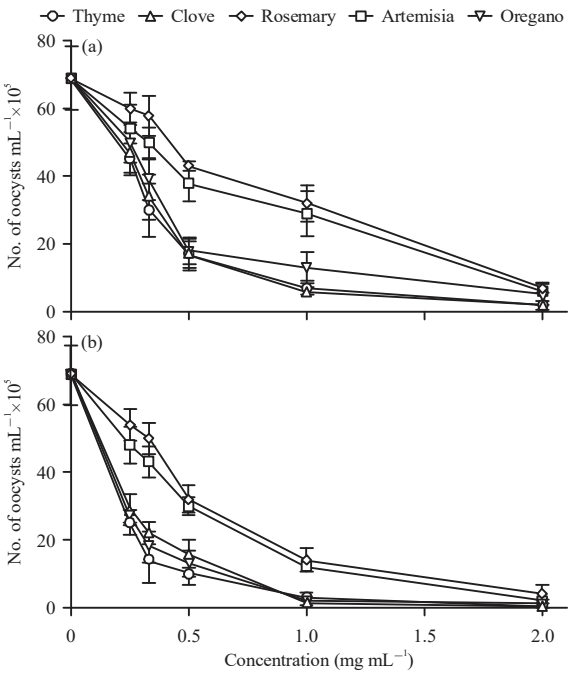

Fig. 1(a-b): The effect of different EOs on the viability of *Cryptosporidium baileyi* oocysts after (a) 3 h and (b) After 24 h of treatment

After treatment with clove oil, oregano oil and thyme oil, 1 mg mL^{-1} was sufficient to destroy all *C. baileyi* (Fig. 1b) and *C. galli* oocysts (Fig. 2b) for a period of 24 h. However, a concentration of 2 mg mL^{-1} was required to obtain an oocysticidal action by the EOs of artemisia and rosemary. When expressed in terms of lethal concentration 50% (LC_{50}), our results show that the most efficacious EOs are thyme (0.31 mg mL^{-1}) followed by clove (0.33 mg mL^{-1}) and oregano oils (0.37 mg mL^{-1}) according to their LC_{50} less than 0.4 mg mL^{-1}, the LC_{50} of artemisia and rosemary is high, measuring 0.69 mg mL^{-1} and 0.88 mg mL^{-1} respectively. It was inferred from the curves expressing the number of oocysts with respect to the concentration of EOs.

Effect of MCs concentrations on *Cryptosporidium* oocysts number: Figure 3 and 4 show that the number of *Cryptosporidium* oocysts decreases after the 3 and 24 h of treatment with the three MCs used in a dose-dependent manner at a concentration that ranges between 0 and 2 mg mL^{-1}.

After 3 h of treatment, the MCs were able to reduce the number of *C. baileyi* (Fig. 3a) and *C. galli* (Fig. 4a) oocysts in a

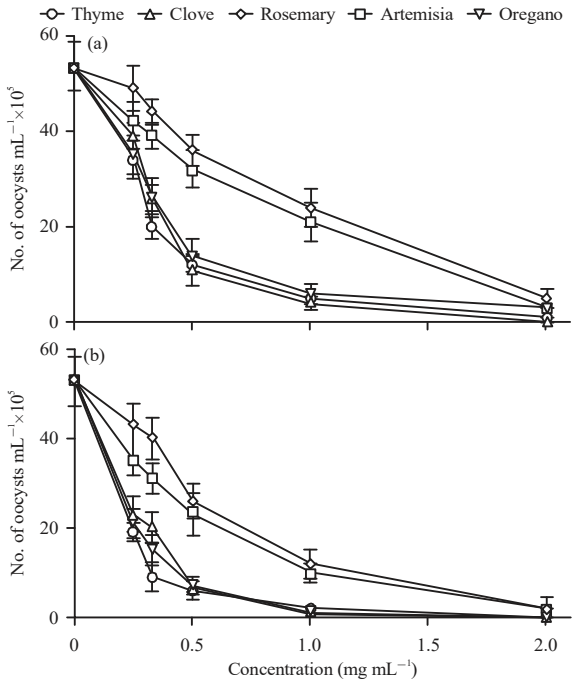

Fig. 2(a-b): The effect of different EOs on the viability of *Cryptosporidium galli* oocysts after (a) 3 h and (b) After 24 h of treatment

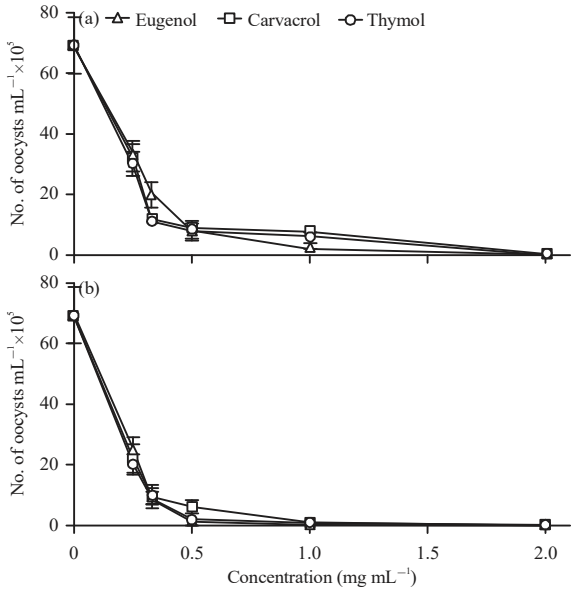

Fig. 3(a-b): The effect of different MCs on the viability of *Cryptosporidium baileyi* oocysts (a) After 3 h and (b) After 24 h of treatment

significant proportion (88%), at a concentration 0.5 mg mL^{-1}. Eugenol can reduce the number of oocysts up to 100% at a concentration of 1 mg mL^{-1}, while no oocysts were visible using carvacrol and thymol at concentrations of mg mL^{-1}.

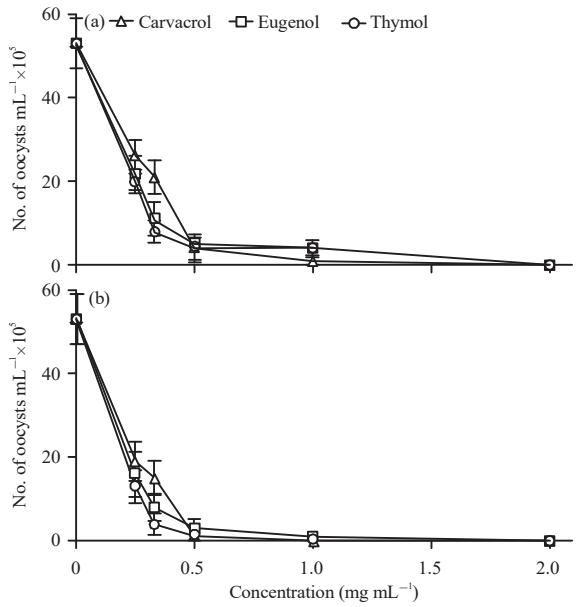

Fig. 4(a-b): The effect of different MCs on the viability of *Cryptosporidium galli* oocysts (a) After 3 h and (b) After 24 h of treatment

After 24 h of treatment (Fig. 3b and Fig. 4b), all oocysts of both *Cryptosporidium* species are destroyed by thymol and eugenol at the concentration of 0.5 mg mL^{-1}. Carvacrol provokes an oocysticidal effect at the high dose of 1 mg mL^{-1}. The expression in terms of lethal concentration 50% (LC_{50}) shows that the three MCs tested, have a practically similar LC_{50} in both *Cryptosporidium* species. Thymol and carvacrol have the same LC_{50} (0.22 mg mL^{-1}), followed by eugenol with an LC_{50} of 0.25 mg mL^{-1}.

Effect of anticoccidial drugs on *Cryptosporidium* oocysts number: Figure 5a and 6a show that the number of *C. baileyi* and *C. galli* oocysts decreased slightly after 3 h of treatment with increasing concentrations of the three antiparasitic drugs tested at the concentrations 0, 0.25, 0.33, 0.5, 1 and 2 mg mL^{-1}. No change in the viability of oocysts was observed even after 24 h of treatment (Fig. 5b and 6b).

Effect of carvacrol and thymol concentrations on the release of 273 nm absorbing material after 24 h of treatment from *Cryptosporidium* oocysts: Figure 7a and b show that after adding carvacrol (A), or thymol (B) in concentrations ranging from 0-2 mg mL^{-1}, the number of oocysts in a mixture of *C. baileyi* and *C. galli* noticeably decreases with very low concentrations (0.25-0,5 mg mL^{-1}). This treatment causes a

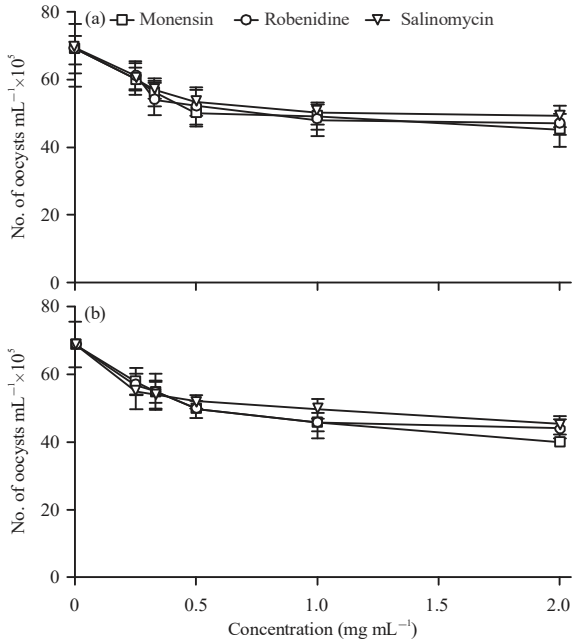

Fig. 5(a-b): The effect of different anticoccidials on the viability of *Cryptosporidium baileyi* oocysts (a) After 3 h and (b) After 24 h of treatment

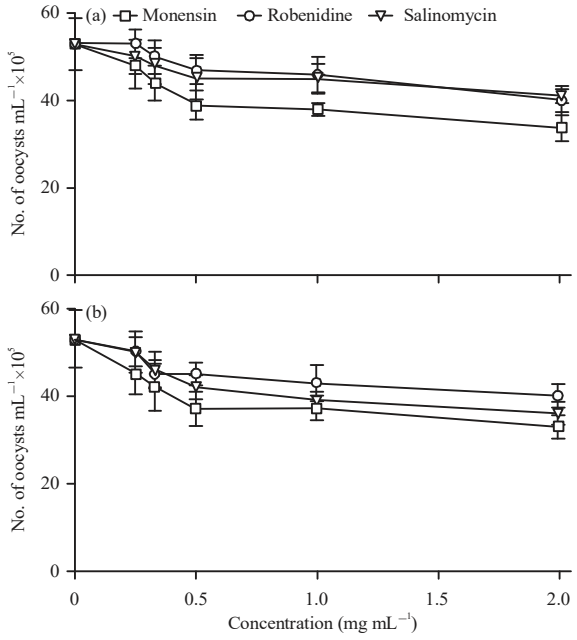

Fig. 6(a-b): The effect of different anticoccidials on the viability of *Cryptosporidium galli* oocysts (a) After 3 h and (b) After 24 h of treatment

considerable release of 273 nm absorbing material that increases in a linear manner according to the concentration of the MC.

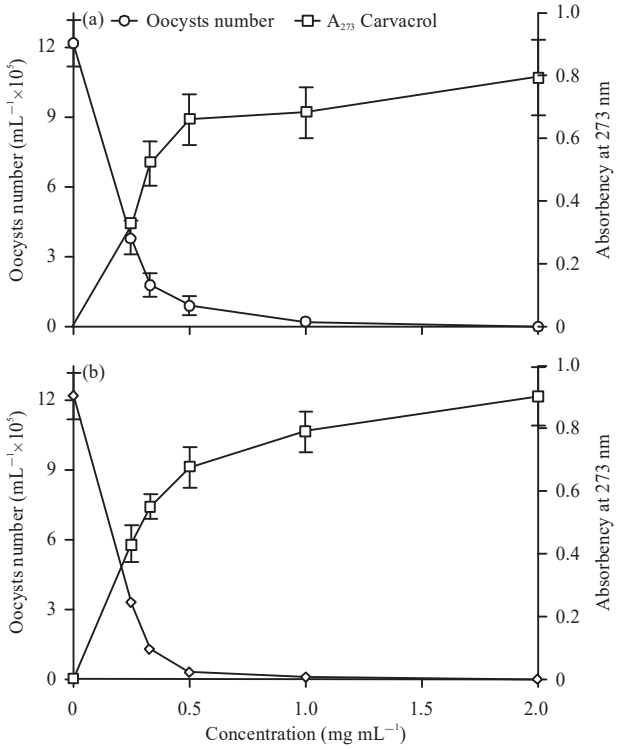

Fig. 7(a-b): The Effect of (a) Carvacrol and (b) thymol concentrations on *Cryptosporidium* oocysts number and the release of 273 nm absorbing material

DISCUSSION

The *Cryptosporidium* parasite is at the origin of the gastroenteritis syndrome (infectious diarrhea) that affects humans and animals[4,7]. The parasite's ability to infect animals in poultry farms and the high mortality that results from it has made of it a major pathogenic agent[30]. In the last decades, researchers have been investigating effective anti-parasitic drugs that could treat Cryptosporidiosis. Today, all solutions rely mainly on symptomatic treatments and preventive measures[18,23]. However, in the case of human medicine, one drug has been approved by the FDA: the Nitazoxanide[31].

Studies have tested the efficiency of natural substances such as curcuma, ginkgolic acids (*Gingko biloba*), onion, cinnamon on oocysts of *C. parvum* or *C. andersoni*, either *in vitro* on cell cultures[32], or *in vivo* on mice[33,34]. The last publication in this context is that of Gaur *et al.*[35], which showed that the EO of oregano and its MC carvacrol can inhibit up to 50% of the infectivity of *C. parvum* on adenocarcinoma ileo-caecal cells HTC-8 to the concentration of 30-60 µg mL^{-1}. However, in this experimental model, it is

not possible to obtain more inhibition owing to the toxicity of this EO and its MC on culture cells. In that regard, we decided to test *in vitro* the anti-parasitic effect of five EOs and their MCs directly on the oocysts of *C. baileyi* and *C. galli* responsible of Cryptosporidiosis in poultry. We, therefore, tested their effectiveness on a mix of *C. baileyi* and *C. galli* oocysts containing approximately 1.22×10^7 oocysts mL^{-1}. The results obtained show that the EOs of thyme, oregano and clove can cause a significant decrease in the number of oocysts of about 85% after 3 h and 98% after 24 h, to the concentration of 1 mg mL^{-1}. At this concentration, the EOs of rosemary and artemisia only cause a decrease of about 60% after 3 h and 80% after 24 h. The results obtained also show that the two species of *Cryptosporidium* used have the same sensitivity to the different EOs after 3 and 24 h.

Since the EOs of thyme, oregano and clove were more efficient and had a LC_{50} inferior to 0.4 mg mL^{-1}, we decided to test the oocysticidic efficiency of their MC which are thymol, carvacrol and eugenol. The results showed that thymol, carvacrol and eugenol have practically the same efficiency from the concentration of 0.5 mg mL^{-1}. Below 0.5 mg mL^{-1}, thymol and carvacrol proved to be more efficient than eugenol.

EOs and their MC have demonstrated an oocysticidal activity *in vitro* on *Eimeria* species as described by Remmal *et al*.[28,29]. These authors have noted the presence of deformed *Eimeria* oocysts with cracked walls and debris after treatment. These publications also identified the mechanism of action of EOs and their MC by measuring the release of the constituents of oocysts absorbing at 273 nm. For this reason, we also measured the liberation of substances absorbing at 273Nm (UV) present in the suspension of *Cryptosporidium* oocysts to gauge the destructive action of these MCs on oocysts. The results obtained show that at the concentration of 0.5 mg mL^{-1}, there is an important decrease in the number of oocysts along with an important increase of absorbing material at 273 nm (UV). This allows us to conclude that the MCs have an oocysticidal action.

Considering that we were working on protozoan oocysts and that Moroccan veterinarians use anticoccidials as a last recourse to fight cryptosporidiosis, or when in doubt in their diagnosis between cryptosporidiosis and coccidiosis. Their doubt can be due to the similarity of the clinical signs of the two diseases, or to the similar oocysts aspect of the two protozoans. Following this reasoning, we decided to test a few anticoccidial agents in order to verify if they have a destructive effect on *Cryptosporidium* oocysts. These agents are usually used to fight avian coccidiosis. They belong to the ionophores family such as salynomycin, monensin, or to the non-ionophores family such as robenidine. The results show that this oocysticide effect is very feeble. This weak sensitivity of *Cryptosporidium* to anticoccidials has already been described in many studies *in vitro* and *in vivo* on other *Cryptosporidium* species[19,36,37].

In order to ascertain the action of EOs and their MC on *Cryptosporidium* oocysts for animals, we have performed trials on 7-day-old broiler chicks infected on a daily basis with *Cryptosporidium* oocysts for 15 days. They were then kept under observation until they reached the age of 42 days. Significant improvements were observed in their zootechnical parameters: body weight, weight gain, feed gain ratio and mortality for the animals treated thymol or carvacrol compared to animals from the control batch. The injuries observed during autopsy were also significantly better (results not shown).

CONCLUSION

We can conclude from this *in vitro* study that the tested EOs and their MC have an oocysticidal activity with regards to *Cryptosporidium* spp for poultry. In parallel, a light decrease in the number of oocysts has been provoked by conventional anticoccidial drugs. To our knowledge, this is the first time that these EOs and their MCs to be tested on avian *Cryptosporidium* oocysts.

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