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The Anticoccidial Effects of the Poultry Bile against *Eimeria papillata* Oocysts of Mice: *In vitro* Study

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ABSTRACT

Background: Coccidiosis is a costly and wide-spread infectious disease in mammals that causes huge losses in the poultry industry worldwide. The present study was conducted to evaluate the potency of intact poultry gallbladder on the sporulation of *Eimeria papillata* oocysts of mice and to determine the best concentration for the activity of poultry bile on the inhibition of sporulation in oocysts.

Methods: In this test, we used potassium dichromate solution (2.5%) as control group. Unpopulated oocysts were exposed (1×10^3/3 ml) to four concentrations of poultry bile (w/v; 10%, 25%, 50% and 100%). It was incubated in petri dishes for 48, 72 and 96 hours at 25-29°C, with 60-80% humidity. The sporulation was examined under an inverted microscope at 40 × to confirm oocyst sporulation.

Result: Showed through infrared spectroscopy of poultry bile that there are many expected active classes of chemical compounds. At a 100% concentration of poultry bile, it was able to inhibit the *Eimeria papillata* oocysts by about 98% in potassium dichromate solution. Also, it observed its ability to inhibit sporulation of oocysts in a dose-dependent manner at concentrations of 50%, 25% and 10% of bile, the rate of inhibition was (71.7%, 33.11% and 19.88%), respectively. We found a direct relationship between inhibition and sporulation related to the increasing time period.

Key words: *Eimeria papillate*, Inhibition, Oocysts sporulation, Potassium dichromate, Poultry bile.

INTRODUCTION

Coccidiosis has spread worldwide, causing tremendous economic losses and is thought to be one of the most serious infectious diseases of birds (Johnson 1923, Chapman 2014, Suohu and Rajkhowa 2021). It is the result of unicellular protozoans of the Eimeria genus, which encompasses a variety of species (Blake and Tomley 2014; Shivaramaiah *et al*. 2014). Clinically, the disease causes digestive issues such as bloody diarrhea, as well as a low feed conversion rate, a slow growth rate and poor weight gain. This has also been identified to be a predisposing factor for infections like bacterial diseases (Collier *et al*. 2008, Bachaya *et al*. 2012, Orengo *et al*. 2012, Kaur *et al*. 2019). When a bird eats a sporulated oocyst from waste, it becomes infected. The development of anticoccidial drug resistance, its lingering effects on bird meat and the harmful effects of disinfectants have prompted the search for alternatives (Abbas *et al*. 2012, Colwell and Gilleard 2012, Blake and Tomley 2014, Shivaramaiah *et al*. 2014). For over 2500 years, researchers of Chinese material medicine have used forty-four different animal bile, including human gallbladder liquid, as pharmaceuticals in traditional Chinese medicine (TCM) to treat patients suffering from a wide range of illnesses (Wang and Carey 2014) because of their detergent properties and membranolytic activity (Zhai *et al*. 1996). Bile acids have microbicidal properties (Binder *et al*. 1975; Begley, *et al*. 2005), a characteristic considered fundamental for restricting the activity of bacteria in the proximal small intestine (Begley *et al*. 2005; de Aguiar Vallim *et al*. 2013). They have demonstrated efficacy in antimicrobial activity and anti-coccidiosis chemotherapeutic effects and are being marketed following a chain of empirical trials for their investigation and are an economically less costly method for the elimination of coccidiosis (Zaman *et al*. 2012; Jones *et al*. 2018).

Traditional Chinese medicine (TCM) has employed gallbladder liquid and some bile ingredients from diverse animals for centuries, together with plant drugs as garlic extracts that performed on rabbits showed the lower number of coccidia oocysts and other materials, to treat infectious and non-infectious diseases, both chronic and acute, including malaria (Cullen and Lo 2004, Tapiñski *et al*., 2018). Based on the chemical composition and pharmacologic actions of bile, such as specific bile salts, the bile pigment bilirubin and its glucuronides, minor bile components such as Vitamins A, D, E, K and melatonin synthetic compounds, and medicinal plants, bile from chickens, cattle, sheep and others may be promising candidates for bear bile's similar and alternative therapeutic purposes (Li *et al*. 2016).

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Animal bile has been shown to improve liver function, dissolve gallstones, suppress bacterial and viral reproduction and have anti-inflammatory, antioxidant, antipyretic, anodyne, anticonvulsive, antiallergic, anti-congestive, antidiabetic and antispasmodic properties (Wang and Carey 2014; Zehua 2015). Modern therapeutic research has suggested that bear bile has a wide range of pharmacological properties, including hepatoprotection, antibacterial, antiviral, anti-inflammation, anti-gallstones, hypolipidemic and other activities (Kou *et al*. 2014; Zhao *et al*. 2015). Goat bile was used in traditional Chinese medicine therapeutically because it was thought to be beneficial in treating optic atrophy, acute hemorrhagic conjunctivitis and several infectious skin diseases (Wang and Carey, 2014). The main components of chicken bile acid were TC: taurocholate, TCDC: taurochenodeoxycholic and TAC: tauroglycocholate (Gu *et al*. 1994). Point studies used carcass residue (bile) in the treatment of coccidiosis in birds (Remmal *et al*. 2013, Jones *et al*. 2018). Since they were first introduced in the West over a century ago (Little *et al*. 1992, Bortolini, Medici and Poli 1997), bile acid precipitated with iron sulfate to form ferrous salts of bile acids known as "Bilron," has been shown to be effective in treating patients with steatorrhea due to bile acid deficiency, especially following ileectomy for Crohn's disease, since they were first introduced in the West over a century ago (Little *et al*. 1992).

The aim of the present study was to evaluate the potential of poultry bile liquid *in vitro* against sporulation and the morphology of oocysts of Eimeria specie.

MATERIALS AND METHODS

Animal material

The experiment was conducted in the Department of Zoology, Parasitology Laboratory, College of Science, King Saud University, for a period of approximately one to two months in October 2021. The preparation and activation of oocytes in mice and then applying the concentrations of the experiment *in vitro*. Poultry bile was obtained from the intact gallbladder of a broiler chicken from a slaughterhouse on a poultry farm in Riyadh (Saudi Arabia). Poultry gallbladders were isolated from 10 healthy female chickens. Gallbladders were sterilized with 70% alcohol before removal of the bile by syringe, transferred and collected to a clean tube and stored at 4°C until they were used on oocysts of Eimeria species.

Infrared spectroscopy

A small portion of the material was combined with an excess of potassium bromide powder (1: 99 wt%) and processed to create a homogenous consistency. The material was then coarsely mashed and placed in a pellet-forming die. The optical spectrometer NICOLET 6700 Fourier-transform infrared spectroscopy from Thermo Scientific was used to examine infrared (IR) (FT-IR). The maximum absorption is expressed as the number of waves (cm^{-1}). At 25°C, spectra with a resolution of 4 cm were recorded, spanning from 4000 cm to 1 to 400 cm^{1}.

Oocyst sporulation test

In this study, *E. papillata* was used as a pathogenic species in the small intestine (Danforth, Entzeroth and Chobotar 1992). Which were obtained mainly from unpopulated oocysts (*Eimeria papillata*) by Prof. Mehlhorn at the University of Duesseldorf (Duesseldorf, Germany), To keep the parasite alive, unpopulated oocysts of *E. papillata* were passed on to mice (Al-Quraishy *et al*. 2011, Dkhil *et al*. 2013). Oocysts were collected from the feces of mice infected with *E. papillata* and surface sterilized with sodium hypochlorite before being washed at least four times in sterile saline, as described. An *in vitro* inhibition test of sporulation was utilized to examine the effect of chicken bile on the oocyst sporulation of *E. papillate* (Schito *et al* 1996). Bile liquid was diluted with distilled water (100%, 50%, 25% and 10%). Then, we used 350 µl of un-sporulated oocysts containing 1×10^3 oocysts were added to 2.5% potassium dichromate solution in petri dishes and exposed to four concentrations of poultry bile with potassium dichromate (w/v; 10%, 25%, 50% and 100%), was used as control group. The petri dishes were partially covered to allow oxygen to pass through and incubated for 48 hours at 25-29°C with water in two petri dishes in the incubator to maintain 60-80 per cent humidity. The petri dish contents were stirred off every now and then (Gadelhaq, *et al*. 2018). Each dilution was examined microscopically after 48 hours, 72 hours and 96 hours in (10 ml) suspension. Then sporocysts were examined under an inverted microscope at 40x to confirm oocyst sporulation. The number of oocysts left in a total of 50000 oocysts was counted to estimate the percentage population of sporulated and inhibitory oocysts (Pieri *et al*. 2014).

For each concentration, three replicates were performed and the experiment was repeated to confirm the results. The oocysts with 4 sporocysts were considered sporulated regardless of the shape and size of the sporocysts. The number of sporocysts within each sporulated oocyst was counted, as well as the number of abnormal sporocysts (in terms of shape and size). The numbers of sporulated and non-sporulated oocysts using a McMaster chamber were counted and the percent sporulation was estimated by counting the number of sporulated oocysts in 100 oocysts for *E. papillate* (Ruiz *et al*., 2006). The percentages of sporulation and inhibition were calculated using the following equations:

$$\text{Sporulation (\%)} = \frac{\text{Number of sporulated oocysts}}{\text{Total number of oocysts}} \times 100$$

As you described (You 2014), sporulation inhibition.

$$\text{(SI\%)} = \frac{\text{Sporulation of control-Sporulation of treated}}{\text{Sporulation of control}} \times 100$$

Statistical analysis

The GraphPad Prism program and one-way ANOVA were used to analyze the data, and Duncan's multiple range test was used to determine significance between groups. Statistical significance was defined as $P<0.05$.

RESULTS AND DISCUSSION

Poultry bile fluid was analyzed with major bands at 3425.53 cm^{-1}, 2093.10 cm^{-1}, 1641.41 cm^{-1}, 1045.64 cm^{-1}, 410.42 cm^{-1}, respectively. Chemicals were present as in the Fig 1, Table 1. Modern physiological, physical-chemical, molecular biological and nuclear receptor regulation, as well as homeostatic research on bile acid and bilirubin metabolism in animal and model bile, have shed light on the possible pharmacolo gical mechanisms involved in the mode of action of various animal bile. These findings support the success of TCM's millennia-old heuristic tactics (Sjövall and Setchell 1988, Vítek and Ostrow 2009, Lefebvre *et al*. 2009). Our findings are consistent with those of Remmal, who discovered that the major components of essential oils tested separately (carvacrol, thymol, isopulegol, eugenol, and carvone) have oocysticidal activity in the fight against coccidiosis (Remmal *et al*. 2013). Furthermore, bile acids have been shown to be potent regulatory agents in the gastrointestinal tract and liver over the last two decades, activating specific nuclear receptors, a G-coupled protein receptor and multiple cell signaling pathways (Mukhopadhyay and Maitra 2004; Hofmann 1963; Lefebvre *et al*. 2009). Generally, the statistical analysis showed that all dilutions of poultry gallbladder (bile) significantly inhibited different levels of sporulation in *E. papillata*, except concentration 100% of bile compared to control group (potassium dichromate solution 2.5%), Which depended on dose concentration and incubation period, which increased sporulation percentage with an increased incubation period at 24, 48, 72 and 96 hrs (Table 2). So, oocyst sporulation incubation in a potassium dichromate solution 2.5% with PB at a concentration of 100% for all periods presented inhibited sporulation. This applies to those results that showed that bile acids play an important role in the regulation of the antimicrobial program of the terminal ileum and suggest that they act as regulators of critical aspects of the intestinal epithelial barrier and immunity. Similarly, animal bile and commercially available bile acids were tested in experimental allergic illness models for their anti-allergic properties. In mouse models of delayed-type hypersensitivity (type IV allergy), picryl chloride-induced contact dermatitis (PC) and sheep red blood cells (SRBC)-induced footpad swelling, pig bile had a significant protective effect. Kubo *et al*. (1989) similarly inhibited PC-CD by the herb Felurs (dried bear gallbladder) (Kubo *et al*. 1989).

After a 48-h incubation were observed significant differences in all concentrations with PB, showed the *E. papillata* oocysts sporulation with potassium dichromate solution 2.5% about 23%, while concentration 100% did not show any sporulation. Whereas the concentrations of PB (50%, 25% and 10%) showed sporulation in varying

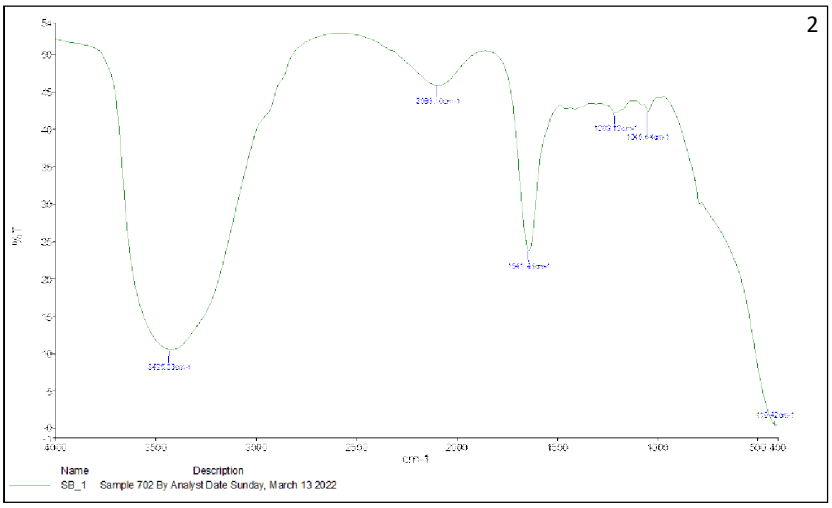


Fig 1: Infrared spectroscopy of poultry bile.

Table 1: IR spectrum of poultry bile liquid by frequency range.

Absorption (cm^{-1})	Appearance	Transmittance (%)	Group	Compound class
3425.53	Medium	12	N-H stretching	Aliphatic primary amine
2093.10	Strong	47	N=C=S stretchy	Isothiocyanate
1641.41	Strong	23	C=C stretching	Alkene
1209.12	Strong	42	C-O stretching tertiary	Alcohol
1045.64	Strong, Broad	43	CO-O-CO stretching	Anhydride
410.42	Strong	3	C-H bending	1,2-disubstituted

Table 2: Shows the rate of non-sporulation in the PDS group and the sporulation rate in the DW.

Treatments	Time	Non-sporulation (%)	Sporulation inhibition (%)	*P. value*
CON+[PDS 2.5%]	48	89.7±4	0	0
	72	56.5±2	0	
	96	9.33±1	0	
100% PB+[PDS 2.5%]	48	100±0.01	100±0.01	**
	72	100±0.01	100±0.01	
	96	100±0.01	100±0.01	
50% PB+[PDS 2.5%]	48	78.67±2.3	79±2.5	*
	72	47.67±2.1	43±2.3	
	96	38.67±1.9	7.7±2	
25% PB+[PDS 2.5%]	48	69.25±3	71±2.5	*
	72	32.33±3	36±3	
	96	20.67±2	6.2±3	
10% PB+[PDS 2.5%]	48	73.24± 3	41±3	
	72	28.67±3	26±2.1	NS
	96	17.66±2	5±1.5	

PB: Poultry bile; CON: Control; PDS: Potassium dichromate solution; NS: No significant differences*: (*p-value*≤0.05); **: (*p-value*≤0.001).

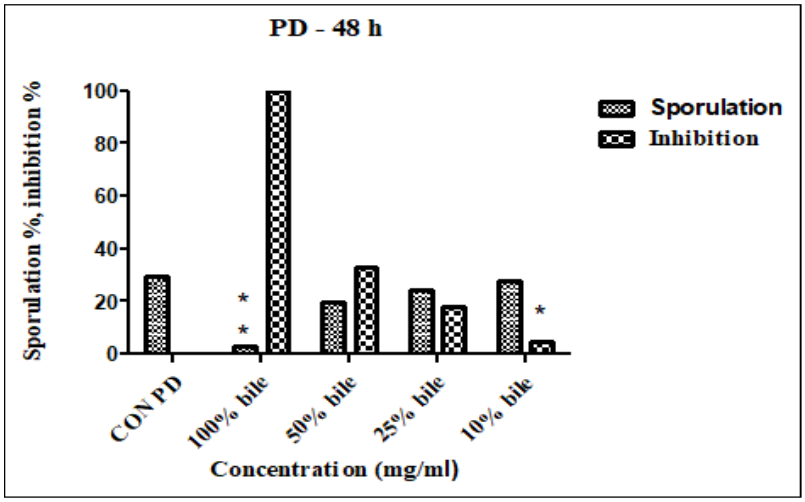


Fig 2: Effects of poultry bile diluting potassium dichromate solution on sporulated oocysts of *E. papillata* of different concentrations, after 48 hours *in vitro*. *: (*p-value*≤0.05); **: (*p-value*≤0.001).

proportions, (Fig 2). While, *E. papillata* oocysts incubation of 72-hour with PB resulted decreased the sporulation ratio with different levels of sporulation in concentrations 50%, 25% and 10% approximately ratios of 26.76%, 42.33%, 63.67% (Fig 2). However, oocysts incubation of 96-hour with PB resulted in increasing sporulation percentage in control group. While, at 100% concentration continue oocysts without sporulation. On the other hand, we noticed that the concentrations (50%, 25% and 10%) increased the percentage of sporulation than the previous ones with increasing time (Fig 4). These findings also point to a potential therapeutic application of bile acids in the treatment of enteric bacterial infections (Tremblay *et al*. 2017). The primary biliary lipids of vertebrates, including cartilaginous and bony fish, reptiles, birds and mammals, contain conjugated steroidal bile acids as soluble salts (Lang *et al*. 2016). Bile alcohol sulfates of C27 and/or C24 bile acids, as well as N-acyl amidites of C27 and/or C24 bile acids, can be employed. Bile acids are always a molecular combination of congeners created in the liver straight from cholesterol. The number of known naturally occurring bile acid species is in the hundreds (Solá *et al*. 2002, Mello-Vieira *et al*. 2013) and is typified by the most evolved species in humans (Zhao *et al*. 2015).

Bile liquid was utilized to dislodge intestinal worms from dogs. As an outcome, it played a critical role in treating infantile undernourishment caused by gastrointestinal disruptions and trematodes. According to paleopathological evidence, these infestations were most probably caused by roundworms (nematodes) (Zehua 2015; Liu 2016).

The main effects of experimental groups and time of sporulation and concentrations ratio on sporulation (%) and

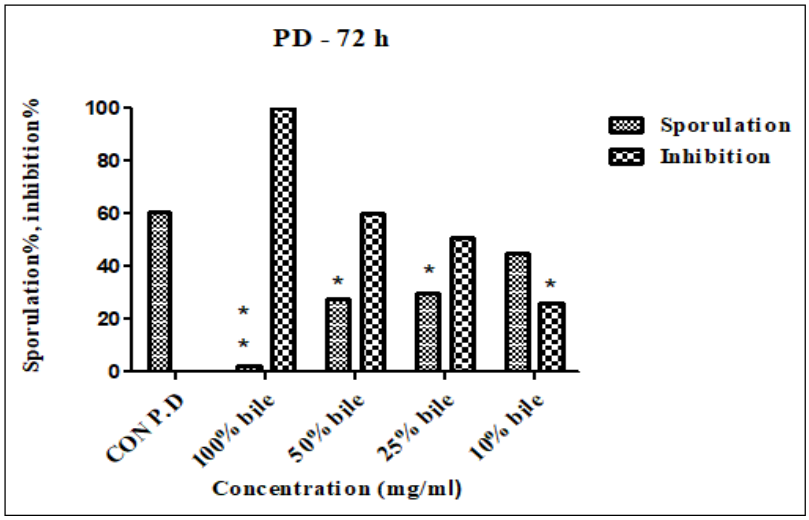


Fig 3: Effects of diluting potassium dichromate on sporulated oocysts of *E. papillata* of different concentrations, after 72 hours *in vitro*. *: (*p-value*≤0.05); **: (*p-value*≤0.001).

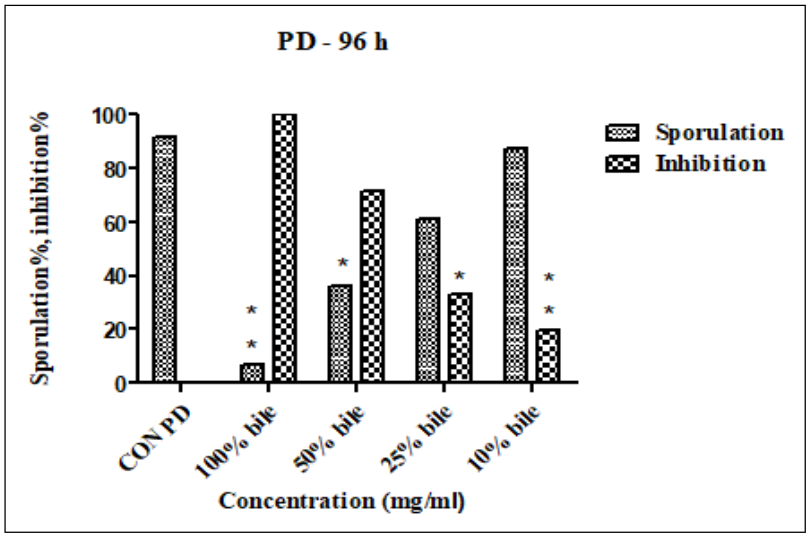


Fig 4: Effects of PB diluting potassium dichromate on sporulated oocysts of *E. papillata* of different concentrations, after 96 hours *in vitro*. *: (*p-value*≤0.05); **: (*p-value*≤0.001).

sporulation inhibition (%) of *E. papillata* oocysts are shown. The sporulation percentage increased with increasing incubation time and conversely that for non-sporulation percentage. The sporulation inhibition rate increased significantly with increasing incubation time up to 72 hours (P<0.05), therefore, the sporulation inhibition rate did not differ significantly between 72- and 96 hour exposure (Fig 3, 4). The sporulation inhibition rate increased significantly with the highest concentration (P<0.05). Therefore, the sporulation inhibition rate disagreed significantly between the concentrations (PB: 50%, 25% and 10%) that *E. papillata* oocysts were exposed (Fig 5, 6).

In Fig 7 PD shows that the percentage of sporulation increased with increasing bile concentration and vice versa for the percentage of no ovulation with the bile dilution visit. The rate of sporulation inhibition increased significantly with a decrease in bile concentration at 10% and 25% (P<0.05), and therefore, the rate of ovulation inhibition differed significantly between exposure to concentrations of 100% and 10%. Bile acids are involved in the regulation of bile acid, glucose, fatty acid and cholesterol synthesis, transport, and metabolism, as well as triglyceride homeostasis and nutrient signaling that controls the utilization of energy, as evidenced by their activation and repression of genes encoding enzymes, other proteins and transporters (Lefebvre *et al*. 2009). Bile acids have also been used for drug delivery to take advantage of their particular physicochemical properties and biocompatibility and as drug absorption enhancers, for both drug solubilization and permeation (Pavlovic *et al*. 2018). Further, UDCA, a bile component, was used in a clinical trial with COVID-19 patients in the USA (Subramanian *et al*. 2020). The usage

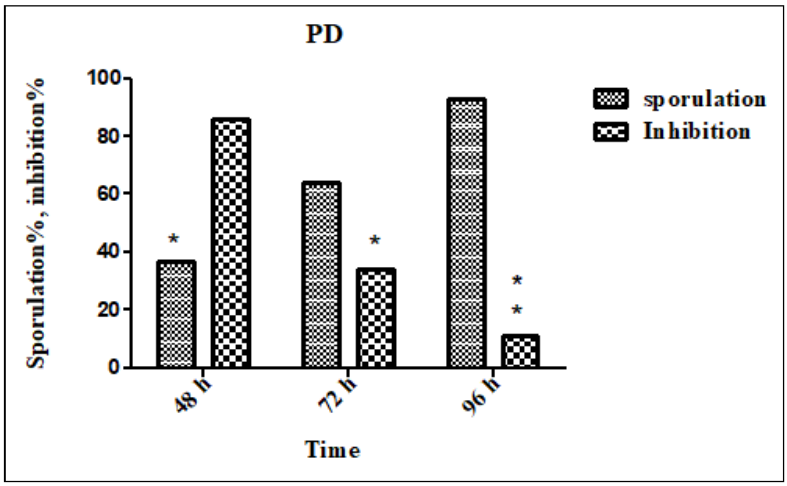


Fig 5: Effects of poultry bile diluting distilled water on inhibition oocysts of *E. papillata* of different times after 48, 72 and 96 hours *in vitro*. *: (*p-value*≤0.05); **: (*p-value*≤0.001).

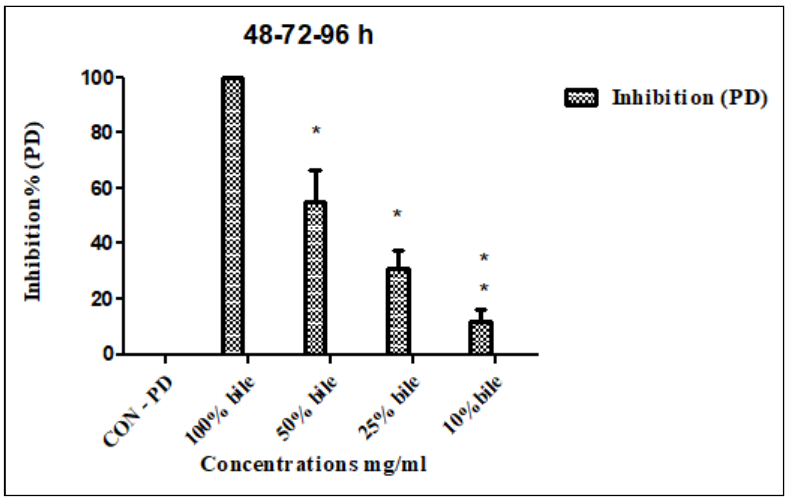


Fig 6: Cumulative effect of poultry bile diluting potassium dichromate on inhibition oocysts of *E. papillata* of different times after 48, 72 and 96 hours *in vitro*. *: (*p-value*≤0.05); **: (*p-value*≤0.001).

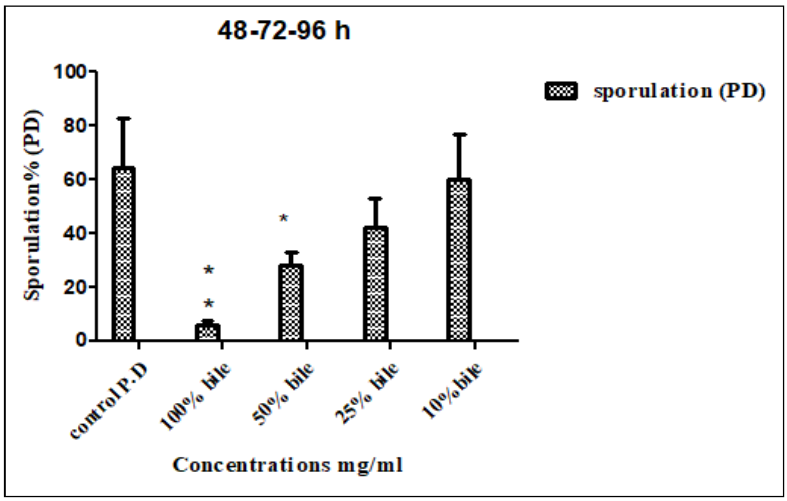


Fig 7: Cumulative effect of poultry bile diluting potassium dichromate on sporulation oocysts of *E. papillata* of different times after 48, 72 and 96 hours *in vitro*. *: (*p-value*≤0.05); **: (*p-value*≤0.001).

of bile compounds in their pure form, in our opinion, gives higher pharmacological and toxicological safety.

More research into gallbladder bile is needed to identify the component(s) responsible for poultry bile activities, as well as *in-vivo* research to understand the histological mechanisms, oxidative stress and molecular of poultry bile induced sporulation inhibition in the animal body and develop the most cost-effective anti-coccidiosis treatment.

CONCLUSION

Conclude that these poultry bile liquid are of particular interest in fighting coccidiosis since they have a destructive effect against sporulation and the morphology of oocysts of Eimeria species. The findings of this study point to the need for necessitate more research into chicken bile to determine the components effective, as well as, more *in vivo* research in order to develop a less expensive and time-consuming coccidiosis treatment. They could also help in the formulation of radical and safe solutions to coccidiosis.

Data Availability

Data supporting this research are available from corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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