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In vitro Studies on Anticoccidial Effects of Healthy Sheep Bile against *Eimeria magna* and *Eimeria exigua* Oocysts and Sporozoites Isolated from Domestic Rabbits

Mutee Murshed[1], Mohammed M. Mares[1], Hossam M.A. Aljawdah[1], Saleh Al-Quraishy[1]

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ABSTRACT

Background: Coccidiosis is one of the most common diseases that hinder the raising of rabbits. The disease causes symptoms such as diarrhea and loss of appetite. Acute infection causes bloody feces and negatively affects fertility. Diarrhea may be bloody due to the intestinal epithelium withering off as many oocysts and merozoites burst out of the cells. This leads to the deaths of rabbits resulting in huge economic losses for commercial rabbit systems.

Methods: The study aims to determine the optimal effective concentration of bile collected from healthy sheep gall bladder inhibiting *in vitro* sporulation and viability of *E. magna* and *E. exigua* oocysts and sporozoites isolated from rabbit intestine. Un-sporulated oocysts were exposed (1×10^2) to six treatments: 2.5% potassium dichromate solution as the non-treated control, four concentrations of bile (12.5%, 25%, 50% and 100%) and Toltrazuril 25 mg/ml as traditional medicine for anti-oocyst activities. In addition, 250, 500, 750 and 1000 µg/mL concentrations of bile were evaluated for effect on sporozoite viability. The oocysts were examined after 25, 50, 75 and 100 hours and sporozoites after 12 and 24 hours of treatment.

Result: GC-Mass analysis showed that sheep bile contains 12 biologically active compounds. In addition, quantitative analysis revealed total phenols (50.65±0.5) and total flavonoids (9.39±0.4). After 100 hours of exposure, sheep bile was able to prevent sporulation of *E. magna* and *E. exigua* oocysts at 100% and 50% concentrations and the rate of inhibition was about 96% and 79%, respectively. Additionally, bile exhibited the least discouraging of 11% at a dose of 125 mg/mL and the highest suppression of 95% of *E. magna* and *E. exigua* sporozoites viable at 1000 µg/ml. Generally, the inhibition rate typically increased with longer incubation times and greater doses. Findings have scientifically validated the use of bile in fighting against coccidiosis. Additional studies are required to separate the active compounds found in bile and their mechanisms of action and potential applications in rabbit farms.

Key words: Anticoccidial, *Eimeria* species, Oocysticidal, Sheep bile, Sporozoiticidal.

INTRODUCTION

Coccidiosis is a parasite that affects mammals, including rabbits and poultry and is familiar in many parts of the world. *E. magna* and *E. exigua* are prevalent coccidia in rabbits. These primarily affect rabbits' jejunum and ileum (Ogolla *et al.*, 2018). The disease causes symptoms such as diarrhea and loss of appetite; acute infection causes bloody feces and negatively affects fertility (Manjunatha *et al.*, 2019). The presence of more than one of these risk factors may cause the animal to have stunted growth and perhaps die, especially when they are young; other clinical signs comprise lethargy, depression and a decrease in usual behaviors (Fang *et al.*, 2019). Diarrhea may be bloody due to the intestinal epithelium withering off as many oocysts and merozoites burst out of the cells (Manjunatha *et al.*, 2019). On the other hand, the peculiarities of the life cycle of species belonging to the genus *Eimeria* contribute to the speed with which young animals become infected (Pyziel and Demiaszkiewicz, 2015; Boyko *et al.*, 2021). Three distinct types of this parasite can affect a rabbit's intestine, liver, or both. They include the intestinal form, the hepatic form and the mixed form, respectively. Occasionally, recordings are made of spasms that occur in the limbs. Pathoanatomical changes show that the duodenum, liver and urinary tract are hurt (Jing *et al.*, 2016; Eladl *et al.*, 2020). Currently, the control of rabbit coccidiosis relies mainly on anticoccidial drugs such as nicarbazin, amprolium, diclazuril, halofuginone, robenidine, toltrazuril and sulfonamides (Mai *et al.*, 2009). Although toltrazuril combination therapy against coccidiosis has shown exceptional responses, the limited medicinal options demand novel therapeutics. Furthermore, drug resistance and

[1]Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia.

Corresponding Author: Mutee Murshed, Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia. Email: mmurshed@ksu.edu.sa

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drug residues remain serious issues in most cases and new medications are proposed to overcome the resistance in addition to conventional therapeutics (Shirley *et al*., 2007). There is an urgent need for safe and effective control mechanisms to reduce or provide alternatives to the use of chemical pharmaceuticals. Researchers have focused on plant-derived compounds because they are generally safer and more prone to degradation than chemical compounds (Benelli *et al*., 2017). People have historically employed natural products to manage pests, which can be utilized as deworming agents (Knaus *et al*., 2021). There are currently many different ways to combat *Eimeria*, which is caused by the microorganisms that cause this condition (Yin *et al*., 2016; El-Ashram *et al*., 2019). Traditional Chinese medicine has been utilized for over 2000 years to treat several ailments and 44 distinct organism bile liquids, including human gall liquid, have been recognized as medicines because of their membrane lytic and detergent properties (Garidel *et al*., 2007; Zhai *et al*., 1996). The antibacterial properties of bile acids are crucial for inhibiting bacterial activities in the intestinal duct (Begley *et al*., 2005). Bile acids are being marketed as therapeutic substitutes due to their antibacterial activity and anti-coccidial chemotherapeutic effects after several experimental studies determined their effectiveness. Also, they are a low-cost way to get rid of coccidiosis. Traditional Chinese medicine uses animal-derived bile and gallbladder secretions to treat chronic and acute illnesses, including malaria and infectious and non-infectious ailments (Murshed *et al*., 2022). Based on their chemical makeup and pharmacological effects, the primary bile constituents may present attractive candidates for equivalent, alternative therapeutic uses (Salim *et al*., 2022). According to studies, animal bile has anti-inflammatory, antioxidant, anodyne, anti-convulsive, antiallergic, anti-congestive, antidiabetic and antispasmodic qualities in addition to improving liver function and dissolving gallstones (Cohen *et al*., 1994). Mineral ions and proteins, such as mucin glycoproteins, are present in significant numbers in bile and it contains high concentrations of antioxidants such as bilirubin, glutathione, vitamin E and melatonin (N-acetyl-5-methoxy tryptamine) (Cohen *et al*., 1994; Tian *et al*., 2022). Bear bile has recently been found to contain a variety of pharmacological characteristics in modern medicinal studies (Tremblay *et al*., 2017). Taurocholate (TC), taurochenodeoxycholic (TCDC) and tauroglycocholate (TAC) are the three main parts of chicken bile acid (Tremblay *et al*., 2017). Studies are currently being done to determine whether carcass bile residue can treat coccidiosis in birds (Remmal *et al*., 2013). Toltrazuril is a valuable medication for avoiding coccidiosis in sheep, cattle, pigs and rabbits (Ayan *et al*., 2020). The first study to use 2.5% toltrazuril to treat rabbit intestinal coccidiosis found that 25 ppm toltrazuril dissolved in water and given to the rabbits for two days helped treat the illness (Singla *et al*., 2000). The study's goal is to find out how well healthy sheep bile works against the growth and shape of *Eimeria* species in the lab, taking these things into account.

MATERIALS AND METHODS

Study area

This experiment was completed in the Lab Parasitology Zoology Department of the College Sciences-King Saud University, during the duration from (3/4/2022 to 6/5/2023).

Collection of bile

Bile was extracted from the entire gall of domestic sheep in the Riyadh Automated butchery in Saudi Arabia. Gallbladders were removed from seven disease-free sheep, then cleaned with alcohol at a concentration of 75%, collected utilizing a syringe, transferred to a clean tube and kept at a temperature of 4°C until experimental usage.

Chemical analysis of compounds from bile utilizing GC-MS

Bile liquid was examined from disease-free sheep using Thermo Scientific, Austin, Texas, USA, trace GC-ISQ Quantum mass spectrometer equipment. The flow rate s used was 1 $mL.mn^{-1}$/min. The material was injected into a GC-MS using a TG-5MS column (30 m × 0.25 mm ID, 0.25 μm film thickness) in about 1 μL. Helium gas was used as a carrier at a constant flow rate of 1.0 mL min^{-1}. The mass spectra were seen in the range of 50 to 500 m/z. The temperature was initially set at 50°C for 10 min, increased to 250°C at a rate of 5°C, then maintained at 300°C for 2 min and then maintained at 350°C for 10 min. By comparing the recorded mass spectra for each compound with the information saved in the earlier libraries, it was possible to identify the phytochemical components using the NIST, Adams, Terpenoids and Volatile Organic Compound libraries. The relative percentage amount for each element was computed using the retention time index and comparing each component's average peak area to the total peak area (Kanthal *et al*., 2014).

Sample collection

The study was carried out in 2023 at the King Saud University Parasitology Laboratory of the Department of Zoology by Veterinary and Sanitary Expertise (Saudi Arabia). The rabbits that were found to be naturally infected with *Eimeria* or suffered from eimeriosis were used for the collection of faecal samples for analysis. The McMaster method was utilized in the examination of faeces for *Eimeria* spp. oocysts. Two types of oocysts (Magna and Exigua oocysts) were distinguished on the basis of their morphological characteristics (Qamar *et al*., 2013).

Application of the experiment

Unsporulated *E. magna* and *E. exigua* oocysts collected from the intestine of infected rabbits were used for oocyst sporulation analysis. *E. magna* and *E. exigua* oocyst sporulation was conducted in an aqueous solution of 2.5% potassium dichromate $K_2Cr_2O_7$ (10^2 oocysts/mL) with or without 100 μg/mL of bile at 25°C for 72 h. Both sporulated and unsporulated oocysts were tallied. The percentage of sporulated oocysts was assessed by microscopic

observation and counting the number of sporulated oocysts in a total of 100 oocysts.

$$\text{Oocysts decrease (\%)} = \frac{\text{Mean oocysts number of control - Mean oocysts number of treatment}}{\text{Mean oocysts number of control}} \times 100$$

Preparation of *Eimeria* sporulated oocysts

Field isolates of *E. magna* and *E. exigua* oocysts were obtained from the small and large intestines. Additionally, samples were taken from the gallbladders and necrotic intestinal lesions of rabbits that had naturally contracted the disease. Using flotation, these oocysts were cleaned and concentrated before experimental use. The sporulated oocysts were kept in 2.5% potassium dichromate ($K_2Cr_2O_7$) and kept at a temperature of 4°C until they were employed for experimental infections. Both the *E. magna* and *E. exigua* field isolates were kept alive by undergoing periodic transmission (passage) through young rabbits in the Zoology Department's Animal House, which also houses the Parasitology Laboratory according to the method (Schito *et al*., 1996).

***In vitro* anti-sporozoites effect of sheep bile**

Phosphate-buffered saline (PBS) with a pH of 7.4 was used to perform multiple washings on oocyst samples kept in potassium dichromate solution. We repeated the process of centrifuging the Falcon tubes with 15 ml of liquid at 1008 g for 10 minutes until all of the $K_2Cr_2O_7$ was removed. Oocysts were shaken for one hour while being incubated in a water bath at a temperature of 41°C. After resuspending the pellet in PBS and giving it a single pass in PBS, the suspension was centrifuged at 1008 g for ten minutes. A McMaster chamber was used to perform the counting of the sporozoites. Plates-well (24) was utilized to test the *in vitro* sporozoite activity. A test solution of 1 mL was mixed with 1 mL of the parasitic suspension containing 1000 sporozoites in order to produce a gross volume of 2 mL of each dose of sheep bile (125, 250, 500 and 1000 μg/ml). To make a comparison, the medicine toltrazuril was utilized at a dose of 50 micrograms per mL as a positive control. At the same time, the potassium dichromate solution served as the negative control. The experiment was done three times for each dose and control treatment under identical conditions. The findings were assessed at 6, 12 and 24 hours. After determining the number of viable and nonviable sporozoites, the percentage of viability was evaluated by counting the number of viable sporozoites among a gross of one hundred sporozoites. The following formula was used to compute the viability percentage inhibition as You described (You, 2014).

$$\text{Inhibition of viability (Vi)\%} = \frac{\text{Vi\% control - Vi\% bile}}{\text{Vi\% control}} \times 100$$

RESULTS AND DISCUSSION

The GC-MS chromatogram of bile showed twelve active chemicals (Fig 1). The chemical constituents obtained were hexadecanoic acid, methyl ester, n-hexadecanoic acid, 9-octadecenoic acid (Z)-, methyl ester, heptadecanoic acid, 16-methyl-, methyl ester, linoelaidic acid, oleic acid, octadecanoic acid, arachidonic acid methyl ester, glycidyl oleate, cholesterol, deoxycholic acid and methyl cholate (Table 1).

In the bile of the sheep, there were a variety of secondary metabolites that were measured. Some of these metabolites included phenolics and flavonoids. The data shown in (Fig 2) shows that the concentration of phenols (50.65±0.5) was higher than the concentration of flavonoids (29.39±0.4).

In vitro, studies were conducted on the influence of sporulation times and groups treated in the experiment with bile on the inhibition and sporulation of *E. magna* and *E. exigua* oocysts which revealed that as incubation time increases, so did the proportion of sporulation and the opposite is true for the percentage of inhibition. By increasing incubation time, the rate of sporulation inhibition rose considerably ($p \leq 0.01$) in groups of treatment. Different

Table 1: Identification of chemical combination by GC-Mass in sheep bile.

$tR_{(min)}$	Proposed compound	MW	Formula	Peak area %
17.50	Hexadecanoic acid, methyl ester	270	$C_{17}H_{34}O_2$	1.81
17.90	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	19.44
19.27	9-Octadecenoic acid (Z)-, methyl ester	296	$C_{19}H_{36}O_2$	3.53
19.51	Heptadecanoic acid, 16-methyl-, methyl ester	298	$C_{19}H_{38}O_2$	1.48
19.61	Linoelaidic acid	280	$C_{18}H_{32}O_2$	13.03
19.67	Oleic acid	282	$C_{18}H_{34}O_2$	42.14
19.86	Octadecanoic acid	284	$C_{18}H_{36}O_2$	7.765
20.75	Arachidonic acid methyl ester	318	$C_{21}H_{34}O_2$	0.79
21.152	Glycidyl oleate	338	$C_{21}H_{38}O_3$	1.33
28.38	Cholesterol	386	$C_{27}H_{46}O$	5.45
32.34	Deoxycholic acid	392	$C_{24}H_{40}O_4$	0.52
38.40	Methyl cholate	422	$C_{25}H_{42}O_5$	2.72

*TR stands for "time retention" "M-H" stands for "protonated molecule" and "MS" stands for "mass acquired range".

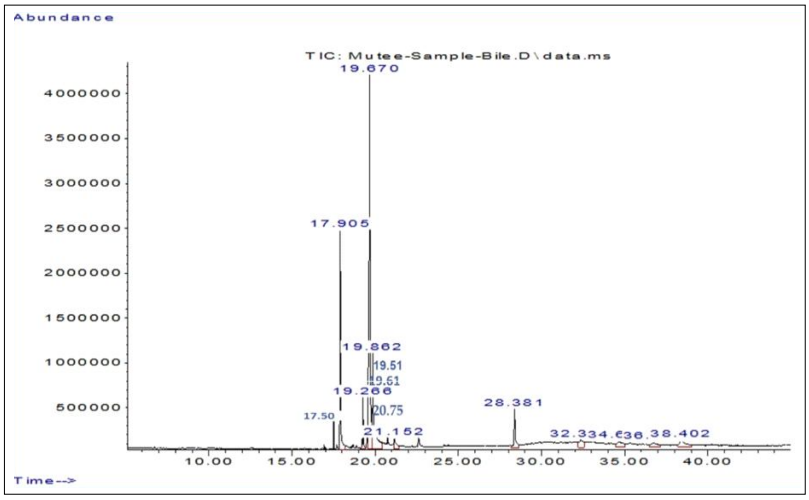


Fig 1: GC-MS chromatogram of the chemical constituents discovered in sheep's bile.

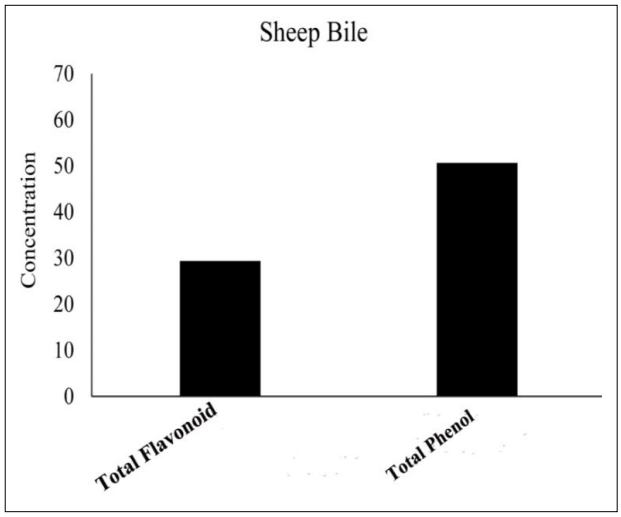


Fig 2: Flavonoids and total polyphenols in the sheep bile.

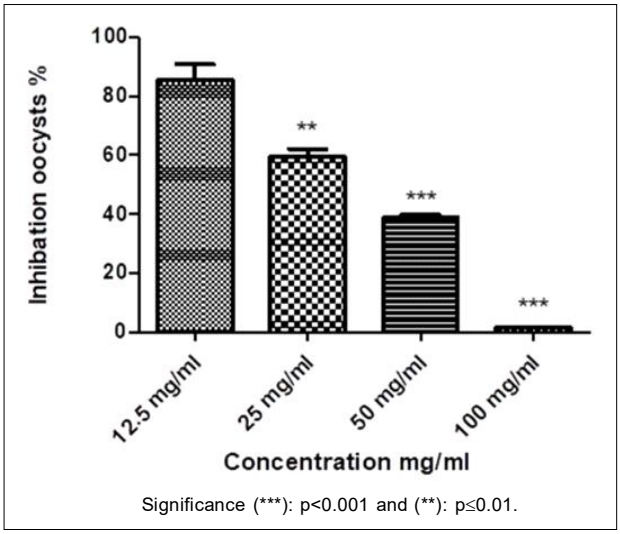


Fig 3: The influence of sheep bile on the sporulation of *E. magna* and *E. exigua* oocysts was tested *in vitro* throughout a variety of time periods for the highest concentration was achieved.

concentrations (12.5%, 25%, 50% and 100%) of bile obtained from disease-free sheep were tested and 100% concentration was the best dose for inhibition of oocysts (Fig 3), compared with the control group (2.5% potassium dichromate), reference therapy and phosphate-buffered saline (Fig 4) at p-value ≤0.001.

The oocysts activity of different doses of sheep bile against the isolated *E. magna* and *E. exigua* oocysts *in vitro* is summarized. The highest efficacy of tested sheep bile doses was recorded after 100 h of exposure at 100% of sheep bile, approximately 91% sporulation inhibition. Compared with the control group ($K_2Cr_2O_7$), which managed to oocyst sporulation significantly. Reciprocally, a low dose of 12.5% of BS lowered the ratio of inhibition of oocysts. Other SB doses (50%, 25% and 12.5 mg/mL) demonstrated varying degrees of effectiveness depending on the tested dose and the time it was incubated. The findings are consistent with those of Tremblay and colleagues, who demonstrated that the bile acid found in the ileum is an antibacterial agent that has the potential to be used as a therapeutic option for bacterial infections that occur within the intestinal tract (Tremblay *et al.*, 2017). Pig bile had a significant defensive impact in mouse models of delayed-type hypersensitivity to containing demonstrated condensed tannins that have the potential to obstruct the activities of endogenous enzymes (including mannitol dehydrogenase, mannitol-1 phosphatase, mannitol-1 phosphate dehydrogenase and hexokinase) (Cedric *et al.*, 2018). Sheep bile liquid (which contains tannins) may reduce the rate of sporulation by inhibiting or inactivating the enzymes responsible for sporulation, as in helminth eggs (Jones *et al.*, 1994).

The un-sporulated oocysts of *E. magna* and *E. exigua* did not exhibit any signs of sporulation after being incubated with sheep bile in a dose of 100% for 48 h. On the other hand, oocysts that were treated with 2.5% potassium dichromate and sheep bile (12.5%, 25% and 50%) displayed sporulation to varied degrees (Fig 5).

The un-sporulated oocysts of *E. magna* and *E. exigua* oocysts were inhibited after incubation with SB at a dose of 100% for 72 h. However, the appearance of sporulated oocysts continued to increase with the lengthening of the incubation periods with 2.5% potassium dichromate solution. At the same time, sheep bile (12.5%, 25% and 50%) demonstrated varying degrees and percentages of sporulation.

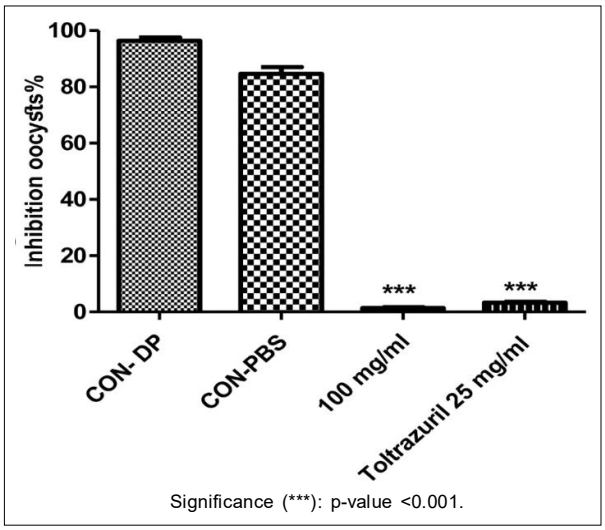


Fig 4: The influence of sheep bile on the inhibition of *E. magna* and *E. exigua* oocysts *in vitro* during different time periods.

After 100 h of incubation with sheep bile at 100% concentration, the *E. magna* and *E. exigua* un-sporulated oocysts demonstrated no sporulation. However, the appearance of sporulated oocysts continued to increase with the lengthening of the incubation periods with 2.5% potassium dichromate and sheep bile (12.5, 25 and 50%), showed varying degrees and percentages of sporulation (Fig 6). In this study, the sheep bile liquid may have gotten into the oocyst during division and hurt the cytoplasm (sporont). This is shown by the fact that oocysts that were exposed to high concentrations of the liquid had abnormal sporocysts form (Jones *et al.*, 1994). Potassium dichromate demolished bacteria in samples that were made up of coccidian oocysts, which led to more coccidial oocyst sporulation (Wang and Carey, 2014). Potassium dichromate might not be able to stop sporulation because it is also a medicine for bacteria, which would make sporulation more likely. Because of this, bacteria might stop oocyst sporulation from happening, probably by competing for food or eating the oocysts. Also, these results suggest that bile acids might be useful for treating enteric bacterial infections in people (Wang and Carey, 2014; Tremblay *et al.*, 2017). Utilizing bile alcohol sulfates of C27 and C24 bile acids and N-acyl amides is a viable option. Bile acid is a molecular mix of congeners formed in the liver from cholesterol. There are hundreds of known naturally occurring bile acid species, with humans being the most developed (Zehua, 2015). The removal of intestinal worms in dogs was accomplished with the help of bile liquid. As a result, bile liquid is crucial in the treatment

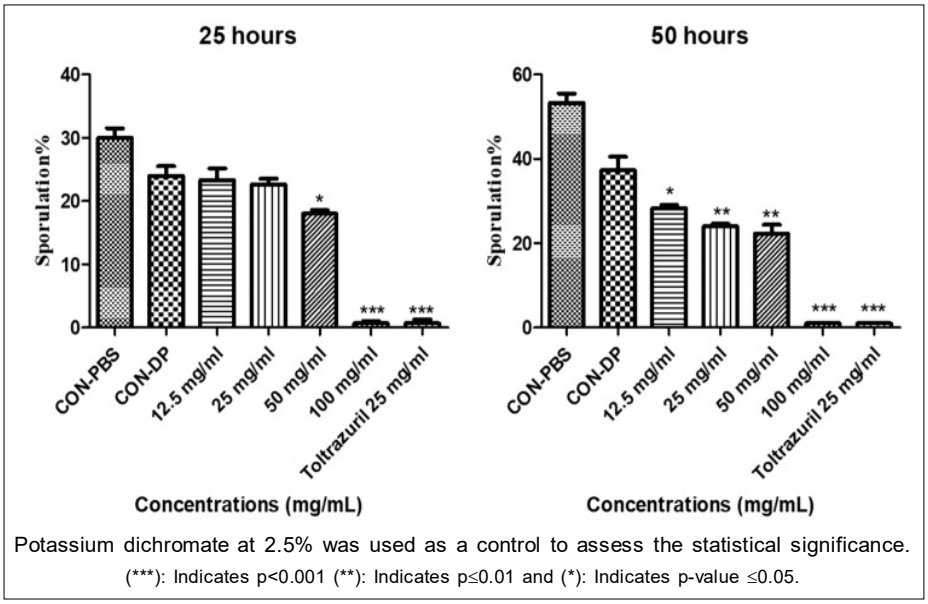


Fig 5: The primary effects of sheep bile on inhibition percent of *E. magna* and *E. exigua* oocysts at varied doses with different contact times and medicament influences at 25 and 50 hours *in vitro*.

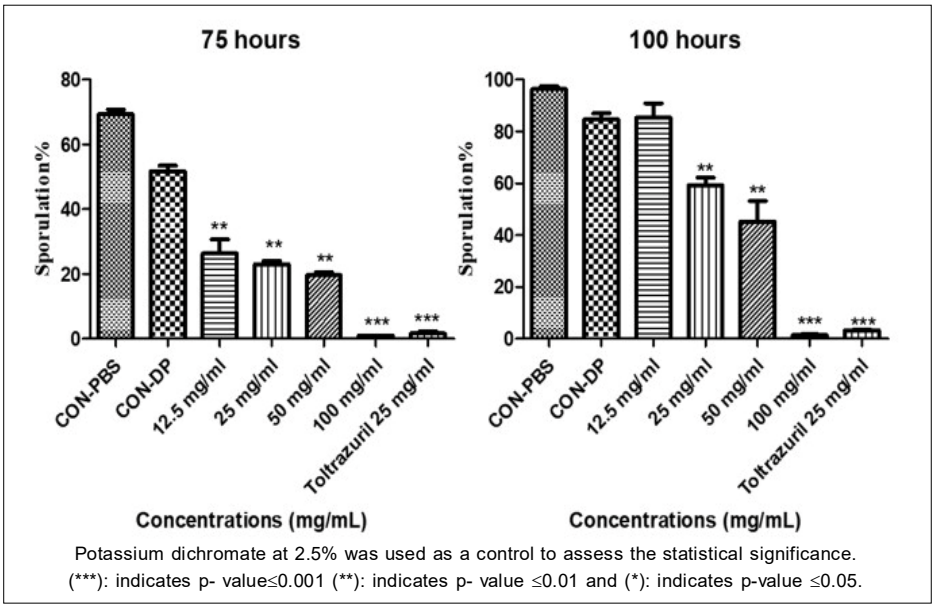


Fig 6: The primary influences of sheep bile on inhibition percent of *E. magna* and *E. exigua* oocysts at varied doses with varying contact times and treatment influence at 75 and 100 h *in vitro*.

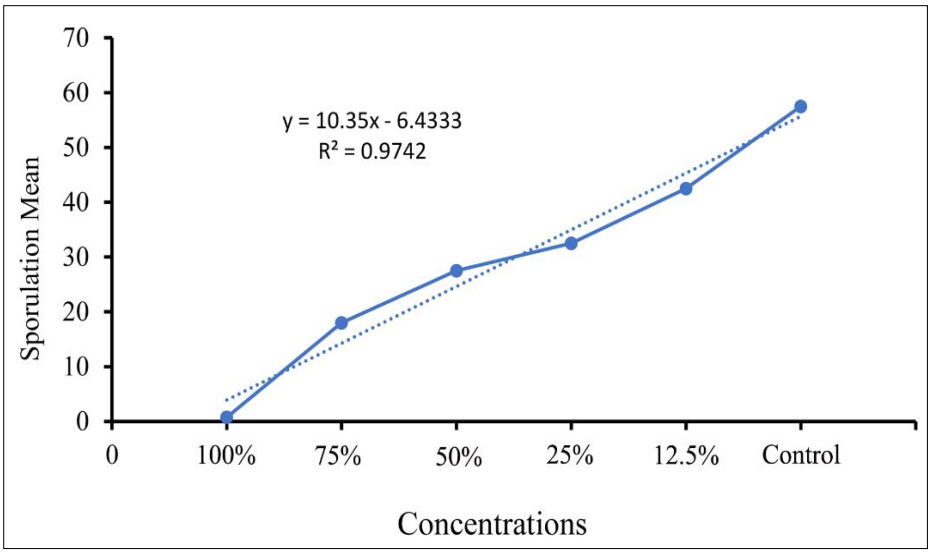


Fig 7: Mean sporulation (%) of *E. magna* and *E. exigua* oocysts at 100 h for various concentrations exposure of SB.

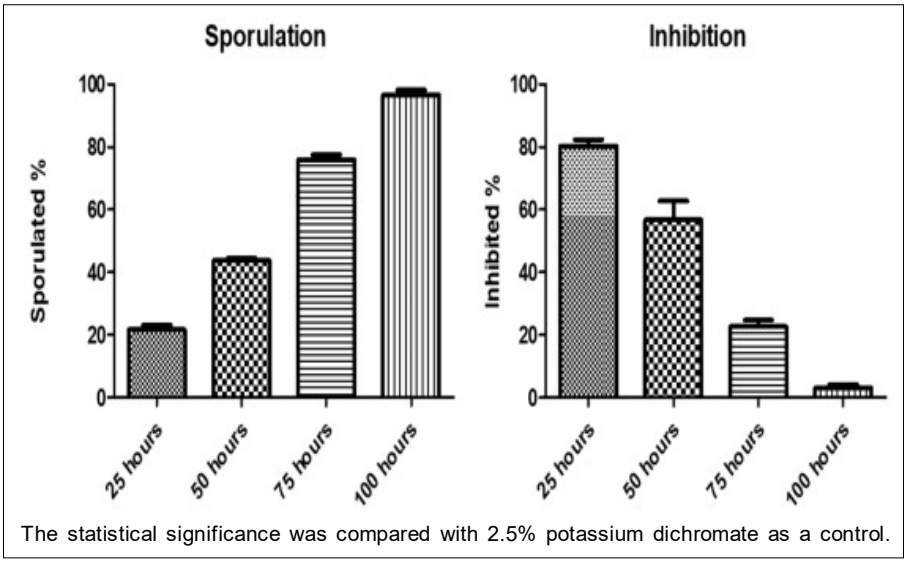
of infantile malnutrition due to trematodes and gastrointestinal disruptions. According to the results of paleopathology, these infestations were most likely brought on by roundworms, also known as nematodes (Sjövall and Setchell, 1988; Liu, 2016).

Fig 7 shows that as the concentration of SB went up, the mean percentage of sporulation in the oocysts of *E. magna* and *E. exigua* went down. The sporulation was highly negatively correlated with concentration percentage (R=-0.96, P≤0.0005) at 75 and 100%, respectively.

In vitro, the study showed the effects of sporulation time and test groups on the percentage of sporulated and non-sporulated *E. magna* and *E. exigua* oocysts. The percentage of sporulated oocysts went up as the incubation time went

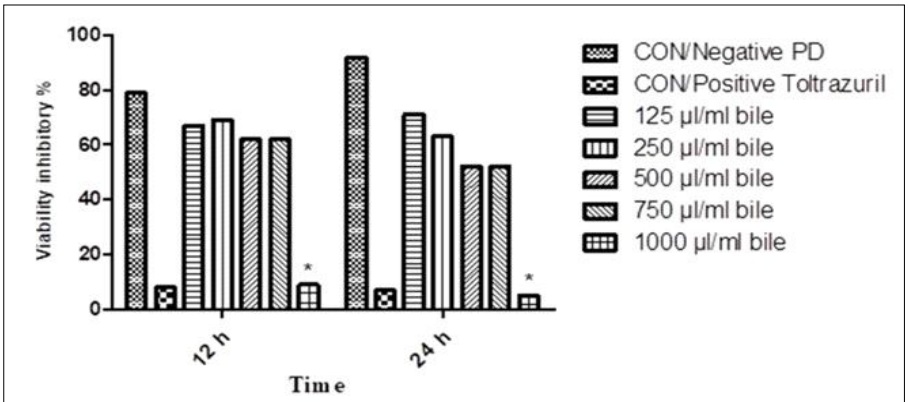
up, but the percentage of non-sporulated oocysts went down. The sporulation inhibition rate increased significantly with increasing incubation time up to 75 h ($p \leq 0.01$); therefore, the sporulation inhibition rate did not differ significantly between 75 and 100 h exposures (Fig 8). Modern physiological, physicochemical, nuclear receptor management and homeostatic research on bile acid and model bile has shed light on the potential pharmaco-logical mechanisms involved in the mode of various animal bile. These findings support the success of TCM's millennial-old heuristic tactics (Lefebvre *et al.*, 2009; Hofmann *et al.*, 2010). Our results are in line with those of Remmal *et al.* (2013), who found that the principal elements of essential oils studied independently, carvacrol, isopulegol, thymol, eugenol and carvone, display oocysticidal activity against coccidiosis. Furthermore, over the past two decades, bile acids have been demonstrated to be potent regulators in the liver and gastrointestinal tract by activating various cell signaling pathways, G-coupled protein receptors and particular nuclear receptors (Hofmann *et al.*, 2010; Khalafalla *et al.*, 2011).

The test groups significantly influenced sporulation (%) and non-sporulation (%) rates. Doses of sheep bile at 100%, 75% and 50% had the highest un-sporulation rate and the lowest sporulation rate ($p \leq 0.05$). Doses of 25% and 12.5% of sheep bile had the highest non-sporulation and inhibition rates of sporulation ($p \leq 0.05$): the lower non-sporulation rate and the high sporulation inhibition rate.



The statistical significance was compared with 2.5% potassium dichromate as a control.

Fig 8: Main influences of SB on inhibition of *E. magna* and *E. exigua* oocysts *in vitro* at different dosages during 25, 50, 75 and 100 h.



The statistical significance was compared with 2.5% potassium dichromate as a control and 25 µL/mL of toltrazuril.

Fig 9: The influences of sheep bile, at varying doses, on the *in vitro* inhibition of the vitality of *E. magna* and *E. exigua* sporozoites after 12 and 24 hours.

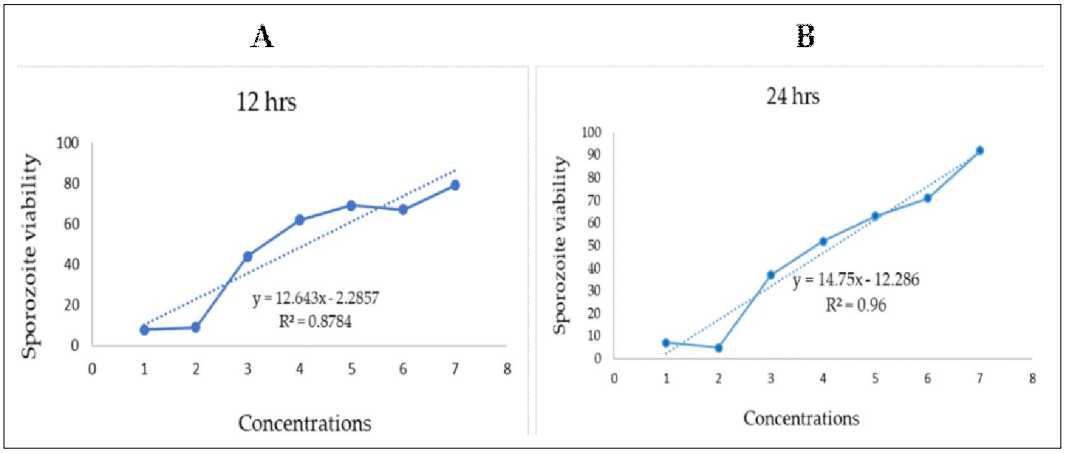


Fig 10: The influences of sheep bile, on the *in vitro* inhibition of the vitality of *E. magna* and *E. exigua* sporozoites after 12 and 24 hours. P≤0.005.

The anti-sporozoite activity of sheep bile

Fig 9 shows the sporozoite viability inhibitory percentage of bile on *E. magna and E. exigua* as a function of concentration, incubation time and standard producer type. It follows from the analysis of this figure that, for bile concentrations, an increase in concentration has enhanced its efficacy. Thus, inhibition rates significantly increased when concentration was increased. The bile, therefore, has the potential to perform better at 1000 µg/mL and probably at higher concentrations. According to our results, most concentrations, including the infusion concentration, exhibited anti-sporozoite activities against *E. magna* and *E. exigua*. In all the *Eimeria* species, 1000 µg/mL of bile concentration showed the highest inhibitory effect at all concentrations compared to the least concentrated (the less active). It also appears that there was an increase in inhibition rate with an increase in incubation time. A high concentration of bile restricted the viability percentage by 83% for *E. intestinalis*. As the concentration of bile decreased, the viability inhibition percentage also decreased accordingly (Fig 9).

The sporozoite viability inhibitory percentage of the *E. magna* and *E. exigua* oocysts decreased with increasing concentrations. The sporozoite viability percentage was highly negatively correlated with increasing exposure periods (R= -0.94, P≤.0005; R= -0.97 and P≤.0006) at 12 and 24 h, respectively (Fig 10 A, B). While there were no significant differences at 100% concentration between SB and the reference drug. Our results support another study on the inhibitory effect of *Curcuma longa* on the activity of *Eimeria tenella* sporozoites and chicken bile secretions on the movement of *Eimeria papillate* (Schubert *et al.*, 2005; Murshed *et al.*, 2022). Schubert and coworkers found that extracellular calcium and Ca^{2}+ signaling are required for the invasion of *E. tenella* sporozoites and their penetration into host cells (Sárközi *et al.*, 2007). Evidence shows that extracts can activate and desensitize calcium channels (Murshed *et al.*, 2023). This corroborates the findings of the research conducted by Cedric *et al.* (2018). Macrophylle extracts that may influence calcium-mediated signaling in the sporozoites, in turn contributing to the observed decrease in sporozoite vitality. In addition, poultry bile against. papillate oocysts of mice may contribute to the observed inhibition in oocyst sporulation (Quiroz-Castañeda and Dantán-González, 2015).

CONCLUSION

Our results demonstrate sheep bile's ability to suppress coccidian oocyst sporulation and antisporozoite actions *in vitro*. Further research is required to demonstrate the efficacy of sheep bile against *E. magna* and *E. exigua-induced* hepatic infections *in vivo* as well as to develop safe, effective compounds that can be synthesized from low-cost natural material.

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Conflict of interest

There are no conflicts of interest between the authors.

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