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In vitro Study: Efficacy of *Artemisia monosperma* Leaves Extract on *Eimeria papillata*

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ABSTRACT

Background: Traditional medicine has long utilized medicinal plants that contain a rich source of biologically active phytochemicals. Many of these have antioxidant and anti-inflammatory properties, which can reduce the risk of many diseases. Numerous animals are afflicted by the disease coccidiosis, which causes significant economic losses. Drug abuse and overuse have led to the emergence of drug-resistant strains of *Eimeria* spp.

Methods: In this study, *Artemisia monosperma* leaf methanolic extract (AMLE) was prepared and tested in vitro as an anticoccidial using the oocyst of *Eimeria papillata*.

Result: Fifteen different functional groups were present in AMLE using infrared spectroscopy. Also, quantitative results showed phenolics and flavonoids 39.7±0.3 and 33.4±0.2 (mg TAE/g DW) respectively in AMLE. Moreover, at 96 h, significant inhibition of process sporulation for *E. papillata* oocyst was observed when exposed to AMLE (300 mg/mL) and formalin 5%, while amprolium, DettolTM and phenol showed different levels of inhibition. Our results showed that AMLE has anticoccidial activity, which promotes the conduct of numerous *in vivo* research to discover an efficient treatment.

Key words: Anticoccidial activity, *Artemisia monosperma*, *Eimeria papillata*.

INTRODUCTION

Infection with apicomplexan parasites of the genus *Eimera* causes coccidiosis in chickens (Chapman *et al*., 2013; Witcombe and Smith, 2014) and ruminants (Khodakaram and Hashemnia, 2017). In ruminants, *Eimeria* sp. leads to poor weight, reduced production and increased mortality in younger stock. Infectious and parasitic illnesses like pneumonia and helminthosis can also made worse (Kanyari *et al*., 1993; Kusiluka *et al*., 1998; Etsay *et al*., 2020).

Globally, this disease has a significant impact on poultry production, resulting in annual economic losses of about US$ 3 billion (Noack *et al*., 2019; Blake *et al*., 2020). It affects the digestive system of the host, which develops in the small and large intestines (Dakpogan *et al*., 2019). It is characterized by diarrhea, dehydration, fluid loss, malabsorption of nutrients, inflammation, oxidative stress and heightened susceptibility to bacterial pathogens such as necrotic enteritis in chickens (Alnassan *et al*., 2014). Due to their negative effects on broilers' final body weight, intestinal health and meat quality, microbial infections significantly affect chicken productivity (Mohamed *et al*., 2021; Swelum *et al*., 2021; Yaqoob *et al*., 2021). Therefore, antibiotics repressed and inhibited microorganisms until the emergence of antibiotic-resistant bacteria (Alagawany *et al*., 2021; Reda *et al*., 2021). This issue is typically resolved by the use of phytogenic substances, such as phenolic compounds in plant extracts (Abou-Kassem *et al*., 2021; El-Saadony *et al*., 2021a; Saad *et al*., 2021).

Many factors aid in the development of coccidiosis, including fecal-oral transmission, high oocyst reproductive potential, the presence of resistant oocysts, the direct life cycle and favorable environmental circumstances for infectivity (sporulation). Since 1939, many ionophore anticoccidial and chemical feed additives have been utilized to combat these potentially harmful parasites in chickens (Nogueira *et al*., 2009). However, drug resistance has emerged (Abbas *et al*., 2011; Hema *et al*., 2015). As a result of this, plant extracts are currently being assessed as viable, sustainable alternatives to new medications.

Recently, there has been a global interest in adopting herbal remedies as secure substitutes to treat different ailments with a reduced danger of the emergence of resistance (Abd El-Hack *et al*., 2020; Abdelnour *et al*., 2020; Ashour *et al*., 2020). Antiprotozoal activity in more than 1200 plants has been reported (Willcox and Bodeker, 2004; Muthamilselvan *et al*., 2016).

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The Arabian Peninsula boasts a rich variety of plants and traditional medical practices centered on foods, spices and plants have been widely used in this region (Maideen, 2020). The Kingdom of Saudi Arabia represents one of the most biodiverse regions on the Arabian Peninsula due to its wide territory and diverse climatic and geographical circumstances (Almehdar *et al*., 2012). There are numerous significant crops and medicinal plants that are traditionally used for medical purposes among Saudi Arabia's flora, such as *A. monosperma, Acacia arabica, Artemisia judaica, Calligonum comosum, Lantana camara, Pulicaria glutinosa*, *etc*. (Ullah *et al*., 2020).

Artemisia is a genus of shrubs and small herbs that includes more than 500 species, which are found in North America, Europe and Asia (Bora and Sharma, 2011). *A. monosperma* is a common desert plant that is used extensively in Saudi Arabian folk medicine to treat muscle spasms, hypertension and parasitic worms (Amin *et al*., 2019). This plant's extracts and essential oils have reportedly demonstrated a range of biological properties, including antibacterial, antimalarial, antioxidant and insecticidal effects (Stavri *et al*., 2004; Stavri *et al*., 2005 and Guetat *et al*., 2017). Also, the medicinal importance of *A. monosperma* is attributed to its anticancer, antispasmodic and antihypertensive properties (Abu-Niaaj *et al*., 1996; Hijazi and Salhab, 2010 and Al-Soqeer, 2011).

Our study's main objective is to assess the anticoccidial activity of *Artemisia monosperma* leaf extract (AMLE) against oocyst sporulation (*Eimeria papillata*) *in vitro*.

MATERIALS AND METHODS

Between January 2023 and August 2023, this experiment was finished in the Zoology Department of King Saud University's College of Sciences. The leaves of the wild plant *A. monosperma* were obtained from Tabuk (Saudi Arabia) and a taxonomist from the Botany and Microbiology Department at the College of Science, at King Saud University (Saudi Arabia) confirmed the plant's botanical identity. Plant specimens were gathered and they were air-dried at room temperature. It was then sieved, ground in an electric mill and stored for extraction.

The method Dkhil (2013) suggested was used to prepare the crude extract. Samples (100 mg) were drenched in 1000 L of 70% methanol for 24 hours at 4°C and kept in a laminated bottle with a 1500 L capacity offered with secure stoppers and overnight shaking. The resulting extract was dried and concentrated in a Japanese Yamato RE300 rotary vacuum evaporator. The resulting crude extract was kept in the deep freezer until to perform various experiments.

KBr pellet method, the plant leaf extract was analyzed using a NICOLET 6700 (Thermo Scientific, Waltham, USA) spectrometer FT-IR with a 400-4000 cm^{-1} range (Abu Hawsah *et al*., 2023).

The phenolic content overall of AMLE was decided by the method according to Singleton *et al*. (1999), with a few modifications. To create a standard curve (25-150 µg/mL), gallic acid solutions were used. Briefly, Folin-Ciocalteu reagent (0.1 mL), ultrapure water (Milli-Q) (1.5 mL) and gallic acid or 0.1 mL of the plant extract (1 mg/mL) were mixed and left for 8 min., then a 20% sodium carbonate (0.3 mL) solution was blended and mixed by a vortex in darkness for 2 h and the mixture was incubated. The absorbance of the resulting blue color was measured with a UV-visible spectrophotometer at 765 nm. Utilizing the equation based on the calibration of the curve, the extracts' overall content of phenolics was calculated as gallic acid equivalent (mg/g DW):

$$y = 0.005 - x - 0.0088$$

Where,

(y) = Absorbance.

(x) = Gallic acid equivalent concentration (mg/g).

The total flavonoids in AMLE were determined using a method reported by Ordonez *et al*. (2006). Briefly, a 2% $AlCl_3$ (1.0 mL) water solution was mixed with 1.0 mL of plant extract (1 mg/mL). At 420 nm, absorbance was measured following an hour of incubation at room temperature. 50-800 g/mL of quercetin solution was used to prepare the standard solution and create a standard curve (R2=0.9996). Using the equation for the calibration curve:

$$y = 0.0011\times + 0.0928$$

Where,

y = Absorbance.

x = Quercetin equivalent concentration (mg/g).

The flavonoids in the extracts were expressed as quercetin (mg/g DW).

From the feces of mice that had caught the infection on their own, *E. papillata* oocysts were isolated.

The non-sporulated oocysts (1×10^5) were incubated in each group, which contained the following: a positive control group (5 ml of 2.5% potassium dichromate), distilled water (5 ml) as the negative control group, a treatment group (5 ml of potassium dichromate 2.5%) with one of the following: AMLE (50, 100, 200 and 300 mg/mL), amprolium (8.3 mg) (Veterinary Agriculture Products Company [VAPCO], Jordan), phenol (25 µL), Dettol TM (109 µL) and formalin (5%). A compound Olympus microscope (Olympus Co., Tokyo, Japan) was used to examine, photograph and track oocyte sporulation. For each treatment, three replicates were carried out and all Petri dishes were incubated for 72 and 96 h at 25 to 29°C (Gadelhaq *et al*., 2018). The sporulation % and sporulation inhibition percentage were computed following Daiba *et al*. (2022) and Cedric *et al*. (2018), respectively.

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

$$\text{Sporulation (Sp) inhibition percentage} = \frac{\text{Sp \% of control - Sp \% of extract}}{\text{Sp \% of control}} \times 100$$

One-way analysis of variance (ANOVA) was used to examine the data in Sigma Plot ® version 11.0 (Systat Software, Inc., Chicago, IL, USA). Differences between groups were considered significant at p-value≤0.01.

RESULTS AND DISCUSSION

Infrared spectroscopy

Data in Table 1 show that the major bands of FT-IR were used to analysis of AMLE. at 3383.5 7cm^{-1}, 2930.29 cm^{-1}, 2231.66 cm^{-1}, 1653.48 cm^{-1},1598.80 cm^{-1}, 1513.75cm^{-1}, 1495.72 cm^{-1}, 1435.09 cm^{-1}, 1361.44 cm^{-1}, 1282.76 cm^{-1}, 1160.39 cm^{-1}, 1123.21 cm^{-1}, 1074.98 cm^{-1}, 820.68 cm^{-1} and 613.27 (Fig 1 and Table 1). N-H stretching was indicated by the band at 3383.57 cm^{-1} confirming the presence of aliphatic primary amine. The band at 2930.29 cm^{-1} implied C-H stretching in the presence of alkane. C=N stretching at 2231.66 cm^{-1} confirms the presence of nitrile. The band at 1653.48 cm^{-1} corresponds to C=N stretching in the presence of conjugated imine/oxime. N-H bending at the band 1598.80 cm^{-1} confirmed the presence of amine. The band at 1513.75 cm^{-1} implied (N-O stretching) for the presence of nitro compound and the band at 1495.72 cm^{-1} corresponds to C-H bending for the presence of alkane. 1435.09 cm^{-1} (O-H bending), 1361.44 cm^{-1} (S=O stretching), 1282.76 cm^{-1} (C-O stretching), 1160.39 cm^{-1} (C-O stretching), 1123.21 cm^{-1} (C-O stretching), 1074.98 cm^{-1} (C-O stretching), 820.68 cm^{-1} (C=C bending) and 613.27 cm^{-1} (C-I stretching) assigned to carboxylic acid, sulfonate, aromatic ester, ester, tertiary alcohol, primary alcohol, alkene and halo compound respectively (Table 1).

Essential oils and plant extracts have gained attention as possible anticoccidial agents in the poultry sector, where coccidial prevention and control are top priorities (Remmal *et al*., 2011; Orengo *et al*., 2012) because they lead to production losses, treatment costs and disease prevention (Quiroz-Castañeda and Dantán-González, 2015). Some factors contribute to the development of coccidiosis, including the presence of resistant oocysts, elevated oocyst reproductive potential and conducive environmental factors for infection (sporulation) (Remmal *et al*., 2011). To effectively manage this parasite, it is essential to block the sporulation process (Mai *et al*., 2009). In addition to increasing the risk of coccidiosis, it can also increase the risk of other contagious diseases and parasitic like pneumonia and helminthosis (Kusiluka *et al*., 1998; Etsay *et al*., 2020). This study's goal was to assess the anticoccidial efficacy *in vitro* of various AMLE doses.

Table 1: FT-IR for *Artemisia monosperma* leaves extract.

Absorption (cm^{-1})	Transmittance (%)	Appearance	Group	Compound class
3383.57	15.30568	Medium	N-H stretching	Aliphatic primary amine
2930.29	3.492565	Medium	C-H stretching	Alkane
2231.66	13.92974	Weak	C≡N stretching	Nitrile
1653.48	3.929176	Medium	C=N stretching	Imine/oxime
1598.80	3.8064	Medium	N-H bending	Amine
1513.75	5.49941	Strong	N-O stretching	Nitro compound
1495.72	5.700059	Medium	C-H bending	Alkane
1435.09	4.588077	Medium	O-H bending	Carboxylic acid
1361.44	4.568289	Strong	S=O stretching	Sulfonate
1282.76	4.530319	Strong	C-O stretching	Aromatic ester
1160.39	5.486414	Strong	C-O stretching	Ester
1123.21	5.430024	Strong	C-O stretching	Tertiary alcohol
1074.98	5.346093	Strong	C-O stretching	Primary alcohol
820.68	9.147205	Medium	C=C bending	Alkene
613.27	9.240555	Strong	C-I stretching	Halo compound

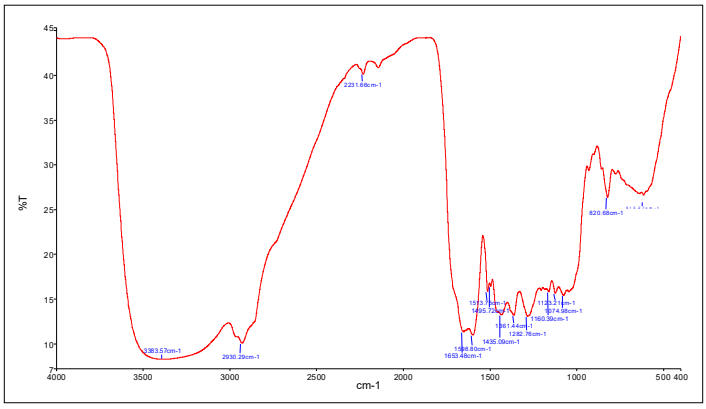


Fig 1: FT-IR of AMLE displays the material's functional properties.

Total phenolics and flavonoid

The amounts of some secondary metabolites in the AMLE were measured, like phenolics and flavonoids. Fig 2 shows that the phenols concentration (39.7±0.3) was high compared to the flavonoids concentration (33.4±0.2). Flavonoids, alkaloids, tannins and phenolic compounds are the most important plant bioactive compounds (Mehmood *et al*., 2015). Several studies have shown that plant extracts consisting of phenolic compounds have inhibitory properties. Components of natural polyphenolic components derived from medicinal plants *in vitro* have been shown to inhibit *E. tenella* sporozoite cell invasion (Arlette *et al*., 2019). These researchers also noted that extracts with compounds of polyphenolic may have the power to inhibit the enzymes necessary for the coccidian oocysts' sporulation process. Some flavonoids affect host-parasite interactions, whereas others disrupt protozoan parasite development or metabolism (Kerboeuf *et al*., 2008), including *Leishmania* sp. and *Trypanosoma* sp. (Fotie, 2008).

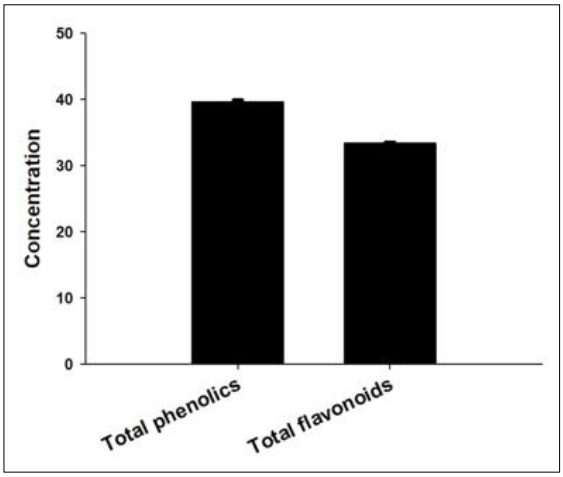
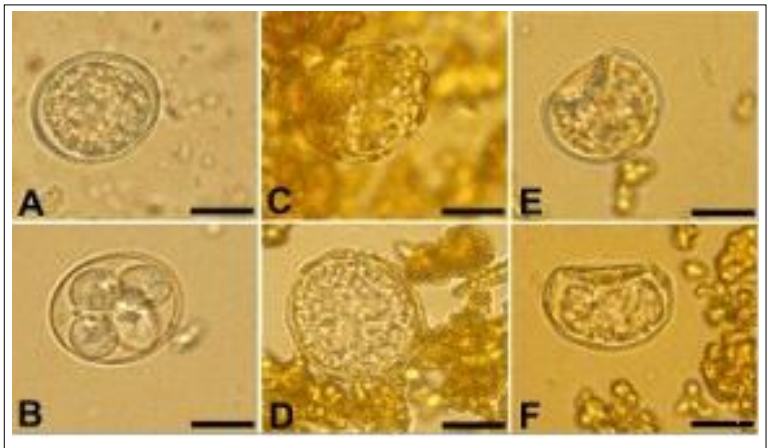


Fig 2: Flavonoids and total polyphenols in the leaves methanolic extract of the *Artemisia monosperma* plant.

Oocyst sporulation

In vitro tests on AMLE and a few other materials revealed sporulation of the oocyst (%) and sporulation inhibition (%) at 72 and 96 h for *E. papillata*. Also, a few changes such as oocyst wall deformation and nuclear material distortion, were noted in oocysts treated with 300 mg/ml AMLE (Fig 3). A significant degree of oocyst sporulation (%) in distributed H_2O was found to be (66.6%) when compared to the AMLE, which had sporulation levels of 0%, 18.83%, 37.04% and 47.5% at 72 h (Fig 4), while at 96 h, were 0%, 67.1%, 78.57% and 81.2% at concentrations of 300, 200, 100 and 50 mg/mL, respectively (Fig 5). Also, the rate of sporulation (%) varied in each of the Dettol™, phenol and formalin 5% were 23.08%, 7.7% and 0%, respectively, at 72 h (Fig 5), while at 96 h, were 18.67%, 10.67% and 0%, respectively (Fig 6). On the other hand, the highest sporulation inhibition (100%) was obtained for AMLE at a concentration of 300 mg in 72 and 96 h (Fig 6 and 7). While the levels of sporulation inhibition for amprolium, Dettol™, phenol and formalin were 37. 33%, 81.33%, 89.33% and 100%, respectively, at 96 h (Fig 7) and at 72 h it was 34.61%, 76.92%, 92.30% and 100% (Fig 6). The findings of this experiment demonstrated that the AMLE ethanol leaf extract has an *in vitro* anticoccidial effect on unsporulated oocysts of *E. papillata* in a concentration-dependent manner, which is attributable to numerous bioactive phytochemical constituents studied (Abdulrahman *et al*., 2023). Likewise, the methanolic extract for AMLE significantly prevented the growth of Blastocystis *in vitro* and changes in Blastocystis shape under the influence of the extract were observed. This may be attributed to Artemisinin has also been shown to affect the operation of oocyst wall construction, resulting in a deficient oocyst wall, mortality of developing oocysts and a decrease in



(A) Normal unsporulated oocysts in H_2O; (b) Normal sporulated oocysts in potassium dichromate; (C-F) Abnormal oocytes in the AMLE (300 mg/mL). Scale bar= 12.5 µm.

Fig 3: Changes that appear on oocytes (*E. papillata*) when exposed to various treatments.

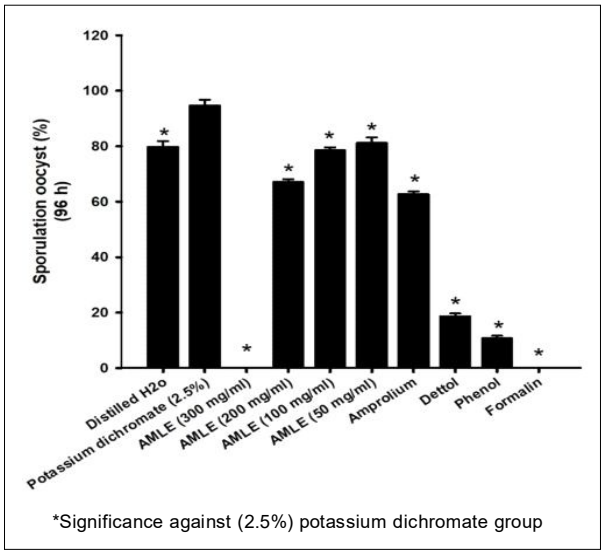


Fig 4: Anticoccidial effects of AMLE on the percentage sporulation of *E. papillata* oocysts at 72 h.

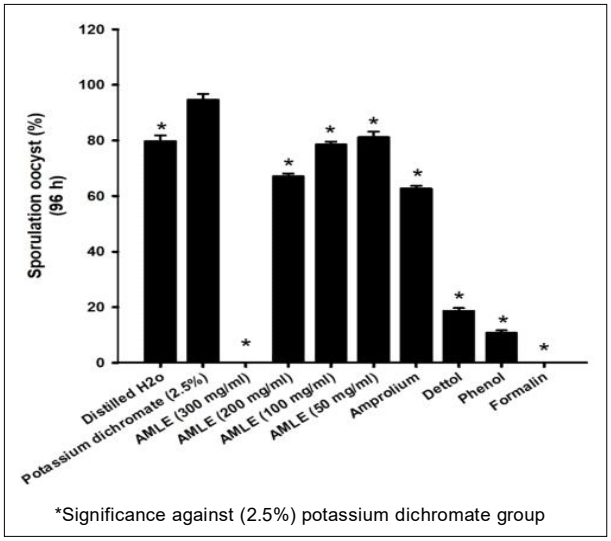


Fig 5: Anti-coccidial effects of AMLE on the sporulation percentage of *E. papillata* oocysts at 96 h.

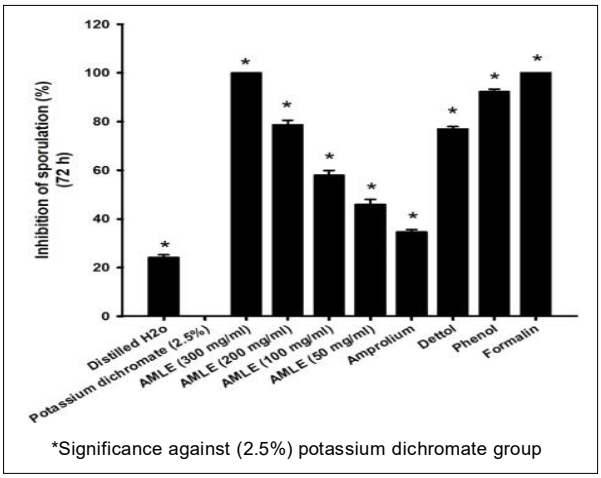


Fig 6: AMLE's anti-coccidial effects on the inhibition of *E. papillata* oocysts sporulated (%) at 72 h.

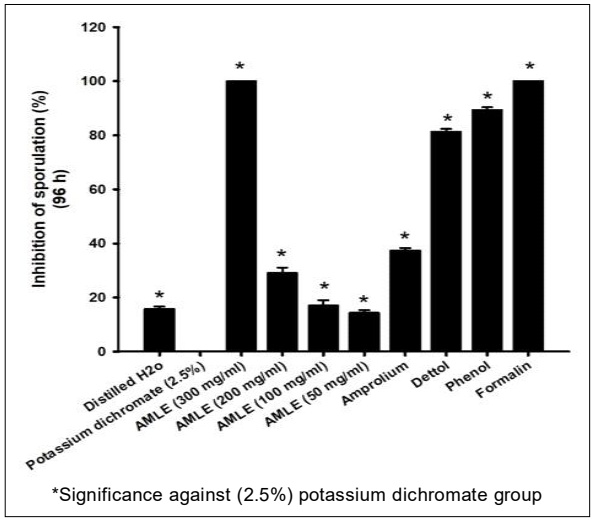


Fig 7: Anticoccidial effects of AMLE on the inhibition of *E. papillata* oocysts sporulated (%) at 96 h.

sporulation rate (Del *et al*., 2010). This alteration is produced by a decrease in the expression of SERCA (Sarco/endoplasmic reticulum calcium ATPase) in macrogametes, which plays a role in calcium homeostasis by influencing the production of wall-forming bodies, a calcium-mechanism dependent (Del *et al*., 2010). Furthermore, the disinfectant formalin (5%) prevented the sporulation of *E. papillata*, which coincided with Thagfan *et al*. (2020) and Abu Hawsah *et al*. (2023). This extremely reactive compound is said to interact with proteins in vitro and hinder sporulation. In addition, Dettol™ and phenol have been reported to prevent sporulation at 96 h by 81.33% and 89.33%, respectively, which is consistent with Mai *et al*. (2009), Gadelhaq *et al*. (2018) and Abu Hawsah *et al*. (2023) who found that the oocyst wall is impermeable to water-soluble substances and resistant to proteolysis.

CONCLUSION

It could be concluded that AMLE has anticoccidial and anthelmintic efficacy *in vitro*. More research should be done to determine the *in vivo* effectiveness of AMLE. This will help guide ongoing studies into the development of AMLE as a novel medication that can be used to treat coccidian infections that affect animals.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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