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Efficacy of *Teucrium polium* leaves extract as anticoccidial and anthelmintic: *In vitro* study

[*Eficácia do extrato das folhas de Teucrium polium como anticoccidiano e anti-helmíntico: Estudo in vitro*]

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ABSTRACT

Natural sources are microbiological species and medicinal plants, which could be potential new sources for development of drugs against different diseases. Coccidiosis affects many animals and leads to great economic losses. Drug-resistant strains of *Eimeria* species have emerged because of overuse and misuse of drugs. *In vitro*, using *Eimeria papillata* oocyst and earthworm (*Eisenia fetida*), we evaluated the anticoccidial and anthelmintic effect of *Teucrium polium* leaves extract (TPLE). Using infrared spectroscopy showed the presence of thirteen compounds for TPLE. Mebendazole (10 mg/mL) caused paralysis and earthworm death by 13.91±0.373 and 18.2±0.980 min, respectively, while, for TPLE (100 mg/ml) were 4.23±0.077 and 4.817±0.386 min. Also, the histological study revealed obvious surface architecture abnormality for treated worms. Moreover, TPLE (300 mg/mL) and formalin (5%) at 72, 96, and 120 hrs led to inhibition of sporulation by approximately 100% with marked deformities, while TPLE (200, 100, 50, and 25 mg/mL), amprolium, Dettol^{TM}, and phenol at 120 hr approximately 65.9%, 23.6%, 4.8%, 3.2%, 12.6%, 68.4%, and 46.6%, respectively. This pilot investigation revealed that TPLE possesses anticoccidial and anthelmintic activity, encouraging additional testing *in vivo* to create a new medication for the treatment of coccidiosis and helminthiasis.

Keywords: *Teucrium polium*, coccidiosis, helminthiasis, treatment

RESUMO

As fontes naturais são espécies microbiológicas e plantas medicinais, que podem ser novas fontes potenciais para o desenvolvimento de medicamentos contra diferentes doenças. A coccidiose afeta muitos animais e causa grandes perdas econômicas. Cepas resistentes a medicamentos de espécies de Eimeria surgiram devido ao uso excessivo e indevido de medicamentos. In vitro, usando oocistos de Eimeria papillata e minhocas (Eisenia fetida), avaliamos o efeito anticoccidiano e anti-helmíntico do extrato das folhas de Teucrium polium (TPLE). O uso da espectroscopia de infravermelho mostrou a presença de treze compostos para o TPLE. O mebendazol (10mg/mL) causou paralisia e morte da minhoca em 13,91±0,373 e 18,2±0,980 minutos, respectivamente, enquanto que para o TPLE (100mg/ml) foram 4,23±0,077 e 4,817±0,386 minutos. Além disso, o estudo histológico revelou uma anormalidade óbvia na arquitetura da superfície dos vermes tratados. Além disso, o TPLE (300 mg/mL) e a formalina (5%) em 72, 96 e 120 horas levaram à inibição da esporulação em aproximadamente 100% com deformidades marcantes, enquanto o TPLE (200, 100, 50 e 25 mg/mL), o amprólio, o DettolTM e o fenol em 120 horas foram de aproximadamente 65,9%, 23,6%, 4,8%, 3,2%, 12,6%, 68,4% e 46,6%, respectivamente. Essa investigação piloto revelou que o TPLE possui atividade anticoccidiana e anti-helmíntica, incentivando testes adicionais in vivo para criar um novo medicamento para o tratamento de coccidiose e helmintíase.

Palavras-chave: Teucrium polium, coccidiose, helmintíase, tratamento

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INTRODUCTION

Intestinal coccidiosis is one of the most important parasitic diseases affecting many animals (Mehlhorn, 2014), which is caused by a protozoan parasite belonging to the genera *Eimeria* species that develops in the small and large intestines and has devastating effects on the younger animals (Kanyari *et al*., 1993; Bakunzi *et al*., 2010; Dakpogan *et al*., 2019). ubclinical manifestations are often associated with poor weight gain, reduced production, and increased mortality in younger animals (Khodakaram-Tafti and Hashemnia, 2017; Macedo *et al*., 2019). Coccidiosis could facilitate the occurrence of other parasitic diseases, such as pneumonia and helminthiasis (Kanyari *et al*., 1993; Kusiluka *et al*., 1998; Etsay *et al*., 2020).

The direct life cycle, fecal-oral transmission, presence of resistant oocysts, lack of cross-protection between *Eimeria* species, high oocyst reproductive potential, high stocking density, and conductive environmental factord for infectivity (sporulation) are effective factors that facilitate the development of coccidiosis (Remmal *et al*., 2011). Therefore, sporulation process disruption is a crucial area where this parasite can be managed (Mai *et al*., 2009). Many drugs are used as coccidiostats (such as toltrazuril, diclazuril, decoquinate, amprolium, and sulfonamide), and these can be either administered orally or through feed and water (Odden *et al*., 2019). However, because of the excessive use and misuse of drugs, have led to the emergence of drug-resistant strains of *Eimeria* species (Hema *et al*., 2015). As a result, developing new drugs from medicinal plants is a potentially sustainable alternative, because they have anti-bacterial and anti-parasitic properties (Cobaxin-Cardenas, 2018), less harmful, and have fewer side effects than conventional chemical agents (Wunderlich *et al*., 2014).

Teucrium is a genus that belongs to the family Lamiaceae. This family is composed of species with exploitable antioxidant activity (Couladis *et al*., 2003). *Teucrium polium* (Labiatae) is a wild-growing flowering plant and is found in southwestern Asia and Europe (Bukhari *et al*., 2015), also it found in the Mediterranean region. The biological activities of *T. polium* are widely reported, and it has been shown to possess anti-inflammatory, antinociceptive, anti-bacterial, anti-hypertensive, hypolipidemic, anti-rheumatoid, and hypoglycemic effects (Tariq *et al*. 1989; Rasekh *et al*. 2001; Abdollahi *et al*. 2003), antioxidant (Couladis *et al*., 2003; Ilhami *et al*., 2003), anticancer (Eskandary *et al*., 2007; Panovska *et al*., 2007), antibacterial (Sarac and Ugur, 2007), antiviral (Alwan *et al*., 1988), and antiparasitic against *Acanthamoeba castellanii* (Tepe *et al*., 2012).

Our research is mainly focused on the *in-vitro* evaluation of the anticoccidial activity of *Teucrium polium* leaves extract (TPLE) against oocyst *Eimeria papillata* sporulation, in addition, to its anthelmintic activity.

MATERIALS AND METHODS

Leaves of *Teucrium polium* were collected from Tabuk, Saudi Arabia. A taxonomist at the Department of Botany and Micriobiology, College of Science, King Saud University (Saudi Arabia), confirmed the identification of the plant. The 150g of leaves were air-dried at 40°C, powdered, and then extracted with 50% ethanol for 24 hr at 4°C. According to Dkhil (2013), the resulting *T. polium* leaves extract (TPLE) was concentrated and dried in a rotary vacuum evaporator (Yamato RE300, Japan).

An excess of potassium bromide powder (1:99 wt%) was added to a tiny portion of TPLE, which was then processed to a homogeneous consistency before being finely ground and placed in a die for pellet formation. The instrument used for the study of Infrared (IR) is Thermo Scientific's optical spectrometer NICOLET 6700 Fourier-transform infrared spectroscopy (FT-IR). Maximum absorption was reported in the number of waves (cm^{-1}). Spectra were registered from 4000 – 400 cm^{-1} at 25°C.

The total phenolic contents of TPLE were determined using the technique according to Singleton *et al*. (1999), with some modifications. Generate a standard curve, gallic acid solutions (25–150µg/mL) were used. Briefly, 0.1mL of Folin-Ciocalteu reagent, 1.5mL of ultrapure water (Milli-Q), and 0.1 mL of TPLE or gallic acid were mixed and left for 8 min, then, 0.3mL of sodium carbonate solution (20%) was added and mixed by a vortex in darkness for 2 hr, the mixture was incubated. A UV-visible spectrophotometer was used to measure the

absorbance of the ensuing blue color at 765 nm. Using the equation based on the calibration curve (y = 0.005 − x − 0.0088), the total phenolic content of TPLE was calculated as gallic acid equivalent (mg/g DW), where (y) absorbance and (x) gallic acid equivalent concentration (mg/g).

The total flavonoids in TPLE were determined using a method reported by Ordoñez *et al*. (2006). Briefly, 1.0 mL of 2% $AlCl_3$ water solution was mixed with 1.0mL of leaves extract (1mg/mL). At 420 nm, absorbance was measured following an hour of incubation at room temperature. A quercetin solution (50-800 g/mL) was used to prepare the standard solution and create a standard curve (R2= 0.9996). Using the calibration curve equation (y = 0.0011 x + 0.0928), where (y) is the absorbance and (x) is the quercetin equivalent concentration (mg/g), the flavonoids in TPLE were expressed as quercetin (mg/g DW).

The antioxidant activities of TPLE were determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (Liyana-Pathirana *et al*., 2005). Briefly, 1mL of TPLE was mixed with 1mL of 0.135mM DPPH at various concentrations (31.25–1000 g/mL). The mixture was held at room temperature in the dark for 40 min while being gently stirred. The absorbance of TPLE (sample) and the control solutions (Ascorbic acid as positive control) was measured at 517 nm, and the percentage of DPPH scavenging activity (%) of TPLE was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{Abs\ control\ -\ Abs\ sample}{\text{Abs control}} \times 100$$

where:

Absorbance of DPPH + methanol (Abs control)
Absorbance of the DPPH radical + sample (Abs sample)

A total of 25 earthworms, *Eisenia fetida*, were collected from agricultural lands and identified by a specialist in the College of Food and Agriculture Sciences (King Saud University). Five worms, approximately of equal size were placed in each Petri dish. Albendazole (10 mg/ml) was used as a positive control, and distilled H_2O was used as a negative control. The extract from TPLE was prepared in distilled H_2O at concentrations of 100, 50, and 25 mg/mL. Time for paralysis was recorded when no movement was observed except when shaken vigorously, while the time of death was recorded when the worms did not show any movement by vigorous shaking nor when dipped in warm water (50°C) (Parida *et al*., 2010).

The small parts of the earthworm body were fixed in 10% buffered neutral formalin, then processed for paraffin embedding, and 4 µm thick sections were stained with hematoxylin and eosin (H&E) (Drury and Wallington, 1973). Sections were examined and photographed using a digital camera (DP 73) fitted on an Olympus B×61 microscope (Tokyo, Japan).

Five Swiss albino mice were inoculated with 1×10^3 sporulated *Eimeria papillata* oocysts via oral gavage. On the fifth day of infection, feces were collected, and oocysts were separated by floatation technique and then used for *in vitro* study.

The non-sporulated oocysts (1×10^5) were incubated in 5mL distilled H_2O (negative control), 5mL potassium dichromate ($K_2Cr_2O_7$) 2.5% (positive control), and finally, 5 mL $K_2Cr_2O_7$ (2.5%) containing one of the following: TPLE (300, 200, 100, 50, and 25 mg/mL), 8.3 mg amprolium (Veterinary Agriculture Products Company [VAPCO], Jordan), 109 µl dettol ™, 25 µl phenol, and formalin (5%). Using an Olympus compound microscope (Olympus Co., Tokyo, Japan), sporocysts were examined, photographed, and monitored the oocysts' sporulation. All Petri dishes were incubated for 72, 96, and 120 hr at 25 to 29°C (Gadelhaq *et al*., 2018). The sporulated and non-sporulated oocysts were counted, and the sporulation (%) was estimated according to Daiba *et al*. (2022), as well as the inhibition (%) of sporulation was calculated according to Cedric *et al*. (2018), as follows:

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

$$\text{Inhibition (\%) of sporulation} = \frac{\text{Sporulation of control- Sporulation of extract}}{\text{Sporulation of control}} \times 100$$

$$\frac{\text{Sporulation of control- Sporulation of extract}}{\text{Sporulation of control}} \times 100$$

SigmaPlot® version 11.0 (Systat Software, Inc., Chicago, IL, USA) was used to analyze the data using the one-way analysis of variance (ANOVA), and the findings were presented as mean ± SD. Differences between groups were deemed significant at a *p*-value ≤ 0.05.

RESULTS

The analysis of TPLE using FT-IR showed major bands at 3418.43 cm^{-1}, 2932.09 cm^{-1}, 1731.73 cm^{-1}, 1617.93 cm^{-1}, 1400.41 cm^{-1}, 1256.87 cm^{-1}, 1047.76 cm^{-1}, 874.42 cm^{-1}, and 601.56 cm^{-1} (Figure 1 and Table 1). O-H stretching was indicated by the band at 3418.43 cm^{-1} confirming the presence of alcohol. The band at 2932.09 cm^{-1} implied C-H stretching for the presence of alkane. C=O stretching to 1731.7 $3cm^{-1}$ confirms the presence of an aldehyde. The band at 1617.93 cm^{-1} corresponds to C=C stretching for the presence of conjugated alkene. O-H bending at the band 1400.41 cm^{-1} confirmed the presence of carboxylic acid. The band 1256.87 cm^{-1} (C-O stretching), 1047.76 cm^{-1} (CO-O-CO stretching), 874.42 cm^{-1} (C=C stretching), and 601.56 cm^{-1} (C-I stretching) assigned to an alkane, aromatic ester, anhydride, alkene, and halo compound, respectively (Table 1).

The phenolic and flavonoid contents in the TPLE were determined. Phenolics were found to be 78.89±0.73 mg gallic acid/g of the sample. In addition, flavonoids were found to be 15.77±0.13 mg quercetin/g of sample (Figure 2).

The antioxidant activities of TPLE were determined using free radical scavenging activity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Overall, the scavenging activity against the DPPH radical increased with concentration increases in TPLE, peaking at 500 g/mL, and after that started to decline (Table 2). The results indicated that TPLE showed the highest percentage inhibition value of DPPH radical at 500µg/mL (70.9%).

TPLE was observed to have anthelmintic activity against *E. fetida*. Where the most effective dose, TPLE (100 mg/mL) showed the time to paralysis and death was 4.23±0.077 and 4.817±0.386 min, respectively. While mebendazole showed less effect (13.91± 0.373 and 18.2±0.980 min for paralysis and death time, respectively) (Table 3). In addition, the dermal layers of the worm cuticle in the control group showed no changes in histological sections stained with hematoxylin and eosin, but the vacuolation was significantly observed in the treated group with TPLE as well as the complete destruction of the upper layer with drug treatment (Figure 3).

A significant level of *in vitro* oocyst sporulation in distilled H_2O was observed to be 75.2±1, at 120 hr. There was no sporulation for oocysts incubated in TPLE (300 mg/ml) and formalin at 72, 96, and 120 hr (Figure 4). At 120 hr, TPLE (200, 100, 50, and 25 mg/mL), amprolium, dettol™, and phenol showed varying levels of inhibition by 65.9%, 23.6%, 4.8%, 3.2%, 12.6%, 68.6%, and 46.6%, respectively (Table 4). Some changes were observed for oocysts treated with 300 mg/ml TPLE including wall deformity of the oocyst and distortion of nuclear material.

Table 1. FT-IR for TPLE

Absorption (cm^{-1})	Transmittance (%)	Appearance	Group	Compound class
3418.43	5.045863	strong, broad	O-H stretching	alcohol
2932.09	9.855134	medium	C-H stretching	alkane
1731.73	10.74595	strong	C=O stretching	aldehyde
1617.93	8.555823	medium	C=C stretching	conjugated alkene
1400.41	11.09049	medium	O-H bending	carboxylic acid
1256.87	11.72453	strong	C-O stretching	aromatic ester
1047.76	8.586036	strong, broad	CO-O-CO stretching	anhydride
874.42	18.81779	strong	C=C bending	alkene
601.56	15.57838	strong	C-I stretching	halo compound

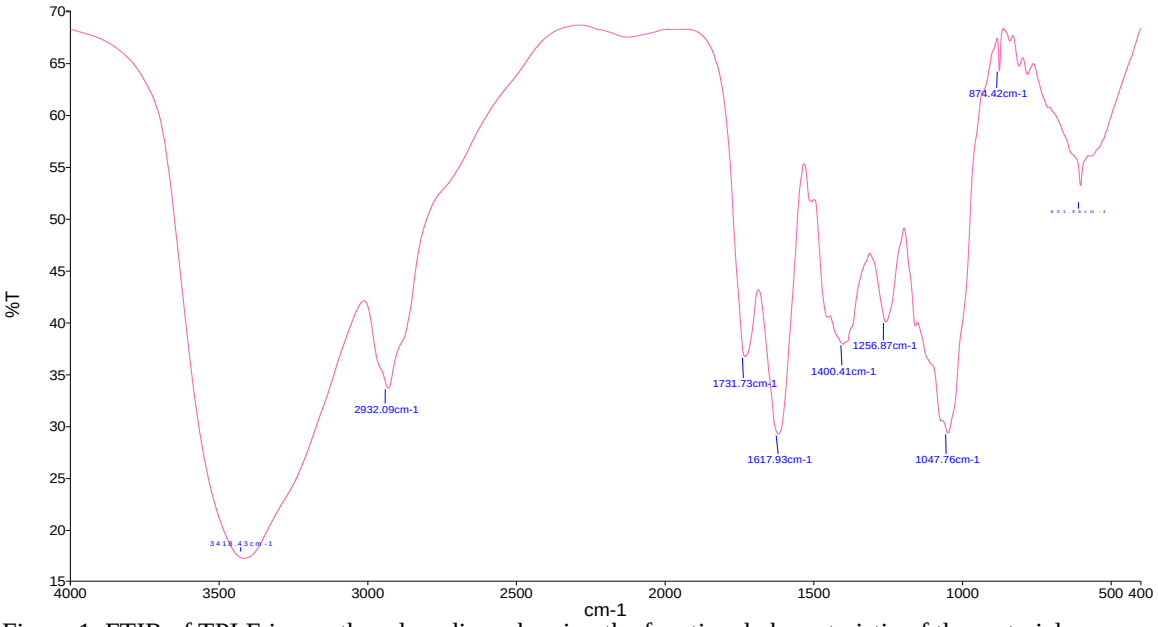

Figure 1. FTIR of TPLE in an ethanol medium showing the functional characteristic of the material.

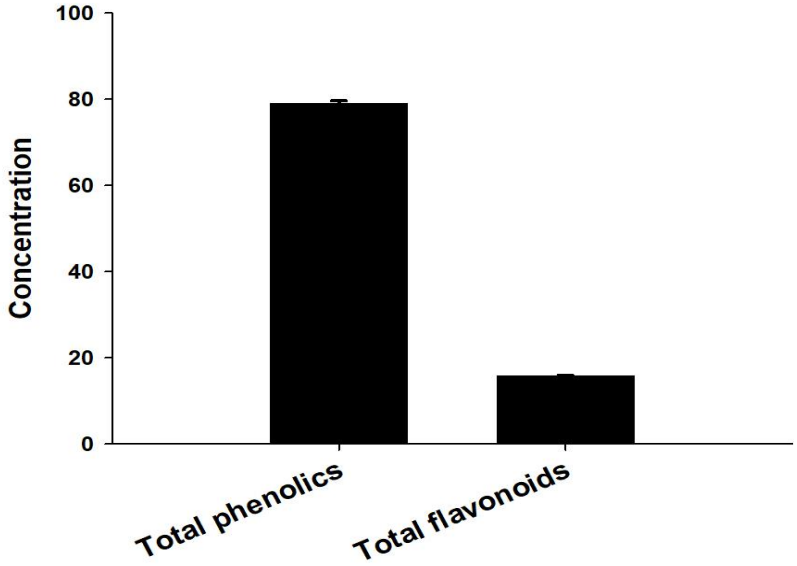

Figure 2. Total polyphenols, flavonoids of the methanolic extract of TPLE.

Table 2. Radical scavenging activity (%) of TPLE

Concentrations (µg/mL)	DPPH Radical Scavenging Activity (%)
31.25	0
62.5	4.2±0.8
125	21.5±1.3
250	51.6±1.5
500	70.9±0.3
1000	79.001±0.6

Values are means ± SEM, n = 3 per treatment group.

Table 3. *In vitro* anthelmintic activity of TPLE

Test samples	Concentration (mg/ml)	Time is taken for paralysis (min.)	Time is taken for death (min.)
Control (H_2O)	--	--	--
TPLE	25 mg/ml	6.365 ± 0.095 $^{*\#}$	8.535 ± 0.0264 $^{*\#}$
	50 mg/ml	6.18 ± 0.187 $^{*\#}$	8.312 ± 0.097 $^{*\#}$
	100 mg/ml	4.23 ± 0.077 $^{*\#}$	4.817 ± 0.386 $^{*\#}$
Mebendazole	10 mg/ml	13.91 ± 0.373 *	18.2 ± 0.980 *

Values are mean ± SD. All superscripts indicate significance at $p \leq 0.05$, * compared to untreated (H_2O), $^{\#}$ compared to mebendazole.

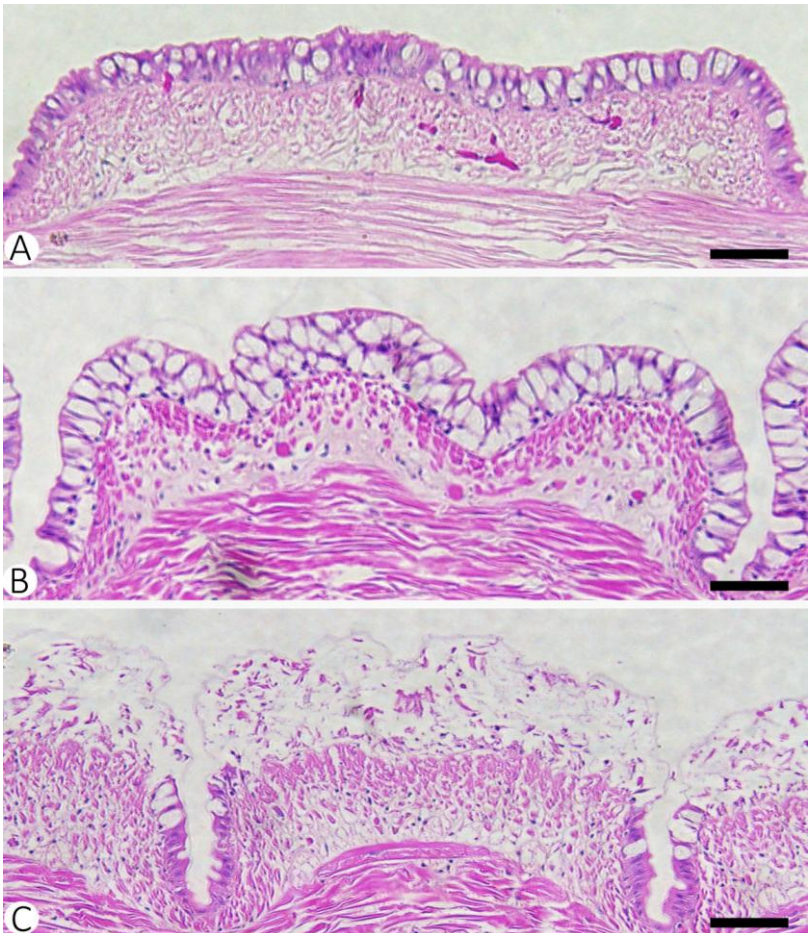

Figure 3. Histological changes in the cuticle of *E. fetida* with various treatments. (A) worms in dist. H_2O (control). (B) worms in TPLE (100 mg/ml). (C) worms in mebendazole. Scale bar = 25µm.

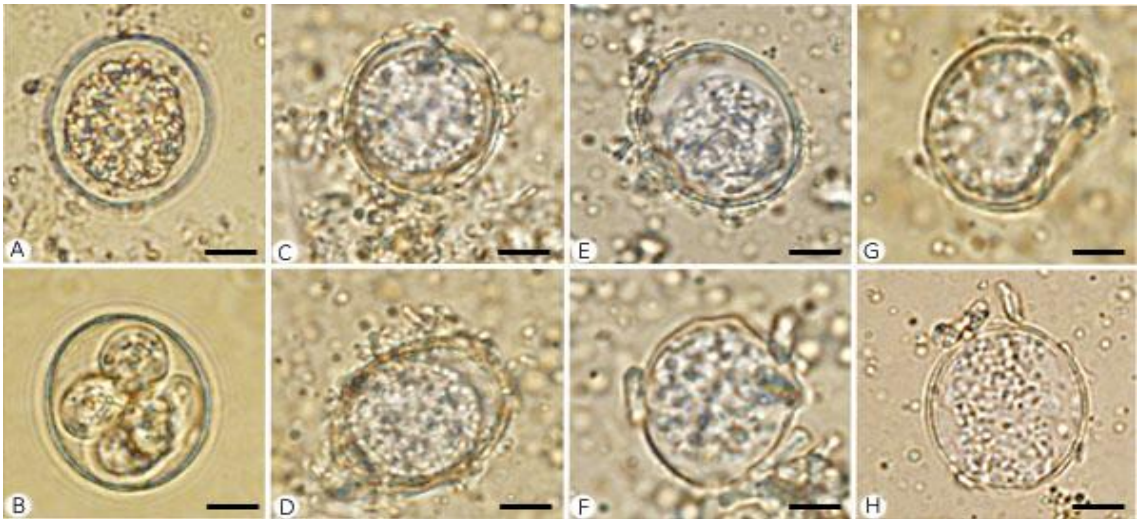

Figure 4. Changes observed after exposure of *E. papillata* oocytes to different treatment. (a) normal non-sporulated oocysts in H_2O; (b) normal sporulated oocysts in $K_2Cr_2O_7$; (c–h) abnormal oocytes in the TPLE (300 mg/mL).

Table 4. *In vitro* anti-coccidial effects of TPLE on the sporulation percentage of *Eimeria papillata* oocysts

Groups	Time	Sporulation of oocyst (%)	Inhibition of sporulation (%)	*p*-value
Distilled H_2O	72 hr	16 ± 2	69.2 ± 2	0.01
	96 hr	23.5 ± 2	72.9 ± 2	0.01
	120 hr	75.2 ± 1	19.1 ±	0.01
Potassium dichromate (2.5%)	72 hr	52.2 ± 2	-	-
	96 hr	86.7 ± 2	-	-
	120 hr	93.2 ± 2	-	-
TPLE (300 mg/ml)	72 hr	0	100 ± 1	0.01
	96 hr	0	100 ± 1	0.01
	120 hr	0	100 ± 1	0.01
TPLE (200 mg/ml)	72 hr	3.9 ± 1	92.4 ± 1	0.01
	96 hr	30.2 ± 1	65.2 ± 1	0.01
	120 hr	31.7 ± 2	65.9 ± 2	0.01
TPLE (100 mg/ml)	72 hr	41.7 ± 1	20.03 ±	0.01
	96 hr	68.7 ± 1	20.7 ± 2	0.01
	120 hr	71.2 ± 1	23.6 ± 1	0.01
TPLE (50 mg/ml)	72 hr	51.5 ± 2	1.4 ± 2	0.01
	96 hr	80.2 ± 1	7.4 ± 1	0.01
	120 hr	88.7 ± 1	4.8 ± 2	0.01
TPLE (25 mg/ml)	72 hr	51.4 ± 1	1.4 ± 1	0.01
	96 hr	81.2 ± 1	6.4 ± 1	0.01
	120 hr	90.2 ± 1	3.2 ± 2	0.01
Amprolium	72 hr	43.6 ± 1	16.5 ± 1	0.01
	96 hr	77.1 ± 1	11.05 ± 1	0.01
	120 hr	81.4 ± 2	12.6 ± 2	0.01
Dettol	72 hr	3.9 ± 1	92.6 ± 1	0.01
	96 hr	26.5 ± 1	69.4 ± 1	0.01
	120 hr	29.2 ± 2	68.6 ± 2	0.01
Phenol	72 hr	25.6 ± 1	50.9 ± 1	0.01
	96 hr	34.7 ± 1	59.9 ± 1	0.01
	120 hr	50 ± 2	46.6 ± 2	0.01
Formalin	72 hr	0	100 ± 1	-
	96 hr	0	100 ± 1	-
	120 hr	0	100 ± 1	-

DISCUSSION

Coccidiosis caused by *Eimeria* parasites affects various animal species and causes considerable economic damage through mortality and reduced weight gains and poor feed efficiencies (Abu Hawsah *et al*., 2023). Previous studies have attempted to determine a solution for this issue. To avoid adverse effects on animal performance, there's a need to develop new agents with minimum side effects against coccidiosis. This study aimed to evaluate the anthelmintic and anticoccidial activities of *T. polium*. Potential anthelminthic medications include ones that harm the parasite but not the host, which causes paralysis of the parasite's musculature either by inhibiting the neuromuscular transition or energy generation enzymes (Andrews *et al*., 1980; Manger, 1991; Veerakumari and Munuswamy, 2000). Also, damage to the body wall allowing rejection or partial digestion by host's immune system (Andrews *et al*., 1980; Cortés *et al*., 2017). It has been proven that benzimidazole and mebendazole affect the energy metabolism of parasites (Kern, 2003).

Our results showed that the concentration (300 mg/ml) of TPLE had anthelmintic efficacy against earthworms compared to Mebendazole, which is attributable to the presence of numerous bioactive phytochemical constituents, this agreed with data of Ali *et al*. (2011) and Boyko and Brygadyrenko (2021). *T. polium* belongs to the family Lamiaceae which contains numerous bioactive phytochemical constituents such as essential oils (Silva *et al*., 2012), tannins, flavonoids, sterols, and saponins (Ali *et al*., 2011), diterpenoids, and iridoids (Fatima, 2016). Saponins from plants sources are responsible for some pharmacological effects like anti-inflammatory (Takagi *et al*., 1980), antimicrobial (Tamura *et al*., 2001), antidiabetic and anticancer (Yuan *et al*., 2010), hypocholesterolemic (Seth *et al*., 2010), antioxidant (Lv *et al*., 2005), anthelmintic, antitussive and cytotoxic activities (Sparg *et al*., 2004). Ali *et al*. (2011) extracted saponin from the plant *T. stocksianum* and found that it has antihelminthic activity. In addition, the aqueous tinctures of *T. polium* showed great activity as a nematicidal effector (Boyko and Brygadyrenko, 2021). Moreover, several studies have indicated that various extracts of *T. polium* exhibit potent antioxidant activity (Couladis *et al*., 2003; Ilhami *et al*., 2003; Ljubuncic *et al*., 2005; Panovska *et al*., 2005; Yazdanparast and Ardestani, 2009).

Our results showed that TPLE (dose-dependent) had a significant effect on the oocyst sporulation of *E. papillata*, which is attributable to numerous bioactive phytochemical constituents studied by Silva *et al*. (2012), Ali *et al*. (2011), and Fatima (2016). *In vitro*, both methanolic extracts of *T. polium* and *T. chamaedrys* showed time- and dose-dependent amoebicidal effects, which led to a decrease in the numbers of viable *Acanthamoeba castellani* trophozoites and cysts (Tepe *et al*., 2012). Regarding the effect of TPLE on the morphology of *Eimeria* oocysts, the deformity of oocysts was observed at a concentration of 300 mg/ml. This result is consistent with a previous study by Abd-Elrahman *et al*. (2022), which reported the effect of natural extracts of allicin and alcoholic garlic on *E. tenella* oocysts in chickens. Also, disinfectant formalin (5%) completely inhibited the sporulation of *E. papillata*, which agreed with Thagfan *et al*. (2020) and Abu Hawsah *et al*. (2023) stated that this highly reactive chemical interacts with proteins *in vitro* and inhibits sporulation. In addition, dettol™, and phenol have been reported to inhibit sporulation at 120 hr by 68.6%, and 46.6%, respectively, which is consistent with Mai *et al*. (2009), Gadelhaq *et al*. (2018), and Abu Hawsah *et al*. (2023) that the oocyst wall is impermeable to water-soluble substances and resistant to proteolysis.

CONCLUSION

This study demonstrated that TPLE has anticoccidial and anthelmintic efficacy, *in vitro*. More research should be done to determine the *in vivo* effectiveness of TPLE. This will inform ongoing studies geared toward the development of TPLE as a novel drug that can be used to manage coccidian diseases that affect animals.

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