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# *In vitro* antiparasitic activity of ethanolic leaves extract of *Anethum graveolens*

[*Atividade antiparasitária* in vitro *do extrato etanólico das folhas de Anethum graveolens*]

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## ABSTRACT

Natural products are safe environmentally friendly agents and have no negative impact on the environment, they can be used to combat parasitic diseases. Helminthiasis and coccidiosis are parasitic diseases that harm both health and the economy. This research aimed to see how *Anethum graveolens* leaves extract (AGLE) worked as an anti-parasitic modulator during oocyst sporulation of an *Eimeria papillata* infection. FT-IR phytochemical analysis revealed the presence of eight compounds. The time required to induce paralysis and death in worms at the highest concentration (200 mg/mL) was 4.57±0.26 and 5.22±0.10 min, respectively. In an *in vitro* study, AGLE (300 mg/ml) inhibited sporulation by approximately 100% after 72 and 96 hr. AGLE (200, 100, and 50 mg/ml), amprolium, Dettol™, and phenol induced variable inhibition levels at 96 hr of 5.54%, 1.01%, 37.33%, 81.33%, and 89.33%, respectively. Our findings suggest that AGLE has potent anthelmintic and anticoccidial properties that could be further developed into a novel therapeutic agent.

Keywords: Eimeriosis, parasite resistance, herbal plants

## RESUMO

*Os produtos naturais são agentes ecologicamente corretos, seguros e não têm impacto negativo sobre o meio ambiente, podendo ser usados para combater doenças parasitárias. A helmintíase e a coccidiose são doenças parasitárias que prejudicam a saúde e a economia. O objetivo desta pesquisa foi verificar como o extrato das folhas de Anethum graveolens (AGLE) funcionou como modulador antiparasitário durante a esporulação de oocistos de uma infecção por Eimeria papillata. A análise fitoquímica FT-IR revelou a presença de oito compostos. O tempo necessário para induzir a paralisia e a morte dos vermes na concentração mais alta (200mg/mL) foi de 4,57±0,26 e 5,22±0,10 minutos, respectivamente. Em um estudo in vitro, o AGLE (300 mg/ml) inibiu a esporulação em aproximadamente 100% após 72 e 96 horas. O AGLE (200, 100 e 50 mg/ml), o amprólio, o DettolTM e o fenol induziram níveis de inibição variáveis de 5,54%, 1,01%, 37,33%, 81,33% e 89,33%, respectivamente, após 96 horas. Nossos resultados sugerem que o AGLE tem propriedades anti-helmínticas e anticoccidianas potentes que poderiam ser desenvolvidas em um novo agente terapêutico.*

*Palavras-chave: eimeriose, resistência do parasita, plantas herbáceas*

## INTRODUCTION

Protozoa of the phylum Apicomplexa, such as *Toxoplasma*, *Plasmodium*, and *Eimeria*, are of great medical and veterinary importance as pathogens that cause various human and veterinary diseases worldwide (Habib *et al*., 2016; Maier *et al*., 2019). One of the most important parasite diseases affecting animals is coccidiosis (Abdel-Gaber *et al*., 2023). It is caused by members of the *Eimeria* genus and has been reported in a variety of species, including chickens, ducks, puppies, rabbits, piglets, dogs, horses, kittens, and birds (Stock *et al*., 2018).

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This disease causes significant economic losses worldwide (Chapman, 2014). It has an asexual and sexual reproduction cycle, and it produces resistant parasite stages known as oocysts that are released into the environment, facilitating the spread of infection. Because of their resistance to environmental factors, controlling *Eimeria* oocysts is difficult (Graat *et al*., 1994). *Eimeria tenella* is one of the most common and dangerous pathogens in the genus *Eimeria*. These parasitic infections cause animals' nutrient uptake to be disrupted, resulting in decreased body weight and increased susceptibility to secondary infections (López-Osorio *et al*., 2020).

Conventional methods for preventing and controlling coccidiosis include anti-coccidian drugs and live vaccines; however, these measures raise concerns about drug resistance, food security, production costs, and species cross-protection (Chapman, 1997; Sharman *et al*., 2010). Therefore, there is an urgent need to seek new ideas and perspectives on coccidiosis prevention and control.

Many plant-derived drugs, such as herbal extracts, have anticoccidial effects (Nweze and Obiwulu, 2009; Youn and Noh, 2011). Anticoccidial herbal medicines typically have fewer drug residues and less drug resistance than chemotherapeutic drugs (Quiroz-Castaneda and Dantan-Gonzáles, 2015). As a result, plant-derived compounds have been developed as an alternative approach to parasitic infection control (Klimpel *et al*., 2011; Amer *et al*., 2015; Elkhadragy *et al*., 2022; Al-Otaibi *et al*., 2023) where, these products have organ-protective properties in *Eimeria*-infected hosts and target parasites (Wunderlich *et al*., 2014).

*Anethum graveolens* (Dill), belonging to the family Apiaceae, is an aromatic plant native to the Mediterranean region that has been widely used as a seasoning in the preparation of various foods (Slupski *et al*., 2005). It is rich in flavonoids, polyphenols, essential minerals, antioxidants, and vital vitamins like riboflavin, folic acid, vitamin A, niacin, vitamin C, and b-carotene (Jana and Shekhawat, 2010). Numerous studies have demonstrated that *A. graveolens* acts against fungi (Kumarasingha *et al*., 2016; Vieira *et al*., 2019), bacteria (Kaur and Arora, 2009), and protozoa (Sahib *et al*., 2014). It has also been utilized in traditional herbal medicine as a diuretic effector and as a treatment for the gastrointestinal disorders (Hosseinzadeh *et al*., 2002).

Given these benefits, the present investigation aimed to evaluate the possible anticoccidial activity of *A. graveolens* leaves extract against *Eimeria papillata* as well as *in vitro* anthelmintic activity.

## MATERIALS AND METHODS

*Anethum graveolens* leaves were collected from the botanical gardens in Riyadh, Saudi Arabia. A taxonomist from the Botany Department (King Saud University, Riyadh, Saudi Arabia) identified and confirmed the plant material. The ethanolic extract of 70% of *A. graveolens* leaves (AGLE) was prepared using the method described by Manikandan *et al*. (2008), with some modifications as follows: air-dried leaves of *A. graveolens* were ground into a powder with an electric blender (Senses, MG-503T, Korea). Dried powder (100 g) of *A. graveolens* leaves was macerated in 70% ethanol for 24 hr at 4 ºC, followed by percolation 5-7 times until complete extraction. Following filtration, ethanol was isolated from extract using a vacuum evaporator at 50 ºC under reduced pressure. The crude extract was lyophilized and stored at -20 ºC until further use.

Fourier-transform infrared spectroscopy (FT-IR) NICOLET 6700 (Thermo Scientific, Waltham, USA) was used for the analysis of the plant extract through the KBr pellet method with a range of 4000 $cm^{-1}$ to 400 $cm^{-1}$, following Al-Quraishy *et al*. (2020).

The phenolic contents of AGLE were determined according to Singleton *et al*. (1999), with some modifications. Briefly, 0.1 mL of Folin-Ciocalteu reagent, 1.5 mL of ultrapure water (Milli-Q), and 0.1 mL of AGLE (1 mg/mL) or gallic acid were mixed and left for 8 min, then, 0.3 mL 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 the extracts was calculated as gallic acid equivalent (mg/g DW),

where (y) absorbance and (x) gallic acid equivalent concentration (mg/g).

The total flavonoids in AGLE were determined using the method of Ordonez *et al*. (2006). Briefly, 1.0 mL of 2% $AlCl_3$ water solution was mixed with 1.0 ml of AGLE (1 mg/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 = 0.0011x + 0.0928), where (y) is the absorbance and (x) is the quercetin equivalent concentration (mg/g), the flavonoids in the extracts were expressed as quercetin (mg/g DW).

The antioxidant activities of AGLE were determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (Liyana-Pathirana *et al*., 2005). Briefly, 1 ml of the extract was mixed with 1 ml of 0.135 mM 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 the samples and the control solutions (Ascorbic acid as positive control) was measured at 517 nm, and the percentage of DPPH scavenging activity of the extracts was calculated using the following equation:

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

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). This study was conducted using three doses of AGLE (50, 100, and 200 mg/ml) against the earthworm by following method of Ajaiyeoba *et al*. (2001). Five worms of roughly the same size were used per dose. As a control, worms in distilled water were used. The reference drug was mebendazole (10 mg/ml). The time it took to reach paralysis and death was measured in minutes (Dkhil, 2013). Before beginning the experiment, all the extracts and drug solutions were freshly prepared.

Small pieces of the earthworm body were removed and fixed in formalin (10%). Following fixation, specimens were dehydrated, embedded in wax, and then sectioned to 5 $\mu$m thicknesses. For histological examinations, sections were stained with hematoxylin and eosin (Drury and Wallington, 1978). Sections were examined and photographed using a digital camera (DP 73) fitted on an Olympus B×61 microscope (Tokyo, Japan).

Five Swiss albino male mice were inoculated with $1\times10^3$ 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 unsporulated oocysts ($1\times10^5$) were incubated at 25-29 ºC for 72 and 96 hs in 5 mL 2.5% potassium dichromate solution $K_2Cr_2O_7$ (positive control), 5 ml distilled $H_2O$ (negative control) and finally in 5 ml $K_2Cr_2O_7$ containing one of the following: AGLE (300, 200, 100, and 50 mg/ml), 109 µl Dettol™, 8.3 mg amprolium (Veterinary Agriculture Products Company [VAPCO], Jordan), 5% formalin and 25 µl phenol. The oocysts' sporulation was monitored by examining sporocysts under an Olympus compound microscope (Olympus Co., Tokyo, Japan). According to Thagfan *et al.* (2020), a total of 100 oocysts were counted in each control and treatment group to estimate the sporulation and inhibition (%) of *E. papillata* oocysts.

Data analysis was performed using SigmaPlot® version 11.0 (Systat Software, Inc., Chicago, IL, USA) and presented as mean ± SD with *p*-value $\leq 0.05$.

## RESULTS

The FT-IR evaluation indicated the presence of 8 expected compounds in AGLE. Some of these compounds have strong appearances including groups with N-O, S=O, C-O, and C-I stretching (Table 1, Figure 1). These expected compounds included nitro compound, sulfonyl chloride, primary alcohol, and halo compound.

Phenolic and flavonoid contents of AGLE were presented in Figure (2). From this Table, it

appears that the concentration of phenolic compounds in the ethanolic extract (1mg/mL) was (32±0.7). while the lowest concentration of flavonoid content was observed in the extract of 23±0.1.

Table (2) showed that the ethanolic extracts had the highest DPPH (65.4±0.9) at the concentration of 1000 µg/mL, while the lowest scavenging percentage (0) was at the concentration of 31.25µg/mL. The ethanolic extracts presented DPPH in a concentration-dependent manner, where the concentrations of the ethanolic extract exhibited good antioxidant properties.

The AGLE had anthelmintic activity against live adult *E. fetida* worms that was comparable to the conventional anthelmintic agent (mebendazole). Table (3) showed that time to paralysis and death were 4.57±0.26 min and 5.22±0.10 min, respectively, for the most effective dose of 200mg/mL. However, compared to the other AGLE concentrations the reference drug mebendazole (10mg/mL) had a lesser effect (Table 3). After receiving AGLE, the cuticle thickness and length of the segment for *E. fetida* was significantly reduced compared to the typical structure for those in water, and upmost layer was destructed in the treated group with mebendazole (Figure 3).

Incubation with AGLE (300 mg/mL) and formalin at 72 and 96 hr inhibited oocysts sporulation by 100% (Table 4). Incubation with AGLE (200 mg/ml), at 72 and 96 hr, inhibited sporulation of *E. papillata* oocysts by 97.52% and 97.09% respectively. At 96 hr, AGLE (100 and 50 mg/mL), amprolium, Dettol$^{TM}$, and phenol induced variable inhibition levels of 5.54%, 1.01%, 37.33%, 81.33%, and 89.33%, respectively (Table 4).

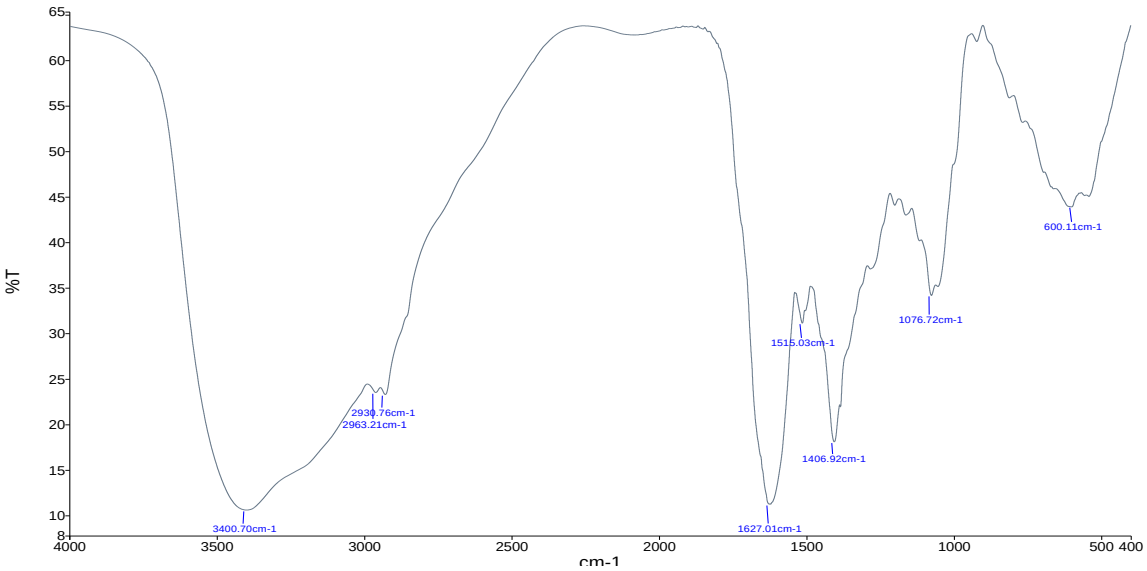


Figure 1. FT-IR of *Anethum graveolens* leaves extract in ethanolic medium showing the functional characteristic of the material.

Table 1. FT-IR for *Anethum graveolens* leaves extract

| Absorption ($cm^{-1}$) | Transmittance (%) | Appearance | Group | Compound class |
|---|---|---|---|---|
| 3400.70 | 21.91108 | medium | N-H stretching | aliphatic primary amine |
| 2963.21 | 19.09228 | medium | C-H stretching | alkane |
| 2930.76 | 18.8832 | medium | C-H stretching | alkane |
| 1627.01 | 10.483 | medium | C=C stretching | alkene |
| 1515.03 | 9.761502 | strong | N-O stretching | nitro compound |
| 1406.92 | 9.064938 | strong | S=O stretching | sulfonyl chloride |
| 1076.72 | 6.937423 | strong | C-O stretching | primary alcohol |
| 600.11 | 3.866574 | strong | C-I stretching | halo compound |

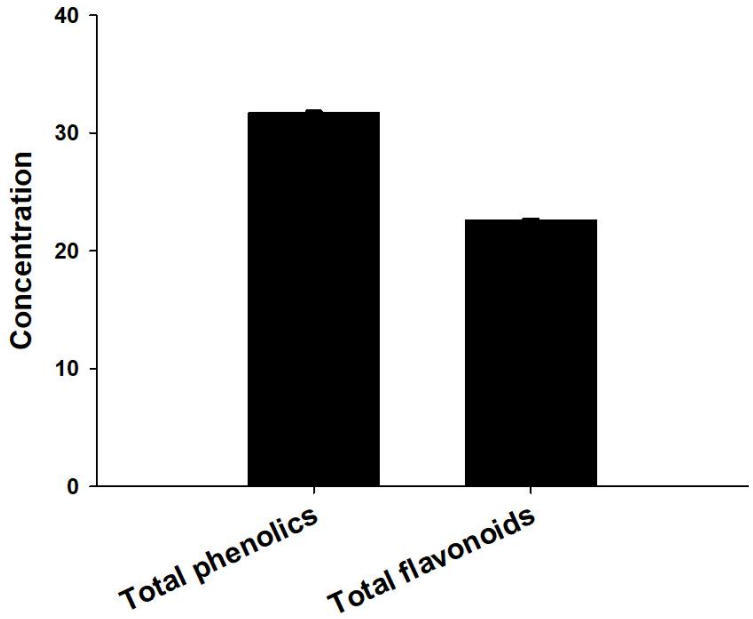


Figure 2. Total polyphenols, flavonoids of the ethanolic extract of *Anethum graveolens* leaves extract.

Table 2. Radical scavenging activity (%) of leaves extract for the *Anethum graveolens* plant

| Concentrations (µg/mL) | DPPH Radical Scavenging Activity (%) |
|---|---|
| 31.25 | 0 |
| 62.5 | 7.1 ± 1.1 |
| 125 | 24.4 ± 0.7 |
| 250 | 56.05 ± 1.2 |
| 500 | 74.8 ± 0.5 |
| 1000 | 65.4 ± 0.9 |

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 ($H_2O$) | -- | -- | -- |
| AGLE | 50 mg/mL | 57.22 ± 1.77 $^{*\#}$ | 58.56 ± 1.81 $^{*\#}$ |
| | 100 mg/mL | 10.67 ± 3.51 $^{*\#}$ | 11.15 ± 3.61 $^{*\#}$ |
| | 200 mg/mL | 4.57 ± 0.26 $^{*\#}$ | 5.22 ± 0.10 $^{*\#}$ |
| Mebendazole | 10 mg/mL | 13.91 ± 0.37 $^{*}$ | 18.2 ± 0.98 $^{*}$ |

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

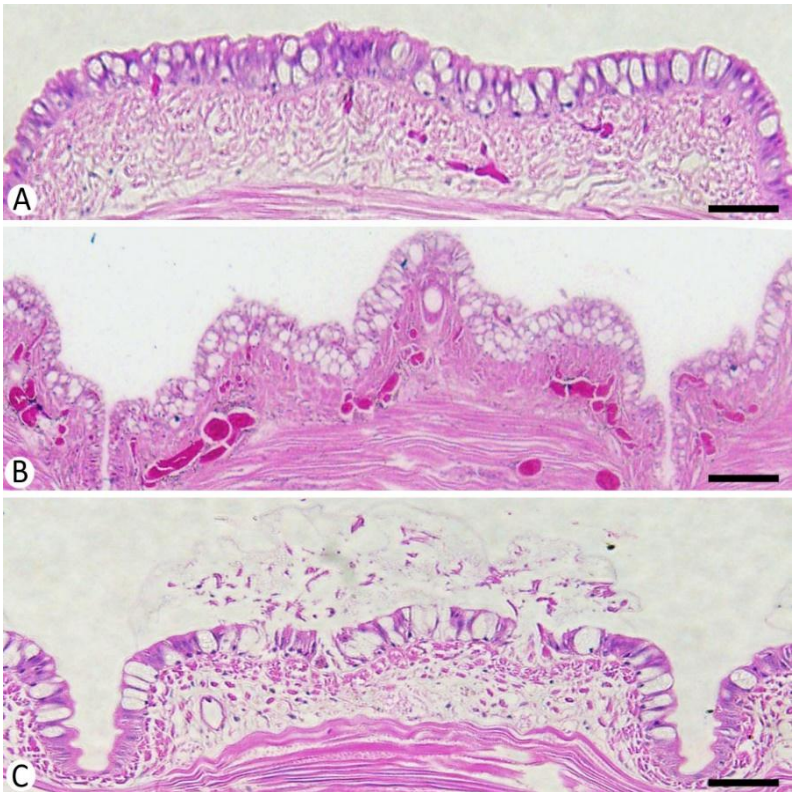


Figure 3. Cuticle thickness of *E. fetida* with various treatments. (A) worms in dist. $H_2O$ (control). (B) worms in AGLE (200 mg/mL). (C) worms in mebendazole. Scale bar = 25µm.

Table 4. *In vitro*, anti-coccidial effects of *Anethum graveolent* leaves extract on the sporulation percentage of *Eimeria papillata* oocysts

| Groups | Time | Sporulation of oocyst (%) | Inhibition of sporulation (%) | *p*-value |
|---|---|---|---|---|
| Distilled $H_2O$ | 72 hr | 66.6 ± 2 | 24.2 ± 1 | 0.01 |
| | 96 hr | 79.78 ± 2 | 15.7 ± 1 | 0.01 |
| Potassium dichromate (2.5%) | 72 hr | 88.03 ± 2 | 0 | 0.01 |
| | 96 hr | 94.67 ± 2 | 0 | 0.01 |
| AGLE (300 mg/ml) | 72 hr | 0 | 100 | - |
| | 96 hr | 0 | 100 | - |
| AGLE (200 mg/ml) | 72 hr | 2.18 ± 1 | 97.52 ± 2 | 0.01 |
| | 96 hr | 2.75 ± 1 | 97.09 ± 2 | 0.01 |
| AGLE (100 mg/ml) | 72 hr | 60.24 ± 1 | 31.57 ± 1 | 0.01 |
| | 96 hr | 89.42 ± 1 | 5.54 ± 1 | 0.01 |
| AGLE (50 mg/ml) | 72 hr | 71.03 ± 2 | 19.3 ± 1 | 0.01 |
| | 96 hr | 93.7 ± 1 | 1.01 ± 1 | 0.01 |
| Amprolium | 72 hr | 65.39 ± 1 | 34.61 ± 1 | 0.01 |
| | 96 hr | 62.67 ± 1 | 37.33 ± 1 | 0.01 |
| Dettol™ | 72 hr | 23.08 ± 1 | 76.92 ± 1 | 0.01 |
| | 96 hr | 18.67 ± 1 | 81.33 ± 1 | 0.01 |
| Phenol | 72 hr | 7.7 ± 1 | 92.30 ± 1 | 0.01 |
| | 96 hr | 10.67 ± 1 | 89.33 ± 1 | 0.01 |
| Formalin | 72 hr | 0 | 100 | - |
| | 96 hr | 0 | 100 | - |

## DISCUSSION

Coccidial and helminth infections constitute public health concerns worldwide. Plant extracts used as mendicants may help to overcome these obstacles. Also, plants have proven to be a promising alternative for controlling small ruminant nematodes. In addition to anthelmintic activity, it reduces the risk of chemical residues in animal products and the environment (Chagas *et al*., 2008). Carvone and limonene are the main chemical components of *A. graveolens* (Jana and Shekhawat, 2010).

The presence of the main chemical components may be responsible for these activities. Meanwhile, other researchers have reported that carvone and limonene have antimicrobial properties (Palmeira *et al*., 2009). Limonene is found as anthelmintic activity in oils from different plants, such as *Cymbopogon martini*, *Lippia sidoides*, *Eucalyptus staigeriana*, and *Mentha piperita* (Katiki *et al*., 2011; Carvalho *et al*., 2012; Ribeiro *et al*., 2013). In the current *in vitro* study, a concentration of AGLE of 200 mg/ml generated significant anthelmintic activity comparable to the conventional anthelmintic agent, mebendazole. Many studies used earth worms as the model for the anthelmintic activity evaluation due to the physiological similarities between the *E. fetida* worms and some intestinal round worms that infect people (Abu Hawsah *et al*., 2023). The carvone and limonene found in AGLE may be the main components of the anthelmintic action observed in this study. Stepek *et al*. (2005) and Fan *et al*. (2023) demonstrated that flavonoid inhibits glycolysis enzymes, disrupts calcium homeostasis, and causes death of the parasite. Parasites cuticle are considered as the target region by which anthelmintic agents interact. In this study, the cuticular surface of the worms treated with AGLE showed a significant shrinking. This agreed with Abu Hawash *et al*. (2023) who described how anthelmintic treatments caused modifications to the worms' body surfaces and might result in paralysis and death of the worm.

Flavonoids, alkaloids, tannins, and phenolic compounds are the most important plant bioactive compounds (Mehmood *et al*., 2015). Several studies have shown that plant extracts containing phenolic compounds have inhibitory properties. Natural polyphenolic components derived from medicinal plants have been shown to inhibit *E. tenella* sporozoite cell invasion *in vitro* (Arlette *et al*., 2019). These researchers also noted that extracts with polyphenolic compounds may have the power to inhibit the enzymes necessary for the coccidian oocysts' sporulation process. It is thus possible that *A. graveolens* extract components exhibited anti-sporulation activity by interfering with the physiological processes necessary for sporulation, thereby inhibiting or inactivating the enzymes responsible for the sporulation process. The finding is supported by a study that linked *Moringa oleifera*'s anticoccidial activity to its biological constituents, which include flavonoids and phenolic compounds with anti-inflammatory and antioxidant properties (Gadelhaq *et al*., 2018).

The results of this experiment demonstrated that AGLE has an *in vitro* anticoccidial effect on unsporulated oocysts of *E. papillata* in a concentration-dependent manner. This is consistent with the findings of Cedric *et al*. (2018), who reported the anticoccidial, antioxidant, and cytotoxicity activity of *Psidium guajava* extracts against four different species of *Eimeria* in a concentration-dependent manner. The effect of antioxidants on DPPH radicals is thought to be due to their ability to donate hydrogen. As a result, DPPH is commonly used as a substrate to assess antioxidant agents' antioxidative or free radical scavenging activity. Mustafa *et al*. (2010) confirmed phenolic compounds' antioxidant role by referring to their redox properties, which are considered free radical scavengers. It is also shown that the commonly used disinfectant formalin (5%) is the most effective in inhibiting *E. papillata* oocyst sporulation, which agrees with Thagfan *et al* (2020). According to Mai *et al*. (2009) and Gadelhaq *et al*. (2018) Phenol and Dettol$^{TM}$ have been reported to inhibit sporulation by 89.33% and 81.33% respectively, and the oocyst wall is resistant to proteolysis and impermeable to water-soluble substances.

## CONCLUSION

It could be concluded that AGLE has anticoccidial and anthelmintic efficacy, *in vitro*. Further studies are recommended to include the *in vivo* effectiveness of AGLE and identify the pathway of its active compounds on parasite and host response.

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