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In vitro studies for the antiparasitic activities of *Azadirachta indica* extract

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Abstract

Coccidiosis and helminthiasis are two parasitic diseases that harm both health and the economy. The present study aimed to assess the effect of *Azadirachta indica* leaf extracts (AILE) as an anti-parasitic modulator during murine coccidiosis as well as helminthic infection. Phytochemical analysis using FT-IR showed the presence of eleven compounds. A dose-dependent efficacy was observed in all experiments. At the highest concentration (200 mg/mL), time consumed to induce paralysis and death for worms was recorded at 9.329 ± 2.183 and 10.024 ± 1.542 min, respectively. Histological study revealed conspicuous deformity of surface architecture in all treated worms. SEM also revealed cuticular shrinkage of the body surface in all treated worms. *In vitro* study showed that incubation with AILE (100 mg/mL) for 96 hr inhibited sporulation by approximately 60%. AILE (50 and 25 mg/mL), amprolium, Dettol™, phenol, and formalin-induced variable inhibition levels at 96 hr of 28%, 44%, 37.33%, 81.33%, 89.33%, and 0% respectively. In addition, IC_{50} of AILE was obtained at 66.214 µg/mL with a percentage of antioxidant activity to be 74.76 ± 2.23. Our results indicate that AILE exhibits powerful anthelmintic and anticoccidial activities and it could be exploited further for the development of a novel therapeutic agent.

Keywords: *Azadirachta indica*; helminths; coccidiosis; medicinal plants.

Practical Application: Efficacy of Azadirachta indica as antihelmintic and anticoccidial effectors.

1 Introduction

Coccidiosis is considered the most important intestinal disease caused by apicomplexan protozoan parasites belonging to the genus *Eimeria* (family Eimeriidae) which spends its life cycle invasive multiplication within the intestinal tract of many species of farm and domestic animals (Allen & Fetterer, 2002; Mehlhorn, 2014; Lai et al., 2018). Gastrointestinal helminths also affect farming systems worldwide (Alzahrani et al., 2016). These parasitic infections disrupt nutrient uptake for animals, resulting in reduced body weight and increased susceptibility to secondary infections (López-Osorio et al., 2020). The economic importance of coccidiosis and helminthiasis is due to production losses and high mortality rates of animals (McDougald, 2003; Dkhil et al., 2013). Most of the research programs for parasite control are focused on the use of anti-parasitic drugs. However, indiscriminate and long-time use of these drugs has led to the emergence of drug resistance and adverse side effects (Abbas et al., 2011; Dkhil, 2013). Plant-derived compounds have been developed as an alternative approach to control parasitic infections (Klimpel et al. 2011; Amer et al., 2015; Elkhadragy et al., 2022). These agents do not target only the parasites but may also have organ-protective properties in the parasite-infected target hosts (Masood et al., 2013; Wunderlich et al., 2014).

Neem (*Azadirachta indica*), which belongs to the family Meliaceae, is one of the most versatile medicinal plants, with a broad spectrum of agricultural and medicinal applications (Anyaehie, 2009; Britto & Gracelin, 2011). This plant has been reported to exert insecticidal, pesticidal, and agrochemical properties. In addition, the active constituents of neem and its derivatives are applied in alternative and modern therapy, such as the treatment of diverse infectious, metabolic, and cancer diseases (Brahmachari, 2004; Ezz-Din et al., 2011; Dkhil et al., 2012; Gotep et al., 2016; Mohamed et al., 2021; Ishaq et al., 2022). Previous studies demonstrated the various multi-targeted biological activities of neem, such as hypoglycemic (Murty et al., 1978), anti-ulcer (Pillai & Santhakumari, 1984), anti-inflammatory (van der Nat et al., 1991), antimalarial (MacKinnon et al., 1997), chemopreventive (Tepsuwan et al., 2002), chemotherapeutic (Paliwal et al., 2005), and antibacterial (Thakurta et al., 2007) properties, as well as an antioxidant (Yanpallewar et al., 2003), and cardioprotective (Peer et al., 2008) effects. Previous studies were recorded for the evaluation of anthelmintic effect of neem leaves on *Haemonchus contortus* in goats (Radhakrishnan et al., 2007; Rahman et al., 2011; Sakti et al., 2018), and bovine strongylosis (Jamra et al., 2015). Moreover, Rabiu and Subhasish (2011) reported the effect of aqueous extract of neem leaves on the adult earthworm (*Pheretima posthuma*), roundworm (*Ascaridia galli*) and tapworms (*Raillietina spiralis*).

The present study has been designed to evaluate the potential role of *A. indica* leaf extracts as an anticoccidial agent against *Eimeria papillata*, as well as its *in vitro* anthelmintic activity.

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2 Materials and methods

2.1 Plant collection and preparation and characterization

Leaves of *Azadirachta indica* were collected from the botanical gardens in Riyadh, Saudi Arabia. Herbal plant identification was made by a botanist at the herbarium of the Botany Department (King Saud University, Riyadh, Saudi Arabia). Methanolic extract of *A. indica* leaves was prepared according to the method of Manikandan et al. (2008) with some modifications, as follows: *A. indica* leaves were washed with water, shade dried, and coarsely powdered using an electric blender (Senses, MG-503T, Korea). The dried powder (100 g) of neem leaves was subjected to maceration extraction technique using 70% methanol at 4 °C for 24 hr followed by percolation 5-7 times till complete extraction. After filtration, methanol was removed from the extract under reduced pressure using a vacuum evaporator at 50 °C. The crude extract obtained was lyophilized and stored at -20 °C until subsequent use.

2.2 Fourier-transform infrared spectroscopy (FT-IR)

Plant extract was analyzed using KBr pellet method with a range of 400-4000 cm^{-1} on NICOLET 6700 (Thermo Scientific, Waltham, USA) FT-IR spectroscopy (Al-Quraishy et al., 2020).

2.3 The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The activity of AILE was determined to scavenge DPPH radicals. Briefly, fresh DPPH radical solution (0.08 mM) in methanol was produced, and 950 mL of DPPH solution was combined with 50 mL of AILE and incubated at 25 °C for 5 min in dark. The absorbance was measured at 515 nm using a microplate reader. The antioxidant activity is expressed as the percent suppression of DPPH radicals according to Akillioglu & Karakaya (2010).

2.4 Anthelmintic activity of A. indica

The anthelmintic study was carried out using three doses (200, 100, and 50 mg/mL) of methanolic *A. indica* leaves extract (AILE) against the earthworm, *Allolobophora caliginosa*, according to Ajaiyeoba et al. (2001). Mebendazole (10 mg/mL) was used as the reference drug. Worms in distilled water were used as a control. Five worms of nearly the same body size were used per dose. The time to reach paralysis and death state was expressed in minutes (Dkhil, 2013).

2.5 Histological examinations

Immediately after paralysis and death experiment, the treated and control worms were prepared for histological study following the method of Drury & Wallington (1973). Briefly, specimens were fixed in 10% formalin for 24 h, then dehydrated by graded ethanol series and embedded in paraffin. Tissues were then cut into thin sections using a microtome, stained with hematoxylin and eosin (H & E), and examined and photography using an Olympus B×61 microscope (Tokyo, Japan).

2.6 Scanning electron microscopy (SEM)

Immediately after paralysis, the treated and control worms were fixed for SEM following a standard method of Roy & Tandon (1991). Briefly, worms were fixed in 3% buffered glutaraldehyde at 4 °C for 2 h, then dehydrated with ascending grades of acetone, air-dried in tetramethylsilane (TMS), and mounted on metal stubs and coated with gold-palladium. Specimens were examined and photographed in Jeol JSM-6060LV at an accelerating voltage of 15 kV.

2.7 Parasite

E. papillata was used as a model coccidial murine parasite. For the propagation of oocysts, five laboratory mice were inoculated with 1×10^5 sporulated oocysts by oral gavage. Feces were collected at 5 days post-infection (p.i.), and oocysts were separated by floatation technique (Kumar et al., 2014). Part of these unsporulated oocysts was used in the *in vitro* study. The remaining oocysts were preserved for further studies.

2.8 In vitro oocyst sporulation

The unsporulated oocysts (1×10^5) were incubated for 72 and 96 hr at 25-29 °C in 5 mL Dist. H_2O (negative control), 5 mL 2.5% $K_2Cr_2O_7$ (positive control), and finally in 5 mL $K_2Cr_2O_7$ containing one of the following: AILE (100, 50, and 25 mg/mL), 8.3 mg amprolium, 109 µL Dettol ™, 25 µL phenol, and 5% formalin. Sporulation of the oocysts was monitored by examining sporocysts using an Olympus compound microscope (Olympus Co., Tokyo, Japan). A total of 100 oocysts were counted per treatment and control group to estimate the sporulation and inhibition (%) of oocysts according to Thagfan et al. (2020).

2.9 In vitro cytotoxicity

The cytotoxicity of AILE was assessed on the human colorectal carcinoma (HCT116) cell line using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoilum bromide (MTT) assay. Cells were cultured in 96-well plates at 2×10^5 cells per well for 24 h at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were then treated with different extract concentrations of 0.0565, 0.1694, 0.5081, 1.5242, 4.5725, 13.7174, 41.1523, and 123.4568 µg/mL. Thereafter, 20 µL of MTT (5 mg/mL) was added to each well and the plate was incubated for another 4 hr at 37 °C. Formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO). Absorbance was recorded at 570 nm measured by a microplate reader (Spectra MAX 190). Untreated cells were used as a control group. Cell viability (%) was calculated according to Shirley & Lillehoj (2012).

2.10 Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) with SigmaPlot® version 11.0 (Systat Software, Inc., Chicago, IL, USA) and presented as mean ± SD. Differences among groups were considered significant at p-value ≤ 0.05.

3 Results

3.1 Infrared spectroscopy

The analysis of AILE using FT-IR showed major bands at 3390.78 cm^{-1}, 2930.29 cm^{-1}, 1622.98 cm^{-1}, 1516.17 cm^{-1}, 1380.62 cm^{-1}, 1273.10 cm^{-1}, 1070.93 cm^{-1}, 852.33 cm^{-1}, 816.67 cm^{-1}, 775.86 cm^{-1} and 598.93 cm^{-1} (Figure 1 and Table 1). N-H stretching was indicated by the band at 3390.78 cm^{-1} confirming the presence of an aliphatic primary amine. The band at 2930.29 cm^{-1} implied C-H stretching for the presence of alkane. C=C stretching at 1622.98 cm^{-1} confirms the presence of conjugated ketone. The band at 1516.17 cm^{-1} corresponds to N-O stretching for the presence of the nitro compound. C-H stretching at the band 1380.62 cm^{-1} confirmed the presence of an aldehyde. The band 1273.10 cm^{-1} (C-N stretching), 1070.93 cm^{-1} (S=O stretching), 852.33 cm^{-1} (C=C stretching), 816.67 cm^{-1} (C-CI stretching), 775.86 cm^{-1} (C-H stretching), and 598.93 cm^{-1} (C-I stretching) assigned to aromatic amine, sulfoxide, alkene, halo compound, and 1,2-disubstituted, respectively (Table 1). In addition, the percentage of DPPH assay was 74.76 ± 2.23.

3.2 Anthelmintic activity of A. indica

The methanolic extract of *A. indica* produced a relatively comparable anthelmintic activity with the conventional anthelmintic agent (mebendazole) against live adult *A. caliginosa* worms (Figure 2). The most efficient dose, 200 mg/kg showed the time to paralysis and death were 9.328 ± 2.183 and 10.024 ± 1.542 min, respectively. However, the reference drug mebendazole (10 mg/mL) showed less effect (9.074 ± 1.355 and 16.748 ± 5.622 for paralysis and death time, respectively) compared to the 200 mg/kg AILE (Table 2).

3.3 Microscopic examinations

LM and SEM studies revealed uniform normal body architecture for control worms, without any alterations to the surface of worms (Figures 3, 4). On the other hand, all worms treated with AILE showed changes in the general topography including a reduction in size through the length and the homogenous body wall shrinkage accompanied by cuticular thickness (Figures 3, 4). All worms treated with the reference drug mebendazole implicated similar kinds of disruption (Figures 3, 4).

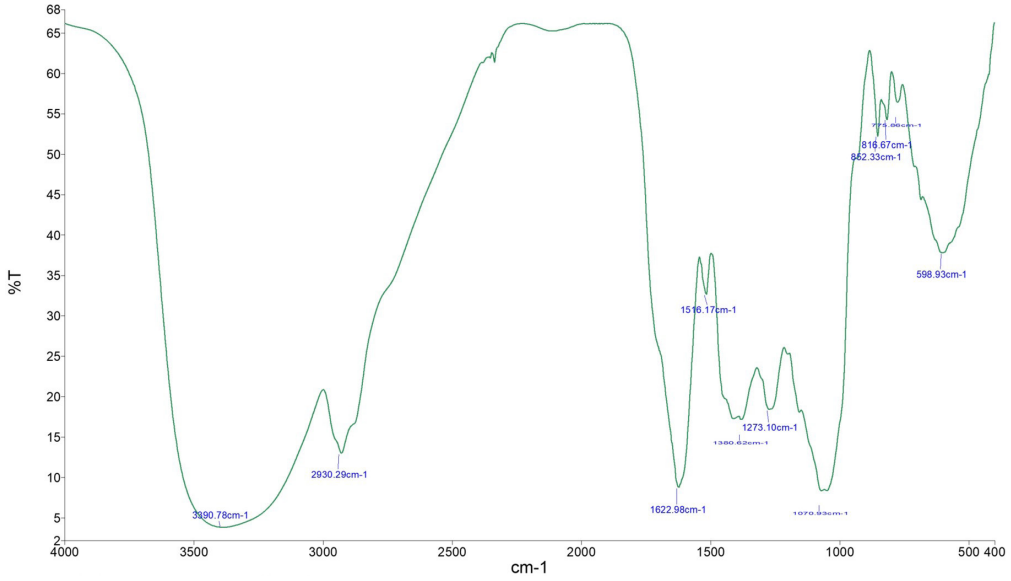

Figure 1. FTIR of *Azadirachta indica* leaf extracts in an aqueous medium showing the functional characteristic of the material.

Table 1. FT-IR for *Azadirachta indica* leaves extract.

Absorption (cm^{-1})	Transmittance (%)	Appearance	Group	Compound class
3390.78	1.259	medium	N-H stretching	aliphatic primary amine
2930.29	4.286	medium	C-H stretching	alkane
1622.98	2.903	medium	C=C stretching	conjugated ketone
1516.17	10.778	strong	N-O stretching	nitro compound
1380.62	5.661	medium	C-H bending	aldehyde
1273.10	6.076	strong	C-N stretching	aromatic amine
1070.93	2.755	strong	S=O stretching	sulfoxide
852.33	17.232	medium	C=C bending	alkene
816.67	17.920	strong	C-Cl stretching	halo compound
775.86	18.624	strong	C-H bending	1,2-disubstituted
598.93	12.475	strong	C-I stretching	halo compound

Figure 2. *In vitro* anthelmintic effect of crude extracts of leaves of *Azadirachta indica* against *Allolobophora caliginosa* in comparison to the reference drug (mebendazole).

Table 2. *In vitro* anthelmintic activity of crude extracts of *Azadirachta indica* leaves.

Test samples	Concentration (mg/mL)	Time is taken for paralysis (min.)	Time is taken for death (min.)
Control (H_2O)	--	--	--
AILE	50 mg/mL	58.835 ±0.657 ^{abde}	75.00 ± 0.211 ^{abde}
	100 mg/mL	27.82 ± 0.773 ^{abce}	28.376 ± 0.662 ^{abce}
	200 mg/mL	9.328 ± 2.183 ^{acd}	10.024 ± 1.542 ^{acd}
Mebendazole	10 mg/mL	9.074 ± 1.355 ^{acd}	16.748 ± 5.622 ^{acd}

Values are mean ± SD. All superscripts indicate significance at $P \leq 0.05$, [a] compared to untreated (H_2O), [b] compared to mebendazole, [c] compared to the lowest concentration of methanolic extract of *A. indica* leaves, [d] compared to the moderate concentration of methanolic extracts of *A. indica* leaves, [e] compared to the highest concentration of methanolic extract of *A. indica* leaves.

3.4 Effect of AILE on oocyst sporulation in vitro

There was no change for oocysts incubation in dist. H_2O (negative control) at both 72 and 96 hr. Oocyst incubation with $K_2Cr_2O_7$ (2.5%), AILE (100, 50, 25 mg/mL), amprolium, phenol, and Dettol™ showed different levels of sporulation (Table 3). After incubation with formalin, the unsporulated *E. papillata* oocysts showed no rate of sporulation. Incubation with AILE (100 mg/mL) for 72 and 96 hr inhibited oocysts sporulation by 23.07% and 60%, respectively. AILE (50 and 25 mg/mL), amprolium, Dettol™, and phenol induced variable inhibition levels at 96 hr of 28%, 44%, 37.33%, 81.33%, and 89.33%, respectively (Table 3).

3.5 AILE cytotoxicity by MTT assay

The cytotoxic effect of AILE on the HCT116 cell line was tested using an MTT assay (Figure 5). The viability of cells has a direct dose-dependent manner. The IC_{50} of AILE was obtained at 66.214 µg/mL. The cell viability was decreased with increasing the AILE dose.

4 Discussion

Search for alternatives to anticoccidial drugs to treat and control coccidiosis is an important field of study. Several studies used plant extracts as antiparasitic agents with minimum side effects different *in vitro* and *in vivo* studies (Aljedaie & Al-Malki, 2020; Yousaf et al., 2021; Qaid et al., 2022). This study assessed the potential role of AILE as anthelmintic and anticoccidial effectors.

Several studies have reported the anthelmintic role of AILE (Radhakrishnan et al., 2007; Priscilla et al., 2014; Jamra et al., 2015). Herein, earthworms were used as a model for the anthelmintic activity of AILE. In the current *in vitro* study, 200 mg/mL concentration of AILE produced a significant anthelmintic activity that is comparable with the conventional anthelmintic agent, mebendazole. This finding is in line with Adjorlolo et al. (2016) and Salma et al. (2021) confirmed the efficacy of AILE due to the presence of active constituents, i.e. alkaloids, phenolics, flavonoids, saponins, tannins. Roy et al. (2010) reported that alkaloids may act on the central nervous system to cause paralysis of the parasite and death. Mustafa et al. (2010) confirmed the antioxidant role of phenolic compounds referred to their redox properties which are considered free radical scavengers. Stepek et al. (2005) and Fan et al. (2023) showed that flavonoid inhibits the enzyme of glycolysis and disturbs the calcium homeostasis and nitrous oxide activity and eventual death of the parasite. Melzig et al. (2001) stated that saponin affects the permeability of the cell membrane of the parasite and causes vacuolization, the disintegration of teguments, and eventual death. Tresia et al. (2016) reported that

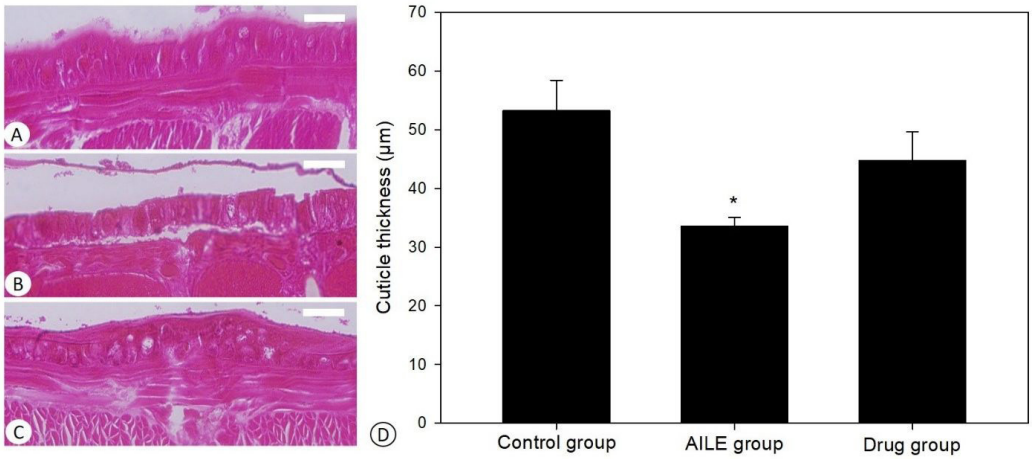

Figure 3. Cuticle thickness of *A. caliginose* with various treatments. (A) worms in dist. H_2O (control). (B) worms in 200 mg/mL *A. indica* leaf extracts. (C) worms in the reference drug (mebendazole). (D) Bar chart is thickness of worm cuticle (µm) among three groups, control group, AILE-treated group, and drug-treated group. Each group represents an average of five different fields of cuticle sections stained with hematoxylin and eosin, Scale bar = 25 µm. * significance change with respect to control group.

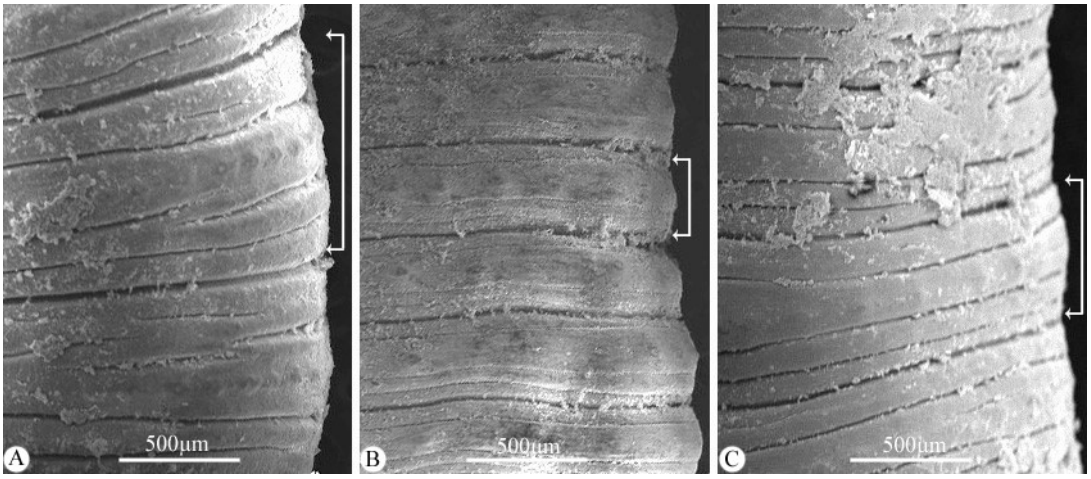

Figure 4. SEM of *Allolobophora caliginose* with various treatments. (A) worms in dist. H_2O (control). (B) worms treated with 200 mg/mL *A. indica* leaf extracts. (C) worms treated with the reference drug mebendazole.

the fatal intracellular consistency occurred due to the inhibition of the enzyme secretion by tannins that would cause paralysis of parasites.

For evaluating anthelmintic action, histopathology and SEM have proved the *in vitro* study and analyzed the topographical effects of AILE on the worms. The cuticle is an important structure of annelids because it provides covering and protection for the worm's body and supports internal organs (Meyer et al., 2021). In the present study, microscopic observations for AILE-treated parasites revealed that remarkable changes occurred on the cuticular surface, with extensive shrinkage. This agreed with Kundu et al. (2012) stated that the cuticle of parasites has associated with one of several target sites by which the anthelmintic products act. Sambodo et al. (2018), and Mrifag et al. (2021) reported the changes in the body surface of the parasites due to anthelmintic agents. Therefore, any destruction caused to the body surface of the parasite due to the treatment of a drug or extract may lead to paralysis and death of the parasite.

Our study demonstrated *in vitro* anticoccidial activity of AILE on the oocyst's sporulation in a dose-dependent manner, which is attributable to numerous bioactive phytochemical constituents studied by Gnanakalai & Gopal (2016), Ibrahim et al. (2017), Abdul Rahman et al. (2020). It is also demonstrated that the commonly used disinfectant formalin (5%) is the most effective in the inhibition of the oocyst's sporulation of *E. papillata*, which agreed with Thagfan et al. (2020). Dettol™ and Phenol have been reported to inhibit sporulation by 81.33% and 89.33%, respectively, which is consistent with Mai et al. (2009) and Gadelhaq et al. (2018) that the oocyst wall is impermeable to water-soluble substances and resistant to proteolysis.

Table 3. *In vitro* anti-coccidial effects of the methanolic extract of *Azadirachta indica* leaves on the sporulation percentage of *Eimeria papillata* oocysts.

Groups	Time	Unsporulation of oocyst (%)	Inhibition of sporulation (%)	P-value
Distilled H_2O	72 h	100 ± 0.2	0	-
	96 h	100 ± 0.2	0	-
Potassium dichromate (2.5%)	72 h	74 ± 1	0	-
	96 h	25 ± 1	0	-
AILE (100 mg/kg)	72 h	80 ± 1	23.07 ± 1	0.01
	96 h	70 ± 1	60 ± 1	0.01
AILE (50 mg/kg)	72 h	81 ± 1	26.92 ± 1	0.01
	96 h	46 ± 1	28 ± 1	0.01
AILE (25 mg/kg)	72 h	85 ± 1	42.30 ± 1	0.01
	96 h	57 ± 1	44 ± 1	0.01
Amprolium	72 h	83 ± 1	34.61 ± 1	0.01
	96 h	53 ± 1	37.33 ± 1	0.01
Dettol™	72 h	94 ± 1	76.92 ± 1	0.01
	96 h	86 ± 1	81.33 ± 1	0.01
Phenol	72 h	98 ± 1	92.30 ± 1	0.01
	96 h	92 ± 1	89.33 ± 1	0.01
Formalin	72 h	100 ± 0.2	100 ± 0.2	0.01
	96 h	100 ± 0.2	100 ± 0.2	0.01

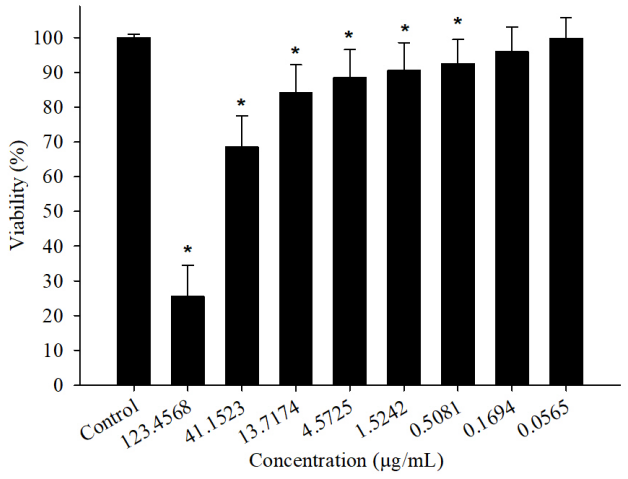

Figure 5. Cytocompatibility evaluation of AILE. * significance change with respect to control group (values are mean ± SD).

Govarthanan et al. (2016) stated the importance of the cytotoxicity test for toxicology investigation to explain the cellular response to toxic material and provide information about cell death and survival. In this study, the cell survival rate was measured by MTT assay. Thus, *in vitro* cytotoxicity of AILE was evaluated against the HCT116 cell line at different concentrations and our results confirmed that cell viability has a direct dose-dependent manner. The cell viability for AILE was found to be 66.214 µg/mL which exhibited no cytotoxic effect on the HCT116 cell line. This study agreed with Ngure et al. (2009), Chaudhary et al. (2017), and Njoga et al. (2022) stated that AILE showed no observable signs of toxicity and proved to be safe for medical use.

5 Conclusion

Recently, medicinal plants have received much attention for their therapeutic uses as an alternative to coccidiostats. Our data indicate that *A. indica* leaves extract exhibited a significant anthelmintic as well as anticoccidial activity. *In vivo* studies on the effect of this extract and its related activities should be included in future.

Ethical approval

This research was approved by the Research Ethics Committee (REC) at King Saud University (approval number KSU-SE-22-66).

Conflict of interest

The author(s) declare that they have no conflict of interest regarding the content of this article.

Availability of data and material

The data used to support the findings of this study are included within the article.

Author contributions

M.A.H., T.A.O., G.A., and R.A.G. contributed to study design. S.A.Q., M.A.H., M.F.E. and R.A.G. contributed to data acquisition. R.A.G., E.M.A.S., and M.A.D. organized the database, performed the statistical analysis. All authors revised, improved, read, and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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