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Effects of ethanolic extract of neem leaves on the sporocyst of *Sarcocystis* availability *in vitro*

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ABSTRACT

The aim of this study was to evaluate the *in vitro* anti-sporocyst efficiency of *Azadirachta indica* (neem) leaves ethanolic extracts against *Sarcocystis* and using it as alternative therapy for Sarcocystis in dogs. 100 g of faeces collected from experimentally-infected puppies with *Sarcocystis* by feeding them on 300 g meat (oesophagus and diaphragm) of sheep infected with *Sarcocystis*. The faeces melted into distilled water and then the sample was centrifuged. After dividing the precipitate into sixteen equal samples, a sample was randomly selected and inspected using the flotation (sugar solution) technique, then the quantity of sporocysts was calculated using a haemocytometer, which was 5000 individuals/g. Afterward, the samples were divided to 3 group each containing on five samples. Finally, 3 mL from the neem extract was added in different concentrations (250, 500, 750 and 1000 mg; C_1, C_2, C_3 and C_4 respectively) and placed in incubator at 37 ºC. The first group was inspected after 24 h, the second after 48 h, and the third after 72 h. A significant reduction was observed in the number of sporocysts in the samples treated with high concentration of neem leaves extract. The number of sporocysts become zero in the sample with the higher dose (C4; 1000 mg mL^{-1}) at 48 h and 72 h of incubation, and also significant reduction in number was observed at 24 h. The number of sporocysts was nil after 72 h of incubation in C_3 (750 mg mL^{-1}), and much lower after 48 and 24 h. In C_2 (500 mg mL^{-1}), the quantity of sporocysts was zero after 72 h of incubation and decreased after 48 h. The minimal impact was observed in C_1 (250 mg mL^{-1}). The best antisporocyst action was observed at C_4 (1000 mg mL^{-1}). As a result, it is suggested that an ethanolic extract of neem leaf at 1000 mg mL^{-1} can be utilized in the *in vitro* therapy of Sarcocystis.

Keywords: Extract, Sporocyst, Plant, *Sarcocystis.*
Article type: Research Article.

INTRODUCTION

Sarcocystosis is a parasitic illness caused by the obligate intracellular coccidian protozoan, *Sarcocystis* (AbdelGaber *et al.* 2020). The parasite of genus *Sarcocystis* is one of the most frequently found cystic parasites of domestic ruminants in slaughtered animal muscles, and certain *Sarcocystis* species can contribute to significant economic losses caused by both clinical and subclinical diseases (Lindsay *et al.* 2020). The clinical manifestations of acute Sarcocystosis in intermediate hosts include brain and spinal cord inflammation, encephalitis, meningitis, and bleeding diathesis. It can result in fetal mortality, early delivery, and abortions in pregnant animals (Dubey *et al.* 2015). Another clinical singes are perivascular monocyte infiltration, petechial haemorrhage, weakness, fever, encephalomyelitis, and also death in cases of massive infestation (Vangeel *et al.* 2013). Generally, *Sarcocystis* doesn't cause illness in the definitive hosts (Fayer 2004). Protozoan parasites of the genus *Sarcocystis* have an obligatory two-host life cycle within the phylum Apicomplexa; requires two separate hosts in a prey-predator relationship: A permanent host in which the sexual stage develops by creating oocysts/sporocysts in the gut mucosa of carnivorous predators after eating sarcocysts, they reproduce asexually forming sarcocysts in the vascular endothelial and striated muscle cells of herbivores prey and omnivores, after ingesting sporocysts in food

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or water contaminated with animal faeces. Humans can serve as intermediate and ultimate hosts for a variety of *Sarcocystis* species (Hoeve-Bakker *et al.* 2019). *Azadirachta indica* (neem) is a species of tree of the mahogany family (Meliaceae), native to Iraq, India, and Pakistan, and spreading in tropical and subtropical climates (Girish *et al.* 2008). From antiquity, every component of the tree has been employed as traditional medicine for domestic remedies against different ailments (Kumar & Navartnam 2013; Ramadass & Subramanian 2018). Many biologically-active chemicals are found in *A. indica* including alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids, and ketones are all found in its neem seed oil, hence its extract has been shown to be effective against some fungi that cause infection in the human body. It is used as an anthelmintic, anti-leprotic (prevents leprosy), antibacterial, and anti-derm-atophytic agent. In addition, it is an active ingredient in the preparation of mosquito repellent coils (Swapna *et al.* 2018). The neem seed extracts (aqueous and ethanol) and *C. albicans* cell wall manno-proteins may serve as immuno-potentiators by boosting microsomal proteins and may be a possible immunological adjuvant for eliciting active immunity against Brucella (Faal *et al.* 2012). Currently, new studies used it as medicinal by anthelmintic effects (Hellawi *et al.* 2021). Therefore, the aim of the study was to evaluate *in vitro* anti sporocyst efficiency of the *A. indica* leaves ethanolic extracts and using it as alternative therapy for *Sarcocystis*.

MATERIALS AND METHODS

Ethical statement

Puppies in this study were managed and treated with all essential approvals and in accordance with all regulations and procedures allowed by the Iraqi Ethics Committee of the College of Veterinary Medicine/ Baghdad University.

Collection of *Azadirachta indica* leaves

Fresh matured *A. indica* (neem tree) leaves were taken from a garden around Baghdad City, iraq. The leaves were cleansed with water and dried in the shade for one week before being crushed to powder with a blender.

Plant materials

Plant categorization was done in Abu Ghraib/Ministry Baghdad's of Agriculture, State Board for Seeds Testing and Certification S.B.S.T.C.

Preparation of *A. indica* extract in ethanol

According to the manufacturer, 50 g air-dried neem leaf powder was placed in the extraction container, followed by 450 mL 98% ethanol, which was firmly sealed and kept in the shade at room temperature for 24 h before filtering with Whatman No.1 filter paper and drying the residue (Manikandan *et al.* 2008).

Dose of neem preparation

The resulting neem leaf extract was diluted with distilled water to generate concentrations of 25%, 50%, 75%, 100%, and pure diluent for 0%. The extracts were kept in clean containers (Table 1).

Table 1. Dose of neem preparation.

Dose	Preparing
100%	Dissolve 4 g of neem extract in 4 mL distilled water
75%	Dissolve 3 g of neem extract in 4 mL distilled water
50%	Dissolved 2 g of neem extract in 4 mL distilled water
25%	Dissolved 1 g of neem extract in 4 mL distilled water

Samples of infected meat with *Sarcocystis* collection

Samples of infected meat with *Sarcocystis* (250-300 g in weight) were collected from oesophagus and diaphragm of slaughtered sheep, then transferred in plastic bags to the Parasitology Laboratory at the College of Veterinary

Medicine, University of Baghdad, Iraq, for detection of microscopic cysts by tracheoscopy, according to Castroforero *et al.* (2020).

Induced infection

Three puppies, at the age of two weeks and local breed, these puppies kept in the experimental unit of the College of Veterinary Medicine at Baghdad University, Iraq. The puppies were gowned in conditions that excluded spontaneous infection. After two weeks of shelter, all puppies were tested for intestinal parasites using conventional faecal examination to confirm they were not infected (Featherstone 1969). Afterward, we infected the puppies by feeding them with sheep muscles (oesophagus and diaphragm) with *Sarcocystis* infection after cutting it into small pieces for two consecutive days. Five days after induced infection, we examined the faeces of infected puppies by flotation technique (Rommel *et al.* 1972). After twelve days of infection, the puppies began shedding sporocysts with faeces.

Collecting sporocyst of *Sarcocystis*

100 g faeces was collected from experimentally infected puppies, then melted into distilled water and centrifuged. Afterward, we divided the precipitate into sixteen equal samples. A sample was obtained at random and studied using the flotation technique, with the number of sporocysts calculated using a hemocytometer (to be 5000 ind/g). Thereafter, we divided the samples to three group each containing on five samples. Finally, we added 3 mL from the neem extract in different concentrations (250, 500, 750 and 1000 mg; C_1, C_2, C_3 and C_4 respectively) and placed in incubator at 37 ºC. Each group was examined after 0, 24, 48 and 72 h.

Statistical analysis

The Statistical Analysis System (SAS 2018) program was used to assess the impact of various components on the study parameters. To compare means in this study, the least significant difference -LSD test (analysis of variance, ANOVA) was used.

RESULT AND DISCUSSION

Extraction of *Azadirachta indica* leaves

The extraction of neem leaves with 100% ethanol produced a dark green extract with a plant powder yield percentage of 9.5%, as estimated by the equation:

Extract yield = Weight of extract (g) / weight of neem leaves powder (g) ×100 = 9.5 (g) / 100 (g) ×100 = 9.5%.

Anti-sporocyst activity of neem leaves ethanolic extract

The ethanolic extract of neem leaves proved to be very efficient against the sporocyst availability. The number of sporocyst become zero with the higher dose (C_4, 1000 mg mL^{-1}) at 48 and 72 h of incubation and there was a significant reduction in number at 24 h. The number of sporocysts was nil after 72 h with C_3 (750 mg mL^{-1}), and much lower after 48 and 24 h. The number of sporocysts was nil after 72 h with C_2 (500 mg mL^{-1}), while there was a decline after 48 h with C_1 (250 mg mL^{-1}). This is consistent with the findings of Yusuf *et al.* (2021), who demonstrated the anticoccidial activity of ethanolic neem leaf extract at various concentrations (50, 40, 30, and 20 mg mL^{-1}). The proportions of live parasites retrieved at 50, 40, 30, and 20 mg mL^{-1}, on the other hand, were 63.0%, 18.2%, 27.6%, 52.9%, and 55.1%, respectively. The results of this study suggest that an ethanolic extract of neem leaf has anticoccidial action at any dose, as it has been found to kill or limit sporozoite growth and development. It also concurred with the findings of Abdullahi *et al.* (2006), who found that aqueous neem leaf extract was more effective against bacteria than Amprolium. Once comparing to amprolium (10 mg L^{-1}), aqueous neem extract (800 mg mL^{-1}) efficiently cured coccidia organisms with 100% survival rates in affected hens. The extract efficacy can be attributed to its ability to penetrate the oocyst cell wall and disrupt the cytoplasm, as seen by the abnormal appearance of oocyst sporozoites in this study (Yamssi *et al.* 2017).

Table 2. Mortality rate of sporocysts after treating with neem leaves extract.

Concentration of neem periods	0 h	24 h	48 h	72 h
250 mg mL^{-1}	0%	0%	0%	50%
500 mg mL^{-1}	0%	0%	50%	100%
750 mg mL^{-1}	0%	25%	75%	100%
1000 mg mL^{-1}	0%	75%	100%	100%
Normal saline	0%	0%	0%	0%

Table 3. Effects of concentration of neem and time in number of sporocysts.

Concentration of neem	mg/ml	Number of sporocysts at different time				
		Zero time	24 h	48 h	72 h	LSD value
Control		5000 A a	5000 A a	5000 A a	5000 A a	0.00 NS
250 mg		5000 A a	5000 A a	5000 A a	2500 B b	127.44 **
500 mg		5000 A a	5000 A a	2500 B b	0 C c	172.03 **
750 mg		5000 A a	3750 B b	1225 C c	0 C d	138.64 **
1000 mg		5000 A a	0 C b	0 D b	0 C b	116.02 **
LSD value		0.00 NS	162.07 **	207.53 **	139.77 **	---

Means with various capital letters in the same column and tiny capital letters in the same row differ substantially; ** ($p < 0.01$).

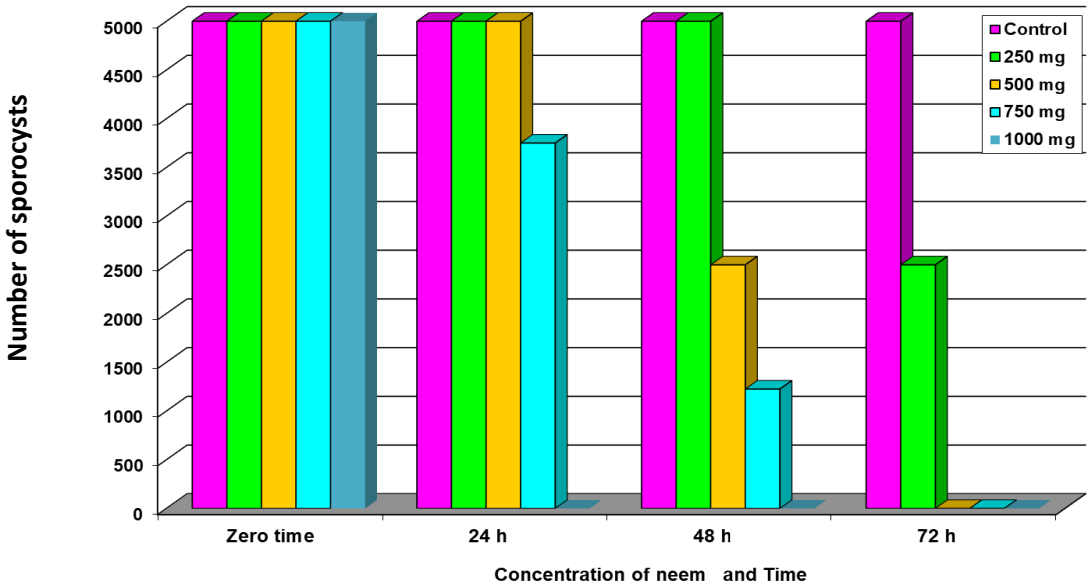

Fig. 1. Effects of neem concentration and time on the number of sporocysts.

Influence of neem leaf extract on sporocysts

When compared to normal sporocyst, there was a significant effect of neem leaves extract on the shape of sporocysts, as shown in Fig. 2. There was a change in the shape, size, and colour of sporocysts treated with C_1 (250 mg neem leaves extract), and also the sporocysts treated with C_2 (500 mg) and C_4 (1000 mg).

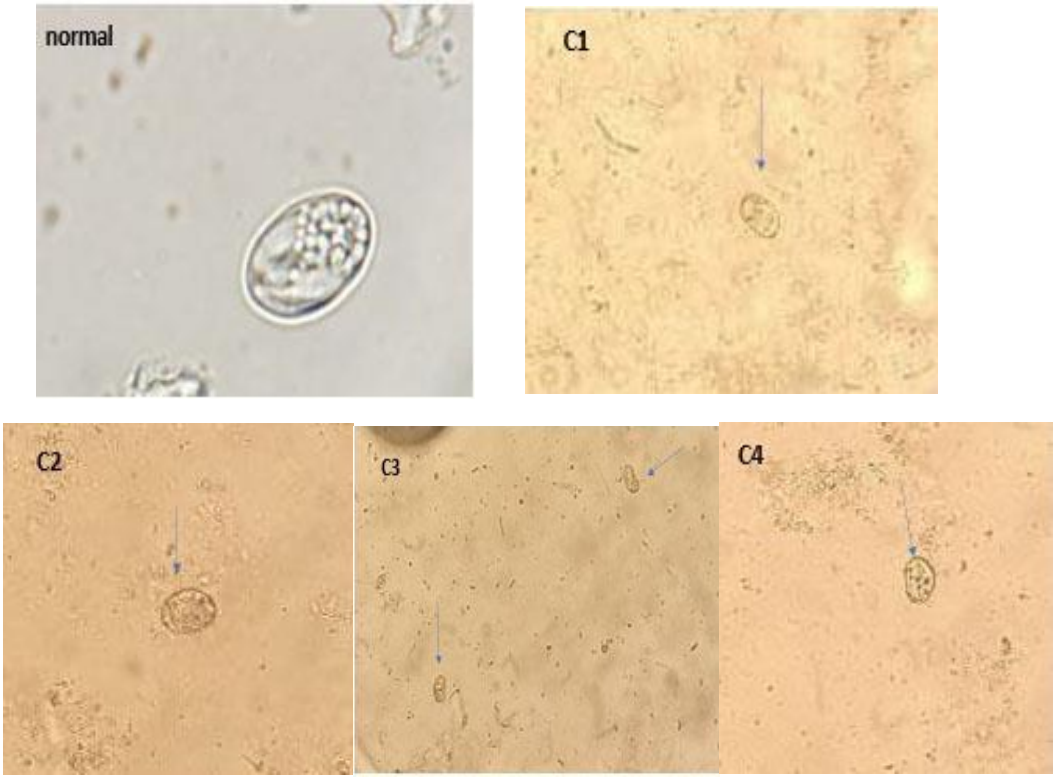

Fig. 2. Normal (Normal sporocyst); C_1, C_2, C_3 and C_4 (250, 500, 750 and 1000 mg neem leaves extract).

CONCLUSION

The purpose of this study was to demonstrate the viability and efficacy of an ethanolic extract of neem leaves on the treatment of *Sarcocystis*, with the objective of providing a viable alternative to synthetic anticoccidial drugs, which are expensive and may have long-term impacts on consumers. At a dose of 1000 mg mL^{-1}, the greatest antisporocyst activity was observed. As a consequence, a 1000 mg mL^{-1} ethanolic extract of neem leaf is recommended for use in the in vitro treatment of coccidia organisms.

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