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Anthelmintic Efficacy of *Eucalyptus globulus* Seeds Extract Against Various Life Cycle Stages of *Haemonchus contortus* in Sheep and Goats

Muhammad Iqbal[1,2], Muhammad Oneeb[2]*, Muhammad Lateef[2], Jawaria Ali Khan[3], Khadija Tariq[2] and Robeela Shabbir[4]

[1]*Livestock and Dairy Development Department, Government of the Punjab, Lahore*
[2]*Department of Parasitology, University of Veterinary and Animal Sciences, Lahore*
[3]*Department of Medicine, University of Veterinary and Animal Sciences, Lahore*
[4]*Department of Zoology, Lahore College for Women University, Lahore*

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Key words
***Haemonchus contortus*, Small ruminants, Food security, *Eucalyptus globulus*, Gastrointestinal nematode, Benzimidazoles, Albendazole**

ABSTRACT

Small ruminants play vital role in the lives of rural and urban populations, providing essential necessities like income, hides, wool and hair, while also ensuring food security through meat and milk. However, gastrointestinal nematodes, particularly *Haemonchus contortus*, pose a significant threat to the small ruminants industry, causing substantial mortality and production losses. To combat these parasites, synthetic anthelmintics are often employed, but their use raises concerns about the emergence of resistant parasite strains, ecosystem disruption, and food safety issues due to drug residues in milk and meat. This has prompted researchers to explore alternative, botanically-derived anthelmintics with no public health hazards or ecosystem concerns. This study aimed to evaluate the in vitro anthelmintic potential of *Eucalyptus globulus* Labill seed extracts against *H. contortus* eggs, infective larvae (L3), and adult stages in sheep and goats. The methanolic extract yield was higher than the aqueous extract, and the chemical composition of both extracts was determined using GC-MS analysis. The aqueous extract (50 mg/mL) inhibited 100% adult worm motility after 24 h. Additionally, the aqueous extract (50 mg/mL) exhibited greater egg hatching inhibition and larval paralysis compared to the methanolic extract. These findings suggest that *E. globulus* seeds possess anthelmintic properties, offering a potential alternative to synthetic anthelmintics. However, in vivo trials are necessary to assess toxicity effects.

INTRODUCTION

The global economy relies heavily on livestock, and small ruminants (sheep and goats) play a vital role in the economic development of developing countries worldwide. They contribute significantly to the economy through the production of meat, milk, wool, hides, and hair, which are major sources of income for rural populations, including women (Ferreira *et al*., 2016; Naeem *et al*., 2021). Small ruminants hold cultural and political significance in various religions and nations, particularly in Muslim communities, where they are used for sacrificial purposes during festivals like Eid-al-Adha (Jabeen *et al*., 2015). Compared to other livestock species, small ruminants can be sold easily to meet basic needs. Additionally, they utilize a limited supply of foodstuffs like wild trees, bushes, crop residues, and agricultural by-products and require minimal space for rearing (Jemberu *et al*., 2022).

The small ruminants industry faces numerous challenges, with health issues caused by gastrointestinal nematodes, particularly *Haemonchus contortus*, being a major concern, especially in tropical and subtropical regions (Knox, 2024). This parasite, found in the abomasum of sheep and goats, is highly pathogenic, consuming approximately 0.05 ml of blood daily, which leads to anemia, retarded growth, loss of appetite, weight loss, early mortality, immune system suppression, and ultimately, production losses in meat, milk, wool, hair, and hides (Bibi *et al*., 2017). Globally, *H. contortus* infections have resulted in significant economic losses, decreased milk production, and substantial mortality rates, posing a considerable threat to food security (Naeem *et al*., 2021).

Benzimidazoles (BZs), macrocyclic lactones (MLs), Imidazothiazoles, and amino-acetonitrile derivatives

* Corresponding author: muhammad.oneeb@uvas.edu.pk
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(AADs) are four chemical groups commonly utilized as anthelmintic drugs. These drugs are widely employed to control *H. contortus* infections in small ruminants (Kotze and Prichard, 2016). However, the prolonged and persistent use of these drugs has led to the emergence of resistance in *H. contortus* (Arsenopoulos *et al*., 2021). Furthermore, the use of these drugs in small ruminants has been linked to adverse effects, including gastrointestinal upset, neurotoxicity, and liver damage (Silva *et al*., 2024). Furthermore, environmental contamination and potential adverse effects on non-target populations have become a major concern (Abubakar *et al*., 2024). In response to these challenges, researchers are increasingly exploring phytotherapeutic alternatives as potential anthelmintics. The concept of using botanical alternatives is gaining popularity, as consumers prefer natural products over synthetic ones (Lone *et al*., 2017). Botanical-derived anthelmintics are generally well-tolerated by animals and leave fewer residues in meat and milk (Ahmed and Al-Jubori, 2020). Moreover, various plant species have been used traditionally to treat human and animal illnesses across different regions (Nyako *et al*., 2016).

Numerous plant-derived compounds, including bioactive molecules present in extracts, powders, and essential oils, are used as pesticide and to inhibit the proliferation of insects and pests. The mechanism of action of plants as biopesticides primarily depends on their active chemical constituents, such as proteins, oxalates, glycosides, terpenes, polyphenols, alkaloids, and anthocyanins. Secondary metabolites, including flavonoids, monoterpenes, and organosulfur compounds, play a crucial role in inhibiting the growth of target insects and parasites (Ismail *et al*., 2020). Various plant extracts have been shown to disrupt the biological functions of nematodes (Chagas, 2015). Plant secondary metabolites can affect fundamental activities essential for the survival, growth, and reproduction of target organisms (Ismail *et al*., 2020).

Eucalyptus sp. have been extensively researched globally, with over 700 species identified, all of which possess medicinal properties. Various extracts, including those obtained from different solvents, as well as essential oils derived from the leaves, flowers, and bark of various *Eucalyptus* sp., have been reported to exhibit a range of biological activities. These include anti-fungal, antibacterial, anti-inflammatory, antioxidant, antimalarial, expectorant, antiseptic, mosquito repellent, acaricidal, insecticidal, and anthelmintic properties (Amri *et al*., 2023; Čmiková *et al*., 2023).

Among the *Eucalyptus* species, *Eucalyptus globulus* Labill is particularly renowned and widely cultivated globally due to its remarkable adaptability to diverse environmental conditions (Kanojiya *et al*., 2015). Preliminary research has demonstrated the effectiveness of *E. globulus* extracts against various diseases; however, their specific impact on *H. contortus*, especially regarding seed extracts, remains understudied. Therefore, this study aimed to evaluate the *in vitro* anthelmintic efficacy of aqueous and methanolic extracts of *E. globulus* seeds against *H. contortus* eggs, infective larvae, and adults in sheep and goats.

MATERIALS AND METHODS

Collection and identification of plant material

The methodology employed in this study was adapted from established protocols (Ferreira *et al*., 2018; Kanojiya *et al*., 2015) with minor modifications. Seeds of *Eucalyptus globulus* were collected from a forest in District Nankana Sahib. The seeds were identified with the assistance of the Department of Botany, Government College University, Lahore, and a voucher specimen (GC. Herb. Bot. 3771) was deposited on May 27, 2021. The seeds were rinsed with distilled water and air-dried for 30 min, followed by shade drying at room temperature for 8-12 days. Subsequently, the seeds were oven-dried at 50 °C for 1 h. The dried seeds were then ground into a fine powder and stored in an airtight container until extraction.

Preparation of plant extracts

Two extraction methods were employed to obtain methanolic and aqueous extracts from *E. globulus* seeds. For methanolic extraction, 100 g of seed powder was subjected to soxhlet extraction. For this, Soxhlet apparatus was set up, with duration of approximately 6 h at a temperature of 64.7 °C, corresponding to the boiling point of methanol. The resulting liquid extract was filtered through Whatman No. 1 filter paper. In contrast, aqueous extraction was performed manually. Seed powder (100 g) was dissolved in 500 ml of distilled water and agitated overnight on a rotary shaker. The solution was then heated and filtered through Whatman No. 4 filter paper. The filtrate was concentrated by boiling until its volume reduced to 150 ml. To remove excess solvent from both extracts, a rotary evaporator was used under reduced pressure (50 mm Hg) at 65°C and 20 rpm for 30 min. The extracts were then oven-dried at 50°C for 3-5 days to obtain solid residues. The resulting dry extracts were weighed, yielding 18.66% and 7.6% for methanolic and aqueous extracts, respectively. Finally, the extracts were stored in an airtight container for further use.

Phytochemical analysis

The methanolic and aqueous extracts were subjected

to Gas Chromatography-Mass Spectrometry (GC-MS) analysis at the Food Chemistry Lab, UVAS, Lahore, and the Chemical Engineering Department, COMSATS University Islamabad, Lahore Campus, respectively. A challan ID (2040088988) was issued for the analysis. The GC-MS analysis was performed using an Agilent Technologies GC 7890B coupled with an Agilent Technologies MS 5977B. The samples were manually injected using a 1 µl glass syringe, with helium (99.999% pure) as the carrier gas. The helium flow rate was 1.2 ml/min, with a split ratio of 1:20. For the methanolic extract, a DB-5MS capillary column (60m × 0.25mm × 0.25µm) was used, while for the aqueous extract, an HP-5 MS capillary column (30m × 0.25m × 0.25µm) was employed. The temperature program for the methanolic extract involved an initial temperature of 50°C, increased to 60°C in 1 min, then to 200°C in 8 min, and finally to 230°C in 2 min. For the aqueous extract, the temperature was held at 40°C for 3 min, then raised to 190 °C at a rate of 5 °C/min, and maintained for 20 min. The injector temperature was set at 270°C. The identification of active constituents was performed using a computer-based library search (NIST 17.L), retention indices, and visual interpretation of the mass spectra.

Collection of adult H. contortus*, harvesting of eggs and culturing of L3 larvae for bioassays*

The culturing of L3 larvae was initiated and maintained. Abomasum from slaughtered sheep and goats were collected from public abattoirs and transported to the Department of Parasitology, UVAS, Lahore. The abomasum were opened, and adult *H. contortus* were recovered, washed, and stored in PBS (pH 7) at 25-30°C until further use. Following separation and identification of female *H. contortus*, eggs were isolated, strained, and ground using a pestle and mortar. The egg suspension was then sieved through 150, 53, and 38 µm sieves. Eggs were washed with distilled water, followed by sterile water. The egg suspension was centrifuged at 1500 rpm for 5 min, and the supernatant was discarded. Eggs were stored under anaerobic conditions for 6 h. The egg suspension was then transferred to a petri plate containing wood sawdust, soaked in distilled water to maintain humidity, and incubated at 25-30°C for 10-12 days. Distilled water was added after 2-3 days to maintain relative humidity. L3 larvae were recovered using the Baermann technique and stored at room temperature until the commencement of the bioassay.

In vitro *anthelmintic assays and preparation of stock solution*

The anthelmintic activity of the test extracts was evaluated using three *in vitro* assays: adult worm motility inhibition, egg hatch, and larval paralysis. For each extract, a 100 mg/ml stock solution was prepared by dissolving 1 g of extract in 9 ml of phosphate-buffered saline (PBS). Subsequent two-fold dilutions yielded five concentrations (50, 25, 12.5, 6.2, and 3.125 mg/ml) for each extract.

Adult worm motility inhibition assay

This test was performed by using 90 mm petri dishes (Techno ware Germany). The five different concentrations (50, 25, 12.5, 6.2, and 3.125 mg) were used, along with a positive control (50 mg albendazole) and a negative control (PBS, pH 7) for the aqueous extract. For the methanolic extract, a negative control was prepared by dissolving 0.5 ml methanol in 9.5 ml PBS (pH 7). Ten actively motile worms, regardless of sex, were inoculated into each petri dish in triplicate for each tested concentration. The dishes were maintained at room temperature (25-30°C). Worm motility inhibition or mortality in response to the tested concentrations indicated anthelmintic activity. Worm motility was observed at 0, 3, 6, 9, 12, 15, 18, 21, and 24-h intervals. Dead worms were identified by their faded body color, flat appearance, and lack of movement in the head and tail regions. Confirmation of mortality was performed using a pinching needle. After 24 h, the extracts and positive control were removed, and the worms were immersed in 50°C PBS for 2-3 min. Worms showing any sign of motility were considered alive; otherwise, they were considered dead. The number of dead and live worms was counted, and the percentage of worm motility inhibition was calculated. The formula is as follows;

$$\%WMI = \frac{\text{No. of motile worms in negative control} - \text{No. of motile worms in treatment}}{\text{No. of motile worms in negative control}} \times 100$$

Eggs hatch assay

This assay was conducted in 24-multiwell plates. An egg suspension (250 µl) containing approximately 100 eggs, stored under anaerobic conditions, was distributed into each well. Five concentrations (50, 25, 12.5, 6.25, and 3.125 mg) of each extract were tested. A positive control consisted of 50 mg albendazole, 1 ml PBS (pH 7), and 0.25 ml egg suspension for the aqueous extract. For the methanolic extract, a negative control was prepared with 0.95 ml PBS (pH 7), 0.25 ml egg suspension, and 0.05 ml methanol. The total volume in each well was 1.25 ml. The plates were incubated at 25-28°C for 48 h. The experiment was performed in triplicate for each concentration, including positive and negative controls. After incubation, the number of hatched larvae (dead or alive) and unhatched eggs was counted under a compound microscope at 4X magnification. The percent inhibition of egg hatching was calculated using the formula: Percent inhibition = (A- B)/A×100, where A is the number of eggs

hatched in the control, and B is the number of eggs hatched in each concentration.

Larval paralysis assay

his assay was conducted to evaluate the larvicidal effects of *E. globulus* aqueous and methanolic extracts. Five concentrations (50, 25, 12.5, 6.25, and 3.125 mg) of each extract were prepared from stock solutions, along with a positive control (50 mg albendazole) and a negative control (PBS for aqueous extract or 0.05 ml methanol + 0.9 ml PBS for methanolic extract). Larval suspension (0.25 ml) was added to each well of a 24-multiwell plate in triplicate. The total volume in each well was 1.25 ml. The plates were incubated at 25-30°C for 48 h. After incubation, the larvae were observed under a compound microscope at 4X magnification for the presence or absence of smooth sinusoidal movements. The results were expressed by counting live (motile) and dead larvae.

Statistical analysis

The data from *in vitro* tests were subjected to statistical analysis using IBM SPSS version 20. One-way ANOVA, followed by Tukey's test ($p < 0.05$), was employed to compare the means. Probit analysis was used to calculate the LC_{50} and LC_{90} values at a 95% confidence interval. The results of the in vitro tests are expressed as mean ± standard error of the mean (SEM). The means were compared using the t-test to evaluate the anthelmintic efficacy at different concentrations.

RESULTS

Phytochemical analysis of extract

Table I shows notable difference in the final yield of the aqueous and methanolic extracts of *E. globulus* seeds. The methanolic extract exhibited a significantly higher yield (18.66%) compared to the aqueous extract (7.6%).

Effect of adult worm motility

Five different concentrations of aqueous and methanolic extracts (50, 25, 12.5, 6.25 and 3.125mg/ml) were tested against adult *H. contortus* irrespective of sexes for 24 h to check their anthelmintic efficacy. The aqueous extract of *E. globulus* seeds was able to deliver good results in killing the *H. contortus* adult worms (Table II). In contrast, the methanolic extract was less effective. The highest concentration (50 mg /ml) of aqueous and methanolic extract significantly inhibited the motility of adult worms after 24 h with mean percent inhibition 100±0 and 63.3±7.3, respectively. Whereas, the lowest concentration (3.125 mg/ml) of aqueous extract showed 3.33±3.33% inhibition after 9 h and methanolic extract had no adulticide activity until 15 h. This dose–dependent efficacy of the aqueous and methanolic extracts was confirmed by the LC_{50} (8.434 mg/ml and 21.996 mg/ml) and LC_{90} (47.530 mg/ml and 360.405 mg/ml) values, respectively (Table IV), which emphasize that low doses may not be sufficient for optimal control. For aqueous extract, 50 mg concentration showed 100±0 % inhibition, while 3.125 mg concentration showed 30±0 % inhibition after 24 h. This indicated that there was no significant difference between these two groups at 24 h despite the large difference in numerical values. In contrast, at earlier time points, 50 mg concentration shows 46.67±3.33 % inhibition and 3.125 mg concentration shows 10±0 % inhibition after 15 h, thus, there is a significant difference between them. This test also helps to observe how the inhibition changes over time at different concentrations, such that for aqueous extract, 50 mg concentration shows 30±0 % inhibition, while 3.125 mg concentration shows 0±0 % inhibition after 9 h, thus revealed statistically significant difference at this time point ($p < 0.05$). However, after 24 h, 50 mg showed 100±0 % inhibition that is significantly different from lower concentrations at but certain lower concentrations (25 mg), it showedthat by this point, their inhibition effect is statistically comparable. When comparing the two extracts, 50 mg of methanolic extract (Table II), showed 63.3±7.3 % inhibition after 24 h, while 3.125 mg concentration showed 11.1±0 % inhibition.

Table I. GC-MS analysis of aqueous of methanolic extracts of *E. globulus* seeds.

S. No.	Active ingredient	Aqueous extract	Methanolic extract
1	Aromandendrene	24.8 %	1.18 %
2	Alpha-Selinene	-	2.59 %
3	Camphor	11 %	-
4	Carvacrol	8.1 %	-
5	Catechol	3.6 %	-
6	Cinnamic acid	13.5 %	-
7	Eucalyptol	9.8 %	-
8	(+)- γ Gurjunene	-	1.38 %
9	m-Anisidine	-	9.24 %
10	3- Methyladipic acid, anhydride with acetic acid	-	84.05 %
11	2-Pentynal	-	1.55 %
12	P- Cymene	4.6 %	-
13	Phenol	9.4 %	-
14	Thymol	13.2%	-
Total		100%	99.99%

Table II. Effect of aqueous and methanolic extracts of *Eucalyptus globulus* Labill seeds on percent inhibition (Mean±SEM) of motility of adult worm.

Time	Extract of *E. globulus* seeds					Control positive
	3.125mg	6.25 mg/ml	12.5 mg/ml	25 mg/ml	50 mg/ml	
Effect of aqueous extract						
3h	0±0	0±0	0±0	6.66 ± 3.33^{H}	16.66 ± 3.33^{H}	63.33 ± 3.33^{C}
6h	0±0	0±0	10±0	16.67 ± 3.33^{G}	26.66 ± 3.33^{G}	80 ± 0^{B}
9h	0±0	3.33 ± 3.33^{F}	13.33 ± 3.33^{F}	20 ± 0^{F}	30 ± 0^{F}	100 ± 0^{A}
12h	3.33 ± 3.33^{E}	10 ± 0^{E}	20 ± 0^{E}	30 ± 0^{E}	40 ± 0^{E}	
15h	10 ± 0^{CD}	16.67 ± 3.33^{D}	26.67 ± 3.33^{D}	36.67 ± 3.33^{D}	46.67 ± 3.33^{D}	
18h	13.33 ± 3.33^{C}	23.33 ± 3.33^{C}	30 ± 0^{C}	46.67 ± 3.33^{C}	60 ± 0^{C}	
21h	20 ± 0^{B}	30 ± 0^{B}	43.33 ± 3.33^{B}	56.67 ± 3.33^{B}	80 ± 0^{B}	
24h	30 ± 0^{A}	43.33 ± 3.3^{A}	53.33 ± 3.33^{A}	73.33 ± 3.33^{A}	100 ± 0^{A}	
Effect of methanolic extract						
3h	0±0	0±0	0±0	0±0	0±0	63.33 ± 3.33^{C}
6h	0±0	0±0	0±0	0±0	6.66 ± 3.33^{G}	80 ± 0^{B}
9h	0±0	0±0	0±0	10 ± 0^{F}	16.67 ± 3.33^{F}	100 ± 0^{A}
12h	0±0	0±0	6.66 ± 3.33^{E}	$16.67\pm3.33_{E}$	30 ± 0^{E}	
15h	0±0	6.66 ± 3.33^{D}	13.33 ± 3.33^{D}	20 ± 0^{D}	40 ± 0^{D}	
18h	3.33 ± 3.33^{C}	13.33 ± 3.33^{C}	20 ± 0^{C}	33.33 ± 3.33^{C}	50 ± 0^{C}	
21h	10 ± 0^{AB}	20 ± 0^{AB}	23.33 ± 3.33^{B}	40 ± 0^{B}	56.67 ± 3.33^{B}	
24h	11.1 ± 0^{A}	22.2 ± 0^{A}	25.9 ± 3.7^{A}	48.13 ± 3.33^{A}	$63.\pm7.3^{A}$	

Notably, this comparison revealed no statistically significant difference between the two concentrations. Meanwhile, at 15 h, same concentrations differ significantly, indicated, the higher concentration was significantly more effective. The Tukey test provides a clearer picture of how effective different concentrations of the extract are at inhibiting worm motility over time. The test also revealed instances where higher concentrations, although numerically superior, did not differ significantly from lower concentrations at specific time points. This refinement helped to clarify the conclusions drawn from the data. Moreover, the statistical test indicated that the observed differences might be attributed to random data variation rather than genuine differences in the extract's efficacy at those concentrations.

Effect on egg hatching

EHA revealed that both extracts hindered embryonic development, with the aqueous extract showed higher % inhibition of egg hatching at all concentrations (50mg-3.125mg), such as 95.17±0.35, 88.96±0.94, 81.38±0.59, 76.54±0.97 and 69.28±0.41, respectively. Alongside, methanolic extract showed 84.46±0.99, 77.97±1.58, 73.28±0.94, 67.13±1.41 and 61±0.74 % inhibition of egg hatching at 50mg-3.125mg concentrations, respectively. In both cases, significant differences between the extracts has been noted, confirming that the aqueous extract was more effective than the methanolic extract in inhibiting egg hatching. Furthermore, higher concentrations of both extracts exhibited significantly more inhibition than lower concentrations, confirming the dose-dependent effectiveness of the extracts. The LC_{50} and LC_{90} (Table IV) of aqueous extract were 0.839 mg/ml and 25.299 mg/ml respectively. For methanolic extract, they were 0.768 mg/ml and 136.490 mg/ml respectively. This indicated that aqueous extract was better in inhibiting egg hatching as compared to the methanolic extract counterpart at same concentration. These results are demonstrated in Table III. Albendazole showed 100±0 % inhibition of egg hatching.

Effect on larval paralysis

The larval paralysis assay (LPA) also showed the same dose-dependent trend as by the above two tests. The aqueous extract (50 mg-3.125mg) showed 84.56±0.28,

Table III. Effect of different concentrations of aqueous and methanolic extracts of *E. globulus* Labill seeds on percent inhibition (Mean±SEM) of hatching of eggs and paralysis of L3 *H. contortus* larvae.

Drug conc.	Aqueous extract	Methanolic extract
Effect on hetching of eggs		
50 mg	95.17 ± 0.35^{AA}	84.46 ± 0.99^{BB}
25 mg	88.96 ± 0.94^{BA}	77.97 ± 1.58^{CB}
12.5 mg	81.38 ± 0.59^{CA}	73.28 ± 0.94^{DB}
6.25 mg	76.54 ± 0.97^{DA}	67.13 ± 1.41^{EB}
3.125 mg	69.28 ± 0.41^{EA}	61 ± 0.74^{FB}
Control + ve	100±0	100±0
Effect on larvae		
50 mg	84.56 ± 0.28^{BA}	81.82 ± 0.49^{BB}
25 mg	80.19 ± 1.71^{CA}	75.75 ± 0.33^{CB}
12.5 mg	75.5 ± 1.15^{DA}	69.74 ± 2.42^{DB}
6.25 mg	70.14 ± 2.53^{EA}	63.27 ± 0.97^{EB}
3.125 mg	63.41 ± 1.41^{FA}	56.02 ± 1.42^{FB}
Control + ve	97.6 ± 0.32^{AA}	97.02 ± 0.32^{AA}

Different superscript letters indicates significant differences ($p < 0.05$) within columns.

80.19 ± 1.71, 75.5±1.15,70.14±2.53 and 63.41±1.41% paralysis of L3 stage larvae. Whereas, methanolic extract showed 81.82±0.49, 75.75±0.33, 69.74±2.42, 63.27±0.97 and 56.02±1.42 % paralysis of L3 larvae, respectively. The statistically significant differences between extracts and concentrations, confirmed that the aqueous extract was generally more effective than the methanolic extract at inhibiting larval motility, particularly at lower concentrations. Meanwhile, the LC_{50} and LC_{90} (Table IV) of aqueous extract were 0.207 mg/ml and 145.550 mg/ml, respectively. Methanolic extract LC_{50} and LC_{90} was 1.015 mg/ml and 165.712 mg/ml, respectively. Aqueous extract was better to develop larval paralysis in L3 stage larvae as compared to methanolic extract counterpart at same concentrations. Albendazole showed 97.6±0.32 % paralysis of L3 stage larvae.

DISCUSSION

In vitro assays, such as egg hatch, larval paralysis, larval development, and adult worm motility inhibition assays, are widely employed in veterinary parasitology to evaluate the anthelmintic activity of botanical extracts and essential oils at various developmental stages of parasites. These assays are prerequisites for developing new anthelmintic drugs, and *H. contortus* is a suitable model organism due to its relatively long survival rate in PBS (Ahmed *et al*., 2020; Coles *et al*., 1992; Davuluri *et al*., 2020; Santos *et al*., 2019). *In vitro* procedures are rapid, simple, time saving, require low cost and have good sensitivity because the botanical extracts or compound to be tested are in direct contact with various life cycle stages of parasites (Davuluri *et al*., 2020). *In vitro* techniques minimize the number of live animals needed for *in vivo* trial (Ahmed and Al-Jubori, 2020). According to World Association for the Advancement of Veterinary Parasitology (WAAVP), it is recommended that the effective anthelmintic should inhibit more than 90 % egg hatching and larval motility and when inhibit 80-90 % it is considered moderately effective (Ferreira *et al*., 2013).

In the present study, the percentage yield of methanolic extract of *E. globulus* seeds was higher as compared to aqueous extract. While, Kanojiya *et al*. (2015) reported that percentage yield of methanolic extract was lower thanaqueous extract of *E. globulus* leaves. However, methanolic extract of *Prunella vulgaris* had more yield as compared to aqueous extract reported by Lone *et al*. (2017).

Table IV. Effect of LC_{50} and LC_{90} of *E. globulus* seeds aqueous and methanolic extract on motility, egg hatching and larval paralysis of *H. contortus*.

E, globulus seeds	Tests	LC_{50}			LC_{90}		
		LC_{50} (mg/ml)	Lower limit (mg/ml)	Upper limit (mg/ml)	LC_{90} (mg/ml)	Lower limit (mg/ml)	Upper limit (mg/ml)
Aqueous extract	AWMI	8.434	5.899	11.388	47.530	29.952	110.912
	EHA	0.839	0.404	1.354	25.299	19.510	36.278
	LPA	0.707	0.207	1.402	145.440	76.292	459.843
Methanolic extract	AWMI	21.996	13.901	46.743	360.405	116.116	8069.665
	EHA	0.768	0.244	1.475	136.490	73.443	404.767
	LPA	1.015	0.374	1.817	165.712	87.193	503.265

AWMI, adult worm motility inhibition assay; EHA, egg hatch assay; LPA, larval paralysis assay.

Phylochamical components of seed extracts

In the present study, GC-MS analysis of both extracts revealed the presence of various active constituents in aqueous and methanolic extract and they have been reported to possess in combination or alone anthelmintic properties. The quality and percentage of active ingredients of the extract or essential oil depends upon various factors like soil in which plant grow, climate, season, parts of plant used, type of solvent and extraction methodology. Like eucalyptol that have been reported by various researchers in different types of botanical extract and essential oil. P-cymene has been reported in different concentration by various researchers. Camphor, thymol, aromandendrene and selinene were present in various concentrations in different extract and essential oils. The concentrations of eucalyptol, P-cymene, camphor, aromandendrene, thymol and selinene of both the extracts were present in the extracts (de Aquino *et al*., 2013; de Araújo-Filho *et al*., 2018; Ferreira *et al*., 2016, 2018; Macedo *et al*., 2010, 2011; Qi *et al*., 2015; Shala and Gururani, 2021; Zhu *et al*., 2013). These compounds have also been reported by different researchers in *E. globulus* while they were also are present in both extracts in the present study.

Anthelmintic effect of extract

The present study depicted, both extracts of *E. globulus* seeds effectively inhibit egg hatching and causing larval paralysis at lower and higher concentrations in dose dependent manner while aqueous extract has better efficacy and LC_{50} and LC_{90} as compared to methanolic extract. Similarly, Kanojiya *et al*. (2015) reported that aqueous extract had better ED_{50} and ED_{90} than methanolic extract of *E. globulus* leaves against gastrointestinal nematodes of sheep and had more egg hatching inhibition as compared to methanolic extract. Likewise, Sastya *et al*. (2018) reported that *Eucalyptus citriodora* crude aqueous extract had better ED_{50} than crude methanolic extract and inhibited 100 % egg hatching at 50 mg/ml while ED_{99} of crude methanolic extract was better than crude aqueous extract against gastrointestinal nematode of goat. However, in present study LC_{50} of methanolic extract was better than aqueous extract and LC_{90} of aqueous extract was better than methanolic extract and aqueous extract causing more egg hatching inhibition as compared to methanolic extract. Contrary to present findings, essential oil of various species of *Eucalyptus* had more efficacy at lower doses than different species of *Eucalyptus* extracts like *Eucalyptus staigeriana* essential oil at 1.35 mg/ml inhibited 99.27±1.09 % egg hatching (Macedo *et al*., 2010), *Eucalyptus citriodora* essential oil (5.3 mg/ml) inhibited 98.8±0.43 % egg hatching (Macedo *et al*., 2011) and *Eucalyptus citriodora* essential oil (2 mg/ml) caused 97.15±1.20 % inhibition of egg hatching of *H. contortus* isolates (de Araújo-Filho *et al*., 2018). Whereas, *E. globulus* essential oil inhibited 99.3 % egg hatching at 21.75 mg/ml of *H. contortus* (Qi *et al*., 2015).

The findings from AWMI, EHA and LPA suggest that the aqueous extract of *E. globulus* seeds is generally more effective than the methanolic extract. The differences in chemical composition between the aqueous and methanolic extracts of *E.globulus* seeds can explain the variations in their anthelmintic efficacy. The aqueous extract, which contains compounds such as eucalyptol, carvacrol, phenol, and thymol, is rich in compounds known for strong anthelminthic, antimicrobial and antifungal properties. These molecules likely contribute to the higher efficacy of the aqueous extract in inhibiting both adult worm motility and egg hatching. Thymol, for example, is a well-known antimicrobial agent that disrupts cell membranes, while phenol has strong toxic effects on a wide range of parasites (Desouky *et al*., 2022).

On the other hand, the methanolic extract, although containing compounds such as alpha-selinene and (+)-γ gurjunene, which may have some degree of biological activity, lacks the more potent anthelmintic compounds present in the aqueous extract. This explains why methanolic extract demonstrated lower efficacy in all assays. Moreover, the lower yield of the methanolic extract might further contribute to its reduced performance, as a lower concentration of bioactive compounds would be available for interaction with the parasites (Mumed *et al*., 2022). Therefore, the differences in chemical composition, extraction yield, and the concentration of bioactive compounds provide a logical basis for the variations in their efficacy against *H. contortus*.

The present study revealed that compounds in the aqueous extract are primarily phenolic compounds and terpenes, which are known for their strong antimicrobial, antifungal, and have anthelmintic activities. Plant extract's anthelmintic activity is mainly due to presence of tannin, alkaloids, terpenoids, saponin, phenolic acids and flavonoids in combination or alone (Davuluri *et al*., 2020; Lone *et al*., 2017; Santos *et al*., 2019). Botanical extracts like terpenoids disrupt energy providing mechanism and inhibited LDH enzyme activity that enhance lactic acid production ultimately decreased ATP synthesis that leads to death of parasites (Ahmed *et al*., 2020; Kanojiya *et al*., 2015).

CONCLUSION

The aqueous extract exhibited far more promising

outcomes, but both extracts showed notable anthelmintic activity against the three life cycle phases of *H. contortus*. This suggests that *E. globulus* seeds may be an efficient herbal remedy for ruminant gastrointestinal parasite management. The aqueous extract's greater effectiveness implies that its active ingredients, which include terpenes and phenolic compounds, are better in destroying the parasites. By suggesting a possible substitute in ethno-veterinary medicine, this is a positive step in tackling the soaring issue of parasite resistance to synthetic anthelmintics.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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