

RESEARCH ARTICLE

Ovicidal potential of *Hedysarum gmelinii* Ledeb. extract against *Toxocara cati* and strongylid eggs with preliminary cytotoxic and antimicrobial evaluation



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ABSTRACT

Background and Aim: *Hedysarum gmelinii* Ledeb. contains bioactive phenolic compounds, including prenylated chalcones, but its biological properties, particularly its ovicidal activity against veterinary parasites, remain insufficiently characterized. This study aimed to evaluate the *in vitro* ovicidal activity of *H. gmelinii* extract against *Toxocara cati* and strongylid eggs and to provide a preliminary assessment of its cytotoxic, antibacterial, and antifungal activities.

Materials and Methods: Aerial parts of *H. gmelinii* were collected in the Akmola Region, Kazakhstan, and extracted with ethanol:chloroform (1:1) using a Soxhlet apparatus. Cytotoxicity was evaluated using *Artemia salina* nauplii at extract concentrations of 1%–10%. Antibacterial and antifungal activities were assessed by agar well diffusion against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. Ovicidal activity was evaluated against *T. cati* eggs at 5% and 10% and strongylid eggs at 10%, 20%, and 30%, with exposure periods ranging from 30 min to 48 h. Egg viability was evaluated after 21 days based on morphological evidence of shell damage, loss of internal contents, and arrested embryonic development.

Results: *A. salina* nauplii showed no mortality at 1%–10% extract concentrations. Antibacterial and antifungal activities were weak and most evident at higher concentrations. At 10%, inhibition zones were 12.0 ± 0.12 mm for *S. aureus*, 14.0 ± 0.14 mm for *E. coli*, and 13.0 ± 0.18 mm for *C. albicans*. Ovicidal activity increased with concentration and exposure time. After 48 h, non-viability of *T. cati* eggs reached $79.0 \pm 1.9\%$ and $89.0 \pm 1.8\%$ at 5% and 10%, respectively. For strongylid eggs, non-viability reached $89.0 \pm 1.2\%$, $92.0 \pm 1.3\%$, and $95.0 \pm 2.2\%$ at 10%, 20%, and 30%, respectively. Differences from the negative control were significant ($p < 0.05$). Microscopy revealed eggshell deformation, disruption of internal structures, and arrested embryonic development.

Conclusion: *H. gmelinii* extract demonstrated pronounced *in vitro* ovicidal activity against *T. cati* and strongylid eggs but limited antibacterial and antifungal activity. These preliminary findings support further phytochemical standardization, mammalian safety assessment, and *in vivo* evaluation before establishing its potential veterinary antiparasitic application.

Keywords: antibacterial activity, antifungal activity, *Hedysarum gmelinii*, *in vitro*, ovicidal activity, plant extract, strongylid eggs, *Toxocara cati*.

INTRODUCTION

Plants contain diverse bioactive compounds and represent an important source of therapeutic agents for pharmacological research. Investigating their chemical composition can help identify and develop biologically active compounds with potential medical and veterinary applications. Plant-derived preparations have long been used in human and veterinary medicine, either independently or as components of combination therapies. The search for plant-derived alternatives is particularly relevant in veterinary parasitology because resistance to major anthelmintic classes, including benzimidazoles, tetrahydropyrimidines, and macrocyclic lactones, is widespread among livestock and companion-animal parasites [1, 2]. Consequently, ethnomedicinal preparations and crude plant extracts are increasingly being investigated as complementary or alternative approaches to parasite control.

Phytochemical characterization is important for interpreting the biological activity of plant extracts. Determining extraction yield, dry residue content, and the qualitative and quantitative composition of major

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compound groups helps standardize phytopreparations, compare studies, and assess relationships between chemical composition and pharmacological activity [3]. Previous successful applications of plant-derived preparations in veterinary medicine have encouraged the continued investigation of new phytopreparations. For example, medicinal preparations derived from plants of the Polygonaceae family have been investigated for their biological and pharmacological activities and subsequently applied in veterinary obstetric practice [4].

Among plants with potential biological activity, *Hedysarum gmelinii* Ledeb. is of particular interest because prenylated and isoprenylated chalcones and other phenolic compounds have been identified in this species [5–7]. Some chalcones isolated from *H. gmelinii* have demonstrated anti-inflammatory and antiproliferative activities. Reported half-maximal inhibitory concentration (IC₅₀) values for inhibition of nitric oxide production ranged from 3.25 to 8.48 µM, whereas IC₅₀ values against A549 and HCT116 cell lines ranged from 7.79 to 9.67 µM. These findings indicate that *H. gmelinii* is a potential source of biologically active natural compounds; however, its chemical composition and pharmacological properties remain incompletely characterized [7–10]. The ethanol:chloroform (1:1) extraction system used in the present study was selected to recover both relatively polar phenolic compounds and flavonoids, as well as more lipophilic prenylated chalcones previously reported in this species [11], rather than targeting a single compound class.

Interest has also increased in plant extracts with antiparasitic properties, including those that reduce helminth egg viability, damage the eggshell, and disrupt embryonic development. This approach is relevant to companion-animal parasitology in Kazakhstan. Surveys in major cities in Kazakhstan found intestinal parasites in 17.7% (230/1301) of examined cats, with *Toxocara cati* as the most frequently detected parasite (6.2%) [12]. Therefore, evaluating the ovicidal activity of *H. gmelinii* against *T. cati* eggs is potentially relevant to veterinary parasitology. A similar rationale applies to equine strongylids, particularly cyathostomins, which are important gastrointestinal parasites of horses. Documented resistance to major anthelmintic classes used in horses further justifies investigating plant-derived compounds active against strongylid eggs.

Although *H. gmelinii* contains biologically active chalcones and other phenolic compounds, evidence on the broader biological activity of its extracts remains limited. Lider *et al.* [13] previously demonstrated an ovicidal effect of *H. gmelinii* extract against *T. cati* eggs using a different concentration and exposure time design. However, that investigation did not evaluate strongylid eggs of veterinary importance in horses or characterize the same extract across complementary cytotoxic, antibacterial, and antifungal assays. Consequently, the extent to which the ovicidal activity of *H. gmelinii* extends across different helminth egg types and how this activity relates to its broader biological profile remains insufficiently characterized. In addition, the phytochemical composition of the extract evaluated in the present study prevents attribution of the observed biological effects to specific constituents. These gaps highlight the need for a broader preliminary evaluation of the biological properties of *H. gmelinii* extract while recognizing the subsequent requirement for phytochemical characterization and standardization.

Therefore, this study aimed to evaluate the biological activity of an ethanol:chloroform (1:1) extract of *H. gmelinii* through complementary *in vitro* assays. Specifically, the study evaluated preliminary cytotoxicity using *Artemia salina*, antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, antifungal activity against *Candida albicans*, and concentration- and exposure time-dependent ovicidal activity against *T. cati* and strongylid eggs. The study further aimed to extend the previous evaluation of *H. gmelinii* against *T. cati* eggs [13] by incorporating strongylid eggs and a broader preliminary biological activity profile. These experiments were designed to provide a basis for subsequent phytochemical standardization, identify constituents associated with the observed biological effects, and further evaluate the extract's potential veterinary applications.

MATERIALS AND METHODS

Ethical approval

All animal-related procedures were reviewed and approved by the local Animal Ethics Committee of the Institute of Animal Science and Veterinary Medicine, Saken Seifullin Kazakh Agrotechnical University, Astana, Kazakhstan (Protocol No. 4, dated May 18, 2024). The study was conducted in accordance with the institution's internal animal welfare guidelines and the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. Biological material used for the parasitological experiments consisted of fecal samples obtained from naturally infected cats and horses. All procedures involving animals and the collection of biological material were performed with measures intended to minimize animal stress and discomfort.

Study period and location

Plant material of *H. gmelinii* was collected during the spring–summer period (May–July) of 2025 in the vicinity of Shchuchinsk, Burabay District, Akmola Region, Kazakhstan (52°55′31.2″N, 70°05′44.7″E). Laboratory experiments were conducted at Saken Seifullin Kazakh Agrotechnical University, Astana, Kazakhstan. The ovicidal experiments were specifically conducted at the N.T. Kadyrov Laboratory of Parasitology of the University. And cytotoxic and antimicrobial evaluation was conducted at the Complex of Laboratories for Program-Targeted Funding (PTF) in the field of Chemistry of Natural Compounds and Pharmaceuticals at K. Kulazhanov Kazakh University of Technology and Business, Astana, Kazakhstan.

Study design

This was an experimental laboratory study designed to characterize the preliminary biological activity of an ethanol:chloroform extract of *H. gmelinii*. The study comprised three complementary experimental components: (i) cytotoxicity assessment using the *Artemia salina* nauplius lethality assay; (ii) antibacterial and antifungal evaluation against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* using agar well diffusion; and (iii) *in vitro* assessment of concentration- and exposure time-dependent ovicidal activity against *T. cati* and strongylid eggs. Appropriate negative and positive controls were included in each assay, and experimental concentrations and exposure periods were defined separately for each biological assay.

Plant material collection and processing

The aerial parts of *H. gmelinii*, comprising leaves, stems, and inflorescences, were collected during active vegetation. Plants were harvested manually under dry weather conditions from areas remote from industrial zones and major roads. Only well-developed plants without visible damage or signs of disease were selected. Botanical identification was confirmed by Zh. Ibatayev, Candidate of Chemical Sciences and Associate Professor at Saken Seifullin Kazakh Agrotechnical University (Astana, Kazakhstan). The total mass of collected aerial plant material was 0.866 kg. A herbarium specimen was deposited under voucher No. NUR003012 to enable verification of the botanical material.

After collection, the plant material was cleaned to remove foreign matter, damaged portions, dust, and plant debris. The material was air-dried in a shaded, dry, well-ventilated room at 20–25°C and 50%–60% relative humidity. It was spread in a thin layer on paper mats, protected from direct sunlight, and turned periodically to ensure uniform drying. Drying was continued for 7–10 days until a stable dry state was achieved.

The dried plant material was standardized according to the applicable pharmacopoeial requirements. Visual appearance, color, odor, purity, homogeneity, degree of comminution, and the presence of organic and mineral impurities were evaluated. The material was then ground to a uniform particle size, sieved, and stored in paper or fabric bags under cool, dry, light-protected conditions until extraction.

Preparation of *H. gmelinii* extract

Standardized and ground aerial material of *H. gmelinii* was extracted using a Soxhlet apparatus (KEX 250, Germany). Ethanol (96.3%; IVK-PLUS LLC, Russia) and chloroform (chemically pure grade; EKOS-1 JSC, Russia) were used as extraction solvents at a 1:1 ratio. The resulting liquid extract was filtered, and the solvents were removed under reduced pressure using a rotary evaporator (IKA HB digital, Germany) to obtain a homogeneous concentrated extract. The extraction yield was approximately 2%–3% (w/w) relative to the air-dried plant material.

Extract quality was evaluated based on organoleptic characteristics, including color, consistency, homogeneity, and absence of visible foreign impurities. The extract met the requirements of ST RK 978-2001, “Extracts from plant raw materials. Specifications.” Analytical-grade dimethyl sulfoxide (DMSO) (99.9%; FLOTANT CHEMICALS LLC, Russia) was used to dissolve the dry extract for experimental use. The final concentration of DMSO in the test solutions was 1%. The prepared extract was dispensed into dark glass vials and stored under conditions protected from light and moisture.

Cytotoxicity assay

Preliminary cytotoxicity of the *H. gmelinii* extract was evaluated using the brine shrimp lethality assay with *A. salina* nauplii. To obtain nauplii, 200 mg of *A. salina* cysts were incubated in 500 mL of artificial seawater at 25°C under continuous aeration and illumination. After hatching, active nauplii that accumulated in the illuminated area were collected using a pipette and used for the assay.

The assay was conducted in 24-well plates. The extract was evaluated at concentrations of 1%, 2.5%, 5%, and 10%, with each concentration tested in triplicate. Each well received 990 µL of artificial seawater containing 20 nauplii, followed by 10 µL of extract solution (final well volume 1000 µL). Stock solutions were prepared so that the 100-fold in-well dilution yielded the reported final test concentrations. Actinomycin D (10 µg/mL) served as the positive control, while DMSO served as the negative control. Samples were incubated for 24 h, after which live and dead nauplii were counted and mortality was calculated for each extract concentration.

Naupliar mortality was calculated according to the following formula [14]:

$$P = [(A - N - B)/Z] \times 100\%$$

where P = Naupliar mortality (%); A = Number of dead nauplii after 24 h; N = Number of dead nauplii before the experiment; B = Mean number of dead nauplii in the negative control; and Z = Total number of nauplii.

Antibacterial and antifungal activity

The antibacterial and antifungal activities of the *H. gmelinii* extract were evaluated using the agar well diffusion method (TM Media/Titan Biotech Ltd., Bhiwadi, Rajasthan, India) [13]. The extract was tested at concentrations of 1%, 2.5%, 5%, and 10%. Gentamicin (10 µg/well) served as the reference antimicrobial for bacterial cultures, whereas fluconazole (25 µg/well) served as the reference antifungal for *C. albicans*.

The test microorganisms comprised *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231, obtained from the Republican Collection of Microorganisms (Astana, Kazakhstan). The cultures were propagated in liquid nutrient media at pH 7.3 ± 0.2 and 30°C for 24–48 h. Meat-peptone broth was used for the bacterial cultures, whereas Czapek medium (Condalab, Madrid, Spain) was used for *C. albicans*. Both media were prepared and autoclaved at 121°C for 15 min according to the manufacturers' instructions. After loading the extract into the wells, plates were incubated at 37°C for 24 h (bacteria) and at 28–30°C for 48 h (*C. albicans*), after which inhibition-zone diameters were measured.

The microbial suspensions were standardized to 2.07×10^6 colony-forming units (CFU)/mL for *S. aureus*, 2.49×10^6 CFU/mL for *E. coli*, and 2.95×10^5 CFU/mL for *C. albicans*.

For each microorganism, 100 µL of the standardized suspension was inoculated onto the surface of nutrient agar in Petri dishes and spread uniformly using the lawn-culture technique. After the agar surface had dried, 8-mm-diameter wells were prepared, and 100 µL of the corresponding extract solution was added to each well.

For each test microorganism, four experimental groups corresponding to the four extract concentrations and one reference-control group were evaluated. Each group was tested in triplicate, resulting in 15 experimental determinations per microorganism. Biological activity was assessed by measuring the diameter of the inhibition zone around each well. Inhibition zones <10 mm were classified as no activity, 10–15 mm as weak activity, 15–20 mm as moderate activity, and >20 mm as pronounced activity. Results were expressed as mean values with standard errors.

Ovicidal activity

The ovicidal activity of the *H. gmelinii* extract was evaluated *in vitro* against *T. cati* and strongylid eggs. Eggs were isolated from fecal samples by a flotation technique using a Sheater's sugar solution with a specific gravity of 1.18–1.20. The egg concentration in the samples was determined as eggs per gram of feces (EPG) using the McMaster technique and was approximately 12,500 EPG for *T. cati* and 650 EPG for strongylids. Eggs were identified by light microscopy (×100) based on their morphological characteristics. The experimental approach was based on methods for assessing helminth egg viability and infective material, consistent with World Association for the Advancement of Veterinary Parasitology (WAAVP) guidance and the methodology described by Easland *et al.* [16].

An aqueous suspension of parasite eggs was used as the biological material. *T. cati* eggs were isolated from fecal samples obtained from naturally infected cats (n=5) that had not received anthelmintic treatment. Strongylid eggs were recovered from fecal samples obtained from naturally infected horses (n=10) from 2 farms in the Akmola Region, Kazakhstan.

For *T. cati*, the extract was evaluated at concentrations of 5% and 10%, whereas concentrations of 10%, 20%, and 30% were evaluated against strongylid eggs. For each treatment, 5 mL of the corresponding extract solution and 1 mL of parasite egg suspension were placed in 20-mL centrifuge tubes. The mixtures were thoroughly agitated and exposed to the treatments for 30 min, 60 min, 24 h, or 48 h. Approximately 400 eggs were evaluated per experimental group.

Following exposure, the suspensions were washed by centrifugation (CLMN-R10/2700 centrifuge, Elecon, Russia) with distilled water at 1,500 rpm ($\approx 441 \times g$) for 5 min to remove residual extract. Subsequently, 1 mL of the washed suspension was transferred to a sterile Petri dish, 3 mL of distilled water was added, and the dishes were incubated at 24°C. Parasite eggs maintained in distilled water served as the negative control, whereas 4% phenol (Honeywell International Inc., USA), with known ovicidal activity, served as the positive control.

Egg viability was evaluated on day 21. An egg was classified as non-viable when one or more of the following morphological criteria were observed: visible deformation or rupture of the eggshell, disorganization or loss of internal cytoplasmic contents, or arrest of embryonic development before reaching the infective larval stage.

The egg viability index was calculated according to the following formula [17]:

$$T = [(A - B)/A] \times 100\%$$

where T = Egg viability (%); A = Total number of eggs examined; and B = Number of non-viable eggs.

Statistical analysis

Statistical analyses were performed using Statistica version 13.0 (TIBCO Software Inc., USA, www.statsoft.pl/en/software/statistica), SPSS version 26.0 (IBM Corp., Armonk, NY, USA, www.ibm.com/us-en), and Microsoft Excel 2023 (Microsoft Corporation, Washington, USA, www.microsoft.com/en-us/microsoft-365/excel). Categorical data were compared using Pearson's chi-square (χ^2) test or Fisher's exact two-sided test, as appropriate, based on the raw egg counts. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Preliminary cytotoxicity of *H. gmelinii* extract

The results of the preliminary cytotoxicity assessment of the *H. gmelinii* extract at concentrations of 1%, 2.5%, 5%, and 10% are presented in Table 1. No mortality or paralysis of *A. salina* nauplii was observed at any tested extract concentration during the 24-h exposure period.

Table 1: Preliminary cytotoxicity assay of *Hedysarum gmelinii* extract against *Artemia salina* nauplii.

Concentration	Live nauplii (control)	Dead nauplii (control)	Live nauplii (test)	Dead/paralyzed nauplii (test)	Mortality (%)
1%	20	0	20	0	0
2.5%	20	0	20	0	0
5%	20	0	20	0	0
10%	20	0	20	0	0

n = 3 replicates per concentration; values are presented as means. Survival was 100% in both control and extract-treated groups at all tested concentrations.

Naupliar survival remained 100% in all control and extract-treated groups, with a mean of 20 surviving nauplii and no mortality at each concentration. Consequently, the extract did not produce detectable lethality in the *A. salina* assay within the tested concentration range of 1%–10%. The median lethal concentration (LC_{50}) could not be determined because no mortality occurred at any tested concentration.

Antibacterial and antifungal activity of *H. gmelinii* extract

The antibacterial and antifungal activities of the *H. gmelinii* extract were evaluated against *S. aureus*, *E. coli*, and *C. albicans* using the agar well diffusion method. The extract was tested at concentrations of 1%, 2.5%, 5%, and 10%, and inhibition zone diameters are presented in Table 2.

Table 2: Antibacterial and antifungal activity of *Hedysarum gmelinii* extract against test microorganisms.

Treatment	<i>Staphylococcus aureus</i> (mm, M $\pm$ m)	<i>Escherichia coli</i> (mm, M $\pm$ m)	<i>Candida albicans</i> (mm, M $\pm$ m)
1%	8.0 $\pm$ 0.05	8.0 $\pm$ 0.61	12.0 $\pm$ 0.48
2.5%	8.0 $\pm$ 0.13	8.0 $\pm$ 0.54	12.0 $\pm$ 0.63
5%	9.0 $\pm$ 0.12	9.0 $\pm$ 0.12	13.0 $\pm$ 0.37
10%	12.0 $\pm$ 0.12	14.0 $\pm$ 0.14	13.0 $\pm$ 0.18
Gentamicin (10 μ g/well)	20.0 $\pm$ 0.00	20.0 $\pm$ 0.00	–
Fluconazole (25 μ g/well)	–	–	33.0 $\pm$ 0.00

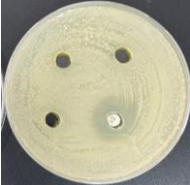
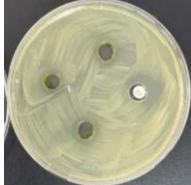
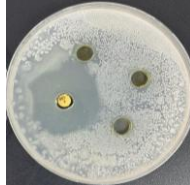
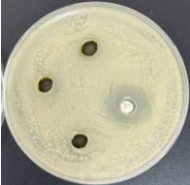
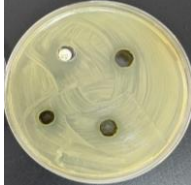
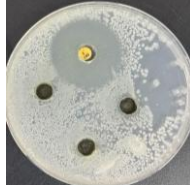
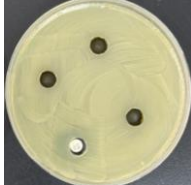
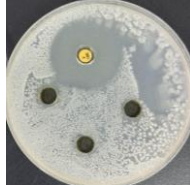
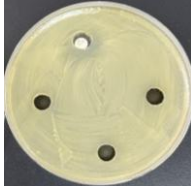
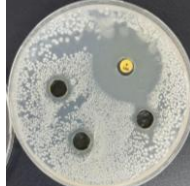
Data are presented as M $\pm$ m. Inhibition zone diameters are expressed in millimeters. Values include the 8-mm well diameter; 8.0 mm therefore denotes no inhibition beyond the well; – = Reference drug not tested against the respective microorganism.

The greatest antibacterial activity was observed at the 10% extract concentration, with inhibition zones of 12.0 $\pm$ 0.12 mm against *S. aureus* and 14.0 $\pm$ 0.14 mm against *E. coli*. At 5%, zone diameters were 9.0 $\pm$ 0.12 mm

for both bacterial species; because the agar wells were 8 mm in diameter and detectable activity was defined as a zone of ≥ 10 mm, these 9.0-mm readings fall within the no-activity category. At 1% and 2.5%, the measured diameters equalled the 8-mm well diameter, indicating no inhibition. Detectable antibacterial activity was therefore confined to the 10% concentration.

Antifungal activity against *C. albicans* was detected at all tested concentrations. Inhibition zone diameters were 12.0 ± 0.48 mm at 1%, 12.0 ± 0.63 mm at 2.5%, 13.0 ± 0.37 mm at 5%, and 13.0 ± 0.18 mm at 10%. Thus, the inhibition zone diameter against *C. albicans* varied only slightly across the tested concentration range (Table 3).

Table 3: Antibacterial and antifungal activity of *Hedysarum gmelinii* extract at concentrations of 1%, 2.5%, 5%, and 10%

Concentration	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>
1%			
2.5%			
5%			
10%			

***In vitro* ovicidal activity of *H. gmelinii* extract**

The ovicidal activity of the *H. gmelinii* extract was evaluated against *T. cati* and strongylid eggs. Final microscopic evaluation was performed 21 days after incubation. Egg viability varied according to extract concentration and exposure duration.

Overall, treatment with the extract reduced the proportion of viable eggs and increased the proportion of non-viable eggs in both parasite groups. At 48 h, the 95% confidence intervals (CIs) for non-viable *T. cati* eggs were 74.4%–83.6% at 5% extract and 85.5%–92.5% at 10% extract (Table 4). For strongylid eggs, the corresponding 95% CIs were 85.5%–92.5% at 10%, 88.9%–95.1% at 20%, and 92.5%–97.5% at 30% extract (Table 5).

For *T. cati* eggs, non-viability generally increased with longer exposure at both tested extract concentrations (Figure 1A).

Table 4: Changes in viability of *Toxocara cati* eggs after exposure to *Hedysarum gmelinii* extract.

Concentration	Exposure time	Viable (%, M $\pm$ m)	Non-viable (%, M $\pm$ m)	95% CI for non-viable eggs
5%	30 min	67.0 $\pm$ 2.7	33.0 $\pm$ 2.6	–
	60 min	52.0 $\pm$ 2.9	48.0 $\pm$ 2.8	–
	24 h	30.0 $\pm$ 2.8	70.0 $\pm$ 1.7	–
	48 h	21.0 $\pm$ 2.3	79.0 $\pm$ 1.9	74.4–83.6
10%	30 min	54.0 $\pm$ 2.5	46.0 $\pm$ 1.8	–
	60 min	56.0 $\pm$ 3.0	44.0 $\pm$ 2.5	–
	24 h	31.0 $\pm$ 1.9	69.0 $\pm$ 2.4	–
	48 h	11.0 $\pm$ 1.8	89.0 $\pm$ 1.8	85.5–92.5
Positive control (4% phenol)	30 min	43.0 $\pm$ 2.9	57.0 $\pm$ 2.0	–

Concentration	Exposure time	Viable (%, M ± m)	Non-viable (%, M ± m)	95% CI for non-viable eggs
Negative control (distilled water)	60 min	21.0 ± 2.4	79.0 ± 2.8	—
	24 h	13.0 ± 1.9	87.0 ± 1.2	—
	48 h	4.0 ± 1.1	96.0 ± 1.7	—
	48 h	96.0 ± 1.4	4.0 ± 1.5	—

Data are presented as M ± m; n = 3 independent replicates. Differences between the experimental groups and the positive control relative to the negative control were statistically significant (χ^2 test or Fisher's exact test, $p < 0.05$). CI = Confidence interval.

Table 5: Changes in viability of strongylid eggs after exposure to *Hedysarum gmelinii* extract.

Concentration	Exposure time	Viable (%, M ± m)	Non-viable (%, M ± m)	95% CI for non-viable eggs
10%	30 min	74.0 ± 2.6	26.0 ± 2.5	—
	60 min	56.0 ± 2.9	44.0 ± 2.6	—
	24 h	23.0 ± 2.4	77.0 ± 2.8	—
	48 h	12.0 ± 1.8	89.0 ± 1.2	85.5–92.5
20%	30 min	34.0 ± 2.7	66.0 ± 2.1	—
	60 min	23.0 ± 1.7	77.0 ± 2.4	—
	24 h	21.0 ± 2.4	79.0 ± 2.2	—
	48 h	8.0 ± 1.6	92.0 ± 1.3	88.9–95.1
30%	30 min	14.0 ± 1.8	86.0 ± 1.9	—
	60 min	8.0 ± 1.6	92.0 ± 1.6	—
	24 h	6.0 ± 1.4	94.0 ± 1.5	—
	48 h	5.0 ± 1.8	95.0 ± 2.2	92.5–97.5
Positive control (4% phenol)	30 min	7.0 ± 1.6	93.0 ± 1.8	—
	60 min	4.0 ± 1.5	96.0 ± 1.4	—
	24 h	4.0 ± 1.2	96.0 ± 1.6	—
	48 h	3.0 ± 1.3	97.0 ± 1.0	—
Negative control (distilled water)	48 h	92.0 ± 1.6	8.0 ± 1.5	—

Data are presented as M ± m; n = 3 independent replicates. Differences between the experimental groups and the positive control relative to the negative control were statistically significant (χ^2 test or Fisher's exact test, $p < 0.05$). CI = Confidence interval.

At 5% extract, the proportion of viable *T. cati* eggs decreased from $67.0 \pm 2.7\%$ after 30 min to $21.0 \pm 2.3\%$ after 48 h, whereas the proportion of non-viable eggs increased from $33.0 \pm 2.6\%$ to $79.0 \pm 1.9\%$. At 10%, the proportion of non-viable eggs increased from $46.0 \pm 1.8\%$ after 30 min to $89.0 \pm 1.8\%$ after 48 h. In the positive control group, 4% phenol resulted in $96.0 \pm 1.7\%$ non-viable eggs after 48 h, whereas $4.0 \pm 1.5\%$ of eggs were non-viable in the negative control group.

The extract also showed ovicidal activity against strongylid eggs. The proportion of non-viable eggs increased with extract concentration and exposure duration (Figure 1B). After 48 h, viable eggs accounted for $12.0 \pm 1.8\%$, $8.0 \pm 1.6\%$, and $5.0 \pm 1.8\%$ at extract concentrations of 10%, 20%, and 30%, respectively. Corresponding non-viability reached $89.0 \pm 1.2\%$, $92.0 \pm 1.3\%$, and $95.0 \pm 2.2\%$.

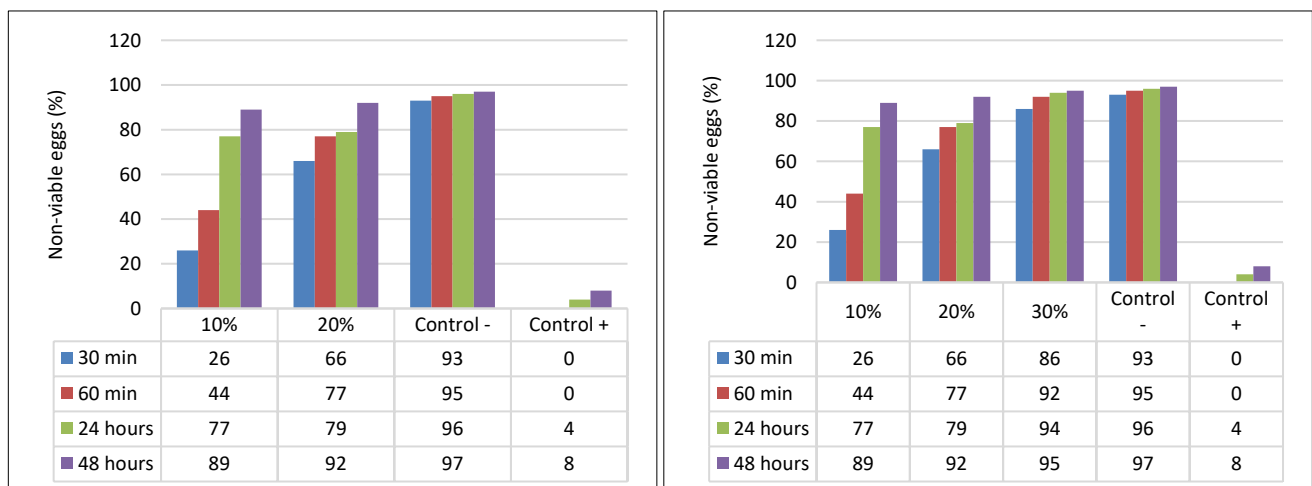


Figure 1: Changes in egg viability after 48-h exposure to *Hedysarum gmelinii* extract and control treatments. (A) Viability of *Toxocara cati* eggs. (B) Viability of strongylid eggs.

Microscopic examination showed partial or complete disruption of the eggshell, loss of internal structure definition, degradation of internal contents, and arrest of embryonic development in extract-treated eggs (Figures 2 and Figure 3). These morphological changes accompanied the reduction in egg viability observed in the quantitative assessments.

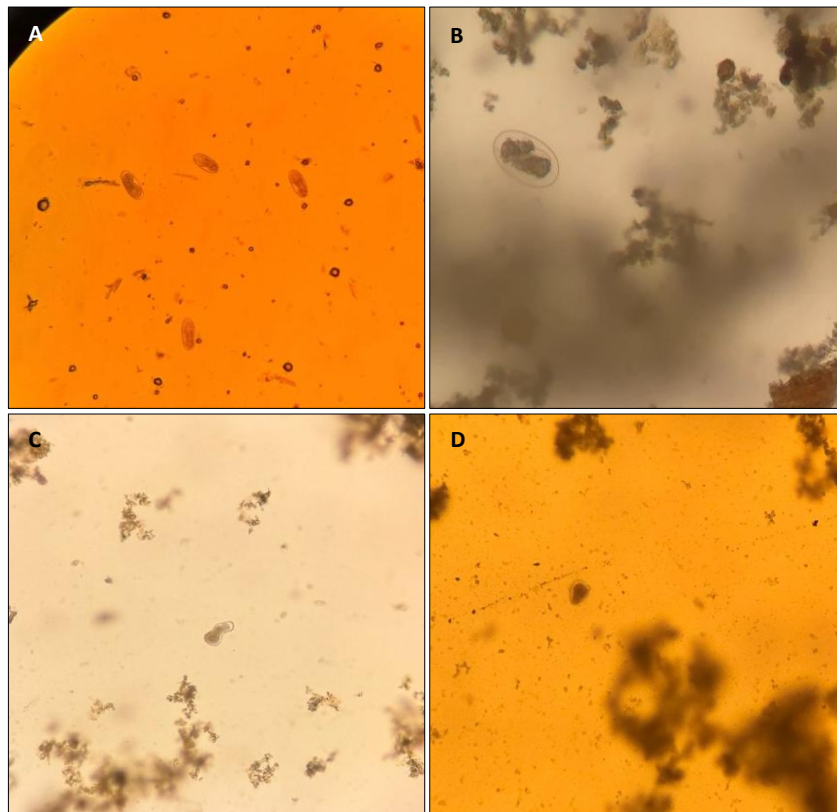


Figure 2. Microscopic appearance of strongylid eggs before and after treatment with 10% *Hedysarum gmelinii* extract. (A) Before treatment; (B) after 30 min; (C) after 60 min; and (D) after 24 h. Magnifications = ×400 and ×1000.

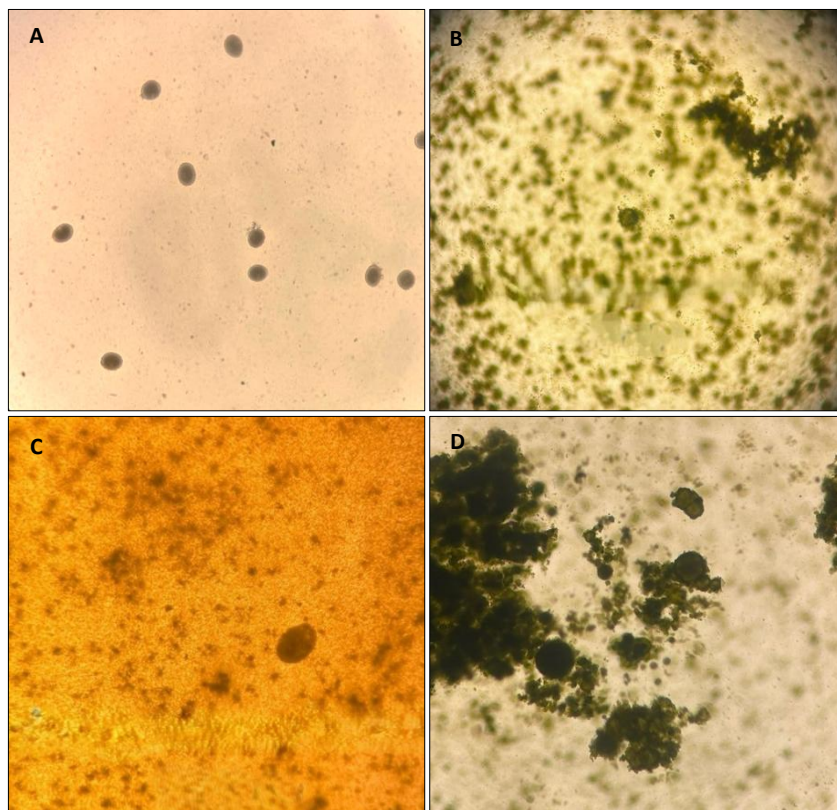


Figure 3: Microscopic appearance of *Toxocara cati* eggs before and after treatment with 10% *Hedysarum gmelinii* extract. (A) Before treatment; (B) after 30 min; (C) after 60 min; and (D) after 24 h. Magnification = ×40.

Overall, the proportion of non-viable eggs increased with extract concentration and exposure duration. After 48 h, non-viability of *T. cati* eggs reached $79.0 \pm 1.9\%$ at 5% extract and $89.0 \pm 1.8\%$ at 10% extract. For strongylid eggs, non-viability reached $89.0 \pm 1.2\%$ at 10% extract and $95.0 \pm 2.2\%$ at 30% extract. These values differed significantly from those in the negative control ($p < 0.05$).

DISCUSSION

Overall biological activity of *H. gmelinii* extract

The biological activity of the *H. gmelinii* extract varied by experimental model. No mortality of *A. salina* nauplii was observed at the tested concentrations, whereas the extract showed limited antibacterial and antifungal activity and substantially greater ovicidal activity against *T. cati* and strongylid eggs. These findings indicate that the biological effects of the crude extract were assay-dependent and were most pronounced in the helminth egg viability experiments.

Preliminary toxicity assessment

In the *A. salina* assay, no naupliar mortality was recorded at extract concentrations of 1%–10% after 24 h of exposure. This finding differs from reports of biological activity in other *Hedysarum* species and isolated constituents. Uyar *et al.* [18] and Keleke *et al.* [19, 20] reported cytotoxic or antioxidant activities of extracts from other *Hedysarum* species, including cytotoxicity of dichloromethane extracts of *Hedysarum aucheri* against tumor cell lines and antioxidant activity of ethanolic extracts of *Hedysarum semenowii*. Liu *et al.* [5] also reported antiproliferative activity of individual prenylated chalcones isolated from *H. gmelinii*, with half-maximal inhibitory concentration values of 7.79–9.67 μM .

Direct comparison with the present findings should be made cautiously because the experimental systems differ substantially. The present study evaluated lethality of a crude extract using an invertebrate nauplius model, whereas previous investigations used mammalian tumor cell lines, different extraction systems, or isolated compounds. Therefore, the absence of mortality in *A. salina* should be interpreted as a preliminary finding restricted to the tested concentrations and assay conditions rather than evidence of general biological safety.

Antibacterial activity

The greatest antibacterial activity was observed at the 10% extract concentration, with inhibition zone diameters of 12.0 ± 0.12 mm against *S. aureus* and 14.0 ± 0.14 mm against *E. coli*. At 5%, inhibition zone diameters decreased to 9.0 ± 0.12 mm for both bacterial species, whereas no inhibition was detected at 1% or 2.5%. According to the predefined interpretation criteria used in this study, the 10% concentration showed weak antibacterial activity, whereas the 9-mm inhibition zones recorded at 5% remained below the threshold defined for detectable activity.

Previous investigations have demonstrated biological activities in different *Hedysarum* species, although their findings are not directly comparable with those of the present study because of differences in species, extraction methods, test organisms, and experimental designs. Dyshlyuk *et al.* [21] and Tian *et al.* [22] reported antioxidant and antibacterial activities of extracts from other *Hedysarum* species, including activity against *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *S. aureus*. Taken together, these studies indicate that the biological activity of *Hedysarum* extracts may vary substantially according to the plant species, extraction procedure, and composition of the resulting extract.

Antifungal activity

The *H. gmelinii* extract showed weak antifungal activity against *C. albicans* across the tested concentration range, with inhibition zone diameters of 12.0–13.0 mm. Unlike the bacterial cultures, where no inhibition was detected at the two lowest concentrations, *C. albicans* remained inhibited at 1% and 2.5%.

Keleke *et al.* [18] and Balturk *et al.* [23] also reported antifungal activity of extracts from *H. semenowii* against *Candida* species. Differences in activity magnitude among studies may reflect variation in plant species, plant material, extraction solvent and procedure, phytochemical composition, and susceptibility of the test microorganisms. Because the present extract was not phytochemically characterized, the specific constituents responsible for the observed antifungal activity cannot be identified.

Possible basis of the antimicrobial activity

Phenolic compounds, flavonoids, isoflavones, and prenylated chalcones have been reported in members of

the genus *Hedysarum* [24–27], and these compound classes have been associated with antimicrobial activity in previous studies. Such compounds may influence microbial membrane permeability and interfere with membrane-associated proteins and enzymes. However, none of these constituents were identified or quantified in the extract evaluated in the present study. Consequently, any attribution of the antibacterial or antifungal effects to specific phytochemical classes remains hypothetical.

The relatively weak antimicrobial activity observed may reflect the composition of the crude extract, the concentration of biologically active constituents, their physicochemical availability, or the methodological characteristics of the agar well diffusion assay. Phytochemical characterization and quantitative antimicrobial assays are required before relationships between individual constituents and biological activity can be established.

Ovicidal activity against *T. cati* and strongylid eggs

Ovicidal activity represented the most pronounced biological effect observed in this study. After 48 h of exposure, the proportion of non-viable *T. cati* eggs reached $79.0 \pm 1.9\%$ at 5% extract and $89.0 \pm 1.8\%$ at 10%. For strongylid eggs, non-viability reached $89.0 \pm 1.2\%$ at 10% and $95.0 \pm 2.2\%$ at 30%. The proportion of non-viable eggs generally increased with both extract concentration and exposure duration.

The ovicidal activity against *T. cati* was broadly comparable to that reported in an earlier study of *H. gmelinii* extract [28], although the studies differed in concentration and exposure-time designs. The present investigation also evaluated strongylid eggs, which the previous study did not include, thereby extending the evaluation of the extract to another group of helminth eggs of veterinary relevance.

Microscopic examination showed eggshell deformation or disruption, degradation of internal contents, and arrest of embryonic development after exposure to the extract. These morphological changes accompanied the decrease in egg viability. However, this study did not directly investigate the molecular or biochemical mechanisms underlying these alterations.

Possible mechanisms of ovicidal activity

Xanthenes and other phenolic compounds have been reported in members of the genus *Hedysarum*, and such constituents could theoretically contribute to biological effects on helminth eggs. One possible explanation is that extract constituents alter eggshell integrity or permeability, thereby facilitating penetration into internal embryonic structures. Nevertheless, the phytochemical composition of the extract was not determined, and no mechanistic assays were performed. Therefore, potential involvement of oxidative stress, membrane disruption, or apoptosis-like processes should be regarded as hypotheses derived from the broader literature rather than mechanisms demonstrated in the present study.

Strongylid eggs showed greater non-viability than *T. cati* eggs under the tested conditions. Differences in eggshell structure and permeability may contribute to this pattern because strongylid eggs generally have structurally different shells from the thick, multilayered shells of ascarid eggs such as *Toxocara*. However, this explanation was not experimentally tested in the present study and should therefore be interpreted cautiously.

Biological significance of the findings

Among the biological activities examined, the principal effect of the *H. gmelinii* extract was its ovicidal activity, whereas antibacterial and antifungal effects were limited under the experimental conditions used. The absence of mortality in the *A. salina* assay provides a preliminary toxicity observation restricted to this single invertebrate model and should not be interpreted as evidence of mammalian safety.

Because the extract was not phytochemically characterized, the compounds responsible for the observed ovicidal and antimicrobial activities remain unknown. Further investigation should therefore include phytochemical profiling, standardization of the extract, isolation or identification of candidate active constituents, and separate evaluation of their biological activities.

Limitations

This study has several limitations. First, although the extraction yield was reported, the extract was not phytochemically characterized by qualitative screening, chromatographic fingerprinting, or quantitative analysis of major constituents. Therefore, the compounds responsible for the observed biological effects could not be identified, and the mechanistic explanations proposed in this Discussion remain hypotheses based on the broader *Hedysarum* literature rather than findings demonstrated for the specific extract evaluated.

Second, preliminary toxicity was assessed only using the *A. salina* invertebrate model. Mammalian cell line cytotoxicity and *in vivo* toxicity were not evaluated; consequently, the absence of naupliar mortality cannot be extrapolated to mammalian safety [29–31].

Third, antibacterial and antifungal activities were evaluated only by agar well diffusion. The study did not determine minimum inhibitory concentration, minimum bactericidal concentration, or minimum fungicidal concentration, limiting quantitative characterization of antimicrobial potency [32, 33].

Finally, the ovicidal findings were obtained exclusively *in vitro*. Further studies are needed to determine whether similar effects occur under biologically relevant conditions and whether the extract or its active constituents can be developed into a reproducible, safe veterinary antiparasitic preparation. Phytochemical standardization, mammalian safety assessment, and controlled *in vivo* efficacy studies are therefore necessary before veterinary application can be considered.

CONCLUSION

The present study demonstrated that the ethanol:chloroform extract of *H. gmelinii* exhibited distinct biological effects depending on the experimental model. No mortality of *A. salina* nauplii was observed at concentrations of 1%–10% during the 24-h exposure period. Antibacterial and antifungal activities were limited, with the most evident inhibition observed at the highest extract concentration. In contrast, the extract showed pronounced *in vitro* ovicidal activity against both *T. cati* and strongylid eggs. After 48 h, non-viability of *T. cati* eggs reached $79.0 \pm 1.9\%$ at 5% extract and $89.0 \pm 1.8\%$ at 10%, whereas non-viability of strongylid eggs reached $89.0 \pm 1.2\%$, $92.0 \pm 1.3\%$, and $95.0 \pm 2.2\%$ at extract concentrations of 10%, 20%, and 30%, respectively. Microscopic examination further showed eggshell deformation, disruption of internal structures, and arrest of embryonic development.

The findings indicate that the principal biological effect of the tested *H. gmelinii* extract was its ovicidal activity rather than its antibacterial or antifungal activity. The observed reduction in egg viability, together with concentration- and exposure time-dependent responses, supports further investigation of this plant as a potential source of compounds with antiparasitic activity of veterinary relevance. However, the present findings are preliminary and should not be interpreted as evidence of clinical efficacy or safety.

A major strength of this study was the use of complementary biological assays to evaluate cytotoxicity, antibacterial activity, antifungal activity, and ovicidal activity within the same experimental framework. The study also assessed ovicidal activity against two distinct helminth egg groups of veterinary importance, *T. cati* and strongylids, and incorporated both positive and negative controls. In addition, the evaluation of multiple extract concentrations and exposure periods allowed characterization of concentration- and time-related patterns in egg non-viability. The use of microscopic assessment further supported the quantitative ovicidal findings by documenting morphological damage to the treated eggs.

The study has several important limitations. The extract was not phytochemically characterized; therefore, the compounds responsible for the observed biological effects could not be identified. The *A. salina* assay provided only a preliminary toxicity screen and does not establish mammalian safety. Antibacterial and antifungal activities were evaluated only by agar well diffusion, without determination of minimum inhibitory, minimum bactericidal, or minimum fungicidal concentrations. In addition, the ovicidal experiments were conducted exclusively *in vitro*, and the findings cannot be directly extrapolated to efficacy under field or clinical conditions.

Further research should include phytochemical profiling and standardization of the *H. gmelinii* extract, followed by isolation and identification of the constituents associated with ovicidal activity. Mammalian cell line cytotoxicity and broader safety assessments are needed before drawing translational conclusions. Quantitative antimicrobial testing should also be performed to determine minimum inhibitory, minimum bactericidal, and minimum fungicidal concentrations. For the antiparasitic effect, controlled *in vivo* studies are needed to evaluate efficacy, dose-response relationships, safety, formulation, and practical applicability under veterinary conditions.

The ethanol:chloroform extract of *H. gmelinii* showed limited antibacterial and antifungal activity but pronounced *in vitro* ovicidal activity against *T. cati* and strongylid eggs. The absence of mortality in the *A. salina* assay provides only a preliminary toxicity observation, whereas the ovicidal findings identify this extract as a candidate for further antiparasitic investigation. Phytochemical characterization, mammalian safety testing, quantitative antimicrobial assays, and controlled *in vivo* studies are required before any veterinary application can be considered.

DATA AVAILABILITY

The supplementary data are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

The authors declare that generative artificial intelligence (AI) tools were used solely to improve the language, grammar, and readability of the manuscript. All scientific content, data analyses, interpretation of the results, and conclusions were developed and verified by the authors. The authors take full responsibility for the accuracy, integrity, and originality of the work, and no AI tool was listed as an author.

AUTHORS' CONTRIBUTIONS

GY: Conceived and designed the study, coordinated the project, and drafted the manuscript. NM and YS: Data collection and database management. AT, MY, and AY: Performed the examinations and contributed to the acquisition of experimental data. LL and GM: Supervised the project, study design, and statistical analyses. All authors have read and approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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