

RESEARCH ARTICLE

In-vitro Larvicidal Potentials of *Achillea* Extracts on Strongylid Larvae

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Abstract: Strongylid infections remain among the most widespread and clinically significant helminthoses in horses, with increasing concerns regarding anthelmintic resistance. Against this background, we investigated the *in-vitro* larvicidal activity of *Achillea salicifolia* and *Achillea setacea* extracts against strongylid larvae. We evaluated concentrations of 20, 30, and 40% at exposure times of 0.5, 1, 2, and 4 h (n = 3). Larval motility was assessed microscopically and expressed as mean $\pm$ standard deviation (M $\pm$ SD). A pronounced dose- and time-dependent reduction in larval motility was observed. For *A. salicifolia* at 40%, motility was $23.0 \pm 3.2\%$ after 30 min, $7.0 \pm 1.8\%$ after 1 h, and 0% after 2 h ($p < 0.001$ vs control). At 30%, motility decreased to $41.0 \pm 4.5\%$, $19.0 \pm 3.7\%$, $4.0 \pm 1.5\%$, and 0% at 0.5, 1, 2, and 4 h, respectively ($p < 0.01$). At 20%, motility values were $58.0 \pm 5.1\%$, $33.0 \pm 4.2\%$, $12.0 \pm 2.8\%$, and 0% at 4 h ($p < 0.05$). For *A. setacea* at 40%, motility was $49.0 \pm 5.4\%$ after 30 min, $13.0 \pm 2.6\%$ after 1 h, and 0% after 2 h ($p < 0.01$). At 30%, values were $61.0 \pm 6.2\%$, $37.0 \pm 4.9\%$, $11.0 \pm 3.1\%$, and 0% at 4 h ($p < 0.05$), whereas at 20%, motility remained at $72.0 \pm 6.8\%$, $54.0 \pm 5.7\%$, $25.0 \pm 4.3\%$, and $4.0 \pm 1.2\%$ after 4 h ($p < 0.05$). The positive control (4% phenol) induced 100% larval immobilization within 1 h ($p < 0.001$), whereas in the negative control, motility remained at 100% throughout the observation period. The results indicate a strong larvicidal effect of the studied extracts, with a more rapid and pronounced activity observed for *A. salicifolia*, supporting the potential of *Achillea* species as a source of natural anthelmintic agents.

Keywords: *Achillea salicifolia*, *Achillea setacea*, *In-vitro*, Larvicidal Activity, Plant Extracts, Strongylid Larvae

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Introduction

Strongylid infections are among the most widespread and epizootologically significant helminthoses in horses worldwide, including Kazakhstan, where prevalence may reach up to 100% in some populations [1-3]. Cyathostomins are considered particularly important due to their high prevalence, pathogenicity, and close association with pasture-based transmission [4]. The epizootic process is sustained primarily by the development and survival of infective larvae in the environment, with these being contingent upon temperature, humidity, and grazing management practices [5, 6].

The effectiveness of conventional anthelmintics has declined considerably because of the widespread emergence of resistance, especially to benzimidazoles and pyrantel [7, 8]. This has increased interest in alternative and environmentally sustainable antiparasitic approaches, including the use of plant-derived compounds and the targeting of free-living larval stages. *In-vitro* larvicidal assays are widely used for the primary screening of biologically active substances [9, 10].

Species of the genus *Achillea* are known for their rich phytochemical composition and diverse biological activities, including antimicrobial and antiparasitic effects [11, 12]. *Achillea salicifolia*, native to Kazakhstan, has demonstrated ovicidal activity against nematodes [13–15], while *A. setacea* has been characterized by significant phytochemical diversity and biological potential [16, 17]. Therefore, the present study aimed to evaluate the *in-vitro* larvicidal activity of extracts from *A. salicifolia* and *A. setacea* against strongylid larvae and to assess their potential application in veterinary parasitology.

Materials and Methods

The study was conducted from October 2025 to February 2026 at the N. T. Kadyrov Laboratory of Parasitology, Institute of Animal Science and Veterinary, Saken Seifullin Kazakh Agrotechnical University, Astana, Kazakhstan. Fecal samples (n = 50) were collected from farms in the Akmola region, from which eggs and larvae of strongylids, predominantly small strongyles (cyathostomins), were isolated and used as biological material. The horses were naturally infected with strongylates and had not undergone anthelmintic treatment before sampling. The animals were maintained under standard farm management conditions, including conventional feeding and watering systems, natural ventilation, and routine veterinary care; no controlled experimental housing conditions were applied. Initial detection of helminth eggs was performed using the flotation method. Briefly, fecal samples were mixed with a sucrose flotation solution with a density of 1.27 g/cm³, followed by microscopic examination to identify characteristic strongylid eggs. The eggs (Figure 1) were oval in shape, measuring 0.07–0.10 × 0.04–0.05 mm, with a thin grayish shell and a morula-stage embryo [18]. Following confirmation of egg presence, positive samples were used for further larval cultivation. For this purpose, fecal material containing strongylid eggs was placed in an incubator and maintained for two weeks under controlled conditions to allow development from eggs to the larval stage. During incubation, adequate moisture was maintained to support embryogenesis and subsequent larval emergence.

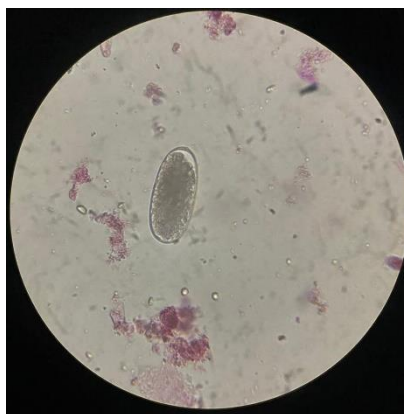


Fig. 1: Representative strongylid eggs observed under light microscopy (x1000)

After completion of cultivation, larvae were isolated from the material using the Baermann–Orlov method, which is based on the active migration of motile larvae from the substrate into a liquid medium. Briefly, the cultured material was placed in the apparatus and incubated for 6–12 h. During this period, larvae migrated from the substrate into water, enabling their separation from fecal debris, eggshells, and other impurities. At the end of the exposure period, the larval suspension was collected and used for further experimental procedures [19]. The larval stage of strongylids (Figure 2) is characterized by an elongated, cylindrical body shape, a transparent cuticle, and active motility [20].

Plant extracts of *A. salicifolia* and *A. setacea* were used as test substances. The extracts were obtained from dried and ground plant material (aerial parts and roots) using a Soxhlet extraction system (KEX 250, Germany). Ethanol (96,3%, «IVK-PLUS» LLC, Russia) and chloroform (chemically pure grade, «EKOS-1» JSC, Russia) were used as extraction solvents. To obtain concentrated extracts, the primary extract was subjected to distillation using a rotary evaporator (IKA HB digital, Germany).

The ratio of plant material to solvent was 1:10. A solvent mixture of ethanol and chloroform (1:1) was used. The extraction duration was 3 h, in accordance with the State Pharmacopoeia of the Republic of Kazakhstan.

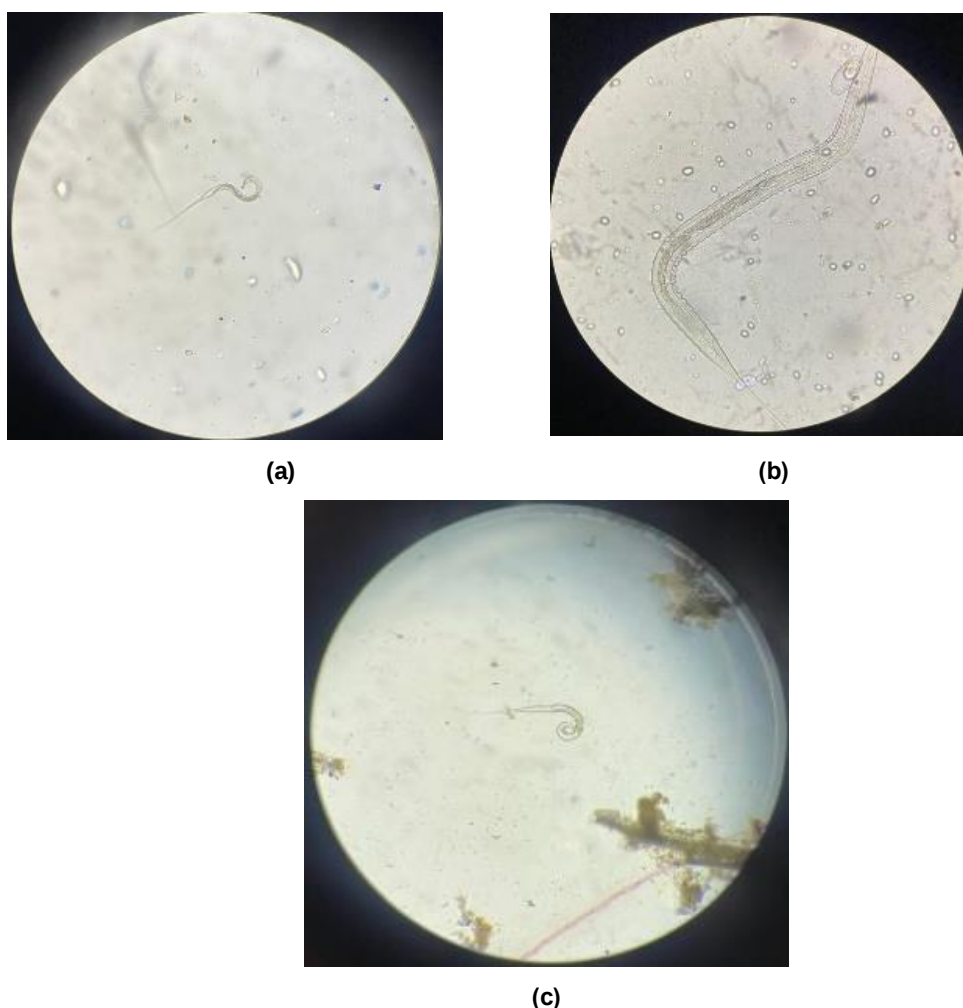


Fig. 2: Morphological characteristics of larval stages of strongylids; a: L1 larva (light microscopy, ×400); b: L2 larva (×1000); c: L3 larva (×400)

To assess the overall biological activity of the extracts, their quality was measured against organoleptic, hygienic, chemical, and microbiological criteria in line with the State Pharmacopoeia of the Republic of Kazakhstan. The obtained extracts complied with current national standards (ST RK 978-2001 "Plant extracts. Technical specifications").

Working solutions of *A. salicifolia* and *A. setacea* extracts were prepared at concentrations of 20%, 30%, and 40%. Dimethyl sulfoxide (DMSO) was used as the solvent. Solutions were prepared immediately before the experiment by diluting the concentrated extract in DMSO to obtain a homogeneous mixture.

For the larvicidal assay, larval suspensions were prepared by dilution with filtered distilled water to obtain a concentration of 50 larvae per 0.5 mL of suspension.

During the experiment, 0.5 mL of larval suspension was transferred into sterile 1.5 mL Eppendorf tubes, followed by the addition of 0.5 mL of the test extract at the corresponding concentration. Thus, the total reaction volume was 1 mL.

A positive control was established using a 4% phenol solution, and a negative control using filtered distilled water.

All experimental samples were incubated at 28 °C in a thermostatic chamber. Larval viability and motility were assessed after 30 min, 1, 2, and 4 h of incubation.

Larval motility was evaluated using a light microscope at 400× magnification. Larvae were considered viable if they exhibited active or reduced movement. Larvae that showed no movement, even after gentle mechanical stimulation with a needle or pipette, were classified as dead.

Larval Mortality (LM) was calculated using Abbott's formula [21]:

$$LM (\%) = (\text{Number of dead larvae} / \text{Total number of larvae}) \times 100$$

Each extract concentration was tested in three independent replicates, and additionally, morphological changes in larvae were recorded, including body deformation, disruption of cuticle structure, body shortening, loss of transparency, and reduced motility, as observed under light microscopy.

The software packages Statistica (version 13.0, TIBCO Software Inc., USA), SPSS (version 26.0, IBM Corp., Armonk, NY, USA), and GraphPad Prism (version 9.0, GraphPad Software Inc., San Diego, CA, USA) were used for statistical analysis of the obtained data. The Shapiro–Wilk test was applied to evaluate the normality of data distribution before analysis.

For data that were normally distributed, a one-way analysis of variance (ANOVA) was conducted, followed by Tukey's post hoc test for multiple between-group comparisons. Results are presented as mean ± standard deviation (M ± SD). Differences were deemed statistically significant when $p < 0.05$.

Results

A comparative assessment of the larvicidal activity of *A. salicifolia* and *A. setacea* extracts against the larval stage of strongylids was conducted under controlled *in-vitro* conditions. The positive control was a 4% phenol solution, and the negative control consisted of untreated larvae.

In addition, larval motility of strongylid larvae was assessed. The most pronounced effect was observed for *A. salicifolia*: at a concentration of 40%, larval motility decreased to 23% after 30 min, to 7% after 1 h, and was completely absent after 2 h.

For *A. setacea*, the reduction in motility occurred more gradually: at 40%, motility was 49% after 30 min, 13% after 1 h, and decreased to 0% after 2 h (Figure 3).

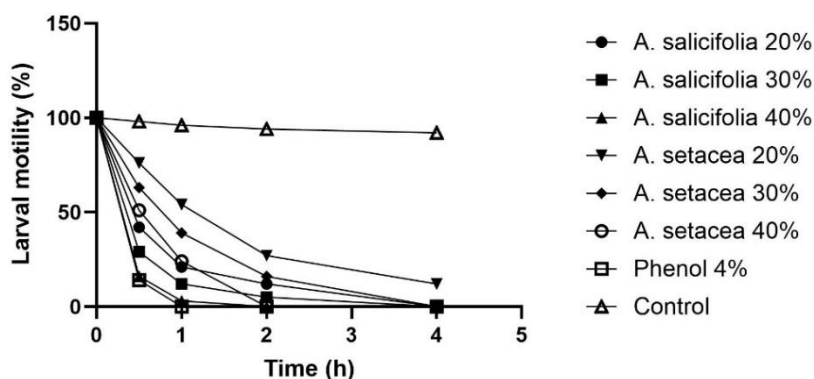


Fig. 3: Time- and concentration-dependent changes in larval motility of strongylid larvae following exposure to *A. salicifolia* and *A. setacea* extracts

The results obtained in this study demonstrated that both plant extracts exerted a larvicidal effect on the larval stage of strongylids. Following treatment, a high level of larval mortality was observed compared to the negative control group.

Exposure to *A. salicifolia* extract at a concentration of 40% resulted in more than 77% larval mortality within 30 min. Furthermore, the use of *A. salicifolia* at concentrations of 20% and 30% led to 100% larval mortality after 4 h of exposure. In the case of *A. setacea*, the extract also demonstrated effectiveness against the larval stage of strongylids. Complete larval mortality was achieved at concentrations of 20% and 30% after 4 h, whereas a concentration of 40% resulted in 100% mortality within 2 h.

The comparative analysis of the obtained data indicates that *A. salicifolia* exhibited a more rapid onset of action, particularly at higher concentrations, as reflected by the faster decline in larval motility and earlier achievement of high mortality rates. In contrast, *A. setacea* demonstrated a more gradual effect, although ultimately reaching comparable levels of efficacy. These differences suggest variation in the dynamics of larvicidal activity between the two extracts, while confirming their overall effectiveness under *in-vitro* conditions.

Summary data on larval mortality and the results of microscopic examination are presented in Table 1.

According to the analysis, *A. salicifolia* exhibited a more pronounced larvicidal activity compared to *A. setacea*. The LC₅₀ values for *A. salicifolia* and *A. setacea* are presented in Table 2.

Table 1: Larval mortality of strongylid larvae under the effect of *A. salicifolia* and *A. setacea* extracts

Tested plant	Extract concentration	30 min (M ± SD, %)	1 h (M ± SD, %)	2 h (M ± SD, %)	4 h (M ± SD, %)
<i>Achillea salicifolia</i>	20%	45 ± 4.5	63 ± 3.2	88 ± 4.6	100 ± 0
	30%	63 ± 5.9	81 ± 7.5	96 ± 5.8	100 ± 0
	40%	77 ± 5.7	93 ± 4.5	100 ± 0	100 ± 0
<i>Achillea setacea</i>	20%	27 ± 6.3	43 ± 3.4	75 ± 5.6	96 ± 4.3
	30%	39 ± 6.2	59 ± 4.6	81 ± 4.4	100 ± 0
	40%	51 ± 7.3	87 ± 5.9	100 ± 0	100 ± 0
Positive control	Phenol (4%)	66 ± 5.7	100 ± 0	100 ± 0	100 ± 0
Negative control	Distilled water	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Note: All values are presented as mean ± standard deviation (n = 3). p-values were calculated in comparison with the negative control (distilled water). Differences were considered statistically significant at p < 0.05

Table 2: Lethal concentrations (LC₅₀ and LC₉₀) of plant extracts against strongylid larvae.

Extract	LC ₅₀ (%)	95% CI	LC ₉₀ (%)	95% CI
<i>A. salicifolia</i>	16.7	13.7–18.9	36.6	33.8–40.7
<i>A. setacea</i>	23.3	14.6–27.9	47.0	36.9–49.6

Note:

LC₅₀ – concentration causing 50% larval mortality

LC₉₀ – concentration causing 90% larval mortality

95% CI – 95% confidence interval

Discussion

The results obtained in the present study demonstrated that both *Achillea* extracts exerted a clear larvicidal effect. Against strongylid larvae under *in-vitro* conditions, with differences in the rate and intensity of action between *A. salicifolia* and *A. setacea*. A dose- and time-dependent decrease in larval motility, followed by complete mortality at higher concentrations, indicates a pronounced biological activity of both plant extracts. These findings suggest that the observed effects are likely associated with the phytochemical composition of the studied species.

The extracts investigated in the present study were obtained from plants of the family Asteraceae, genus *Achillea*, which are known to contain a wide range of biologically active compounds, including flavonoids, the alkaloid achilleine, sesquiterpene lactones, phenolic acids, and essential oils. The presence of these compounds suggests that the observed larvicidal activity may be associated with their toxic effects on parasites, including disruption of cell membranes, impairment of energy metabolism, and inhibition of neuromuscular activity.

The larvicidal activity of the tested plant extracts may be associated with the presence of biologically active compounds, including flavonoids, terpenoids, phenolic acids, and alkaloid-related constituents reported in species of the genus *Achillea*. Previous studies have demonstrated that plant secondary metabolites can affect nematode viability through disruption of membrane integrity, interference with energy metabolism, and impairment of neuromuscular activity. In particular, phenolic compounds and flavonoids have been associated with reduced motility and paralysis in helminth larvae [22, 23].

In this study, exposure to *A. salicifolia* and *A. setacea* extracts resulted in reduced larval motility and a subsequent larvicidal effect, which may indicate a disruption of key physiological functions in strongyle larvae. Such effects may contribute to the impaired motility and paralysis observed in strongyle larvae. (Figure 4).

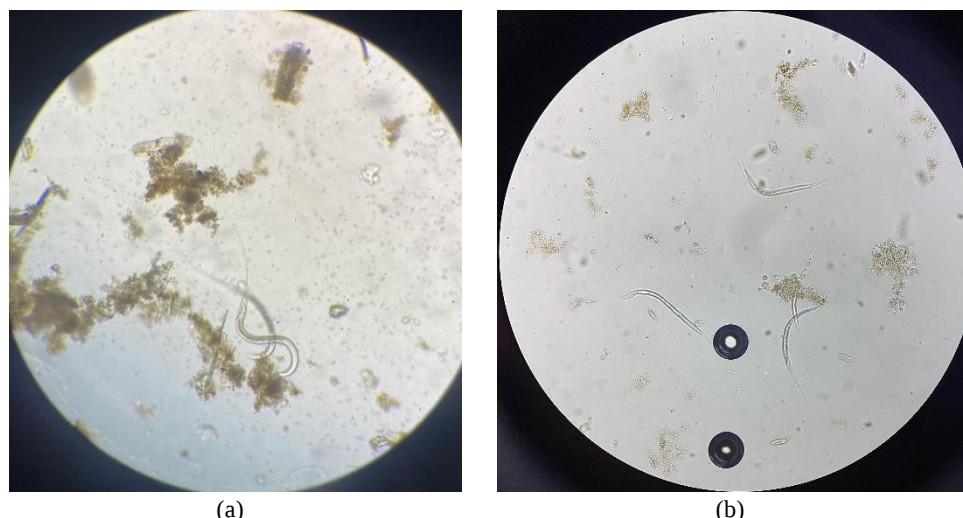


Fig. 4: Morphological alterations associated with strongyle larvae death following exposure to 40% *Achillea salicifolia* extract; a: untreated larvae (light microscopy, ×400); b: dead larvae after 2 h exposure (light microscopy, ×400)

The present findings are consistent with previously published data on the anthelmintic activity of plants rich in phenolic compounds, flavonoids, essential oils, and terpenoids, which are known to disrupt nematode metabolism, impair motility, and damage the integrity of the cuticle [9, 10].

Natural plant-derived compounds may be applied either as standalone agents or as components of integrated parasite control strategies, targeting free-living larval stages and reducing pasture contamination [6,24].

Thus, the obtained data support the potential of *Achillea* extracts as a source of natural larvicidal compounds. However, further studies are required for a comprehensive evaluation of their efficacy and safety, including the isolation of active constituents, investigation of mechanisms of action, and in vivo validation.

Conclusion

Extracts of *A. salicifolia* and *A. setacea* demonstrated high larvicidal activity against strongyle larvae under *in-vitro* conditions, with *A. salicifolia* showing greater efficacy, as evidenced by lower LC50 and LC90 values. Plant extracts of the genus *Achillea* represent a promising basis for the development of alternative phytotherapeutic agents in veterinary practice. Further research should focus on the identification of active compounds, elucidation of their antiparasitic mechanisms, and in vivo studies to assess efficacy and safety. The extracts of *A. salicifolia* and *A. setacea* may serve as a foundation for the development of novel agents aimed at reducing parasite burden and improving control strategies for strongyle infections in horses.

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Author's Contributions

Lyudmila Lider: Conceptualization, methodology, formal analysis, data curation, project administration, writing original draft.

Yerlan Suleimen: Project administration, resources, supervision.

Gulnur Mamytbekova: Conceptualization, methodology.

Madiyar Yessimkhanov: Conceptualization, methodology, formal analysis, data curation, writing review and editing.

Nellya Mannapova & Amanbol Talgat: Conceptualization, methodology, formal analysis, data curation.

Gulzhan Yeszhanova: Conceptualization, methodology, formal analysis, data curation, resources, supervision, writing original draft.

Ethics

This paper is an original contribution containing unpublished material. The published version of the manuscript has been read and agreed upon by all authors. Approval for the animal procedures was granted by the local Animal Ethics Committee at the Institute of Animal Science and Veterinary, Saken Seifullin Kazakh Agrotechnical University, Astana, Kazakhstan (Protocol No. 4, issued May 18, 2024). The research was carried out in compliance with the institution's internal guidelines on animal welfare and with the ARRIVE Guidelines.

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