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Evaluation of methanol extracts of *Zanthoxylum alatum* as a topical repellent against terrestrial leech (*Haemadipsa montana*) in rabbits

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In the present study, methanolic extracts of *Zanthoxylum alatum* fruits prepared into petroleum jelly-based ointments were evaluated for its leech repellent effect on New Zealand white rabbits. A widely prevalent terrestrial leech species of the humid forest areas, *Haemadipsa montana*, known to parasitise animals and humans, causing blood loss, skin irritation, and secondary infections in animals due to prolonged bleeding from bite wounds, was used for the study. There exist concerns regarding long-term use of synthetic leech repellents for environmental safety and toxicity. Plant-based repellents provide a promising alternative because of traditional use and eco-friendliness. Three concentrations of the extract at 10%, 20%, and 30% (w/w) incorporated into ointments were investigated on 20 rabbits randomly distributed into five groups, including a negative (plain petroleum jelly) and a positive (19% DEET ointment) control. Abdominal fur of the rabbits was shaved, and test ointments were applied. Ten terrestrial leeches per rabbit were then allowed to attach onto the prepared site and attachment behaviour, time to attachment, feeding success, and post engorgement weight were recorded. Results showed that the methanolic extracts had a dose-dependent leech repellent effect against *H. montana*. The highest concentration (30%) showed best repellence. No adverse skin reactions were noted in any treatment group. These findings support the efficacy of *Z. alatum* extracts as effective leech repellents. The study validates the traditional use of the plant and suggests its potential for developing natural, safe, and sustainable topical repellents for veterinary use.

Keywords: *Haemadipsa montana*, Indigenous Traditional Knowledge, Methanolic extract, Rabbit bioassay, *Zanthoxylum alatum*

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Application of synthetic repellents has proven effective in preventing leech attachment in humans[1], their use in animals is not possible due to concerns of oral ingestion by licking causing toxicity. Moreover, accessibility and affordability of synthetic repellents in remote rural areas of Northeast India are often limited[2]. Traditional knowledge systems among indigenous communities have long employed various plant-based remedies for repelling terrestrial leeches. Extensive studies have documented the anti-leech effects of plant extracts on important aquatic leeches causing internal hirudiniasis in livestock, *Tyrannobdella rex*[3] and *Limnatis nilotica*[4,5]. Leech-repellent properties of *Zanthoxylum alatum* against the buffalo leech (*Poecilobdella manillensis*) was reported[6]. However, research on repellents effective against terrestrial leeches remains limited. Ethnobotanical practices across India and Southeast Asia highlight the use of various plants—such as *Zanthoxylum* spp.[2], *Solanum* spp.[7], *Nicotiana tabacum*, *Careya arborea*[8], and *Gymnocladus assamicus*[9]- as terrestrial leech repellents by indigenous communities. Among these botanicals, *Zanthoxylum* spp. (family: Rutaceae) has demonstrated insecticidal and repellency properties in earlier studies, attributed to its rich phytochemical composition[10]. It's essential oils have shown strong activity against mosquitoes and other pests[11,12].

Despite a wide array of traditional plant-derived extracts[13] and synthetic repellents tested globally like N,N-diethyl phenylacetamide (DEPA)[14], Dimethyl phthalate, N, N-diethyl-m-toluamide (DEET), there is limited *in vivo* evidence on the efficacy of botanical formulations against terrestrial leeches. Previous studies predominantly evaluated the efficacy of essential oils of *Callistemon rigidus*, *Z. armatum*,

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Azadirachta indica[15,16], or aqueous extracts of *Catunaregam spathulifolia*, *Vernonia elaeagnifolia*, and *Sapindus rarak*[13] through cloth impregnation methods, with limited research exploring direct topical applications or *in vivo* testing. Repellent activity is reported for essential oils from three plant species through direct application on human volunteers, against mosquitoes against mosquitoes, black flies and land leeches[17].

This study was designed to evaluate the leech-repellent efficacy of methanolic fruit extracts of *Z. alatum* formulated as a topical ointment, and to compare its effectiveness with DEET against *H. montana* using a rabbit model. By focusing on a regionally significant parasitic problem and investigating an indigenous plant species with traditional use, this study aims to contribute to the development of eco-friendly, veterinary-relevant leech repellents suited for local conditions in Northeast India.

Materials and Methods

Collection of plant material

Fresh fruits of *Z. alatum* were collected during October (post-monsoon season) from the West Kameng district of Arunachal Pradesh. The samples were transported to the laboratory in clean plastic bags and air-dried in the shade to prevent degradation of active constituents. The dried fruits were powdered using a laboratory-grade feed grinder. Plant identification was done using standard taxonomic keys[18] and confirmed by the Botanical Survey of India, Itanagar.

Preparation of plant extracts

A total of 500 g of powdered sample underwent Soxhlet extraction using 2.5 L of methanol as the solvent[19]. The extraction was carried out at 40°C for 48 h, until the solvent in the siphon tube became colourless. After completion, the methanol was evaporated in a hot air oven at 50°C. The resulting extract formed a thin film, which was scraped off (yield: 15.2%) using a sterile scalpel and stored in tightly sealed glass bottles at room temperature until use.

Formulation of ointments

Ointments were prepared using petroleum jelly as a base with plant extract (Supplementary Fig. S1) concentrations of 10%, 20%, and 30% (w/w). DEET (19% w/w; Sigma, USA) was used as the positive control, while plain petroleum jelly served as the negative control.

Experimental animals and leech challenge

Twenty clinically healthy New Zealand white rabbits of both sexes, aged 4-6 months, weighing 2.3-2.8 kg maintained on a standard diet were used for the study. Terrestrial leeches (*H. montana*) were collected in the morning from the forest floor of Nyukmadung village (27°25′N, 92°08′E), West Kameng district, Arunachal Pradesh. Small (15.88±0.13 mg), uniform-sized (1.3-1.7 cm), pre-engorged active leeches were gathered and transported to the laboratory in a glass flask (Supplementary Fig. S2). In the laboratory, individual leeches were weighed and stored in 15 ml centrifuge tubes containing moist cotton at the bottom. The tube lids were perforated with tiny holes to allow ventilation and prevent escape. The tubes were kept in a shaded area of the laboratory to maintain ambient conditions.

For preparation of the experiment, a 12×6 cm abdominal region on one side was shaved on each rabbit. The abdominal region was selected because it provides a large, flat, and sparsely furred surface that allows uniform application of ointment and easy observation of leech attachment and behaviour. On the next day, four uniform-sized terrestrial leeches (*H. montana*) were allowed to feed on each rabbit to establish baseline attachment behaviour (Supplementary Fig. S3-S6). The attachment time, feeding duration and weight of engorged leeches were recorded (Supplementary Fig. S7). Rabbits were restrained while the leeches were allowed to attach and post attachment, they were returned to their respective cages for observation. No discomfort or irritation-related behaviours like scratching or grooming was noticed. Post-feeding, the bite sites were cleaned and treated with potassium permanganate and iodine ointment. Healing was monitored for 10 days before the start of the efficacy experiment.

Experimental design

The rabbits were randomly divided into five groups (A to E; n = 4 per group): Group A (Positive control): Treated with 19% DEET ointment, Group B (Negative control): Treated with plain petroleum jelly, Group C to E (Test groups): Treated with methanol extract of *Z. alatum* at varying concentrations (10%, 20%, 30%). Prior to each trial, the abdominal region of each rabbit was re-shaved to expose a consistent 12×6 cm area. One gram of the assigned ointment was

applied uniformly to the shaved site and allowed to air-dry for 15 min to ensure absorption and minimize transfer. Ten leeches with average weights of 15.88 mg were used per rabbit. The leeches were placed individually in 15 mL centrifuge tubes for confinement and standardization of the exposure. The tube cap was removed and gentle puffs of air were blown into the tubes to stimulate the leech, and they were released onto the treated area and allowed to attach spontaneously for up to 10 min. When spontaneous attachment did not occur within the time frame, the leeches were carefully removed using sterile forceps and manually placed on the treated site to assess forced contact repellency. Leech behaviour, time to attachment, feeding success, and detachment responses were observed and recorded throughout the exposure period. Following the completion of each trial, the treatment site on each rabbit was cleaned with 70% ethanol followed by sterile distilled water to remove any residual extract.

Statistical analysis

Pre- and post-engorgement weight of all leeches (n=80) were compared using a paired t-test to assess weight gain after feeding. Mean blood intake per rabbit was analysed using one-way ANOVA followed by Tukey's post-hoc test at $p<0.05$ in Microsoft Excel.

Table 1 — Baseline attachment and feeding characteristics of *Haemadipsa montana* on New Zealand white rabbits during preparatory trial

	Mean±SE	Range	Units
Pre-engorgement weight	15.88±0.13	13.60 - 19.00	mg
Post-engorgement weight	254.81±3.44	183.33 - 342.00	mg
Time of attachment	2.09±0.09	1.00 - 4.00	min
Feeding duration	29.21±0.66	19.00 - 42.00	min
Blood intake	238.93±3.32	169.73 - 323.00	mg
Blood intake as fold of body weight	15.00±0.11	12.48 - 17.00	fold
Healing duration of bite wound	6.83±0.14	5.00 - 10.00	days

Results

In the preparatory phase, baseline parasitic behaviour of *Haemadipsa montana* was characterized prior to repellent efficacy trials (Table 1). The average time to attachment was 2.09±0.09 min. Feeding duration ranged from 19 to 42 min (29.21±0.66). The mean pre-engorgement weight of leeches was 15.88±0.13 mg, which increased significantly to 254.81±3.44 mg post-engorgement ($t=72.04$, $p<0.001$). One-way ANOVA revealed no significant variation in mean blood intake among rabbits ($f=0.79$, $p=0.71$), indicating consistent feeding behaviour across replication. The blood intake as fold of body weight ranged from 12.48 to 17.00 (average 15.04) with larger leeches ingesting more. Continuous bleeding from bite wounds was observed. Post-feeding, leech bite wounds were treated with potassium permanganate and iodine ointment. Complete healing was observed within 10 days, with no adverse dermatological responses noted.

Complete repellency (100% efficacy) was observed in DEET-treated group (Group A). Leeches showed no attachment during the 10-min observation period. Manual placement attempts with forceps resulted in instant aversion, with leeches remaining tightly attached to the forceps and displaying aversion from the test site. In contrast, the leeches in the negative control group (Group B) demonstrated quick and complete attachment, and all leeches successfully started feeding within 2-5 min, confirming the baseline leech behaviour in the absence of any repellent.

In the test groups (Group C to E), which received *Z. alatum* extract-based ointments, a clear concentration-dependent repellent effect was observed (Table 2).

At 10% concentration, a modest repellent response was recorded. There was no spontaneous attachment at 10% and moved away from the site when manually placed without initiating attachment for engorgement (Supplementary Fig. S8). At 20% concentration, the

Table 2 — Repellent efficacy of methanolic extract of *Zanthoxylum alatum* and control treatment against *Haemadipsa montana* in rabbits

Group	Treatment	Leech attachment	Repellency behaviour	Response to manual placement	Response to air puff stimulation
A	19% DEET (Positive Control)	No attachment	Strong repellency; complete aversion	Instantly dropped off and died	Not Applicable
B	Petroleum jelly (Negative Control)	2.13±0.08 min	No repellency	Not Applicable	Not Applicable
C	10% *Z. alatum* ointment	No attachment	Modest repellency, no spontaneous attachment	Moved away naturally without feeding.	Responsive to air puffs
D	20% *Z. alatum* ointment	No attachment	Pronounced repellency; clear head aversion	Wriggled away from the site.	Dormant; no response to air puffs
E	30% *Z. alatum* ointment	No attachment	Strong repellency; immediate head withdrawal	Instantly wriggled and dropped	Dormant; no respond to air puffs

repellent effect was more pronounced. None of the leeches attempted attachment even when placed directly on the treated site using forceps. They responded with immediate withdrawal and wriggled away without attacking their suckers to move indicating strong behavioural deterrence. No leech attached themselves spontaneously, moved away spontaneously and dropped off when manually placed skin without initiating attachment. At 30% concentration, the extract showed best efficacy. When manually attempts were made to place them to the site they showed marked aversion even detaching itself to the forceps as it was brought near the ointment applied site.

No adverse skin reactions or irritation were noted in rabbits treated with any concentration of *Z. alatum* extract, supporting its dermatological safety. The rabbits remained healthy throughout the trial, and healing of the control leech bites was uneventful, ensuring valid baseline comparisons.

Discussion

This study demonstrated the strong hematophagous capacity of *H. montana*, with an average blood ingestion of 15.00 times its body weight. This substantial blood loss, combined with continued oozing blood after the dropping of the leech under natural field conditions, attracting flies and increasing the risk of bacterial infections. In highland livestock, especially in free ranging ruminants such as yaks and mithun, repeated infestations by terrestrial leeches can result in anemia[20], weakness, and reduced productivity, particularly during the monsoon season when leech activity peaks[21]. Therefore, preventive measures are needed, especially in regions like Northeast India where leeches are abundant in the environment and veterinary services are limited.

Dose-dependent leech repellent activity of the methanolic extract of *Z. alatum* was evident and the 30% (w/w) formulation was nearly as effective as the standard 19% DEET ointment. This is consistent with previous studies demonstrating the insecticidal and repellent potential of *Zanthoxylum* species, particularly their essential oils and seed extracts, against various arthropods and leech[11,12,15]. The efficacy of our methanol-based formulation further supports its utility as a viable botanical repellent. Bioactive monoterpenes[21,22] were present in *Z. armatum* interferes with olfactory and chemosensory pathways of hematophagous parasites producing a strong deterrent effect[23]. In *Paramphistomum cervi*, anthelmintic potential of *Z. armatum* seed extracts was shown disrupt energy generation, resulting to parasite paralysis[24]. With 30% *Z. limonella* essential oil in impregnated textiles, complete mosquito repellency (zero mosquito landings) was also observed, indicating that *Zanthoxylum* species have strong bioactivity across parasite taxa and application techniques[25]. In the fields of veterinary medicine and public health, surface-functionalized textiles or topical ointments are potential vector protection delivery methods.

In comparison to essential oils, methanolic extracts provide a low-cost and sustainable extraction method appropriate for rural or low-resource environments. In endemic areas, where synthetic repellents such as DEET or DEPA are either unaffordable or environmentally unsustainable[26], this could be crucial. According to studies, DEET is frequently detected in surface waters, where it could disturb microbial ecosystems and could have an impact on non-target species[27]. Further ecological hazards are raised by its neurotoxic effects and disruption of insect chemosensory systems[28]. Our topical formulations did not elicit any adverse skin reactions in rabbits, underscoring their potential safety for veterinary application congruent with pharmacological safety profiles previously reported for *Z. armatum*[29]. The repellent effects of the *Zanthoxylum* sp. is not restricted to mosquitoes and leeches, essential oils from *Z. myriacanthum* have been reported to have repellent and insecticidal activity against stored product pests such as *Tribolium castaneum* and *Lasioderma serricorne*, suggesting its potential broad-spectrum application in pest control[30].

The usefulness of plant-based leech repellents is further supported by ethnoveterinary studies. Seven plant species, including Piper beetle and *Brugmansia suaveolens*, were found to be frequently used as leech repellents in a field ethnobotanical study among estate workers in the Nilgiris, India[31]. Such information validates the cultural significance of botanical repellents in leech-infested areas. These observations highlight the importance of integrating local practices into formal research and formulation development. The actual success of such botanical repellents depends on field validation. Further research is needed to evaluate the formulation's stability under high humidity conditions and its user acceptability and long-term safety. Field evaluation on high-risk

species of livestock (e.g., yaks, mithun, and cattle) and human occupational groups (e.g., herders, plantation workers) are needed.

Conclusion

The methanolic extract of fruit of *Z. alatum* demonstrated a strong, dose-dependent leech repellent effect against *H. montana*. Ointment formulation with 30% extract was found most effective. The formulation did not cause any visible irritation on rabbit skin, confirming that it was well tolerated on rabbit skin and may be safe for external use. The results highlight the potential of *Z. alatum* as a safe, eco-friendly, and locally available botanical repellent suitable for use in leech-prone regions. Further research is needed to assess formulation stability, long-term safety, and field efficacy under natural conditions using livestock and human volunteers.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at https://nopr.niscpr.res.in/jinfo/ijtk/IJTK_25(3)(2026)239-244_SupplData.pdf

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Author Contributions

JB: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, SI: Writing – review & editing, Supervision, PP: Investigation, Writing – original draft, DB Writing – review & editing, Supervision, Funding Acquisition.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Approval

Prior approval was obtained from the Institutional Animal Ethics Committee (Approval No. 770/ac/CPCSEA/FVSc/AAU/IAEC/13-14/207).

Data Availability

All data supporting the findings of the study is available within this article.

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