RESEARCH ARTICLE

# *In Vitro* Assessment of Acaricidal and Reproductive Inhibitory Effects of *Allium Sativum* Clove Extracts against *Rhipicephalus microplus*

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## Abstract

The present study was carried out to investigate the *in vitro* efficacy of crude aqueous, ethanolic, and hydroethanolic extracts of *Allium sativum* (garlic cloves) against *Rhipicephalus microplus* ticks, with a focus on their potential as novel botanical agents for tick control. Bioassays were conducted on both fully engorged adult female ticks and larval stages using extract concentrations ranging from 6.25 mg/mL to 100 mg/mL. While adult and larval mortality was limited, a notable dose-dependent reduction in egg-laying capacity was observed in treated female ticks compared to the untreated control group. This reproductive inhibition, revealed through the adult immersion test (AIT), highlights a promising sublethal effect of *A. sativum* extracts. The larval packet test (LPT) yielded $LC_{50}$ values of 88.43, 44.04, and 171.1 mg/mL for aqueous, ethanolic, and hydroethanolic extracts, respectively. Despite the modest direct acaricidal activity, these findings underscore the potential of *A. sativum* to impair tick fecundity and contribute to integrated tick management strategies. The observed interference with tick reproduction warrants further research into optimizing extract formulation and exploring synergistic combinations for enhanced efficacy.

**Key words:** Acaricidal, AIT, *Allium sativum,* Crude extracts, LPT, *Rhipicephalus microplus.*

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## Introduction

*Rhipicephalus microplus*, a widely prevalent ixodid tick in tropical and subtropical regions, poses a significant threat to cattle and other livestock by causing direct damage through blood loss, irritation, and reduced productivity, as well as by serving as a vector for economically important haemoparasitic diseases such as babesiosis and anaplasmosis (Ghosh *et al.,* 2007; Mandloi *et al.,* 2016). The control of *R. microplus* has traditionally relied on the extensive use of synthetic acaricides. However, the emergence of resistance in tick populations (Sangwan *et al.,* 2021; Bhowmik *et al.,* 2024) concerns over chemical residues in animal-derived food products, and environmental toxicity have necessitated the search for safer, sustainable, and eco-friendly alternatives (Abbas *et al.,* 2014; Benelli *et al.,* 2016). In this context, botanical acaricides have gained considerable attention due to their biodegradability, reduced likelihood of resistance development, and relatively lower environmental impact (George *et al.,* 2004).

Among plant-based candidates, *Allium sativum* (garlic) is recognized for its broad-spectrum bioactivity, including antimicrobial, antiparasitic, and insecticidal properties, largely attributed to its sulfur-containing compounds such as allicin, ajoene, and diallyl disulfides (Ankri and Mirelman, 1999; Amagase *et al.,* 2001). Previous studies have reported variable efficacy of *A. sativum* extracts against tick species, often with limited direct mortality (Iqbal *et al.,* 2014; Kumar *et al.,* 2022). However, recent emphasis has shifted towards evaluating sublethal effects, such as interference with tick fecundity, egg hatching, and reproductive physiology, which could contribute significantly to long-term tick population suppression (Rodriguez-Vivas *et al.,* 2018). Extracts of *A.*

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*sativum* have demonstrated insecticidal effects against a variety of ectoparasites and arthropod pests (El-Kamali, 2009; Meriga *et al,* 2012; Iqbal *et al.,* 2014), but their acaricidal and sublethal effects on *R. microplus* remain relatively underexplored.

Hence, the present study was designed to assess the *in vitro* acaricidal and reproductive inhibitory effects of crude aqueous, ethanolic, and hydroethanolic extracts of *A. sativum* against both larval and adult stages of *R. microplus*. By exploring both lethal and sublethal outcomes, the study seeks to provide a more comprehensive understanding of the bioefficacy of *A. sativum*, and its potential role as a botanical intervention within integrated tick management strategies.

## Materials and Methods

### Collection and Processing of Plant Materials

Healthy, disease- insect- and weed-free *A. sativum* (cloves) were procured from the local market of Mathura district, Uttar Pradesh (India). The cloves of *A. sativum* were thoroughly washed and cleaned with running water. The cloves were spread out on paper sheets and left to dry in a shaded area at room temperature. Once dried, the materials were finely ground using a grinder. The resulting powder was stored in separate airtight containers at 4°C and labelled with the plant name and the date of preparation.

### Preparation of Aqueous, Ethanolic and Hydroethanolic Extracts

Aqueous, ethanolic, and hydroethanolic cloves' extracts of *A. sativum* were prepared. For the aqueous extract, 10 g of finely powdered cloves were soaked in 100 mL of distilled water at room temperature for 48 h on a magnetic stirrer. The extract was then filtered through Whatman No. 1 filter paper and dried at 37°C. Ethanolic and hydroethanolic (20:80) extracts were obtained by the Soxhlet extraction method (Redfern *et al.,* 2014). Approximately 10 g of cloves was placed in a cellulose thimble within the extractor. The solvents (400 mL) were heated using an isomantle, and the extraction was conducted for 10 h. After extraction, the solvent was evaporated, and the dried extracts were stored in glass vials at 4°C. The extract yield was calculated using the standard formula.

### Estimation of Total Phenolic and Flavonoid Content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu colorimetric method (Ainsworth and Gillespie, 2007). Gallic acid served as the standard, with absorbance measured at 760 nm. Extract concentrations (500, 1000, and 2000 µg/mL) were analyzed in triplicate, and results were expressed as gallic acid equivalents (GAE).

Total flavonoid content (TFC) was assessed using the aluminium chloride colorimetric assay (Zhishen *et al.,* 1999; Yang *et al.,* 2004). Quercetin was used as the standard, with absorbance recorded at 510 nm. Extract concentrations (2500, 3000, and 4000 µg/mL) were evaluated in triplicate, and results were expressed as quercetin equivalents (QE). Standard curves for both gallic acid and quercetin were plotted to calculate the respective contents.

### Collection and Handling of Ticks

Samples were gathered from unorganized cattle farms in Mathura district, Uttar Pradesh, India, ensuring a diverse representation. Fully engorged adult female ticks were collected from naturally infested cattle with the help of forceps and placed in well-ventilated, wide-mouth containers covered with muslin cloth. The ticks were transported to the laboratory for further analysis. Ticks were collected only from cattle that had not been treated with acaricides for at least 30 days to avoid confounding acaricide residues. The ticks were washed three times with phosphate-buffered saline (PBS, pH 7.2) to remove dirt and debris, and then dried using blotting paper. Identification was conducted using a stereomicroscope based on morphological characteristics, following standard classification methods (Walker 2003). For egg-laying and larval development, a subset of engorged female ticks was incubated under controlled conditions at 27 ± 2°C with 80 ± 5% relative humidity (RH). The resulting larvae were subsequently used for the larval packet test to evaluate acaricidal efficacy.

### Adult Immersion Test (AIT)

The Adult Immersion Test (AIT) was conducted according to the guidelines of the Food and Agriculture Organization (FAO, 1984). Eggs laid by treated ticks were incubated under the same conditions, and the hatching percentage was visually assessed. The percentage inhibition of oviposition, reproductive efficiency and product effectiveness were calculated by the method of Drummond *et al.* (1973).

### Larval Packet Test (LPT)

The Larval Packet Test (LPT) was conducted in accordance with the FAO (1984) guidelines with minor modifications. Rectangular filter paper pieces (3.75 cm × 8.5 cm, Whatman No. 541) were utilized in place of the standard square filter papers prescribed in the original protocol. This alteration was made to increase the surface area for uniform impregnation of test compounds and to provide improved spatial accommodation for larval movement within the packet, and the percentage mortality was calculated.

### Statistical Analysis

All the collected data was statistically analysed using the probit method and GraphPad Prism 8.0 software.

## Results and Discussion

The highest extract yield of *A. sativum* cloves was observed in the aqueous extract (50%), followed by the hydro-ethanolic (13%) and ethanolic (4.6%) extracts. The phenolic content

**Table 1:** Dose dependent response of adult immersion test (AIT) to aqueous, ethanolic and hydroethanolic extract of *Allium sativum* (cloves) against *R. Microplus*

| Extract | Concentration (mg/mL) | ATR ± SE | MAM15 ± SE | MEMR ± SE | RI ± SE | IO % | Hatching % (visual) | EPI ± SE | OR ± SE | PE ± SE |
|---|---|---|---|---|---|---|---|---|---|---|
| Aqueous | 100 | 1.94 ± 0.02 | 0.00 ± 0.00 | 0.99 ± 0.01 | 0.51 ± 0.01 | 22.27 | 85.18 | 51.22 ± 0.86 | 22.28 ± 1.24 | 33.80 ± 1.07 |
| | 50 | 1.87 ± 0.01 | 0.00 ± 0.00 | 1.00 ± 0.01 | 0.53 ± 0.01 | 18.97 | 89.30 | 53.40 ± 0.71 | 18.99 ± 0.50 | 27.65 ± 0.70 |
| | 25 | 1.86 ± 0.03 | 0.00 ± 0.00 | 1.14 ± 0.04 | 0.61 ± 0.02 | 7.35 | 92.13 | 61.06 ± 2.45 | 18.99 ± 0.50 | 14.64 ± 3.41 |
| | 12.5 | 1.86 ± 0.04 | 0.00 ± 0.00 | 1.14 ± 0.00 | 0.61 ± 0.01 | 6.78 | 93.10 | 61.44 ± 1.31 | 7.36 ± 3.69 | 13.19 ± 2.06 |
| | 6.25 | 1.80 ± 0.03 | 0.00 ± 0.00 | 1.15 ± 0.00 | 0.64 ± 0.01 | 2.91 | 95.21 | 63.99 ± 1.23 | 6.75 ± 2.24 | 7.56 ± 1.77 |
| | Control | 1.75 ± 0.01 | 0.00 ± 0.00 | 1.16 ± 0.00 | 0.66 ± 0.00 | 0.00 | 100.00 | 65.91 ± 0.49 | 2.91 ± 1.86 | 0.00 ± 0.00 |
| Ethanolic | 100 | 1.19 ± 0.00 | 13.33 ± 0.00 | 0.57 ± 0.01 | 0.48 ± 0.01 | 7.00 | 89.71 | 48.31 ± 1.18 | 6.93 ± 1.78 | 16.51 ± 1.58 |
| | 50 | 1.19 ± 0.00 | 6.66 ± 0.00 | 0.58 ± 0.01 | 0.48 ± 0.01 | 6.95 | 91.64 | 48.34 ± 0.96 | 6.73 ± 2.81 | 14.51 ± 2.70 |
| | 25 | 1.20 ± 0.01 | 0.00 ± 0.00 | 0.59 ± 0.01 | 0.49 ± 0.01 | 4.73 | 93.21 | 49.50 ± 0.68 | 6.73 ± 2.81 | 11.02 ± 2.05 |
| | 12.5 | 1.22 ± 0.01 | 0.00 ± 0.00 | 0.62 ± 0.00 | 0.51 ± 0.00 | 2.70 | 95.20 | 50.55 ± 027 | 4.54 ± 2.13 | 7.14 ± 2.32 |
| | 6.25 | 1.23 ± 0.01 | 0.00 ± 0.00 | 0.63 ± 0.02 | 0.51 ± 0.03 | 1.98 | 95.69 | 50.92 ± 1.59 | 2.45 ± 2.50 | 5.74 ± 4.90 |
| | Control | 1.25 ± 0.01 | 0.00 ± 0.00 | 0.65 ± 0.02 | 0.52 ± 0.01 | 0.00 | 100.00 | 51.96 ± 1.36 | 1.52 ± 5.00 | 0.00 ± 0.00 |
| Hydro-ethanolic | 100 | 1.89 ± 0.03 | 0.00 ± 0.00 | 1.10 ± 0.00 | 0.58 ± 0.00 | 11.29 | 89.25 | 58.11 ± 0.26 | 11.26 ± 0.78 | 20.80 ± 0.82 |
| | 50 | 1.88 ± 0.01 | 0.00 ± 0.00 | 1.12 ± 0.00 | 0.60 ± 0.01 | 9.04 | 90.43 | 59.58 ± 0.16 | 8.97 ± 2.40 | 17.66 ± 2.32 |
| | 25 | 1.88 ± 0.03 | 0.00 ± 0.00 | 1.13 ± 0.00 | 0.60 ± 0.01 | 8.57 | 91.86 | 59.89 ± 0.85 | 8.97 ± 2.40 | 16.00 ± 0.98 |
| | 12.5 | 1.85 ± 0.02 | 0.00 ± 0.00 | 1.14 ± 0.00 | 0.62 ± 0.01 | 5.97 | 94.91 | 61.59 ± 0.60 | 8.55 ± 1.18 | 10.69 ± 1.68 |
| | 6.25 | 1.84 ± 0.02 | 0.00 ± 0.00 | 1.15 ± 0.00 | 0.62 ± 0.01 | 4.75 | 95.75 | 62.39 ± 0.53 | 5.90 ± 1.75 | 8.77 ± 0.94 |
| | Control | 1.76 ± 0.01 | 0.00 ± 0.00 | 1.15 ± 0.01 | 0.66 ± 0.01 | 0.00 | 100.00 | 65.51 ± 0.77 | 4.73 ± 0.83 | 0.00 ± 0.00 |

ATR: average tick weight per replicate (gm); SE: standard error; MAM15: mean adult mortality within 15 days (%); MEMR: mean eggs mass per replicate (gm); RI: reproductive index; IO (%): percent inhibition of oviposition; EPI: egg production index; OR: oviposition reduction; PE: product effectiveness (%).

was found highest in the hydro-ethanolic extract (123.80 mg/mL), followed by the ethanolic (68.42 mg/mL) and aqueous (25.93 mg/mL) extracts. Conversely, flavonoid content was highest in the aqueous extract (74.29 µg/mL), followed by the ethanolic (27.99 µg/mL) and hydro-ethanolic (11.91 µg/mL) extract.

The results of Adult Immersion Test (AIT) are depicted in Table 1. The results indicated that the ethanolic extract of *A. sativum* induced 13.33% adult tick mortality at the highest tested concentration (100 mg/mL), whereas the aqueous and hydroethanolic extracts showed no mortality at the same concentration. On the contrary, Aboelhadid *et al.* (2013) reported 100% tick mortality with garlic oil. This variation may be due to differences in formulation, solvent penetration, compound concentration, species susceptibility, and the potential antagonistic effect of antioxidant properties.

The highest Oviposition Reduction (OR) was found in aqueous extract (22.28) followed by hydroethanolic (11.26) and ethanolic (6.93) at 100 mg/mL, respectively. Finally, the product effectiveness (PE) values were calculated as 33.80%, 16.51%, and 20.80% for aqueous, ethanolic, and hydro-ethanolic extracts, at the concentration of 100 mg/mL respectively (Table 1). The $LC_{50}$ values for AIT could not be determined due to insufficient adult tick mortality data.

The Larval Packet Test (LPT) was also conducted to see the acaricidal efficacy of selected extracts on *R. microplus* larvae. The percent larval mortality at different concentrations of aqueous, ethanolic and hydroethanolic extracts is depicted in Table 2. At the highest tested concentration (100 mg/mL), mean larval mortalities were observed to be 4.14% for both aqueous and ethanolic extracts, and 3.94% for the hydroethanolic extract. Based on the larval mortality data, the calculated $LC_{50}$ values were 88.43 mg/mL for the aqueous extract, 44.04 mg/mL for the ethanolic extract, and 171.10 mg/mL for the hydroethanolic extract (Table 3), indicating comparatively higher larvicidal potential of the ethanolic extract. The present study demonstrates that aqueous, ethanolic, and hydro-ethanolic extracts of *A. sativum* exhibited limited acaricidal efficacy against *R. microplus*, particularly in oviposition inhibition and larval mortality. On the contrary, Shyma *et al.* (2014) and Nasreen *et al.* (2020) reported significantly higher tick mortality using methanolic extracts, underscoring the importance of solvent selection.

**Table 2:** Dose-dependent response of larval packet test (LPT) to aqueous, ethanolic and hydroethanolic extract of *Allium sativum* (cloves) against *R. microplus*

| Concentration (mg/ml) | % Larval mortality ± SE | | |
|---|---|---|---|
| | Aqueous | Ethanolic | Hydroethanolic |
| 100 | 4.14 ± 0.04 | 4.14 ± 0.07 | 3.94 ± 0.04 |
| 50 | 3.12 ± 0.08 | 2.06 ± 0.04 | 3.78 ± 0.10 |
| 25 | 0.00 ± 0.00 | 1.10 ± 0.06 | 1.14 ± 0.10 |
| 12.5/6.25 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Control | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |

Bozin *et al.* (2008) reported that phenolic compounds in *A. sativum* act as strong antioxidants, which may reduce oxidative stress in ticks and thereby lessen acaricidal efficacy. Efficacy also varies with tick species, as shown by 65% larval mortality in *Hyalomma anatolicum* (Noaman and Bahreininejad, 2024), suggesting physiological and genetic differences. These findings highlight the complexity of botanical acaricides and emphasize the need for optimized extraction methods, standardized formulations, and species-specific evaluations for effective tick control.

## Conclusion

The present investigation revealed limited acaricidal activity of aqueous, ethanolic, and hydroethanolic extracts of *A. sativum* cloves against both adult and larval stages of *R. microplus* collected from cattle of Mathura, Uttar Pradesh. The low adult tick mortality and modest larval response observed suggest that the crude extracts, in their current form, possess suboptimal efficacy. The findings highlight the complex interactions between solvent selection, extraction techniques, species-specific susceptibility, and environmental factors in influencing the efficacy of *A. sativum* extracts.

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**Table 3:** Log $LC_{50}$, $LC_{50}$, Log $LC_{90}$, $LC_{90}$, Mortality slope, $R^2$, against aqueous, ethanolic and hydroethanolic extract of *Allium sativum* (cloves) as determined by LPT of *R. microplus*

| Variable | Log $LC_{50}$ (95% CI) | $LC_{50}$ (95% CI) | Log $LC_{90}$ (95% CI) | $LC_{90}$ (95% CI) | Slope ± SE (95% CI) | $R^2$ |
|---|---|---|---|---|---|---|
| Aqueous | 1.95 (1.88 to 2.02) | 88.43 (75.36 to 104.20) | 2.16 (1.91 to 2.43) | 145.5 (81.56 to 265.80) | 1.23 (1.05 to 1.46) | 0.94 |
| Ethanolic | 1.64 (1.61 to 1.68) | 44.04 (40.95 to 47.29) | 2.53 (2.28 to 3.05) | 339.4 (191.6 to 1111) | 2.63 (2.27 to 3.07) | 0.97 |
| Hydroethanolic | 2.23 (2.17 to 2.29) | 171.1 (148.8 to 195.6) | 7.33 (7.64 to 52.06) | 990.5 (28.72 to 995.52) | 1.76 (1.39 to 2.33) | 0.91 |

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