INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print); 2222-5234 (Online)
Website: https://www.innspub.net
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Vol. 28, Issue: 4, p. 17-22, 2026

RESEARCH PAPER OPEN ACCESS

In vitro anthelmintic efficacy of betel leaf (*Piper betle*) ethanolic extract against *Ascaridia galli* at varying concentrations

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Key words: *Ascaridia galli*, *Piper betle*, Anthelmintic, Phytochemical, Poultry

Received: March 17, 2026 **Accepted:** March 31, 2026 **Published:** April 05, 2026

DOI: https://dx.doi.org/10.12692/ijb/28.4.17-22

ABSTRACT

The rising incidence of anthelmintic resistance in chicken parasites has necessitated the urgent development of sustainable, environmentally acceptable alternatives to traditional synthetic medications. Ethnoveterinary medicine presents a viable approach by employing bioactive plant extracts that proficiently control helminth infections while reducing chemical residues. This study assessed the *in vitro* anthelmintic efficacy of Betel Leaf (*Piper betle*) Ethanolic Extract (BLEE) against *Ascaridia galli* to combat the rising anthelmintic resistance in chicken. Adult nematodes were subjected to a completely randomized design (CRD), with exposure to BLEE doses of 40, 80, and 120 mg/ml, alongside a Levamisole control. The results indicated a substantial response that was dependent on both dosage and duration. At 2 hours, 120 mg/ml BLEE resulted in 83.33% mortality, demonstrating no significant difference from Levamisole. After 6 hours, all BLEE treatments had cumulative fatality rates ranging from 90.00% to 96.67%. Phytochemical analysis revealed that tannins and saponins are the principal bioactive chemicals responsible for inducing parasite paralysis. The research suggests that *P. betle* extract at 120 mg/ml serves as a powerful botanical substitute for synthetic anthelmintics, endorsing its incorporation into sustainable poultry health management practices.

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INTRODUCTION

The global poultry industry faces a continuous threat from gastrointestinal helminthiasis, with *Ascaridia galli*—the large intestinal roundworm-serving as one of the most economically devastating parasites in avian species. Infections are characterized by reduced feed conversion efficiency, weight loss, intestinal obstruction, and increased vulnerability to secondary pathogens, particularly in smallholder and free-range systems where environmental exposure is high (Belot and Bernard, 2021). While synthetic anthelmintics like Albendazole have traditionally been the primary line of defense, their efficacy is increasingly compromised by the rapid emergence of multidrug-resistant parasitic strains and rising concerns over chemical residues in animal-derived food products.

This growing challenge has revitalized interest in ethnopharmacology, specifically the search for bioactive botanical compounds that offer sustainable and eco-friendly parasite control.

Piper betle, or Betel leaf, is a climbing vine native to Southeast Asia with a rich history in traditional medicine. It is known to contain a complex matrix of secondary metabolites, including eugenol, hydroxychavicol, and various flavonoids, which have exhibited significant antimicrobial and antioxidant properties (Ahmad *et al.*, 2013). Recent preliminary studies suggest that these phytochemicals may also interfere with the neuromuscular coordination of helminths. Consequently, this study evaluates the in vitro anthelmintic efficacy of betel leaf (*Piper betle)* ethanolic extract against *Ascaridia galli* to determine its potential as a natural phytotherapeutic agent. These findings contribute to the growing body of evidence supporting plant-derived anthelmintics as viable, sustainable alternatives for parasitic management in veterinary medicine and livestock production.

MATERIALS AND METHODS

Materials

This study used betel leaves, ethanol (95%), an empty container for maceration, a blender, a rotary evaporator for concentration, Petri dishes, PBS, a micropipette, an incubator, and levamisole.

Plant collection and identification

Fresh leaves of betel (*Piper betle* L.) were collected and taxonomically identified by Prof. Hemres Alburo at the Biodiversity, Environment, and Natural Resources Research Center, Cebu Technological University – Argao Campus.

Preparation of leaf extract

Freshly collected leaves were meticulously rinsed with water to eliminate surface contaminants. The cleaned leaves were subsequently air-dried in the shade to avoid direct sunlight exposure. Subsequent to air-drying, the leaves were severed into smaller fragments with scissors and subsequently pulverized into a fine powder utilizing an electric grinder. Fifty grams of dried and powdered betel leaves will undergo Soxhlet extraction with 300 milliliters of ethanol for approximately 16 hours. The crude plant extract obtained via solvent extraction was removed from the Soxhlet apparatus and concentrated to dryness in a rotary vacuum evaporator at temperatures below 50 °C, then stored at -4 °C until required for bioassays (Arani Datta *et al.,* 2011).

Phytochemical screening betel leaf extract

The ethanolic extract samples were submitted to the Applied Microbiology Laboratory at the Department of Biology, University of San Carlos-Talamban Campus, for phytochemical screening of tannins, saponins, flavonoids, alkaloids, and glycosides using various test methods.

Collection and preparation of *Ascaridia galli*

Live adult nematodes were harvested from the small intestines of freshly slaughtered native chickens (*Gallus gallus domesticus*) at a local slaughterhouse in Dumanjug, Cebu. To maintain physiological viability, the collected *A. galli* worms were immediately transferred into Petri dishes containing sterile Phosphate-Buffered Saline (PBS) at a pH of 7.4. Only active, adult worms of both sexes with uniform size were selected for the bioassay to ensure experimental consistency. The use of motility as a primary indicator for anthelmintic efficacy follows established protocols in ethnopharmacological research, allowing for the observation of neuromuscular interference (Ahmad *et al.*, 2013).

Experimental design

The *in vitro* bioassay was conducted using a Completely Randomized Design (CRD). A total of 120 worms were randomly assigned to four treatment groups, with three replicates per group. Each replicate was suspended in 10 ml PBS of the respective test solution within 100 mm Petri dishes. The treatment groups were established as follows:

Group 1 (Positive control): Worms exposed to a standard concentration of levamisole (synthetic anthelmintic).

Group 2: 40 mg/ml *Piper betle* ethanolic leaf extract.
Group 3: 80 mg/ml *Piper betle* ethanolic leaf extract.
Group 4: 120 mg/ml *Piper betle* ethanolic leaf extract.

Assessment of mortality

Worm motility was assessed every two hours over a six-hour period (2, 4, and 6 hours) to determine the effectiveness of the treatment. Following the method of Ahmad *et al.* (2013), worms that stopped moving were initially classified as immobile. To separate temporary paralysis from death, these worms were placed in lukewarm phosphate-buffered saline (PBS) for 30 minutes (Iqbal *et al.*, 2004; Ogedengbe *et al.*, 2019). "Time of Paralysis" (P) was recorded if the worm did not respond to shaking but began moving again after the recovery period (Ahmad *et al.*, 2013). Conversely, Time of Death was recorded if the worm remained still and showed signs of fading or loss of body firmness, indicating a permanent loss of function (Ahmad *et al.*, 2013; Ogedengbe *et al.*, 2019; Taki *et al.*, 2021).

Calculation of mortality index

The efficacy of the *Piper betle* extract was quantified using the Mortality Index (MI), expressed as a percentage. The formula used for this calculation is:

% MI= {Total number of immobile worms (dead)/ Total number of worms per Petri dish}×100

Parasite morphology verification

Collected worms were identified at the College of Veterinary Medicine, Visayas State University (ViSCA), by Dr. Harvie P. Portugaliza using microscopic examination of key morphological traits to ensure precise species confirmation.

Statistical analysis

Data obtained from the *in vitro* bioassay were expressed as mean percentage mortality of *Ascaridia galli* across different treatment concentrations and observation periods (2, 4, and 6 hours). The experiment followed a Completely Randomized Design (CRD) with four treatments and three replicates per treatment. Analysis of Variance (ANOVA) was performed to determine significant differences among treatment means at each observation time. When significant differences were detected, treatment means were compared using an appropriate post hoc test (e.g., Least Significant Difference, LSD) at a 5% level of significance ($p < 0.05$). The coefficient of variation (CV%) was also calculated to assess the variability within the experimental data.

RESULTS AND DISCUSSION

Mean percentage of mortality of *Ascaridia galli*

In Table 1, the *in vitro* bioassay revealed that BLEE possesses significant anthelmintic properties, characterized by a distinct dose-dependent and time-dependent response. Such patterns are common in botanical studies, where increasing concentrations of secondary metabolites—such as alkaloids, flavonoids, and tannins-lead to higher larval or adult mortality (Khan *et al.*, 2024; Zirintunda *et al.*, 2022).

At the 2-hour observation, the highest concentration (T4: 120 mg/ml) and the positive control (T1: Levamisole) both achieved a mortality rate of 83.33%. This statistical parity indicates that high-concentration BLEE can induce rapid parasitic mortality comparable to synthetic agents. Levamisole typically acts by inducing neuromuscular paralysis through nicotinic acetylcholine receptor agonism (Ranasinghe *et al.*, 2023). The rapid onset of mortality in T4 suggests that the extract may contain bioactive compounds, like terpenoids or alkaloids, which similarly disrupt neurotransmission or energy generation in the early stages of exposure (Zirintunda *et al.*, 2022).

Table 1. Mean percentage (%) of mortality of *Ascaridia galli* at varying betel leaf extract concentrations across different observation periods

Treatments	Observation period		
	2 hours	4 hours	6 hours
T1 (Levamisole)	83.33 [a]	96.67 [a]	96.67
T2 (40 mg/ml BLEE)	63.33 [b]	83.33 [bc]	90.00
T3 (80 mg/ml BLEE)	63.33 [b]	76.67 [c]	93.33
T4 (120 mg/ml BLEE)	83.33 [a]	90.00 [ab]	93.33
CV (%)	7.87	8.16	5.36

Table 2. Screening of phytochemical compounds in betel leaf ethanolic extract

Phytochemical analysis	Method	Positive result	Extract: Betel leaf
Flavonoids	Alkaline reagent test	Colorless	-
Tannins	Ferric chloride test	Brownish green or Bluish black color	+
Saponins	Foam test	Foam formation	+
Alkaloids	Keller-Kiliani test	Brown-reddish precipitate	-
Glycosides		Brown-ring color at the interface	-

By the 4-hour mark, Levamisole reached near-maximum efficacy (96.67%), while T4 increased to 90.00%. Although T4 remained statistically similar to the synthetic control, the lower doses (T2 and T3) lagged behind. This delay in lower concentrations is often attributed to the time required for phytochemicals to penetrate the nematode cuticle, which serves as a significant protective barrier against drug permeability (Soares, 2021). Bioactive compounds such as cysteine proteinases or phenolic compounds are known to act by physically damaging this cuticle or causing its detachment, a process that can be slower than the direct neurotoxic action of synthetic drugs (Espino Ureña *et al.*, 2025; Soares, 2021).

By the final 6-hour interval, all treatments converged toward high efficacy, with mortality rates ranging from 90.00% to 96.67% and no significant statistical differences remaining. This stabilization suggests that while lower concentrations require a longer duration to breach the worm's protective layers, they eventually reach a lethal threshold. The ability of a botanical extract to match the cumulative performance of a standard synthetic dewormer highlights its potential as a "green" alternative in sustainable poultry management, offering a solution to concerns regarding anthelmintic resistance and chemical residues in poultry products (Nxumalo *et al.*, 2021; Zirintunda *et al.*, 2022).

Phytochemical screening

Phytochemical screening of the betel leaf (Table 2) identified the presence of tannins and saponins, whereas flavonoids, alkaloids, and glycosides were absent. The presence of tannins is particularly significant, as these polyphenolic compounds are known to bind to free proteins in the gastrointestinal tract of the parasite or directly to its cuticle, causing structural damage and death. Simultaneously, saponins can disrupt the cell membrane integrity of the nematode, leading to vacuolization and paralysis (Rola *et al.*, 2015). The synergistic action of these two compounds likely accounts for the high mortality indices observed, even in the absence of alkaloids.

CONCLUSION

The findings confirm that BLEE is a potent phytotherapeutic agent, with its efficacy rooted in the presence of tannins and saponins. The study demonstrates that while Levamisole remains the most consistently rapid treatment, 120 mg/ml *Piper betle* ethanolic extract (T4) serves as a statistically comparable natural alternative. The anthelmintic activity of BLEE is clearly concentration-dependent; higher doses produce immediate results similar to synthetic drugs, while lower doses require extended exposure to achieve maximum lethality. By the end of the 6-hour observation period, the natural extract demonstrated over 90% efficacy across all tested levels.

RECOMMENDATIONS

Future studies should conduct *in vivo* validation: Clinical trials involving live poultry are necessary to assess the extract's safety, palatability, and metabolic impact on the host.

ACKNOWLEDGEMENTS

The author sincerely thanks DOST Strand N for their support and Cebu Technological University- Barili Campus and Argao Campus for providing laboratory accommodations and assistance in plant species identification.

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