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Phytochemical composition, miticidal and pediculicidal efficacy of ethanolic leaf extracts of neem (*Azadirachta indica*) and tobacco (*Nicotiana tabacum*) against *Pterolichus obtusus* and *Goniodes dissimilis*

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ABSTRACT

Ectoparasite infestations cause indirect losses to animals due to weakened health, augmented death rates, and an amplified menace of disease transmission. Botanical insecticides work well and improve the immune system, which may help avert chemical resistance; however, using these results in clinical or veterinary practice requires preclinical and clinical studies to ensure they elicit the desired effect, establish the right dose, and guarantee the formulation is safe and consistent. The current study was performed to evaluate the efficacy of *Azadirachta indica* leaf and *Nicotiana tabacum* leaf ethanolic extracts *in vitro* against *Pterolichus obtusus* and *Goniodes dissimilis*. The stock solutions had a concentration of 100 mg/ml using an aqueous solvent. Qualitative phytochemical screening has demonstrated the presence of secondary metabolites encompassing coumarins, flavonoids, phenols, saponins, and tannins. Statistically, no notable response was recorded against *Goniodes dissimilis*, which appeared almost unaffected by treatment type and duration. Moreover, findings infer that the individual activity of both plants, and even when combined together, yields an intensely substantial effect on mortality against *Pterolichus obtusus*, with death rates evidently mounting as contact extends from 3 to 15 hours. Mortality rates at 9, 12, and 15 hours are significantly raised in contrast to 3 and 6 hours (all $p < .001$), and a modest yet remarkable increase is observed between 12 and 15 hours ($p = .034$). The result demonstrates that prolonged exposure is essential for attaining significant mortality against susceptible parasites. Strong statistical proof that plant-based extracts, both alone and in combination, have a big effect on how mites respond. The results show that botanical acaricides can be used as an alternative to synthetic ones, considering the pharmacological activities of plant metabolites, thus stressing the essential of improving botanical formulation and longer exposure time to attain the desired specific effect.

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INTRODUCTION

Mite infestations can make birds irritable, stressed, anemic, eat less, gain weight more slowly, and lay fewer eggs. Stress from mites not only affects physical health, but it can also lead to violent behaviors like feather pecking and cannibalism. *Pterolichus obtusus* is a feather mite that probably doesn't feed on blood, while the northern fowl mite (*Ornithonyssus sylviarum*) and the poultry red mite (*Dermanyssus gallinae*) are the two types of mites that have the biggest effect on the economy. Hematophagous mites are common ectoparasites in Asian poultry systems. Recent studies have shown that they are found all over the world, including in Malaysia, Myanmar, Vietnam, and China, where more than 46% of people are infected. In China, 64% of commercial laying hens and 37% of breeder hens are affected, with layer hens having a particularly high prevalence (85.2%) compared to broilers (0.6%) (Sparagano and Ho, 2020). Mites and other ectoparasites, such as lice, fleas, and ticks, can infest as much as 70% of commercial hen populations. These infestations have a big impact on the economy and people's well-being. Hematophagous mites are linked to a drop in the quality and quantity of egg and meat production, which causes big financial losses and worries about animal welfare. Disease transmission makes control efforts even harder, especially since acaricide resistance is on the rise (Sparagano and Ho, 2020). For instance, *D. gallinae* has been shown to spread Chicken Infectious Anemia Virus (CIAV) through both transovarial and transstadial pathways. This shows how dangerous these mites are to the health and productivity of flocks, as well as how important it is to have good management plans to stop the spread of viral pathogens (Xu *et al.*, 2025). In addition to mites, even small infestations of other ectoparasites, like the chicken body louse (*Menacanthus stramineus*), in cage-free systems have led to more preening and moderate to severe skin lesions. This is likely bad for the birds' health, feed efficiency, egg production, and overall flock health (Murillo *et al.*, 2024). A study by Stockdale and Raun (1960) in the Journal of Economic Entomology found no significant differences in production between infested and uninfested birds, but its relevance to current poultry management practices is limited. Because hematophagous mites and other ectoparasites are so common, have such a big effect on the economy, and are becoming harder to control, we need to learn more about their ecology, effects, and management in modern poultry systems.

As more and more people worry about how chemical pesticides hurt people's health, animals' welfare, and the environment, it's more important than ever to find safer and more environmentally friendly ways to control pests. This problem is especially bad for people with low incomes, who often have a hard time finding cheap and effective solutions. Dealing with this common problem is important for moving forward with sustainable farming and making sure that people from all socioeconomic backgrounds have fair access to safe pest control methods. Traditional knowledge of plant-based medicines and ethnobotanical practices, which have been used in both human and animal medicine for a long time, could be a valuable but underexplored source for new ways to control pests. Phytochemical compounds and their chemical analogs, which come from these practices, have already helped a lot in finding new drugs and treating both chronic and acute diseases. But even though these ethnobotanical alternatives look promising, there hasn't been enough systematic scientific research and validation of them yet (Benelli *et al.*, 2016; Niroumand *et al.*, 2016).

The world is asking for more environmentally friendly ways to control parasites, but there are still not enough good alternatives to synthetic products that have been studied enough.

Recently, research has changed from only looking at secondary metabolites to also looking at some primary metabolites as bioactive substances (Cvjetko *et al.*, 2018). Researchers are still looking for new plant-based medicines to help with different health problems (Ahmad *et al.*, 2018; Mahamat *et al.*, 2025). Tobacco (*Nicotiana tabacum*) and neem are two plants that are known for having a lot of different bioactive compounds. This makes them good candidates for finding new medicines and pest control agents. These metabolites, which include alkaloids, polysaccharides, terpenoids, phenolic compounds, phytosterols, and a number of aromatic and volatile compounds, have a wide range of biological activities (Selwal *et al.*, 2023; Cvjetko *et al.*, 2018; Shen *et al.*, 2006; Gozan *et al.*, 2014; Leal, 2023; Zou, 2021; Popova, 2019; Liu, 2008). *Nicotiana tabacum* has been used in traditional medicine for a long time to treat a wide range of diseases in both people and animals. Its phytochemicals can have a wide range of effects, from making people healthier to killing pests. There are more than 4,000 compounds in the leaves alone (Rodgman and Perfetti, 2013). Many of these compounds help protect plants by stopping or slowing the growth of pathogens and insects (Cvjetko *et al.*, 2018; Shen *et al.*, 2006; Gozan *et al.*, 2014; Zou *et al.*, 2021; Eshetu *et al.*, 2015). Many studies have shown that high-nicotine extracts from *Nicotiana tabacum* and related species are very good at killing parasites and pests. For example, Schorderet Weber *et al.* (2019) found that aqueous tobacco extracts killed more than 90% of the invasive snail *Pomacea canaliculata*, while Guo *et al.* (2023) found that the tick *Rhipicephalus sanguineus* was very resistant to them. Kanmani *et al.* (2021) also showed that tobacco leaf extracts worked against adult rice weevils (*Sitophilus oryzae*). Other studies have shown that they are very effective at killing *Malacosoma neustria* (testaceous form) larvae and stopping their enzymes from working, and that microencapsulation makes them even more effective. These results show that Nicotiana extracts could be useful against a wide range of agricultural and veterinary pests. This supports the idea that they should be studied more as long-term options for integrated pest management.

Neem (*Azadirachta indica*) is a well-known medicinal plant with a very wide range of phytochemicals. Numerous studies have substantiated that nearly all parts of the neem tree—including the leaves, flowers, seeds, fruits, roots, and bark—are abundant in various bioactive constituents such as alkaloids, steroids, flavonoids, terpenoids, fatty acids, carbohydrates, glycoproteins, triterpenes, limonoids (e.g., azadirachtin and nimbin), phenols, tannins, saponins, carotenoids, glycosides, catechins, and phenolic acids (such as gallic, tannic, ferulic, and chlorogenic acids) (Malakar *et al.*, 2025; Abbas *et al.*, 2020; Sandhir *et al.*, 2021; Andersa *et al.*, 2024; Khare *et al.*, 2025; Singh *et al.*, 2005). Scientists have found more than 130 different bioactive compounds in neem so far. Some of the most well-known ones are azadirachtin, salannin, nimbidin, nimbolinin, nimbidol, nimbin, and quercetin. These compounds have led to neem being called a "miracle herb" or "panacea" in scientific literature (Tufail *et al.*, 2025; Batra *et al.*, 2022). Neem's wide use in traditional medicine systems like Ayurveda, Unani, Homeopathy, and Chinese medicine shows how versatile it is as a medicine. Neem has been used in these systems for a long time because of its pharmacological effects, which include killing insects, lowering blood sugar, reducing inflammation, protecting the brain, heart, and liver, healing wounds, and changing the immune system. Comprehensive reviews have shown that neem can control many biological processes, which adds to its effectiveness and safety profile. It is generally thought to have a wide range of effects without causing major problems (Kumar *et al.*, 2018). Neem is still a major focus of pharmacological research because of its complex and structurally intricate chemical makeup and its wide range of biological activities. It has a lot of potential for new therapeutic uses.

With the increasing demand for eco-friendly methods of managing pests in food animals, it is important to look into natural options instead of chemical pesticides. In this case, it makes sense and is necessary to do in vitro studies on tobacco and neem leaf extracts to see how well they work against chicken lice and mites. Researchers can accurately evaluate the efficacy and safety of these botanical extracts in controlled laboratory settings, guaranteeing that only the most promising treatments progress to in vivo studies or practical application. This strategy not only helps lower the risks to the health of animals and people, but it also helps the poultry industry reach its main goal of promoting sustainable practices.

MATERIALS AND METHODS

Plant preparation

Nicotiana tabacum and *Azadirachta indica* were selected as plant species for experimental assays owing to their extensive utilization and acknowledged significance in diverse traditional medicinal practices. To make sure that each plant was correctly identified, its voucher specimen was sent to the Bureau of Plant Industry (BPI) in Manila, Philippines, for authentication and verification. All procedures for treating animals followed the rules set by ethical standards of the Institutional Animal Care and Use Committee (IACUC) of Cagayan State University.

Plant extraction

Leaf samples were obtained from healthy, vigorous plants and thoroughly washed with distilled water (Wilkins®) to remove any foreign materials. The samples were air-dried at room temperature for 7 days before being cut into small pieces and pulverized with an electric blender. The resulting powder was sieved through a flour strainer for a fine consistency before being sealed and labeled in separate jars and stored at 4°C until needed.

To make ethanol plant extracts, 100 grams of each powdered plant material were combined in an Erlenmeyer flask with 1000 mL of 95% ethanol. The mixtures were macerated at room temperature for 16 hours (27°C). The flasks' openings were covered with aluminum foil and agitated to ensure that all active constituents were extracted. The extracts were three times filtered through Whatman No. 1 filter paper. To remove the solvent, the filtrates were concentrated using a rotary evaporator (Genevac EZ-2 series). The crude extracts were then stored at subzero temperatures until needed for further analysis.

Qualitative phytochemical analysis

Phytochemical screening of ethanolic extract samples was examined at Cagayan State University's Analytical Laboratory in the Philippines for qualitative phytochemical analysis.

Specimen collection

Adult lice and mites were taken from infected *Gallus gallus domesticus*. To avoid contamination and sampling bias, sterile, powder-free gloves were worn the whole time.

Using a sterilized fine-toothed brush, the parasite species were carefully removed in a consistent pattern to make sure that all specimens were sampled evenly. This methodical approach is in line with the best ways to reduce sampling bias in entomological studies (for example, consistent removal protocols lower variance caused by handling) (Miller *et al.*, 2010). Collected specimens were carefully placed in their assigned chambers.

Treatment application

One mL of each solution was evenly spread out in a 30 mm Petri dish. To create realistic exposure scenarios, treated filter papers were air-dried for 10 minutes in a sterile environment before being covered with a tea bag containing mites and lice. This way of pre-drying makes sure that the dose is always the same and that the conditions of exposure can be repeated.

Test chamber setup

Microporous tea bags with 10 µm pores were used to hold lice and mites while letting treatment compounds pass through for the controlled diffusion bioassay. This choice takes advantage of the ability of these membranes to hold active agents in one place while allowing them to spread quickly, which is similar to how controlled-release and containment assays work. We put each tea bag in one of six test solutions: ethanolic extracts of tobacco leaves and neem leaves, citronella essential oil, a mix of all three botanicals, and two store-bought anti-louse shampoos.

Experimental design

There were 180 lice in total, and they were split up into five treatment groups, with 30 lice in each group in a complete randomized design setup. There were three biological replicates in each group, with each replicate containing 10 lice. This replication structure ensures statistical robustness and aids in the precise estimation of treatment effects, a common practice in toxicological bioassays to reduce Type I/II errors (Miller *et al.*, 2010). On the other hand, mites were set up in an arena assembly. In each dish, there is a teabag that has been soaked in the right solutions. Collected mites also encompassed five distinct treatment groups (labeled T1 to T6), each executed in triplicate (R1-R3), culminating in a total of 18 arenas. Each arena was clearly marked with the right treatment and replicate number so that tracking and documentation could be done correctly. Five to ten adult mites were carefully moved into each arena with fine brushes to keep them from getting hurt. This method of introducing mites is in line with the standard procedures for handling mites in controlled in vitro bioassays (Sioutas *et al.*, 2023). After that, all the arenas were put in a controlled-environment chamber that kept the temperature and relative humidity at levels that were good for mite activity and survival.

Exposure and monitoring

Bioassays were tested at a temperature of 27 ± 2 °C and a relative humidity of 70 ± 5%. Every three hours for 24 hours, standard procedures were used to check on the mites and lice's health. Every 24 hours, for example, a stereomicroscope was used to check on the ectoparasites' deaths. Dead parasites were found by looking for ones that weren't moving, which could have been on their own or because of a light touch. Death is recorded if they don't move after being poked gently. To make time-mortality assessments accurate, it is necessary to standardize the environment and keep track of time intervals in a systematic way.

Data collection and analysis

ANOVA was used to figure out how important the data were at the 5% level, and employed a Generalized Linear Model (GLM) to look at both the main effects and the interaction effects of extracts on killing mite and louse over time.

RESULTS AND DISCUSSION

Qualitative phytochemical screening

The qualitative phytochemical screening of ethanolic leaf extracts of neem (*Azadirachta indica*) and tobacco (*Nicotiana tabacum*) revealed the presence of several biologically active secondary metabolites (Table 1). Both extracts tested positive for coumarins, flavonoids, and tannins. These classes of compounds are well documented for their antioxidant, antimicrobial, and anti-inflammatory properties (Bubols *et al.*, 2013; Chagas *et al.*, 2022; Jeba Malar *et al.*, 2020), which may contribute to the observed biological activities of the extracts.

Table 1. Qualitative phytochemical constituents identified in garlic and ginger extracts

Phytochemicals (Secondary metabolites)	Neem (*Azadirachta indica*) ethanolic extract	(*Nicotiana tabacum*) ethanolic extract
Anthocyanin	(-)	(-)
Coumarins	(+)	(+)
Flavonoids	(+)	(+)
Phenols	(+)	(-)
Saponins	(+)	(-)
Steroids	(-)	(-)
Tannins	(+)	(+)
Terpenoids	(-)	(-)

Legend: (+) indicates presence, (–) indicates absence

In addition to the shared metabolites, the neem leaf ethanolic extract uniquely contained phenols and saponins, which were absent in the tobacco extract (Table 1). Phenolic compounds are known for their strong free-radical scavenging capacity, while saponins exhibit antimicrobial and immunomodulatory effects (Mohideen *et al.*, 2022; Ouerfelli *et al.*, 2022; Prommaban *et al.*, 2022;

Singaravelu *et al.*, 2019; Sarkar *et al.*, 2021). The presence of these additional constituents may partly explain the relatively higher acaricidal activity associated with neem-based treatments observed in this study.

Anthocyanins, steroids, and terpenoids were not detected in either extract (Table 1). Their absence may reflect genuine lack in the plant material or limited extraction efficiency due to solvent polarity, as ethanol may not effectively solubilize certain phytochemical classes.

In vitro miticidal and pediculicidal evaluation

Representative images of the target ectoparasites used in the bioassays are presented to facilitate species identification and contextual interpretation of the mortality results (Fig. 1 and 2).

Fig. 1. *Pterolichus obtusus*

Fig. 2. *Goniodes dissimilis*

Miticidal activity against _Pterolichus obtusus_

A highly significant treatment effect on mite mortality was observed for *Pterolichus obtusus* (F = 22.0, $p < .001$), indicating substantial differences among treatment groups. Citronella essential oil (CEO; Treatment 3) and the combined botanical formulation (Combo solution; Treatment 4) produced significantly higher mortality rates and were not statistically different from each other. These findings are consistent with earlier studies demonstrating the acaricidal activity of citronella oil and its monoterpenoid constituents against mites (Saad *et al.*, 2006; Hanifah *et al.*, 2011; Tang *et al.*, 2024), as well as synergistic interactions between citronella compounds and neem-derived phytochemicals (Chikhi-Chorfi *et al.*, 2025).

Neem leaf ethanolic extract (NLEE) and tobacco leaf ethanolic extract (TLEE) also induced mortality, though their effects were comparatively slower and less pronounced. Similar acaricidal effects of neem-based formulations have been reported against *Sarcoptes scabiei* and *Rhipicephalus sanguineus*, supporting the role of neem phytochemicals in mite control (Seddiek *et al.*, 2013; Chen *et al.*, 2014; Corpuz *et al.*, 2025).

Exposure duration exerted a highly significant influence on mite mortality (F = 44.89, $p < .001$). Mortality increased progressively as exposure time extended from 3 to 15 hours, with significant differences emerging at 9 hours and continuing thereafter. Mortality rates at 9, 12, and 15 hours were significantly higher than those observed at 3 and 6 hours (all $p < .001$), and a modest but significant increase was observed between 12 and 15 hours ($p = .034$). These results emphasize the importance of prolonged exposure for achieving effective acaricidal action, particularly for botanical formulations whose active compounds may act cumulatively or require extended contact (Isman, 2008; Pavela, 2016).

A significant interaction between treatment and exposure duration (F= 8.88, $p < .001$) further demonstrates that treatment efficacy varied over time.

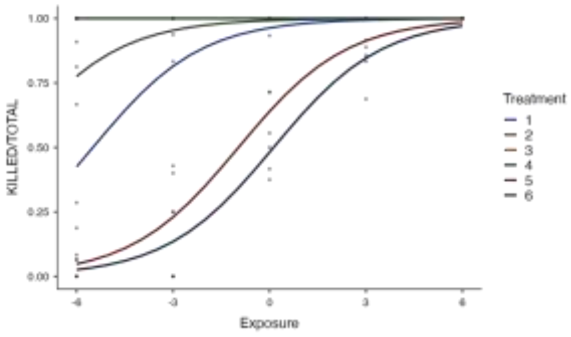

Fig. 3. The interaction between time exposure and solution (treatments) on the proportion killed against *Pterolichus obtusus*

Treatment 1 : Neem leaf ethanolic extract (NLEE)
Treatment 2 : Tobacco leaf ethanolic extract (TLEE)
Treatment 3 : Citronella essential oil (CEO)
Treatment 4 : Mixture of Neem leaf ethanolic extract and Tobacco leaf ethanolic extract (Combo solution)
Treatment 5 : Commercial Miticide A
Treatment 6 : Commercial Miticide B

Fig. 3 illustrates the interaction between exposure duration and treatment on the proportion of *P. obtusus* killed. The model indicates a strong dose-time relationship, with mortality increasing by approximately 3–4% per unit increase in exposure. CEO and the Combo solution increased mortality by approximately 16 percentage points, whereas Commercial Miticide A and Commercial Miticide B reduced mortality by 28% and 35%, respectively. These findings suggest that certain commercial formulations may act slowly or be less effective under short-term exposure conditions, as previously reported (Huang *et al.*, 2013).

Pediculicidal activity against *Goniodes dissimilis*

In contrast to the pronounced miticidal effects observed against *P. obtusus*, none of the treatments produced a statistically significant effect on *Goniodes dissimilis* mortality. Neither exposure duration (F = 1.000, p = 0.337), treatment type (F = 1.000, p = 0.458), nor their interaction (GLM p = 0.525) significantly influenced mortality outcomes.

Fig. 4 shows the interaction between exposure duration and treatment on the proportion killed against *G. dissimilis*, confirming consistently low mortality across all treatments and time points. These results suggest that the botanical extracts tested lacked sufficient bioactive concentration or penetration to exert pediculicidal effects under the experimental conditions. Similar findings have been reported for botanical pediculicides with low essential-oil content or suboptimal extraction efficiency (Heukelbach *et al.*, 2008; Anbu Jeba Sunilson *et al.*, 2009; Yingklang *et al.*, 2023).

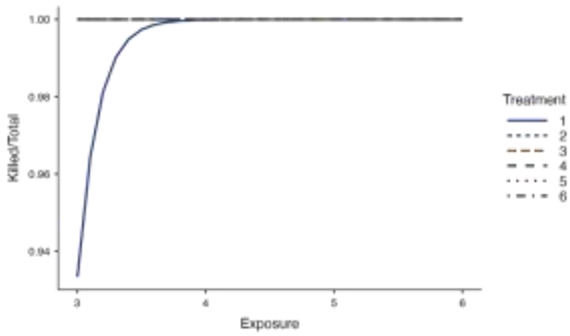

Fig. 4. Interaction between exposure and treatment on the proportion killed against *Goniodes dissimilis*

Treatment 1 : Neem leaf ethanolic extract (NLEE)
Treatment 2 : Tobacco leaf ethanolic extract (TLEE)
Treatment 3 : Citronella essential oil (CEO)
Treatment 4 : Mixture of Neem leaf ethanolic extract and Tobacco leaf ethanolic extract (Combo solution)
Treatment 5 : Commercial Pediculicide A
Treatment 6 : Commercial Pediculicide B

Furthermore, the negligible efficacy of Commercial Pediculicide A and B aligns with growing evidence of resistance in louse populations, often associated with mutations in the knockdown resistance (kdr) gene and reduced susceptibility to pyrethroids (Bouvresse *et al.*, 2012; Mohammadi *et al.*, 2021; Ghavami *et al.*, 2023; Abbasi *et al.*, 2023; Alghashmari and Zelai, 2025).

Overall, the results demonstrate a clear species-specific response to botanical treatments. Ethanolic leaf extracts of neem and tobacco, particularly when combined with citronella essential oil, exhibited significant miticidal activity against *Pterolichus obtusus*, with efficacy strongly dependent on exposure duration (Fig. 1 and 3). In contrast, *Goniodes dissimilis* showed minimal susceptibility to both botanical and commercial treatments (Fig. 2 and 4), highlighting biological and physiological differences between mites and lice.

These findings support previous assertions that plant-based acaricides represent promising alternatives to synthetic products, while also underscoring the necessity of optimizing formulation, exposure time, and target-species specificity to achieve effective ectoparasite control (Isman, 2017).

CONCLUSION

The results indicate, both statistically and biologically, that ethanolic leaf extracts of neem (*Azadirachta indica*) and tobacco (*Nicotiana tabacum*) possess miticidal efficacy against *Pterolichus obtusus*, either singularly or in synergy. The other hand, the activity against *Goniodes dissimilis* is very low. The presence of substantial secondary metabolites in these plant extracts supports the documented pharmacological efficacy against these parasites.

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