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Original Article

In Vitro Acaricidal Efficacy of Neem (*Azadirachta indica*) Oil against Ear Mites (*Otodectes cynotis*)

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ABSTRACT

Neem tree (*Azadirachta indica*) is an herbal plant that has unique properties against insects. This study aimed to assess the in vitro acaricidal efficacy of Neem seed oil against the ear mite (*Otodectes cynotis*). A preliminary test was conducted to determine the LC_{50} of Neem seed oil immersed in distilled water in DMSO. Mites were exposed with approximately 2ml of varying concentrations (0.39%, 0.44%, 0.50%, 0.56%, and 0.62%) of Neem seed extract and were observed at 12 and 24 hours post-exposure. Results of the study showed that the highest concentration (0.62%) of Neem seed oil mixture is considered highly effective against *O. cynotis* with 86% mortality rate at 12 hours exposure. At 24 hours exposure, the mortality was 98% and was comparable to 2% Ivermectin at 100%. The LC_{50} of the Neem seed oil against *O. cynotis* were 0.54% and 0.49% at 12 hours and 24 hours, respectively. The acaricidal activity of the Neem seed oil may be an effect of the secondary metabolites as bioactive compounds that were found during qualitative phytochemical analysis (alkaloids, flavonoids and saponins). The present study showed the acaricidal efficacy of the Neem seed oil in O. *cynotis*. Its application to other external parasites can be explored.

KEYWORDS: *in vitro*, *Otodectes cynotis*, Neem oil, acaricidal, secondary metabolites

1 INTRODUCTION

Ear mite infestation or otocariosis is caused by *Otodectes cynotis*, an obligate parasite that inhabits the vertical and horizontal ear canals of dogs, cats and wild carnivores (Scott and Horn, 1987). It induces various degrees of pruritus and symptoms such as scratching, rubbing of the ears or violent shaking of the head leading to hematoma and serious complication on the host's health (Baker, 1999). Infested animals develop otitis externa, characterized by vertical and horizontal canal erythema and a dark brown, ceruminous otic exudates (Herwick, 1978).

The use of synthetic pesticides is the most common control and treatment of otocariosis (Paradis, 1998). However, these drugs are expensive and sometimes unavailable (Huang *et al.*, 2000).

Furthermore, the wide spread use of synthetic pesticide has commonly lead to multiple resistance (Coles and Stafford, 1999). These, in turn, lead to the development of alternative strategies for ear mite treatment and control. Hence, preparations from the plant have been widely used and developed (Traina *et al.*, 2005).

Neem tree (*Azadirachta indica*) is a herbal plant that has many potential uses, including its medicinal applications and its unique properties against insects (Nisbet, 2000). Its main component is a complex tetranortriterpenoid limonoid called Azadirachtin, which is responsible for both anti-feedant and toxic effects in insects (Subrahmanyam *et al.*, 1989). In this study, the potential of Neem seed oil against ear mites was determined.

2 MATERIALS AND METHODS

Preparation of Neem Seed Oil. A commercially cold pressed 100% Neem seed oil preparation was utilized. DMSO (Dimethyl sulfoxide) and distilled water were utilized to create different concentrations of the extract. The different concentrations were determined after the LC_{50} was established. The initial concentrations for the LC_{50} of Neem seed oil were 0.50% and 1.0%. After establishing the LC_{50}, which was at 0.50% concentration, two higher and two lower concentrations were computed. The different concentrations (0.39%, 0.44%, 0.50%, 0.56% and 0.62%) were computed as previously described (Guevara and Recio, 2005).

Collection of Ear Mites. Ear mites were collected from a naturally infested dog and cat using previously described procedures (Soulsby, 1982). Briefly, samples were collected through ear swabbing using cotton buds and transferred into a glass slide with a mineral oil. At least ten adult ear mites were picked individually using a single brush strand and transferred into a clean, dry Petri plate. Samples were examined using a microscope (at 100×) and were identified based on its characteristics.

Assay of Acaricidal activity. The experiment was conducted as previously described (Seddiek *et al.*, 2013). Different concentrations of the Neem seed oil mixture were applied (approximately 2 ml) to Petri

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plates with at least ten ear mites and were covered with a nylon mesh. The ear mites were checked under the microscope at 12 and 24 hours after exposure.

The mortality and efficacy were determined (Reik and Keitz, 1954) as follows:

$$\text{Percent Mortality} = \left(\frac{Total\ number\ of\ dead\ mites}{Total\ number\ tested\ mites}\right) x\ 100$$

Wherein: 70% mortality is ineffective;
71-80% mortality is effective;
81-100% mortality is said to be highly effective.

Experimental Design Trial. The experiment was laid out using completely randomized design (CRD) with eight treatment groups (representing the five concentrations and the controls) with five replicates each were used. Each replicate had at least ten ear mites and was impregnated uniformly with the varying concentrations of Neem seed oil mixture. Also, two negative controls, the T01 $_{(-)}$ 10% DMSO, and T02 $_{(-)}$ distilled water; and T0 $_{(+)}$ as a positive control with 2% Ivermectin were used.

Phytochemical Analysis of the Plant Oil. A qualitative phytochemical analysis of the Neem seed oil was conducted to test the following secondary metabolites: alkaloids (Mayer's test, Wagner's test), tannins (Tannins test), saponins (Froth test), flavonoids (Alkaline Reagent test, Lead acetate test, Ferric Chloride test), and terpenoids (Chloroform and sulfuric acid test). The qualitative grading system of the phytochemicals was described in the following manner: (+) positive, (-) traces and (0) undetected (Senguttuvan *et al.*, 2014).

Statistical Analysis. The mean differences between treatments were analyzed using the One- way Analysis of Variance (ANOVA). Tukey's HSD test (Honest Significant Difference) was used to evaluate for the significant differences among treatment groups. Probit analysis was performed to estimate the lethal concentration 50 (LC_{50}) and the 95% confidence limit of the Neem seed oil mixture.

Ethical Considerations.The procedures performed in this study were guided by the principles of animal welfare, Animal Welfare Act of the Philippines (RA 8485) and Administrative Order No. 45 of the Bureau of the Animal Industry of the Philippines.

3 RESULTS AND DISCUSSION

The characteristics of the ear mites (Figure 1) were depicted by their sexual differences. As cited by Souza *et al.* (2008) and described by Soulsby (1982), the adult male is 274 to 362 μm while the ovigerous female is between 345 to 451 μm in length. In the distal portions, pretarsi of the anterior pairs of the legs reveal a wine-glass shaped caruncle on a short pedicel. The male mites have a caruncle on all four pairs of legs, while the third and fourth pairs of legs on the female terminate in long hairs or setae. The posterior body of the male also possesses two vent ventrally situated suckers that are two used for the attachment of the male for copulation.

After twelve hours of exposure, variation in the number of dead mites was observed and recorded. The different treatments were dose-dependent. The efficacy of the Neem seed oil mixture according to percent mean mortality rate and its equivalent percent efficacy were: 0.62% (T5) with the highest mortality rate (86%) and was highly effective; 0.56% (T4) with 54% mortality; 0.50% (T3) with 22% mortality; 0.44% (T2) with 15% mortality; and 0.39% (T1) with 11% mortality. The last four treatments were considered ineffective.

The mean percent efficacy of the Neem seed oil mixture at 24 hours exposure of the ear mites was similarly dose-dependent. T5 had the highest mean mortality (98%) and was considered highly effective (Reik and Keitz, 1954). This was followed by 77% (T4), which was effective. The remaining Neem seed oil mixtures (T3, T2, and T1) were considered ineffective. The efficacy of T5 was statistically comparable with 2% Ivermectin (Table 1). The LC_{50} of Neem oil mixture were 0.538 and 0.486 based on Probit analysis at 95% confidence level.

The Neem seed oil mixture exhibited potential acaricidal activity against *O. cynotis* at 12 and 24 hours exposure. The efficacy based on mite's mortality of T5 in 24 hours exposure was found to be comparable with the commercially available acaricide (2% Ivermectin). The efficacy based on the mortality may be an effect of the secondary metabolites that were identified during the qualitative grading system of the phytochemicals tests, wherein flavonoids, alkaloids and saponins were detected (Table 2). Figure 2 shows the results of the qualitative phytochemical tests (flavonoids as indicated with intense yellow color; alkaloids with reddish brown and cream-colored precipitate for Mayer's and Wagner's tests, respectively; and the presence of froth for saponins).

Furthermore, the main component of Neem oil is a complex tetranortriterpenoid limonoid called Azadirachtin, which is responsible for both anti-feedant and toxic effects to organisms (Nisbet, 2000; Subrahmanyam *et al.*, 1989). The effects of other secondary metabolites were: 1) For flavonoids, it affects insects and bacterial microorganisms (Havsteen, 2002). Its insecticidal activity is mainly inhibition to enzymatic pathways, such as the action of a cytochrome P-450 dependent oxidase (Kumar and Pandey, 2013). In another study, the acaricidal activity of flavonoids was demonstrated against *Psoroptes cunicula* (Macchioni *et al.*, 2004). 2) Alkaloids can act as defense compounds of plants, which can be efficient against pathogens due to their toxicity (Anitha Sri, 2016). It produces multiple toxic effects on insects by inhibiting choline acetyltransferase, affecting neurotransmission and several other neuroreceptors and DNA synthesis (Wink *et al.*, 1998; Wink, 2000). Also, in a review of Chowański *et al.*, (2016), alkaloids were reported to be effective against insects. 3) Saponins have biological and pharmacological activities (Shibata, 1977). It has a pesticidal and insecticidal potential (Chaieb, 2010). The Insecticidal activity is due to its interaction with

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cholesterol, causing a disturbance of the synthesis of ecdysteroids. These substances are also protease inhibitors or cytotoxic to certain insects. Furthermore, Podolak *et al.* (2010) found that saponins are natural glycosides that possess a wide range of pharmacological properties including cytotoxic activity. Also, Pelah *et al.* (2002) extracted saponins from quillaja bark and proved its biologic effects by exposing to *Aedesaegypti* and Culexpipiens that resulted in its high mortality.

However, the level of the secondary metabolites depends on the season wherein the seeds were collected (Azmir *et al.*, 2013). The type of soil and other factors, such as temperature, altitude, radiation, atmosphere, light exposure, and nutrition can also influence the variability in plant metabolite levels which can affect the safety and the reliability of plant extract's efficacy (Pavarini *et al.*, 2012).

Table 1. Mean % mortality of Neem seed oil mixture against *O. cynotis* at 12 and 24 hours exposure

Treatment (concentrations)	Mean number of mites exposed	Mean Number of Dead Mites at 12-hour Exposure	% Mortality	Mean Number of Dead Mites at 24-hour Exposure	% Mortality
$T0_{(-)1}$	12	0	0%[a]	0	0%[a]
$T0_{(-)2}$	11	0	0%[a]	0	0%[a]
$T0_{(+)}$	14	14	100%[e]	14	100%[f]
T1 (0.39%)	11.6	1.4	11%[ab]	1.6	13%[b]
T2 (0.44%)	10.8	1.6	15%[b]	2.8	26%[c]
T3 (0.50%)	12.8	2.8	22%[b]	6.4	50%[d]
T4 (0.56%)	12.6	6.8	54%[c]	9.6	77%[e]
T5 (0.62%)	13.6	11.8	86%[d]	14	98%[f]

% Mortality with the same letters are not statistically significant

Table 2. Qualitative phytochemical tests of Neem seed oil

Secondary metabolites	Phytochemical results
Alkaloids	+
Tannins	0
Terpenoids	0
Flavonoids	+
Saponins	+

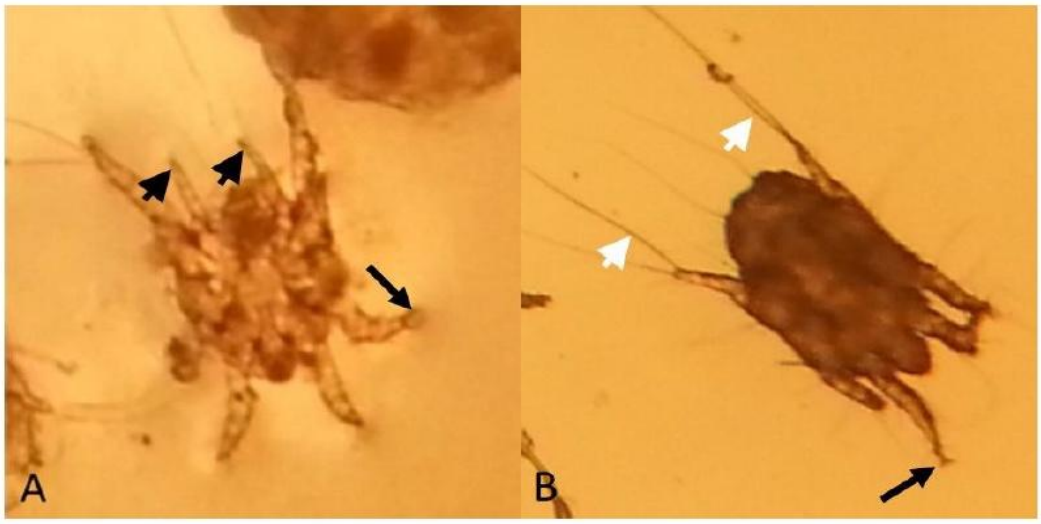

Figure 1. Images of the collected male (A) and female (B) *O. cynotis* showing ventral suckers (arrowhead, black), wine-glass shaped caruncle (arrow) and hairs or setae (arrowhead, white). Magnification= 100×

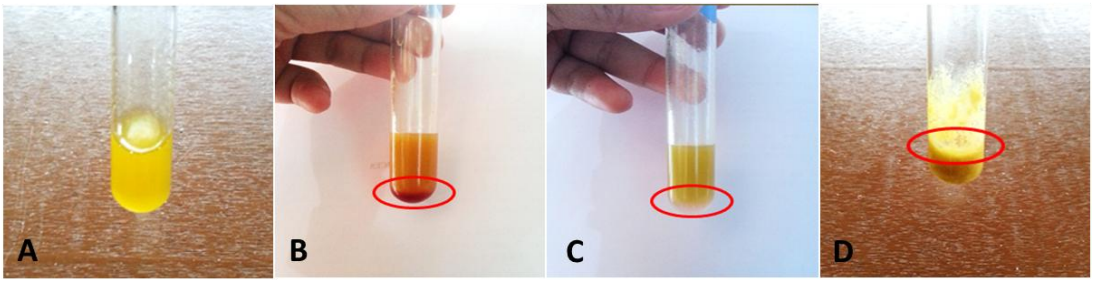

Figure 2. Positive results of the qualitative phytochemical tests for flavonoids (A= Alkaline reagent test); alkaloids (B= Meyer's test; C= Wagner's test); and saponins (D= Froth test)

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4 CONCLUSION

The effective concentration of Neem seed oil mixture that showed highly effective acaricidal activity against ear mites that was comparable to Ivermectin was 0.62%. Moreover, the LC_{50} of Neem seed oil *in vitro* against ear mites of dog and cats were 0.538% and 0.486% at twelve and 24 hours, respectively. The secondary metabolites that were found to be present qualitatively in the Neem seed oil were flavonoids, alkaloids, and saponins. These components may influence the acaricidal efficacy of the Neem seed oil.

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