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The Optimal Concentrations of Ethanolic-Extracted Propolis for a Reduction of *Nosema ceranae* Infection in Western Honey Bees, *Apis mellifera*

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The Optimal Concentrations of Ethanolic-Extracted Propolis for a Reduction of *Nosema ceranae* Infection in Western Honey Bees, *Apis mellifera*

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Simple Summary: The optimal concentration of propolis extracts of the Western honey bee, *Apis mellifera* that can reduce spore loads and increase survival in *A. mellifera* workers was investigated. Newly emerged bees were infected with *N. ceranae* spores (10^5 *N. ceranae* spores per bee) and treated with 25%, 50%, and 75% ethanolic extracted propolis in 50% sucrose solution (v/v). Our data suggest that 50% and 75% propolis extracts are appropriate concentrations for lowering *N. ceranae* infection in honey bees. Therefore, the optimal concentrations of propolis extracts for promoting honey bee survival are 50% and 75%, which causes a reduction in bee mortality.

Abstract: Ethanolic extracts of propolis collected from *Apis mellifera* hives have been shown to reduce *Nosema ceranae* infection in honey bee workers. This study aimed to determine the propolis concentration that optimizes the control of *N. ceranae* infection in honey bee workers. Newly emerged worker bees were individually fed 2 µL of 50% sucrose solution (w/v) containing 10^5 *N. ceranae* spores per bee, and treated with 0%, 25%, 50%, and 75% ethanolic extracted propolis in 50% sucrose solution (v/v). The treated bees were maintained in cages at 34 ± 2°C and 55 ± 5% RH throughout the study. The number of dead bees was counted and removed from the cages daily for 30 days to generate survival curves. The number of *N. ceranae* spores per bee (infectivity) was quantified using a hemocytometer, and the number of infected bees per hundred bees (infection rate) was determined. The survival rates of *N. ceranae* infected bees fed 25%, 50%, and 75% propolis (NP1, NP2, NP3, respectively) were significantly higher than those of infected bees not fed propolis (NO). Furthermore, the infectivity and infection rates of NP2 and NP3 groups were significantly lower than those of the NO group. The infectivity and infection rates of the NP1 group were not different than those of the NO group. Our data suggest that 50% and 75% propolis extracts are appropriate concentrations for treating *N. ceranae* infection in honey bees. Ethanolic extracted propolis can be an alternative treatment for *N. ceranae* infection in Western honey bees.

Keywords: *Apis mellifera*; ethanolic extracted propolis; infectivity; infection rate; *Nosema ceranae*

1. Introduction

Apis mellifera, the Western honey bee, plays a pivotal role as a pollinator of several crops for which it increases yield and quality [1–3]. Recently, a combination of factors, including nutrition, queen quality, pests, and pathogens, was believed to have caused significant yearly losses of managed honey bee colonies [4–6].

Nosema disease, or nosemosis, is a honey bee disease caused by three species of microsporidia: *Nosema apis*, *N. ceranae*, and *N. neumanni* [7–9]. Microsporidia are related to fungi based on phylogenetic analysis [10–12]. [There has been a recent proposal to reclassify *Nosema* spp. to *Vairimorpha* spp. [13]. We continue the use of '*Nosema* spp.' in this study until the genus name is

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resolved]. *Nosema ceranae* is a strain distributed in honey bee populations globally [14]. The disease transmission pathways include trophallaxis, fecal-oral, oral-oral, and sexual routes [8,15,16]. *Nosema* spp. can infect all castes of honey bees (drones, queens, and workers) and honey bee larvae [17–20].

Nosema spp. attack and reproduce in cells that compose the epithelial lining of the midgut, an area where enzymes are produced for the digestion of pollen, nectar, and other foodstuffs [8,16,21–24]. Ingested *Nosema* spp. spores germinate in the midgut. During this process, the polar filament is activated, penetrating the epithelial cells to release the sporoplasm [25,26]. The sporoplasms develop into meronts that multiply (by cell division) into sporonts, sporoblasts, and mature spores by utilizing the host cell contents as a food supply. This reduces the midgut cells' efficiency in food digestion and nutrient absorption [25]. Following this, the mature spores are released into the lumen of the host digestive tract, where they can infect neighboring epithelial cells and reproduce, contributing to their high pathogenicity [8]. Finally, *Nosema*-infected bees can develop dysentery and die, leading to declining bee populations that affect pollination services and the beekeeping industry [8,27–29].

Propolis is a natural resinous mixture produced by honey bees via the mixing of glandular secretions (saliva and wax) with plant resins (substances collected from parts of plants, buds, and exudates) [30,31]. It comprises 50-70% resins, 10% essential oils, 30-50% wax, and 5-10% pollen [32]. Honey bees use propolis to seal openings and cracks in the nest, smooth internal walls, and reduce the nest entrance to prevent invading enemies [33,34]. Propolis also has potent antioxidant, anti-inflammatory, anticancer, antifungal, and antibacterial activities [35–41]. The chemical constituents of propolis vary depending on the honey bee species creating it and the plant source from which they collected the resins [42]. The primary chemical constituents include flavonoids, phenolics, and aromatic compounds [43].

Recent reports showed that 50% ethanolic extracted of propolis (v/v) collected from stingless bee, *Tetrigona apicalis*, nests can reduce the mortality, spore loads, and infection ratio (% infected cells per non-infected cells) and increase the protein contents of hypopharyngeal glands and trehalose levels in the hemolymph of *N. ceranae* infected *A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera* workers compared to the same parameters of untreated bees [24,27,28,44–46]. Moreover, 50% ethanolic extracts of propolis (v/v) collected from *A. mellifera* hives can reduce the mortality/spore loads and increase the protein contents of hypopharyngeal glands and in the hemolymph of *N. ceranae* infected *A. mellifera* workers compared to the exact parameters of untreated bees [44,45,47]. These authors all tested 50% ethanolic extracts of propolis. It is possible, however, that a different extract level will prove to be more efficacious as a *Nosema* spp. control.

This study aimed to determine the propolis concentration that optimizes the control of *N. ceranae* infection in honey bee workers. We determined propolis efficacy by calculating the survival rate, infection rate (number of infected bees per non-infected bees for a hundred bees), and infectivity (number of *N. ceranae* spores per bee) of *N. ceranae* treated bees.

2. Materials and Methods

2.1. Honey Bees

Frames containing sealed brood were collected from three *A. mellifera* colonies maintained according to management practices standard for the region (fed and requeened when necessary, pest/pathogens controlled, etc.). The colonies were kept at the Honey Bee Research and Extension Laboratory (H.B.R.E.L.) research apiary, Entomology and Nematology Department, University of Florida, Gainesville, Florida, U.S.A. The brood frames were collected and kept in an incubator (BINDER, BD 400, Germany) at 34 ± 2°C and 50 – 55% relative humidity (RH) for 24–48 hrs to allow adult worker honey bees to emerge from the combs for use in the experiments. The source colonies were confirmed *N. ceranae* free following the procedure of Fries et al. (2013)[48]. Briefly, we dissected midguts from 50 adult worker honey bees from each colony, pooled the midguts by colony, and homogenized them. Then, we checked for the presence of *N. ceranae* spores using a light microscope (Z.E.I.S.S., A.X.I.O. Lab.A1, Germany). We repeated this procedure on ten newly emerged bees to confirm the absence of *N. ceranae* spores.

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2.2. Preparing Ethanolic-Extracted Propolis

Propolis was collected from ten colonies of *A. mellifera* in an apiary located at the UF H.B.R.E.L. It was dried in an oven (Fisher Scientific, 650D, U.S.A.) at 80 °C for 72 hrs, and then 60 g of dried propolis was extracted in 100 mL of 70% ethanol for three days [45,46]. The resulting solution was gravity-filtered using a Whatman no. 4 filter paper. The liquid extract (100% ethanolic-extracted propolis: the stock solution) was stored in a dark bottle at room temperature. The stock solution was diluted with distilled water to make 25%, 50%, and 75% concentrations (v/v) that were used in the following experiment as ethanolic extracted propolis treatments.

2.3. Nosema Ceranae Spore Preparation

Nosema ceranae spores were extracted from *A. mellifera* workers collected from *N. ceranae* infected colonies in an apiary located at the UF H.B.R.E.L. The spores were propagated in adult *A. mellifera* workers following the procedure described by Naree *et al*. (2021) [45]. Briefly, fifty worker bees were group fed with *N. ceranae* spores in sugar water (w/v) at a dosage of 5×10^7 spores per cage (polystyrene cage size; h = 10 cm, r = 4 cm). The cages were maintained at 34 ± 2 °C and 50 – 55% RH for 14 d to increase the number of *N. ceranae* spores available for the experimental infection. Following this, the midguts of the *N. ceranae* infected bees were removed and transferred to 1.5 mL microcentrifuge tubes containing 100 µL of distilled water. Afterward, the midguts were homogenized and centrifuged at 6,000 g for 10 mins [45,46]. The supernatant was discarded, and the white sediment was collected into the new microcentrifuge tubes to resuspend with distilled water. Following this, the number of *N. ceranae* spores was counted under a light microscope (Z.E.I.S.S., A.X.I.O. Lab.A1, Germany) using a hemocytometer (Neubauer, U.S.A.) following the procedure described by Cantwell, 1970 [49]. Finally, the *N. ceranae* spores were resuspended in 50% (w/v) sucrose solution at a concentration required to feed 10^5 spores per bee (5×10^4 spores per µL). The sucrose solution containing *N. ceranae* spores was kept at room temperature until needed for inoculation the next day.

2.4. Ultrastructure of Nosema Ceranae Spores

The ultrastructure of *N. ceranae* spores was studied following the procedure described by Suwannapong *et al*. (2011) [19]. The midguts of *N. ceranae* infected *A. mellifera* workers (10 d post infection or d p.i.) were fixed in Karnovsky's fixative for three hours at 4°C. Then, the tissue was washed with washing buffer (pH 7.4) three times (ten minutes each) at 4°C. After that, they were post-fixed in 2% osmium tetroxide for one hour at 4°C and washed with washing buffer (pH 7.4) for ten minutes at 4°C. Next, the tissue was dehydrated in an alcohol series, 30%, 50%, 70%, 90%, and 100%, for ten minutes each percentage at 4°C. Then, it was agitated for 12 hrs in a mixture of propylene oxide and embedding media. The sample was embedded in capsules in an Epon 812-aradite mixture. Then, it was kept in an incubator (Memmert UN 55, Germany) for polymerization at 35°C for 24 hrs, 45°C for 48 hrs, and 65°C for 72 hrs, respectively. Following this, the tissue was semi-thin sectioned (1 µm) using an ultramicrotome (Leica, Leica Ultracut R, Germany) and stained with 1% toluidine blue in sodium borate solution to select target areas. Furthermore, the tissue was ultra-thin sectioned (90 nm) using an ultramicrotome (Leica, Leica Ultracut R, Germany) and floated in distilled water. Tissues were collected on 200 mesh grids, stained with uranyl acetate, and contrast-enhanced using lead citrate. The samples were examined by transmission electron microscope (Philips, Tecnai 20, U.S.A.).

2.5. Nosema Ceranae Inoculation and Bee Rearing

Newly emerged bees (*N. ceranae* free) were confined to cages (polystyrene cage size; h = 10 cm, r = 4 cm) in groups of 50 and divided into fourteen treatment groups, with three replicate cages per treatment (Table 1). The first seven treatment groups were randomly selected to be inoculated with *N. ceranae*. *Nosema ceranae* inoculation was accomplished by force-feeding individual bees with 2 µL of 50% sucrose solution (w/v) containing *N. ceranae* spores at a dosage of 10^5 spores per bee.

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Table 1. The fourteen treatment groups with their corresponding experimental manipulations. *Nosema ceranae* spores collected from adult honey bees (*Apis mellifera*) were used to inoculate *N. ceranae* free workers. Propolis collected from *A. mellifera* hives was used to treat *N. ceranae* infected honey bee workers. All treatment groups were provided with the same diet, and each cage began with 50 adult bees. Each group comprised one cage from each of the three source colonies (three replicate cages per group).

Treatment groups	*N. ceranae* inoculation (spores/ bee)	Propolis treatment (% v/v)	Ethanol exposure (% v/v)	Food provided
CO	0	0	0	50% sucrose solution (w/v)[a]
CP1	0	25	17.5	25% propolis in sucrose solution (w/v)[b]
CP2	0	50	35	50% propolis in sucrose solution (w/v)[c]
CP3	0	75	52.5	75% propolis in sucrose solution (w/v)[d]
CE1	0	0	17.5	17.5% ethanol in sucrose solution (w/v)[e]
CE2	0	0	35	35% ethanol in sucrose solution (w/v)[f]
CE3	0	0	52.5	52.5% ethanol in sucrose solution (w/v)[g]
NO	10^5	0	0	50% sucrose solution (w/v)[a]
NP1	10^5	25	17.5	25% propolis in sucrose solution (w/v)[b]
NP2	10^5	50	35	50% propolis in sucrose solution (w/v)[c]
NP3	10^5	75	52.5	75% propolis in sucrose solution (w/v)[d]
NE1	10^5	0	17.5	17.5% ethanol in sucrose solution (w/v)[e]
NE2	10^5	0	35	35% ethanol in sucrose solution (w/v)[f]
NE3	10^5	0	52.5	52.5% ethanol in sucrose solution (w/v)[g]

a= 1 mL of 50% sucrose solution contained 0.5 g of sucrose + 1 mL of distilled water (w/v). b= 1 mL of 25% propolis in sucrose solution contained 0.5 g of sucrose + 0.75 mL of distilled water + 0.25 mL of 100% ethanolic extracted propolis (v/v). c= 1 mL of 50% propolis in sucrose solution contained 0.5 g of sucrose + 0.5 mL of distilled water + 0.5 mL of 100% ethanolic extracted propolis (v/v). d= 1 mL of 75% propolis in sucrose solution contained 0.5 g of sucrose + 0.25 mL of distilled water + 0.75 mL of 100% ethanolic extracted propolis (v/v). e= 1 mL of 17.5% ethanol in sucrose solution contained 0.5 g of sucrose + 0.75 mL of distilled water + 0.25 mL of 70% ethanol (v/v). f= 1 mL of 35% ethanol in sucrose solution contained 0.5 g of sucrose + 0.5 mL of distilled water + 0.5 mL of 70% ethanol (v/v). g= 1 mL of 52.5% ethanol in sucrose solution contained 0.5 g of sucrose + 0.25 mL of distilled water + 0.75 mL of 70% ethanol (v/v).

Four of the *N. ceranae* inoculated treatment groups were provided with either 0%, 25%, 50%, or 75% propolis extract in 50% sucrose solution (v/v). These groups were defined as NO, NP1, NP2, and NP3, respectively (Table 1). The other three *N. ceranae* inoculated treatment groups served as ethanol/*N. ceranae* controls (i.e. to determine if ethanol by itself impacted *N. ceranae* spore loads). These groups received either 17.5%, 35%, or 52.5% ethanol in 50% sucrose solution (v/v), defined as NE1, NE2, and NE3, respectively (Table 1). Bees in the remaining seven treatment groups were force-fed 2 µL of 50% sucrose solution (w/v) that did not contain *N. ceranae* spores. Four of these seven groups were provided with 0%, 25%, 50%, and 75% propolis extract in 50% sucrose solution (v/v), defined as C.O., CP1, CP2 and CP3, respectively (Table 1). The remaining three treatment groups were provided with 17.5%, 35%, and 52.5% ethanol in 50% sucrose solution (v/v), defined as CE1,

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CE2, and CE3, respectively. Each cage was fitted with two gravity feeders, one containing distilled water and the other 50% (w/v) sucrose solution provided *ad libitum* during the experiment. Each cage was also provided with a pollen patty (60 g pollen mixed with 17 mL of 50% sucrose solution (w/v)) *ad libitum*. All treatment cages were kept in an incubator (BINDER, BD 400, Germany) at 34 ± 2 °C, with an RH ranging from 50 to 55% [45,46].

2.6. Survival Analysis

Dead bees were collected and counted daily for 30 d. Survivorship curves of *N. ceranae* infected honey bees treated with different doses of propolis and those of control bees were generated using the Kaplan-Meier approach by plotting the number of surviving bees against days from initiation of the experiment [46]. The percentage of surviving bees was compared across the treatment groups using a non-parametric, univariate analysis of variance and a corresponding post hoc test (Kruskal-Wallis test and Mann-Whitney U test).

2.7. Infection Rate (Number of N. Ceranae-Infected Bees per 100 Exposed Bees)

For all cages, all dead honey bees were collected daily, and all remaining living honey bees were collected 30 d p.i. to determine the number of *N. ceranae* infected bees per 100 bees (infection rate). To do this, the guts of all individual honey bees were removed and homogenized in 100 µL of distilled water [45,46]. Then, the presence/absence of *N. ceranae* spores was determined using a light microscope (Z.E.I.S.S., A.X.I.O. Lab.A1, Germany) at 400× magnification. The infection rates were compared across the treatment groups using a non-parametric, univariate analysis of variance and a corresponding post hoc test (Kruskal-Wallis test and Mann-Whitney U test)

2.8. Infectivity (Number of N. ceranae Spores per Bee)

All dead bees were collected daily and all living bees were collected at 30 d p.i. from each group to determine the number of *N. ceranae* spores per bee (infectivity). The guts of all honey bees were removed and homogenized in 100 µL of distilled water [45,46]. Then, the number of *N. ceranae* spores was counted for each bee using a light microscope (Z.E.I.S.S., A.X.I.O. Lab.A1, Germany) and a hemocytometer (Neubauer, U.S.A.) at 400× magnification following the procedure described by Cantwell, 1970 [49]. The infectivity was compared across the treatment groups using a non-parametric, univariate analysis of variance and a corresponding post hoc test (Kruskal-Wallis test and Mann-Whitney U test). The number of spores per bee was calculated with the following formula:

$$\text{number of spores per bee} = (a \times 5 \times b \times c)/ d$$

a= number of spores from 5 blocks, b= dilution factor (if used), c= volume of bee gut suspension (100 µL), d= volume in 25 blocks of hemocytometer (0.1 µL).

3. Results

3.1. Survival Analysis

The survival of N. ceranae infected bees treated with 25% (NP1), 50% (NP2), and 75% (NP3) propolis was significantly higher than that of infected bees not fed propolis (NO) (χ^2= 66.55, df= 13, p< 0.0001; Figures 1 and 2). However, the survival of infected bees treated with propolis was significantly lower than that of uninfected bees (C.O., CP1, CP2, CP3). Moreover, the survival of *N. ceranae* infected bees treated with 75% (NP3) and 50% (NP2) propolis was significantly higher than that of infected bees treated with 25% (NP1) propolis. The highest survival (mean ± *S.E.*) at the end of the experiment was found in the CP3 group with 79.2 ± 1.2% survival, followed by CP2: 75.6 ± 1.72%, CP1: 75.6 ± 0.75%, CO: 72.0 ± 2.45%, CE1: 68.0 ± 1.41%, and CE2: 64.8 ± 1.02% survival. Moreover, the survival of NP3, NP2, and NP1 was 51.6 ± 0.75%, 45.2 ± 1.02%, and 28.8 ± 0.89% survival, respectively followed by NE1: 9.6 ± 0.75%, NO: 8.0 ± 0.89%, NE2: 0.8 ± 0.49%, and CE3: 0.6 ± 0.24%. No *N. ceranae* infected bees survived in the NE3 group.

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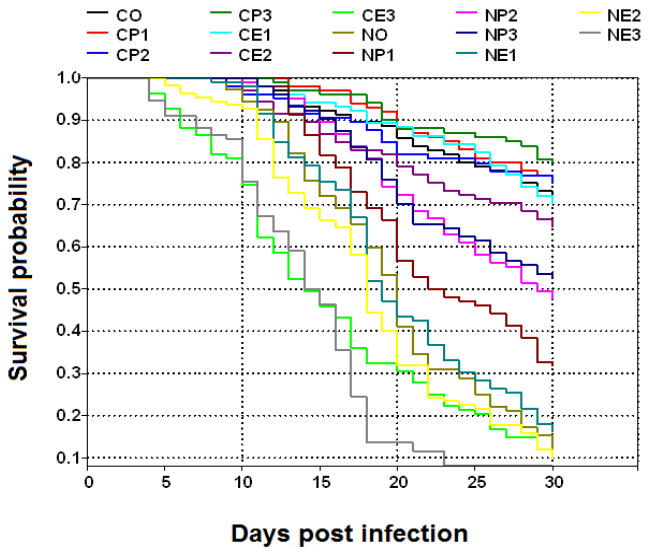

Figure 1. Thirty-day probability of survival of *Apis mellifera* workers from the fourteen treatment groups (see Table 1 for the explanation of treatments). C.O. (uninfected bees fed 50% sucrose solution); CP1, CP2, and CP3 (uninfected bees fed 25%, 50%, and 75% propolis, respectively); CE1, CE2, and CE3 (uninfected bees fed 17.5%, 35%, and 52.5% ethanol, respectively); NO (*Nosema ceranae* infected bees fed 50% sucrose solution); NP1, NP2, and NP3 (*N. ceranae* infected bees fed 25%, 50%, and 75% propolis, respectively); NE1, NE2, and NE3 (*N. ceranae* infected bees fed 17.5%, 35%, and 52.5% ethanol, respectively).

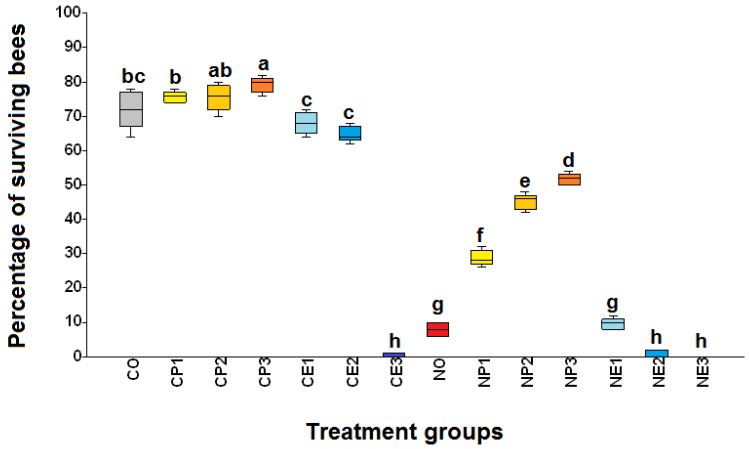

Figure 2. Box plots of 30 d percentage survival of *Apis mellifera* workers from the fourteen treatment groups (see Table 1 for the explanation of treatments). C.O. (uninfected bees fed 50% sucrose solution); CP1, CP2, and CP3 (uninfected bees fed 25%, 50%, and 75% propolis, respectively); CE1, CE2, and CE3 (uninfected bees fed 17.5%, 35%, and 52.5% ethanol, respectively); NO (*Nosema ceranae* infected bees fed 50% sucrose solution); NP1, NP2, and NP3 (*N. ceranae* infected bees fed 25%, 50%, and 75% propolis, respectively); NE1, NE2, and NE3 (*N. ceranae* infected bees fed 17.5%, 35%, and 52.5% ethanol, respectively). The boxes, whiskers, and lines are interquartile ranges from the first to the third quartile, the lowest to the highest observations, and medians, respectively. Treatment means with different letters are significantly different (Kruskal-Wallis test and Mann-Whitney U test; χ^2= 66.55, *df*= 13, *p*< 0.0001).

3.2. Infection Rate (Number of N. ceranae-Infected Bees per 100 Exposed Bees)

No N. ceranae spores were detected in any bees from uninfected groups (groups with "C" as the first letter of the group name) throughout the study, while bees from N. ceranae infected groups ("N" as the first letter of the group name) were positive with mature spores (Figures 3 and 4). The N.

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ceranae infected bees treated with 50% (NP2) and 75% (NP3) propolis showed a significantly reduced infection rate compared to infected bees not fed propolis (NO) and that fed 25 % propolis (NP1) (χ^2= 58.42, df= 13, p< 0.0001; Figure 3). The highest infection rate (mean ± S.E.) was found in the NE2 group with 99.6 ± 0.04%, followed by NE1: 99.2 ± 0.49%, NE3: 98.8 ± 0.49%, and NO: 98.2 ± 0.49%, respectively. Moreover, the infection rates of NP3, NP2, and NP1 were 72.6 ± 1.33%, 73.2 ± 1.50%, and 91.8 ± 4.48%, respectively (Figure 3).

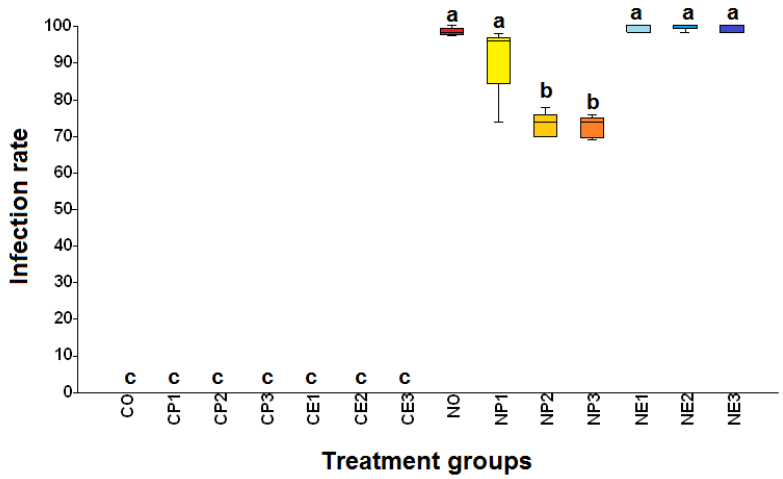
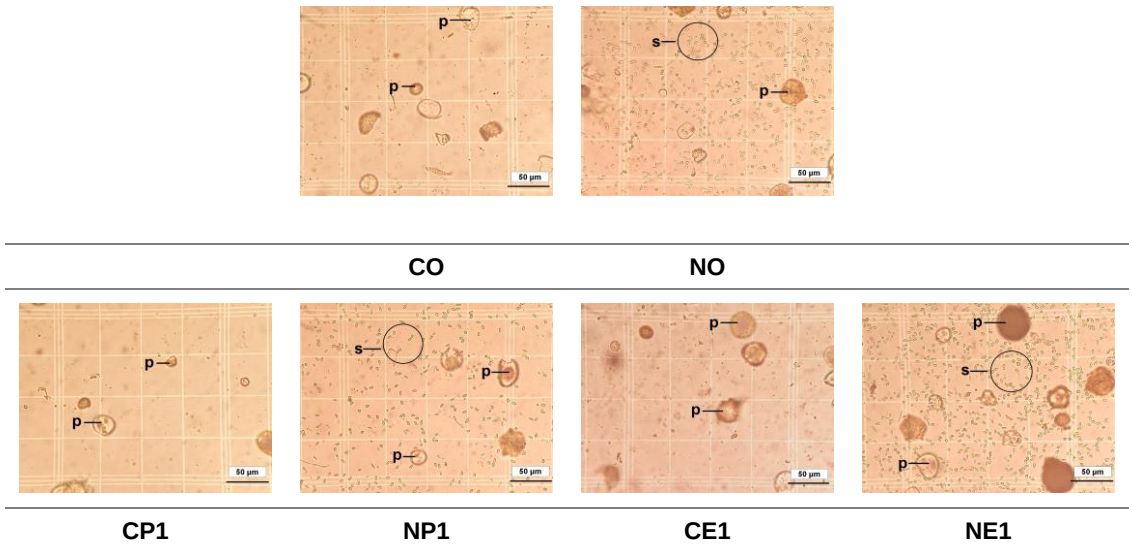


Figure 3. Box plots of infection rate (number of *Nosema ceranae*-infected bees per 100 exposed bees) of *Apis mellifera* workers from the fourteen treatment groups (see Table 1 for the explanation of treatments). C.O. (uninfected bees fed 50% sucrose solution); CP1, CP2, and CP3 (uninfected bees fed 25%, 50%, and 75% propolis, respectively); CE1, CE2, and CE3 (uninfected bees fed 17.5%, 35%, and 52.5% ethanol, respectively); NO (*N. ceranae* infected bees fed 50% sucrose solution); NP1, NP2, and NP3 (*N. ceranae* infected bees fed 25%, 50%, and 75% propolis, respectively); NE1, NE2, and NE3 (*N. ceranae* infected bees fed 17.5%, 35%, and 52.5% ethanol, respectively). The boxes, whiskers, and lines are interquartile ranges from the first to the third quartile, the lowest to the highest observations, and medians, respectively. Treatment means with different letters differ significantly (Kruskal-Wallis test and Mann-Whitney U test; χ^2= 58.42, *df*= 13, *p*< 0.0001).



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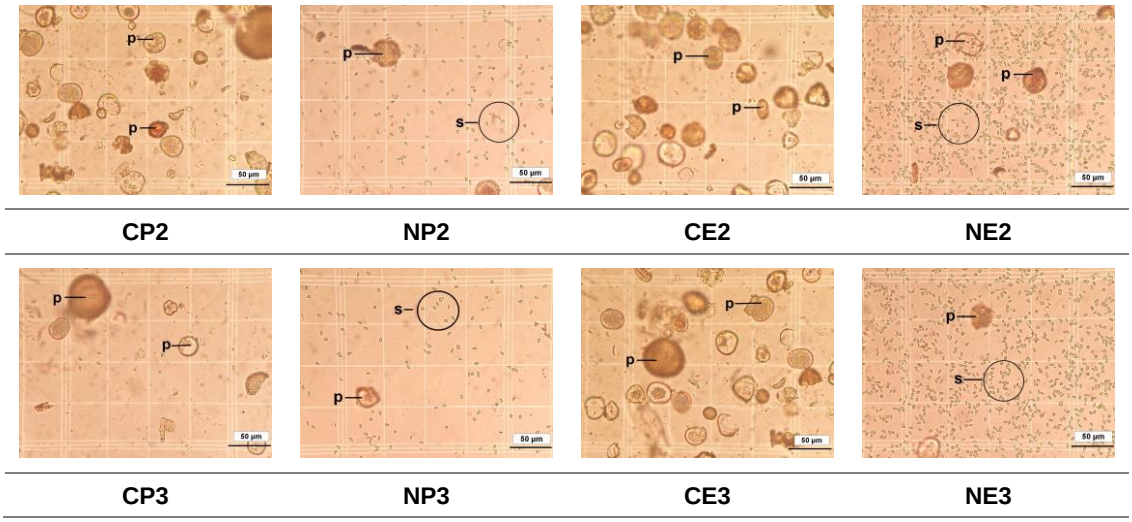

Figure 4. Light micrographs of *Nosema ceranae* spores isolated from the midguts of *Apis mellifera* workers from the fourteen treatment groups (see Table 1 for the explanation of treatments). C.O. (uninfected bees fed 50% sucrose solution); CP1, CP2, and CP3 (uninfected bees fed 25%, 50%, and 75% propolis, respectively); CE1, CE2, and CE3 (uninfected bees fed 17.5%, 35%, and 52.5% ethanol, respectively); NO (*N. ceranae* infected bees fed 50% sucrose solution); NP1, NP2, and NP3 (*N. ceranae* infected bees fed 25%, 50%, and 75% propolis, respectively); NE1, NE2, and NE3 (*N. ceranae* infected bees fed 17.5%, 35%, and 52.5% ethanol, respectively). Pictures were taken of the hemocytometer placed on a light microscope at 400× magnification. We used five blocks within the grid to count spores. Each picture represents one block containing 4 × 4 grids (16 squares). p, pollen grain; s, *N. ceranae* spores.

3.3. Infectivity (Number of N. Ceranae Spores per Bee)

Honey bees from the uninfected groups (groups with "C" as the first letter of the group name) did not have N. ceranae spores throughout the study, while all other groups ("N" as the first letter of group name) were positive for N. ceranae infection (Figure 5). The infectivity of N. ceranae infected bees treated with 50% (NP2) and 75% (NP3) propolis was significantly lower than those of infected bees not fed propolis (NO) or fed 25% propolis (NP1) (χ^2= 994.9, df= 13, p< 0.0001; Figure 5). The highest infectivity (mean ± SE) was found in the NE3 group with 87.33 ± 24.34 × 10^6 spores per bee, followed by NE2: 86.18 ± 5.22 × 106, NE1: 78.97 ± 4.49 × 106, and NO: 70.3 ± 4.36 × 106 spores per bee, respectively. Moreover, the infectivity of NP1, NP2, and NP3 were 61.92 ± 3.97× 106, 28.76 ± 3.21× 106, and 27.75 ± 2.92 × 106 spores per bee, respectively (Figure 5). The individual infectivity of N. ceranae infected bees showed a trend of increasing spore loads over time after honey bees were inoculated with N. ceranae spores (Figure 6). The first deaths of bees from the N. ceranae infected groups treated with no (NO), 25% (NP1), 50% (NP2), and 75% (NP3) propolis occurred on days 8, 8, 10, and 11 p.i., respectively. Moreover, the first deaths of bees from the NE1, NE2, and NE3 groups occurred on days 8, 4, and 5 p.i. (Figure 6)

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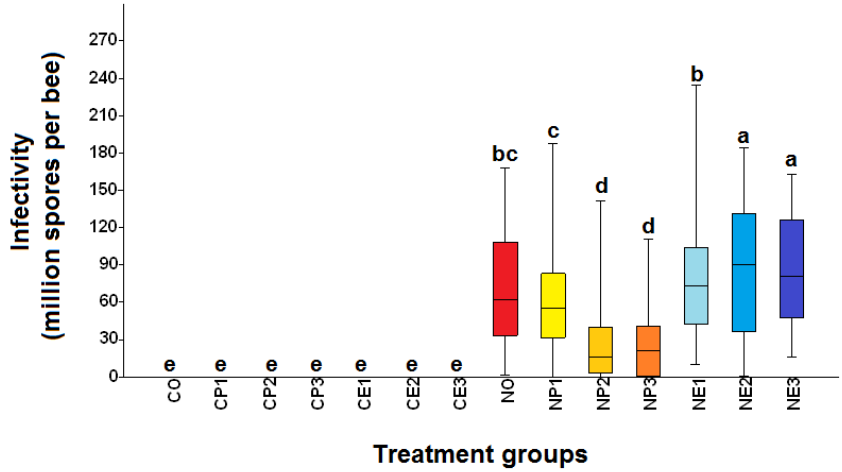
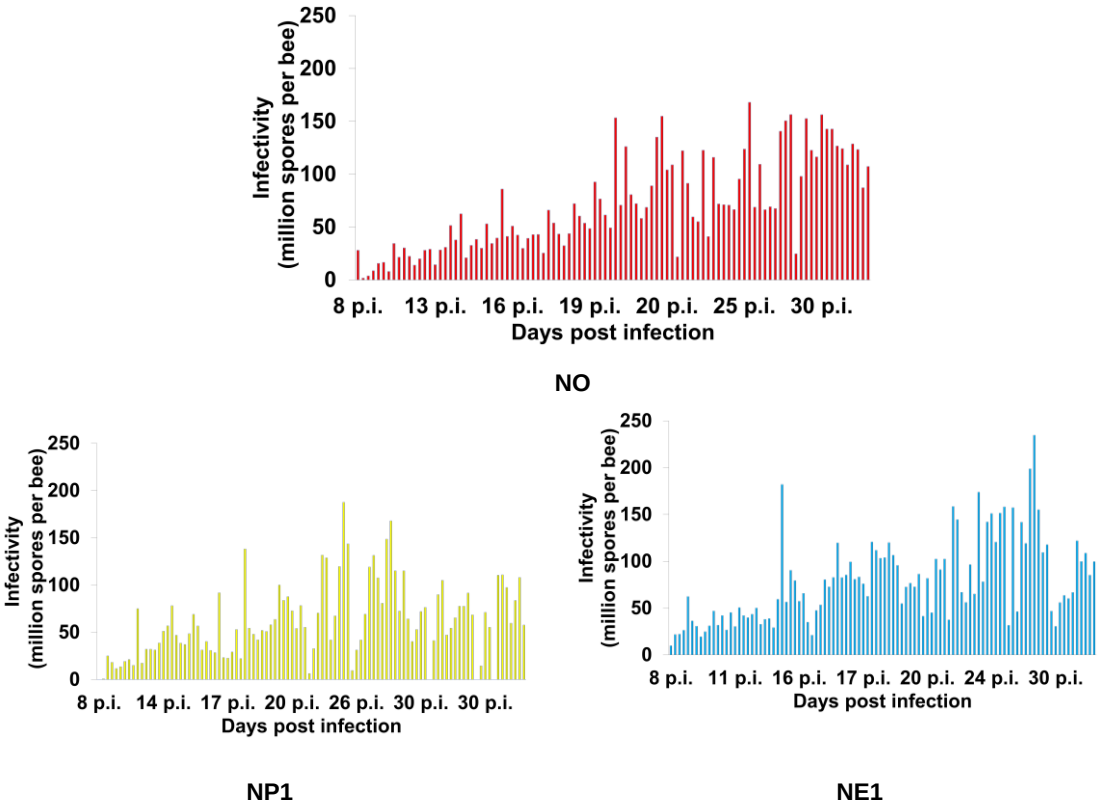


Figure 5. Box plots of infectivity (number of *Nosema ceranae* spores per bee) of *Apis mellifera* workers from the fourteen treatment groups (see Table 1 for the explanation of treatments). C.O. (uninfected bees fed 50% sucrose solution); CP1, CP2, and CP3 (uninfected bees fed 25%, 50%, and 75% propolis, respectively); CE1, CE2, and CE3 (uninfected bees fed 17.5%, 35%, and 52.5% ethanol, respectively); NO (*N. ceranae* infected bees fed 50% sucrose solution); NP1, NP2, and NP3 (*N. ceranae* infected bees fed 25%, 50%, and 75% propolis, respectively); NE1, NE2, and NE3 (*N. ceranae* infected bees fed 17.5%, 35%, and 52.5% ethanol, respectively). The boxes, whiskers, and lines are interquartile ranges from the first to the third quartile, the lowest to the highest observations, and medians, respectively. Treatment means with different letters differ significantly (Kruskal-Wallis and Mann-Whitney U tests; $\chi^2 = 994.9$, $df = 13$, $p < 0.0001$).



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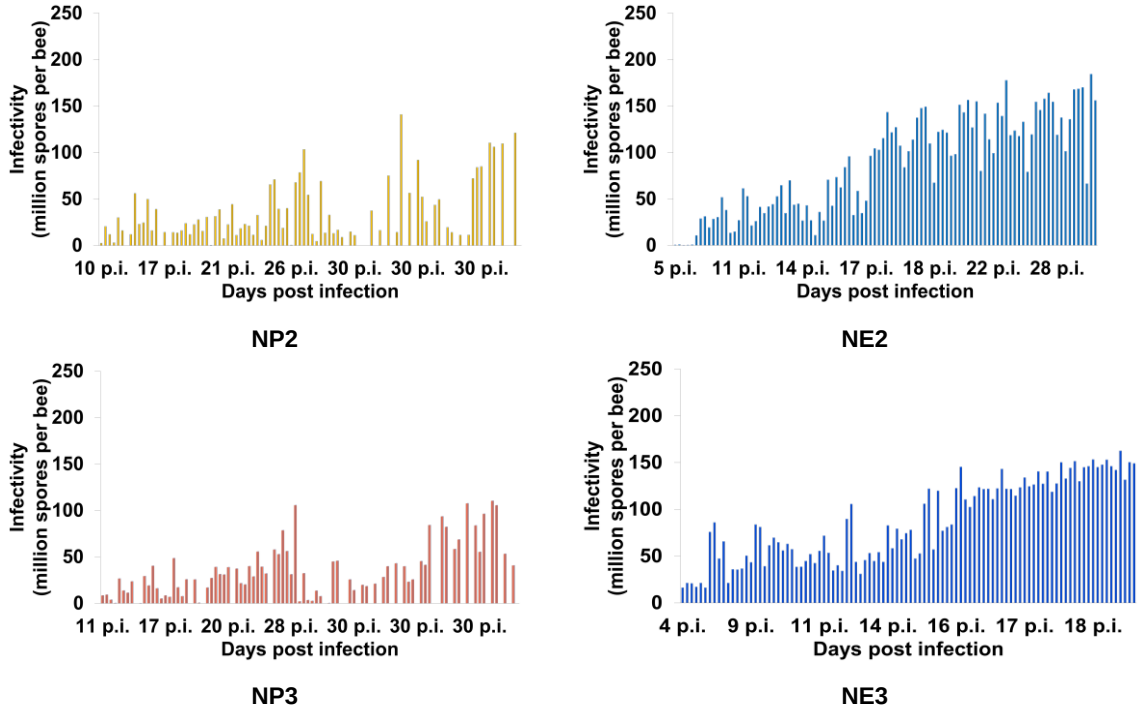

Figure 6. Infectivity (number of *Nosema ceranae* spores per bee) of individual *Apis mellifera* workers from the treatment groups (see Table 1 for the explanation of treatments) sampled daily throughout the project. NO (*N. ceranae* infected bees fed 50% sucrose solution); NP1, NP2, and NP3 (*N. ceranae* infected bees fed 25%, 50%, and 75% propolis, respectively); NE1, NE2, and NE3 (*N. ceranae* infected bees fed 17.5%, 35%, and 52.5% ethanol, respectively).3.4. Ultrastructure of Nosema Ceranae Spores

Transmission electron micrographs of A. mellifera worker midguts infected with 10^5 N. ceranae spores/bee at 10 d p.i. show N. ceranae spores embedded in the cytoplasm of ventricular cells. The spore in Figure 7 was oval. It contained an endospore, exospore, double nuclei, 17 to 20 polar filament coils, and an anchoring disk at the apical part of its spore. The length and width of all measured N. ceranae spores were 4.12 ± 0.51 µm and 2.19 ± 0.16 µm, respectively. Moreover, the polar filament diameter was 63.09 ± 1.23 nm (Figure 7).

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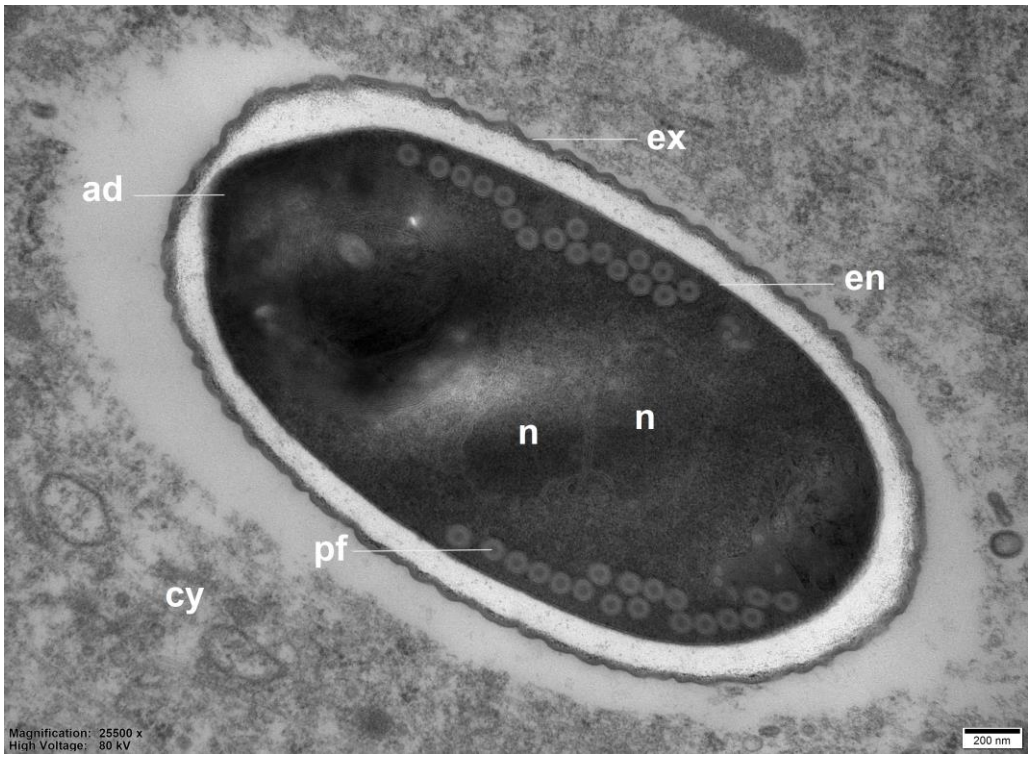
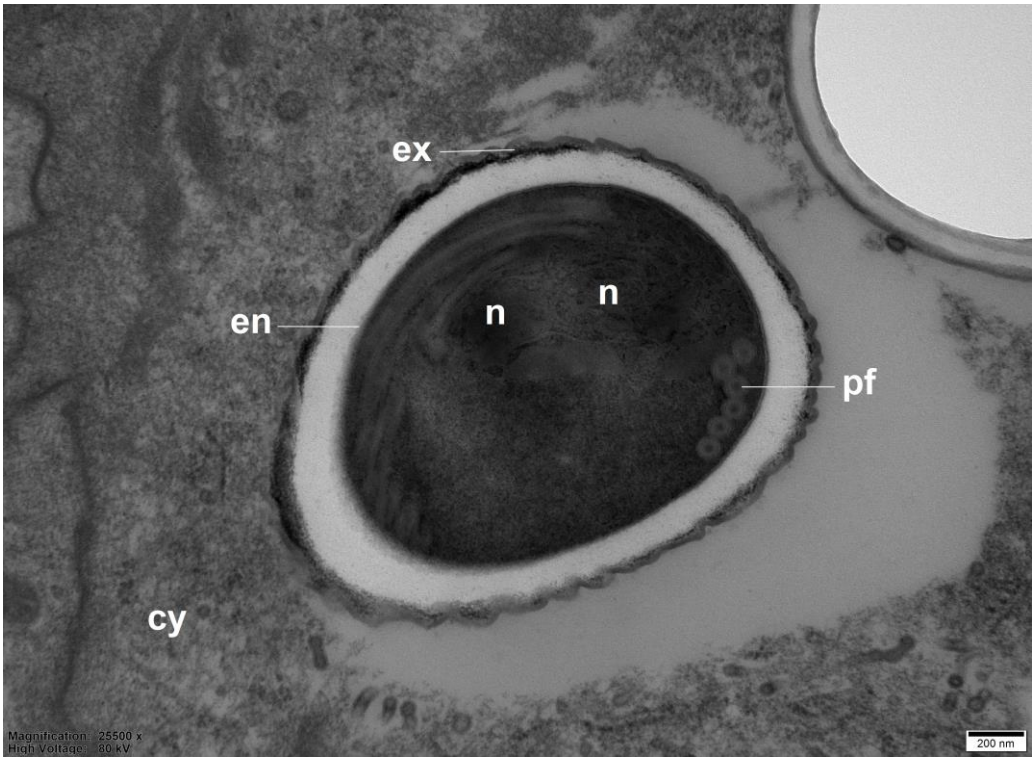


(a)



(b)

Figure 7. Transmission electron micrographs of *Nosema ceranae* spores embedded in a ventricular cell of *N. ceranae* infected *Apis mellifera* workers. (a) long section and (b) cross section of *N. ceranae* spores showed 17 to 20 polar filament coils. The mean ± s.e. length and width of *N. ceranae* spores were 4.12 ± 0.51 μm and 2.19 ± 0.16 μm, respectively. The polar filament diameter was 63.09 ± 1.23 nm. Abbreviations: ad, anchoring disk; cy, the cytoplasm of a ventricular cell; en, endospore; ex, exospore; n, nucleus; pf, polar filament.

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4. Discussion

Nosema ceranae can cause severe nosemosis disease in honey bees, leading to declining bee populations that affect both pollination services and the beekeeping industry [8]. Using an antibiotic to control this disease in honey bees can lead to antibiotic resistance and contamination of hive products [50–56]. Our results in this study demonstrate an alternative way to control *N. ceranae* in honey bees, namely by using 75% propolis (v/v) collected from *A. mellifera* hives and fed to bees. The results showed that the propolis extract could reduce, though not eliminate entirely, the negative consequences of *N. ceranae* infection (mortality, infection rate, and infectivity) in treated *A. mellifera* workers. Our study also demonstrated that high (75% propolis (v/v)) and medium concentrations (50% propolis (v/v)) of propolis extract were more effective against *N. ceranae* infection (mortality, infection rate, and infectivity) in *A. mellifera* workers than was a lower concentration (25% propolis (v/v)) of propolis extract. Our results were similar to those of others using 50% ethanolic extracted propolis (v/v) from *A. mellifera* and stingless bees (*Tetrigona apicalis*) to treat and reduce mortality and spore loads in *N. ceranae* infected bees [44,45,47].

The CE1/NE1 (17.5% ethanol), CE2/NE2 (35%), and CE3/NE3 (52.5%) treatments did not contain propolis and served as solvent controls in our study, the "C" treatments representing bees not infected with *N. ceranae* and the "N" treatments including bees infected with N. *ceranae*. These groups allowed us to distinguish effects caused by the propolis from those, if any, caused by the solvent ethanol. The CE1/NE1 and CE2/NE2 treatments did not impact bee survival or any *N. ceranae* related parameters. Others have found similar results [57]. Interestingly, a high concentration of ethanol (52.5%) in the CE3 group negatively impacted honey bee survival to match that of the NE3 group (52.5% ethanol + *N. ceranae*). This was not seen in the other treatment groups (CP3, NP3) containing 52.5% ethanol (52.5%). This implies that high concentrations of ethanol lead to reduced bee survival [58] and that treatment with propolis may offset these impacts.

Our results showed that feeding *A. mellifera* workers propolis at medium and high concentrations (50% and 75% propolis (v/v)) can decrease *N. ceranae* infection. Our results suggest that 35% and 52.5% ethanol in sugar water can increase the virulence of *N. ceranae* infection in infected *A. mellifera* workers, complimenting the results of Naree et al. [44]. In their research, the infectivity (# *N. ceranae* spores per bee) of *N. ceranae* infected bees treated with 35% ethanol (v/v) ($92.2 \pm 14.18 \times 10^6$ spores per bee) was significantly higher than that of *N. ceranae* infected bees not feed ethanol ($78.28 \pm 13.42 \times 10^6$ spores per bee) [44].

Propolis has antioxidant, anti-inflammatory, anticancer, antifungal, and antibacterial properties [35–41]. The bioactive compounds of propolis vary depending on bee species and their plant source [42]. Propolis includes flavonoids, phenolics, and aromatic compounds [43]. It is unclear how propolis affects *N. ceranae* infection. However, a study by Mura et al. [47] found that ethanolic propolis extracts from *A. mellifera* contain almost 50 phenol and flavonoid family compounds. These included flavones, flavonols, caffeic acid, and ferulic acid [47]. Flavonoids inhibit fungal growth via mechanisms such as plasma membrane disruption and the inhibition of cell wall formation [59]. Ergosterols are necessary components for cell membrane manufacturing. Flavonoids can inhibit ergosterol biosynthesis, resulting in the disruption of cell membrane integrity. This can lead to the leakage of intracellular components [60,61]. The cell wall of *N. ceranae* is primarily composed of glucans and chitin. The antifungal mechanism of flavonoids is cell wall deformation caused by inhibiting glucans and chitin synthesis [61,62]. Cell wall deformations and the membrane damage caused by flavonoids can contribute to malfunctions of the membrane, leading to depolarization, K+ leakage, reduction in membrane fluidity, and ultimately cell death [63,64]. These reasons may partially explain how ethanolic-extracted propolis can reduce *N. ceranae* infection in honey bees.

One can use the number of polar filament coils to distinguish the *Nosema* species [65]. *Nosema apis* contains 30 to 44 polar filament coils [66–68], while *N. ceranae* and *N. neumanni* include 20 to 23 polar filament coils [69] and 10 to 12 polar filament coils [7], respectively. Our polar filament coil data

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allow us to confirm *N. ceranae* identity, given that the analyzed spores had 17 to 20 polar filament coils [69].

5. Conclusions

Using ethanolic-extracted propolis collected from *A. mellifera* hives and formulated at suitable concentrations (50% and 75% propolis) to treat *N. ceranae* infection can decrease the mortality, infectivity, and infection rate of *N. ceranae* infection in honey bee workers. These findings suggest an alternative way to control *N. ceranae* in honey bees using propolis collected from *A. mellifera* hives and extracted with suitable concentrations (50 and 75%) of propolis. This is especially important given the relative inefficacy of various controls available to treat *N. ceranae* infection in honey bees [70]. Admittedly, more work is needed before this treatment recommendation can be made. Nevertheless, our data suggest that propolis use as a preventative of and treatment for *N. ceranae* infection in honey bees is promising and worth pursuing through additional research.

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Institutional Review Board Statement: The animal study protocol was approved by the Ethics Committee of Burapha University (protocol code: IACUC 021/2562 and date of approval: 28 December 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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