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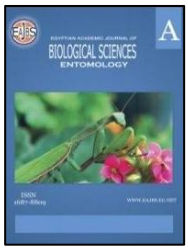

Efficiency of Thymol and Propolis Extract in Controlling Gut Parasite *Vairimorpha (Nosema) ceranae* in Honey Bee, *Apis mellifera*

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ABSTRACT

Honey bees, vital agricultural pollinators, suffer significant losses due to the pathogen *Nosema ceranae*. Fumagillin, utilized to prevent nosemosis, poses a hazardous risk. This study aims to investigate the potential of natural thymol and propolis extract in combating *Nosema* infection in honey bee colonies. The evaluation will be conducted through qPCR to quantify the effects at the molecular level, and histopathological analysis to examine tissue damage and cellular responses, providing a comprehensive understanding of the extracts' efficacy in controlling this parasitic infection. In this experiment, infected honey bee colonies were divided into four treatment groups: (1) untreated, (2) propolis treatment (3 g/L), (3) thymol treatment (0.1 g/L), and (4) a combined treatment with both thymol and propolis. Molecular identification of *Nosema* was carried out using specific primers and reduction in infection was measured using qPCR of the copy number gene for *Nosema*. Histopathological changes were performed in honey bees midgut. Results showed *N. ceranae* detection in an untreated colony using PCR, with amplified fragment 220bp, the sequence of the amplified fragment was assigned in GenBank with accession number PP239351 and *N. ceranae* was closely related to other *N. ceranae* strains. Gene expression of *N. ceranae* decreased in honeybee colonies treated with thymol and propolis extracts, and their mix of both extracts showed better results. The light and TEM micrographs of midgut cells demonstrated that treatment with propolis and thymol improved the midgut epithelial cells of honey bees, reduced parasite infection, and led to the disappearance of various spores and the presence of proliferating small epithelial digestive cells containing the empty spores of the parasite. It can be concluded that thymol and propolis extract show potential in combatting *Nosema* and have positive effects on honey bee health.

INTRODUCTION

Honey bee, *Apis mellifera* L., is an invaluable resource that benefits humans and environment. They serve as pollinators of crops and plants. Moreover, they are efficient biological indicators of environmental pollutants. Honey bees produce a wide variety of

products, including honey, royal jelly, propolis, pollen, honey bread, venom, and wax **(El-**Seedi *et al*., 2022; Salama *et al*., 2022). Nowadays, managed honeybee colonies face many challenges. Pesticide exposure, environmental stress, and poor nutrition are all factors that can endanger bees, while parasites and diseases not only reduce honey production but also kill off entire colonies (Crenna *et al*., 2020).

Bee colony death can be caused by various diseases, including parasites like *Nosema* (Marín-García *et al*., 2022). Nosemosis is a frequent and dangerous disease affecting adult honey bees. Many species of *Nosema*, including *N. ceranae*, *N. apis*, and *N. neumanni*, can cause this microsporidian infection. In the Asian honey bee, *Apis cerana*, the genus *N. ceranae* was discovered for the first time, followed by its identification in the western honey bee *A. mellifera* (Higes *et al*., 2019). It is currently endemic worldwide (Fries *et al*., 2013). It is an obligatory intestinal pathogen of certain *Apis* species (true honey bees) (Goblirsch, 2018). The disease can spread orally when bees ingest infected nectar, pollen, or water. Infected bees' feces contain spores that facilitate the transmission of the pathogen (MacInnis *et al*., 2022). Many *Nosema* species have spores that accumulate on flowers and are transported to bee colonies by pollen. Infected colonies contain this endoparasite throughout the year and multiply to epidemic proportions between the beginning of spring and the end of winter (Gisder *et al*., 2017). Infected bees release many infectious agents in their waste, which can readily infect healthy bees. Various physiological and behavioral changes can occur in infected honey bees (Li *et al*., 2018). Most research on nosemosis therapies has ignored the potential negative consequences for honeybees.

Treatments that effectively prevent *Nosema* infections are necessary for beekeeping. Fumagillin was successfully used for decades to treat colonies infected with *N. apis* (van den Heever *et al*., 2014). Nevertheless, numerous investigations indicated that this drug could be ineffective against *N. ceranae* (Mendoza *et al*., 2017). Fumagillin's toxicity to humans constitutes an additional limitation regarding its use in beekeeping (van den Heever *et al*., 2014). More research is needed to discover new strategies for solutions for controlling *N. ceranae* infections (Burnham, 2019). Numerous compounds were evaluated in the lab or field settings for their effectiveness in controlling *N. ceranae* infestation, yielding promising outcomes (Maistrello *et al*., 2008; Porrini *et al*., 2017). Among the several potential agents for managing *N. ceranae* infections, natural supplements and organic extracts taken orally warrant special consideration because of their presumed minimal toxicity and advantageous impacts on improving bee lifetime and reducing *Nosema* spore burdens ((Maistrello *et al*., 2008; Costa *et al*., 2010; Porrini *et al*., 2017).

Thymol, derived from the thyme plant (*Thymus vulgaris*), is a popular ingredient in many pharmaceutical products (Hossain *et al*., 2013). Thymol and its derivatives are utilized in food, agriculture, medicine, and cosmetics (Sobczak *et al*., 2014). Moreover, its beneficial antimicrobial and antiparasitic activities have also been documented in animals and bees (Maistrello *et al*., 2008; Costa *et al*., 2010; van den Heever *et al*., 2016; Glavinic *et al*., 2022). Thymol's inhibitory effect on the proliferation of harmful bacteria and fungi was recognized for numerous years (Maistrello *et al*., 2008; Kovacevic *et al*., 2021). Thymol was utilized for decades in beekeeping for controlling the honey bee mite *(Varroa destructor*) (Imdorf *et al*., 1995), with variable success (Stanimirovic *et al*., 2019). In the early 21st century, the first investigations were carried out to investigate the potential effect of thymol in controlling *Nosema* infections in hives (Yücel and Doğaroğlu, 2005). Thymol belongs to the few substances demonstrated for reducing *N. apis* and *N. ceranae* spore burdens as well as decreasing mortality in bees infected with *Nosema* (Maistrello *et al*., 2008; Costa *et al*., 2010; van den Heever *et al*., 2016; Glavinic *et al*., 2022).

Honey bees additionally gather several substances that have the potential to favorably affect the well-being of the colony (Erler and Moritz, 2016). Propolis is one

example; it is a resinous combination of chemicals that have antimicrobial properties that many Hymenoptera species use to safeguard their nests from pathogens and diseases (Simone-Finstrom and Spivak, 2010). Propolis extract from stingless bees is proving to be a successful remedy for controlling *N. ceranae* in three species of honey bee: *A. cerana*, *A. florea*, and *A. mellifera* (Yemor *et al*., 2015; Arismendi *et al*., 2018; Suwannapong *et al*., 2018; Naree *et al*., 2021).

Molecular techniques are required to accurately identify *Nosema* at the species level (Michalczyk *et al*., 2011). The detection of *N. ceranae* in *A. mellifera* and the execution of samples retrospectively have been greatly facilitated by novel, extremely sensitive, precise molecular technologies, proving that *N. ceranae* has infected *A. mellifera* multiple times during the previous 20 years and does not represent a novel microsporidian parasite in this host (Guerrero-Molina *et al*., 2016). Regular microscopy for *Nosema* spore identification is probably to blame for the lag in correctly identifying *N. ceranae* in *A. mellifera* (Higes *et al*., 2010a). Beekeepers should benefit greatly from molecular technologies that accurately identify the species of *Nosema* cells.

This study aims to identify *Nosema* using specific primers and to highlight the importance of using natural products (thymol and propolis) to manage apiaries rather than using conventional chemical products. This can be achieved by quantifying *Nosema* infection in honey bee workers using quantitative real time - polymerase chain reaction (qRT-PCR) and by examining the effects of such products on the midgut histology of workers using light and transmission electron microscopy (TEM).

MATERIALS AND METHODS

Preparation of Ethanolic Extract of Propolis:

During the spring season of 2023, the propolis was gathered from the apiary of El-Dokki Honeybee Research Department, Plant Protection Research Institute, Agriculture Research Center, Giza, Egypt. To facilitate easy crushing, the propolis was promptly placed in the freezer at -20 °C. One hundred grams of crushed frozen propolis was mixed with one liter of 80 % ethanol and stored at room temperature in the dark until further use as stock solution. To prepare a 0.3 % working solution, 30 ml of the stock extract was diluted in 1 L of sugar syrup (1:1 w/v sugar: water) (Ghallab *et al*., 2021; Sebak *et al*., 2025).

Thymol Preparation:

Thymol (99 % purity) was purchased from Al-Gumhuriya Pharmaceutical Company, Cairo, Egypt. Thymol concentration of 0.1 g/L (0.01 %) was achieved by dissolving firstly 0.1g of thymol in 1ml of absolute ethanol 99.9 %, then brought to a final volume of 1 L with 1:1 (w/v) sugar: syrup **(**Sánchez *et al*., 2021; Sebak *et al*., 2025).

Preparation of Thymol and Propolis Mix Extract:

The same concentration of thymol and propolis extract (0.01 % and 0.3 %, respectively) were added together and were mixed well, then diluted to 1 L with sugar syrup (Sebak *et al*., 2025).

Colony Selection and Management:

This study was conducted in the summer from June to September 2023, an apiary consisting of 8 asymptomatic colonies of *A. mellifera* infected with *N. ceranae* and exhibiting low propolis levels was chosen for the experiment at the Honeybee Research Department, Plant Protection Research Institute. All colonies of identical strength were utilized in this study, comprising two frames per colony: one containing honey and pollen and one with both sealed and unsealed brood. Larvae were reared to produce virgins (unfertilized queens) of the same age. They were then mated to be queens of the same age from the same queen mother. Only one month old, a new Carniolan queen hybrid replaced their former queens.

The colonies were divided into four experimental groups (2 colonies each) following the methodology of Sebak *et al*. (2025). Two colonies of honeybees for control, 2 honeybee colonies for Egyptian propolis ethanolic extract treatment 3g/L concentrated (0.3 %) equal to 30 ml of ethanolic extract (Seyam *et al*., 2022; Sebak *et al*., 2025), 2 colonies for thymol treatment 0.1 g/L (0.01 %) (Glavinic *et al*., 2022; Sebak *et al*., 2025) and 2 colonies for mix treatment (propolis and thymol together). The different concentrations were added weekly for three months with the treatments to sugar syrup concentration (sugar: water, 1:1) (w/v) in the feed container inside each colony. The colonies were examined weekly and the consumption of all the feed before adding the new nutrition was noted.

Collection of Honeybee Samples:

Three biological replicates, each comprising 10 randomly selected workers were taken from each colony of both control and treatments. The selected workers stored at -20 °C until DNA isolation.

DNA Extraction:

DNA was isolated from ten abdomens of honey bee workers for *N. ceranae* detection using a QIAamp DNA mini kit (Qiagen, Valencia, CA, USA), adhering to the manufacturer's guidelines.

Conventional PCR-Based Detection of *Nosema ceranae* in Honey Bees:

The ribosomal RNA gene of *N. ceranae* was amplified using a specific pair of primers, according to Chen *et al*. (2008). The forward primer: (5′ -CGGATAAAAG AG TCCGTTACC-3′) and the reverse primer: (5′ - TGAGCAGGGTTCTAGGGAT -3′). PCR products of *N. ceranae* were determined using the PCR program in the following manner: for the initial denaturation, only one cycle at 94 °C for three minutes and 35 cycles for 45 seconds at 94 °C for denaturation, 1 minute at 47 °C for annealing, and 1 minute at 72 °C for extension, followed by a final extension of 7 minutes at 72 °C. The predicted size of the *N. ceranae* fragments was 220 bp. The 1.5% agarose gels were stained with ethidium bromide used to visualize PCR products. Following the manufacturer's instructions, the sequencing cycle was carried out using Big Dye Terminator version 3.1 kit (Applied 78 Biosystems, Foster 7 City, CA). The Bioedit program, version 7.0.0, was utilized to collect and analyze the data. Analyses of nucleotide sequence were performed using ExPasy database (http://expasy.org/tools/dna.html). A blast search was performed using the NCBI GenBank database to align the identified sequence with previously published ones (http://blast.ncbi.nlm.nih.gov/Blast.cgi). MEGA7.0 program was used to create the phylogenetic tree, and using the CLC sequence viewer program, the sequence alignment was compared with other genes accessible in the Genbank database.

Quantifying *Nosema ceranae* Infection (Quantitative Real-Time PCR (qRT-PCR)):

The genomic DNA of *Nosema* from ten bees from the studied colonies (untreated and treated colonies) was extracted as described above, used as PCR template and were used to quantify *N. ceranae* infection by the same specific primers of *N. ceranae* (Chen *et al*., 2008), also ß-actin was utilized as a housekeeping gene (Cunha *et al*., 2005). The ß-actin forward primer: (5′ -TGCCAACACTGTCCTTTCTG-3′) and the reverse primer: (5′ -AGAA TTGACCCACCAATCCA-3′). Quantitative real-time PCR has been accomplished in triplicate using Quantifast SYBR Green PCR 2x master mix kit (Qiagen, Germany, cat no. 1044152) using specific primers of *N. ceranae* and incubated in Proflex thermal cycler (Applied Biosystems). The PCR reaction mixture contains 10 µl SYBR master mix, 1 µl of each primer, 2 µl of genomic DNA and 6 µl sterile distilled water. The qRT-PCR was programed to initial denaturing at 95 °C for three minutes; subsequently, a series of 40 cycles of denaturation was conducted at 95 °C for 15 seconds, annealing at 60 °C for ten seconds, followed by extension at 72 °C for ten seconds. Negative controls for the PCR reactions were used. Then PCR amplification and melting curve analysis were performed with

accepted PCR efficiencies between 90% and 100%. The fungal loads were analyzed by $2^{-\Delta\Delta CT}$ method, as a relative quantification strategy for quantitative real-time polymerase chain reaction (qPCR) data analysis.The difference between the threshold cycle (Ct) number for honey bee β-actin and that of *Nosema* of interest was calculated. The control (untreated samples at zero time) means functioned as a calibrator. Each treatment sample as well as untreated sample was replicated thrice.

Preparation of *Apis mellifera* Midgut for Light and Transmission Electron Microscopy:

This study involved midgut of honey bees naturally infected with *N. ceranae*, and midgut of honey bees treated with propolis and thymol. The dissected midgut specimens had been fixed in phosphate-buffered glutaraldehyde (pH 7.4 at 4 °C for 2 hours), washed with 0.1 M phosphate buffer (pH 7.4), and postfixed in 1% osmium tetroxide. Epoxy resin was utilized for embedding the samples, and semithin sections were meticulously prepared utilizing a Leica Ultracut UCT ultramicrotome. Toluidine blue (1x) was used to stain semithin cells, and sections were examined using a Leica ICC50 HD camera. Ultrathin slices (75–90 nm thick) were mounted, put on copper grids, and stained with uranyl acetate and lead acetate. They were examined with a transmission electron microscope, JEOL (JEM-1400 TEM).

Statistical Analysis:

Results of RT- PCR were presented as the mean ± standard error (SE). The statistical significance of honeybee colony samples that were either untreated or treated was assessed using one-way ANOVA. We used Duncan's analysis **(**Duncan, 1955) to determine if the treatments differed significantly. Significance level was set at $P<0.05$.

RESULTS

PCR, Sequencing and Phylogenetic Analysis:

The PCR product amplified fragment of *N. ceranae* of 220bp from the abdomen of an infected untreated colony (Fig. 1).

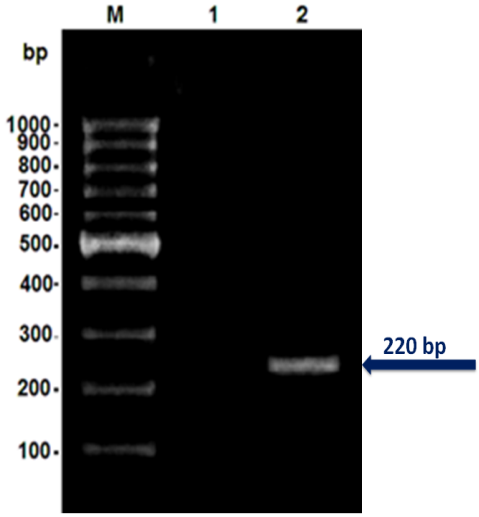

Fig. 1. Ethidiumbromide-stained 1.5% agarose gel-electrophoresis of PCR amplified ribosomal RNA gene using specific *N. ceranae* primers. Lane M: DNA ladder 100bp, lane1: Negative control, and lane2: represent *N. ceranae* from untreated colonies.

The sequence of the amplified fragment of *Vairimorpha (Nosema) ceranae* was assigned in GenBank (https://ncbi.nlm.nih.gov/ genbank/flatfile_changes/) with accession number PP239351.

On comparing the present *N. ceranae* nucleotide sequence of a small subunit ribosomal RNA gene with other ribosomal RNA genes of other *Nosema* species (*N. ceranae* KC295450.1, *N. ceranae* OQ550097.1, *N. ceranae* OQ550100.1, *V. ceranae* MZ576091.1 and *N. ceranae* KC708001.1), some different nucleotides were observed throughout the six sequences **(**Fig. 2**).**

The phylogenetic relationship between current *N. ceranae* and others from the GenBank Database was illustrated by constructing a maximum likelihood tree (Fig. 3). The genetic distance value was found to be low (0.0-1.082) within the sequence of *N. ceranae* and others on GenBank (Fig. 3).

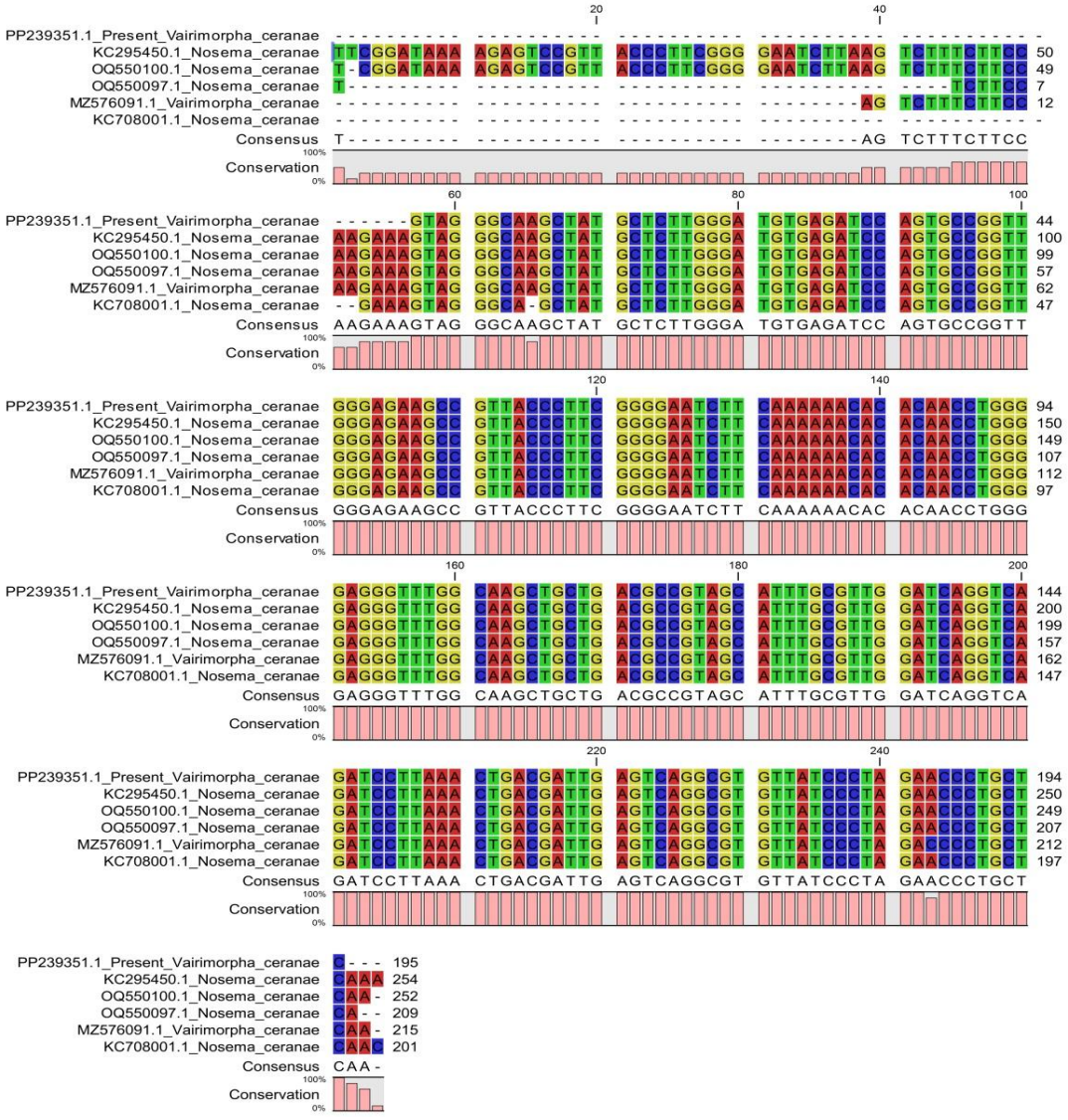

Fig. 2. Multiblesequene alignments for the present *N. ceranae* nucleotide sequence with other *Nosema* species sequences registered in NCBI.

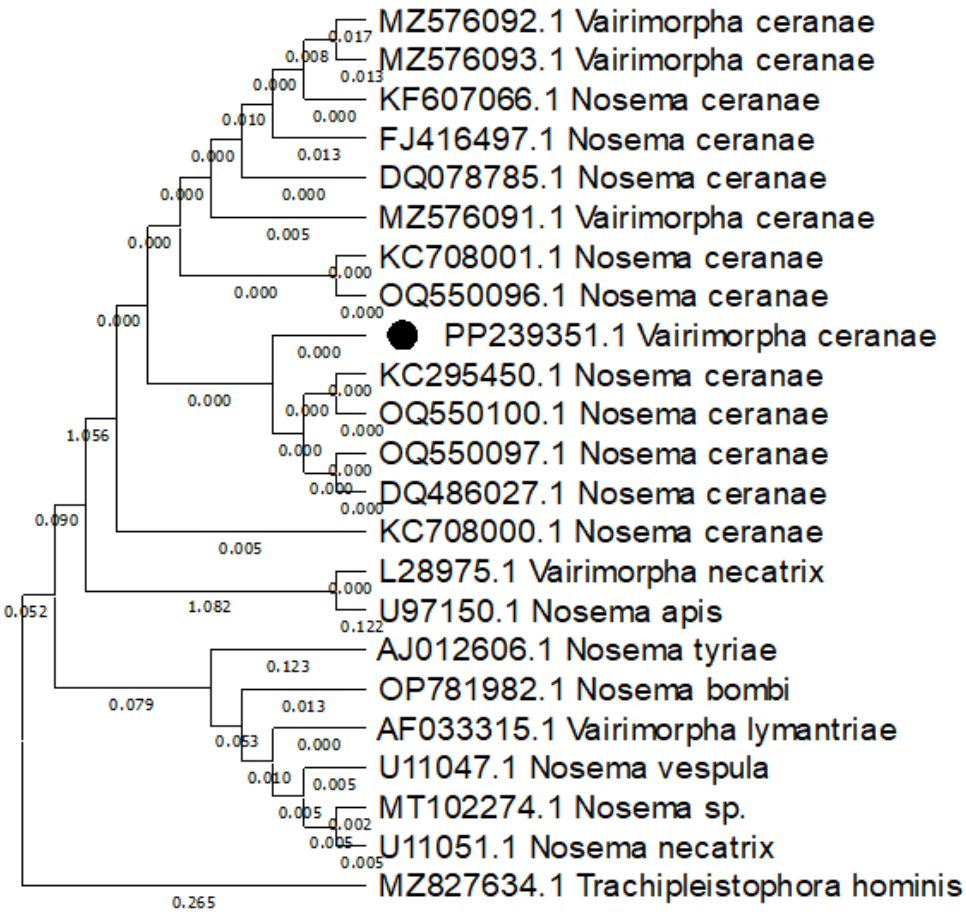

Fig. 3. Phylogenetic tree of the *Vairimorpha (Nosema) ceranae* based on 16S ribosomal RNA gene, partial sequence. The tree has been created with the maximum likelihood tree.

Quantifying *Nosema ceranae* Infection (Quantitative Real-Time PCR (qRT-PCR)):

Samples of *N. ceranae* from infected honeybees were successfully amplified in all samples. One peak in analyzing the melting point curve verified the amplified products' specificity. In general, the gene expression of *N. ceranae* showed a reduction in both treatments (using thymol and propolis extract) of honeybee colonies compared to untreated colonies. By comparing all samples (treated and untreated) using post hoc Duncan's analysis significant downregulation were observed between all samples except between propolis and thymol extract treatments where insignificant differences were observed ($P>0.05$). In comparison between zero time and untreated colonies, significant downregulation ($P<0.05$) was found, as the untreated samples recorded 0.8407±0.34 fold change ($P<0.05$) while in case of ethanolic propolis (3 g/L) and thymol extract (0.1 g/L) treatments where was found that ribosomal RNA gene of *N. ceranae* copies down-regulated significantly in treated than untreated to 0.1023±0.03 and 0.1403±0.05 copy using expression fold (2^-ΔΔCT) for thymol and propolis extracts treatments, respectively ($P<0.05$). Furthermore, in mix treatment of propolis and thymol extracts using the same concentrations, more significant reduction than both treatments separately and untreated samples and downregulated significantly to 0.06633±0.01 copy by expression fold (2^-ΔΔCT) was obtained ($P<0.05$) (Fig. 4).

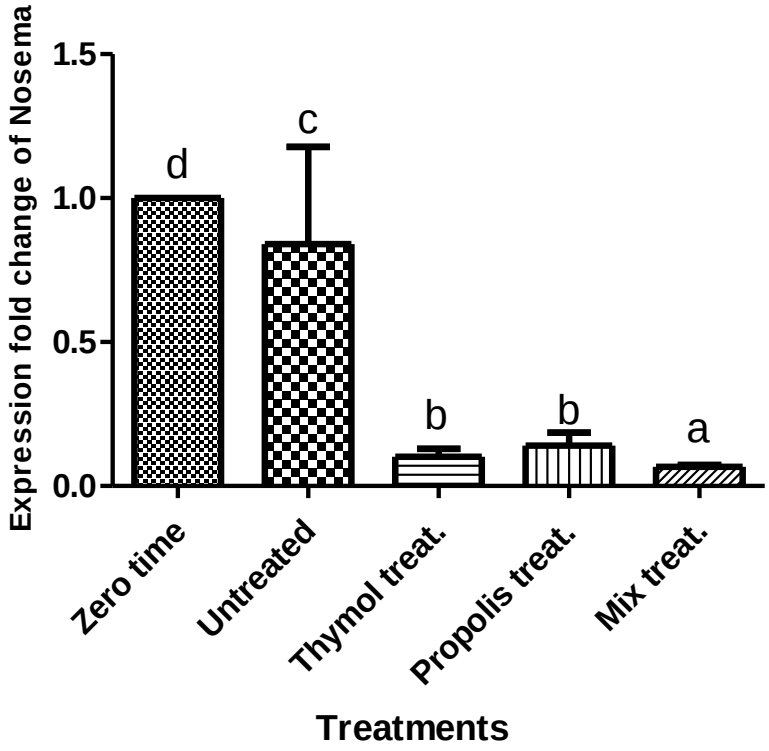

Fig. 4. The impact of propolis and thymol extract treatments on the expression folds of *N. ceranae* copies for infected honey bees with ± SE carried out in triplicate**.** Using Duncan's test, there is a significant difference in the means among columns with different letters ($P<0.05$).

Light and Transmission Electron Microscope:

Midgut epithelial cells have a striated border (Sb) at the apex, a normal nucleus, homogeneous cytoplasm inclusion, and an intact and regular cell boundary (Figs. 5 C& D). The most common kinds of epithelial cells are columnar cells that rest on a basement membrane and regenerative cells, which do not have terminals that reach the lumen (Figs. $5A_1$, $5B_2$, $5C_1$ & $5D_1$).

Figures $5B_2$, $5C_1$ & 5 D_2 show that the midgut epithelium was encircled by a layer of muscles, which include circular muscles (Cmu) and longitudinal muscles (Lmu). Figures 5 D_1, 5 D_2, 7D, 8A, 8D & 9C illustrate proliferating tiny epithelial digestive cells (Pf) as little buds originating from the midgut epithelium. The presence of several small vesicles of zymogen granules within epithelial cells facilitates the secretion of digestive enzymes (Figs. 6 B-D & 8 A-D).

The honey bees that were sampled did not show any obvious signs of anatomical alterations when examined under a microscope. Infected cells were found all over the midgut epithelium in honey bees that were infected naturally, according to the histological study. Parasites at different developmental stages were identified in certain cells at the base of the cytoplasm, while in numerous cells, the apical cytoplasm was significantly enlarged and entirely occupied by spores (Figs. $5A_1$, $5A_2$ & 6 A-D). The spores exhibited an ovoid morphology, demonstrating uniform shape and size with heterogeneous staining characteristics. Mature spores exhibited a surrounding wall and contained a basophilic elongate mass, whereas immature spores appeared optically empty (Figs. 6 A-D). In cells with significant infection, various intracellular parasitic stages, including mature and immature spores, were observed within the same cells (Figs. 5 A_2 & 6 B-D). The infected

epithelial cells exhibited signs of degeneration and lysis only after being filled with spores (Figs. 5A_1, A_2 & 6A-D). As shown in Figures 6 A-D, the nucleus of infected epithelial cells appeared abnormal and small because of degeneration.

After treatment with thymol and propolis (for 3 months), the midgut epithelial cells recovered more from parasite infection and the disappearance of different spores (Figs. 5 B_1, B_2, C_1, C_2, D_1 & D_2). The presence of large vacuoles in the midgut epithelial cells that are treated with a mixture of thymol and propolis as shown in Figures 5 D_1, 5 D_2 & 9 C-F. The empty spores of *N. ceranae* are present in the treated midgut epithelial cells with thymol and propolis, as shown in Figures 7 A-D, 8 A-D & 9 A-F. The treated midgut epithelial cells with thymol and propolis showed proliferating small epithelial digestive cells containing the empty spores of the parasite, as shown in Figures 5 D_1, 5 D_2 7D, 8A, 8D & 9C.

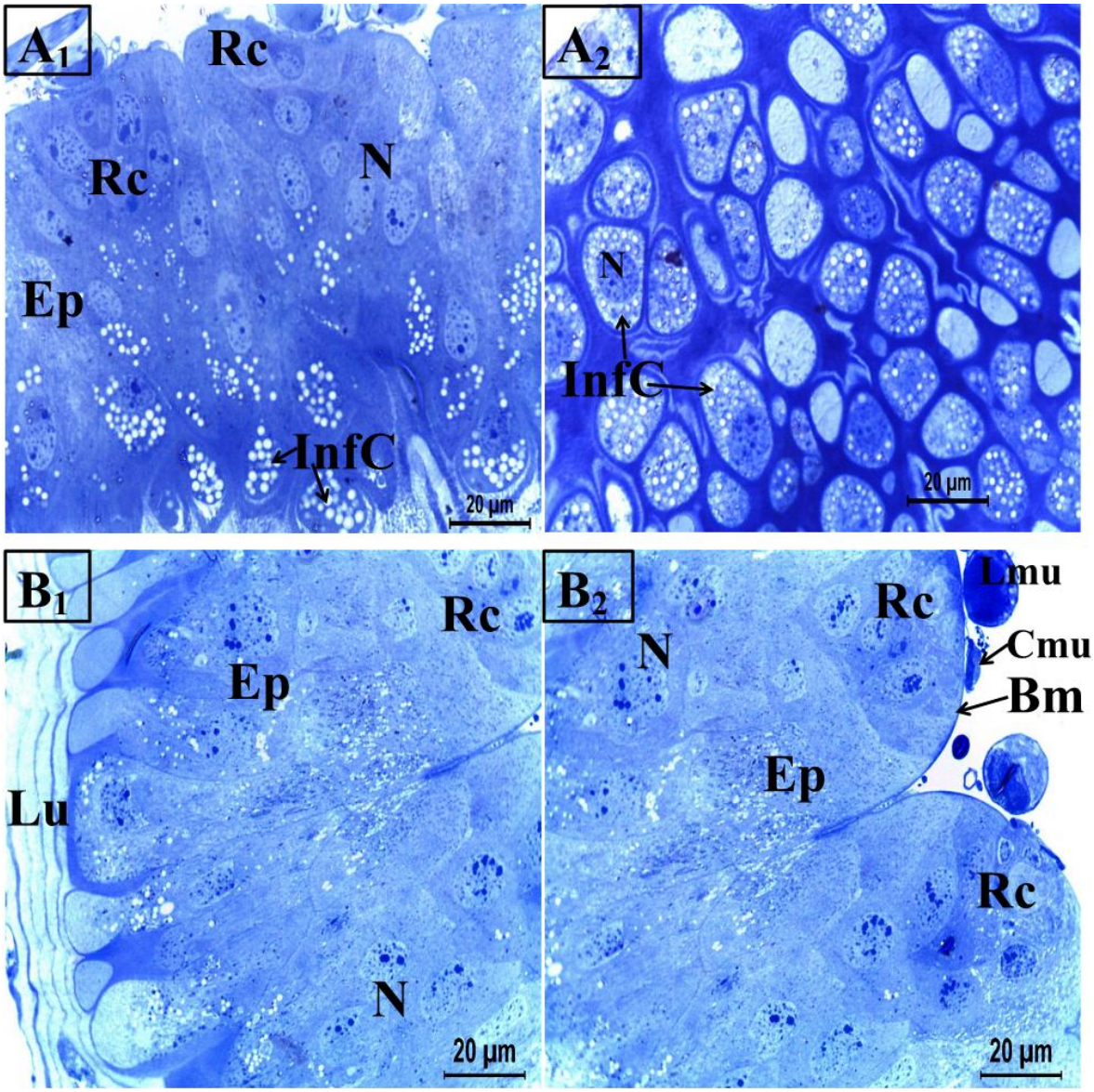

Fig. 5. Semithin micrographs of honey bee midgut. (**A_1, A_2**) Heavily infected midgut epithelial cells with *N. ceranae* in various stages of development and spores. (**B_1, B_2**) Honey bee midgut cells treated with thymol (for 3 months). (BM): Basement membrane, (Cmu): Circular muscles, (Ep): Epithelium cells, (InfC): Infected cells with *N. ceranae*, (Lmu): Longitudinal muscles, (Lu): Lumen, (N): Nucleus, (Rc): Regenerative cells. (Toluidine blue stain).

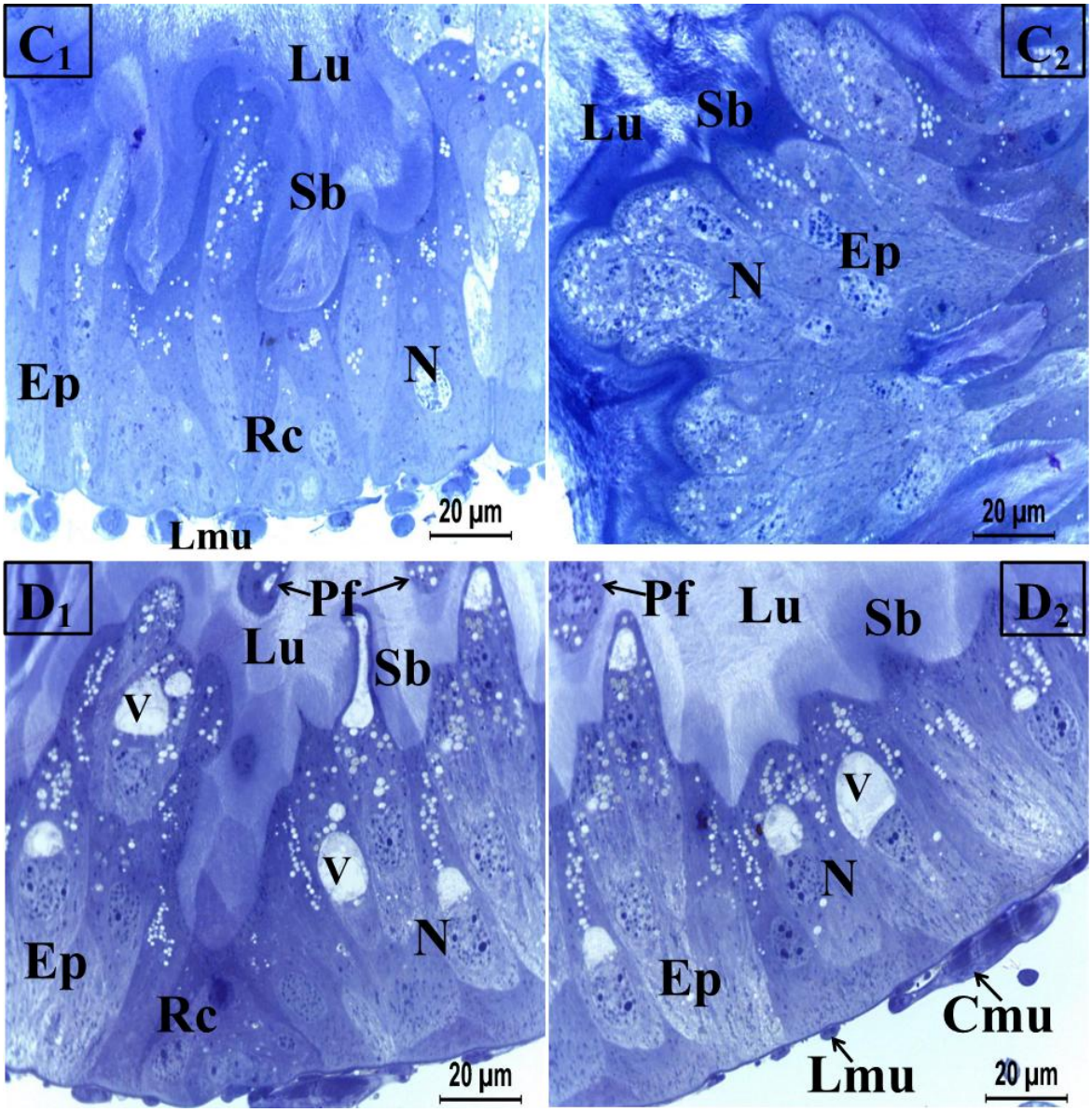

Fig. 5. (Continued). Semithin micrographs of honey bee midgut. (**C1, C2**) Honey bee midgut cells treated with propolis (for 3 months). (**D1, D2**) Honey bee midgut cells treated with a mixture of propolis and thymol (for 3 months). (Cmu): Circular muscles, (Ep): Epithelium cells, (Lmu): Longitudinal muscles, (Lu): Lumen, (N): Nucleus, (Pf): Proliferating small epithelium digestive cells, (Rc): Regenerative cells, (Sb): Striated border, (V): Vacuoles. (Toluidine blue stain).

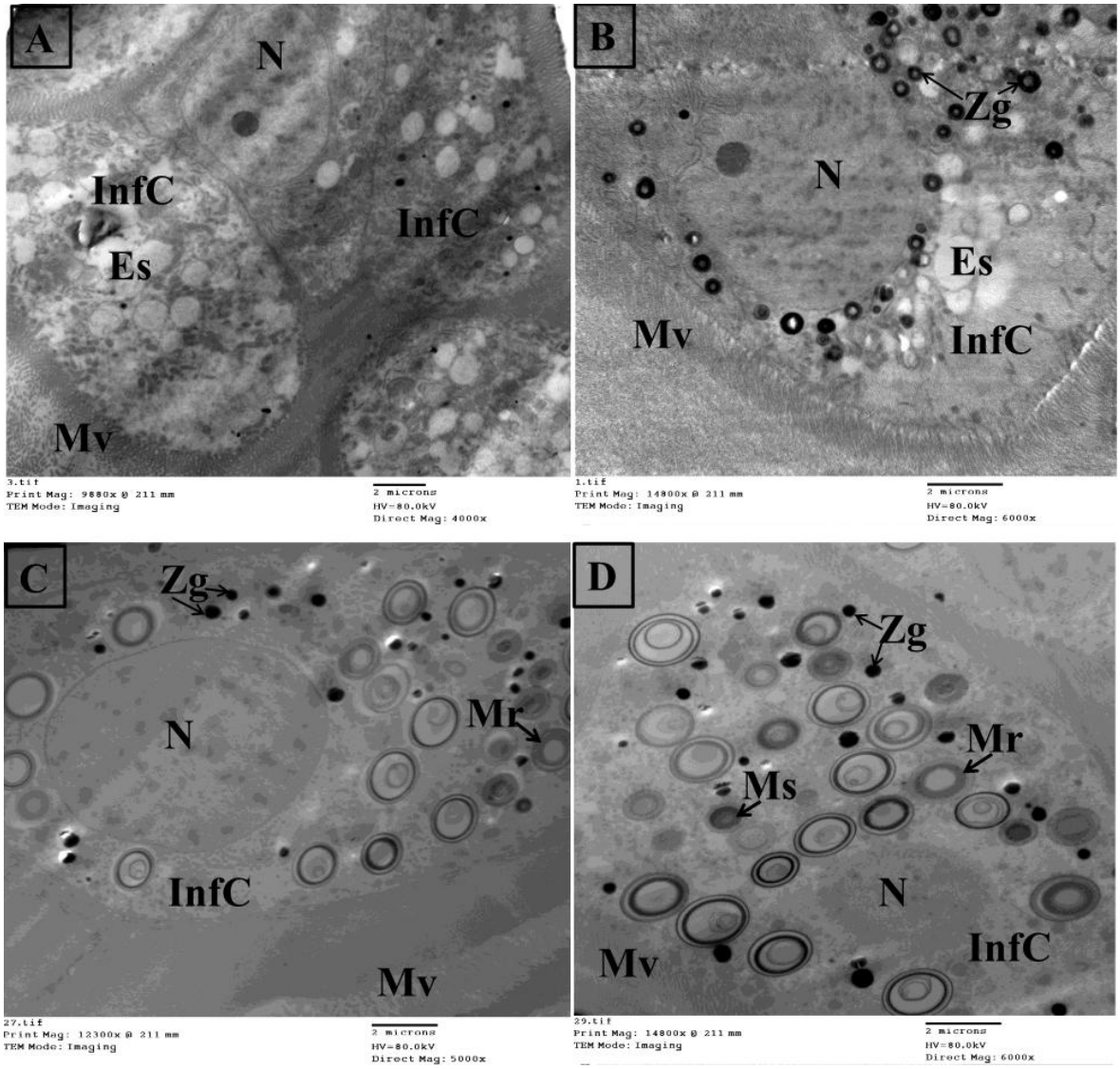

Fig. 6. (A-D) TEM micrographs of naturally infected midgut epithelial cells with *N. ceranae* in various stages of development and spores. (Es): Empty spores, (InfC): Infected cells with *N. ceranae*, (Mr): Meronts, (Ms): Mature spores, (Mv): Microvilli, (N): Nucleus, (Zg): Zymogen granules. (Uranyl acetate and lead citrate acetate stain).

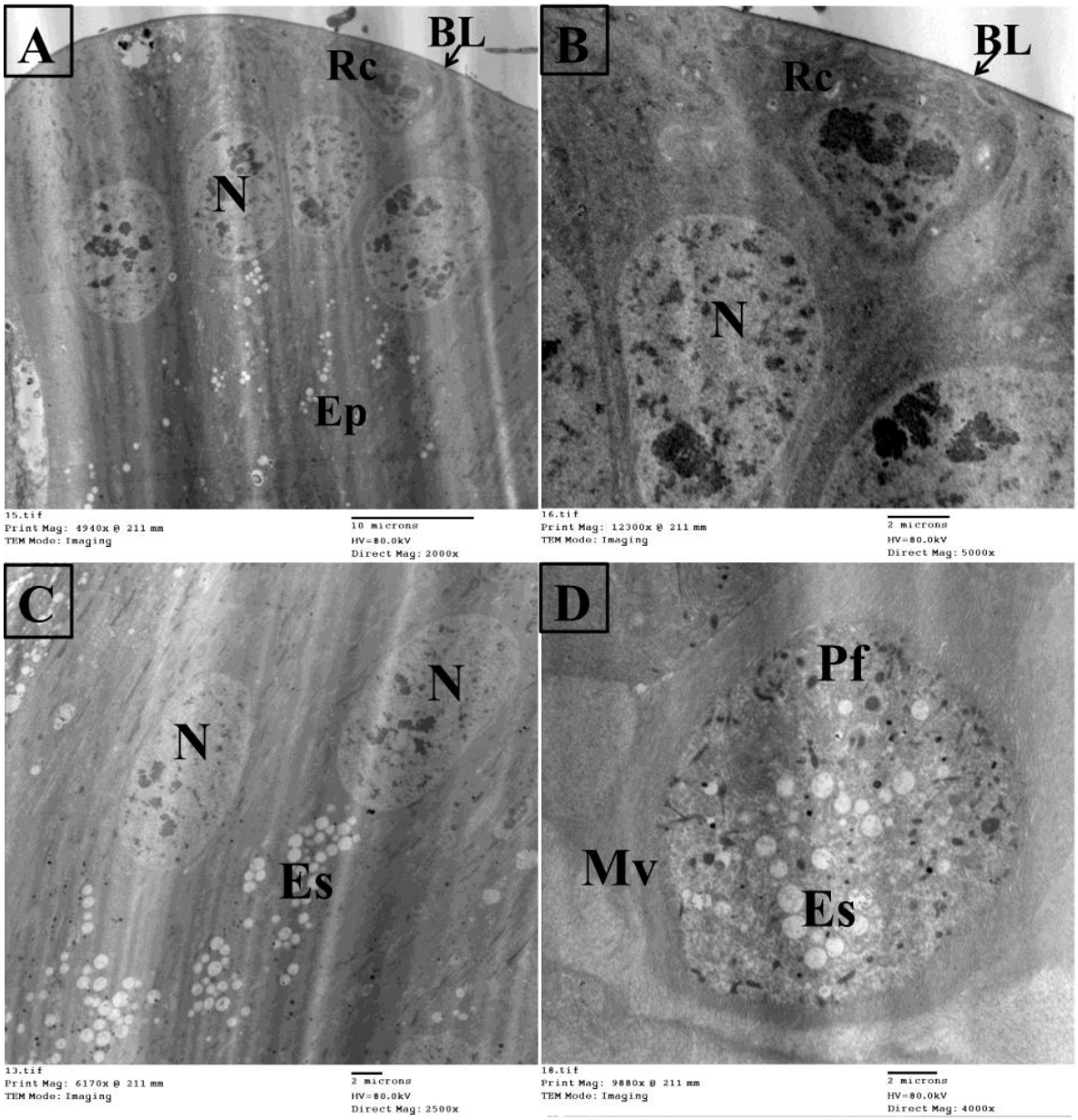

Fig. 7. (A-D) TEM micrographs of honey bee midgut epithelial cells treated with thymol (for 3 months). (BL): Basal lamina, (Ep): Epithelial cells, (Es): Empty spores, (Mv): Microvilli, (N): Nucleus, (Pf): Proliferating small epithelium digestive cells, (Rc): Regenerative cells. (Uranyl acetate and lead citrate acetate stain).

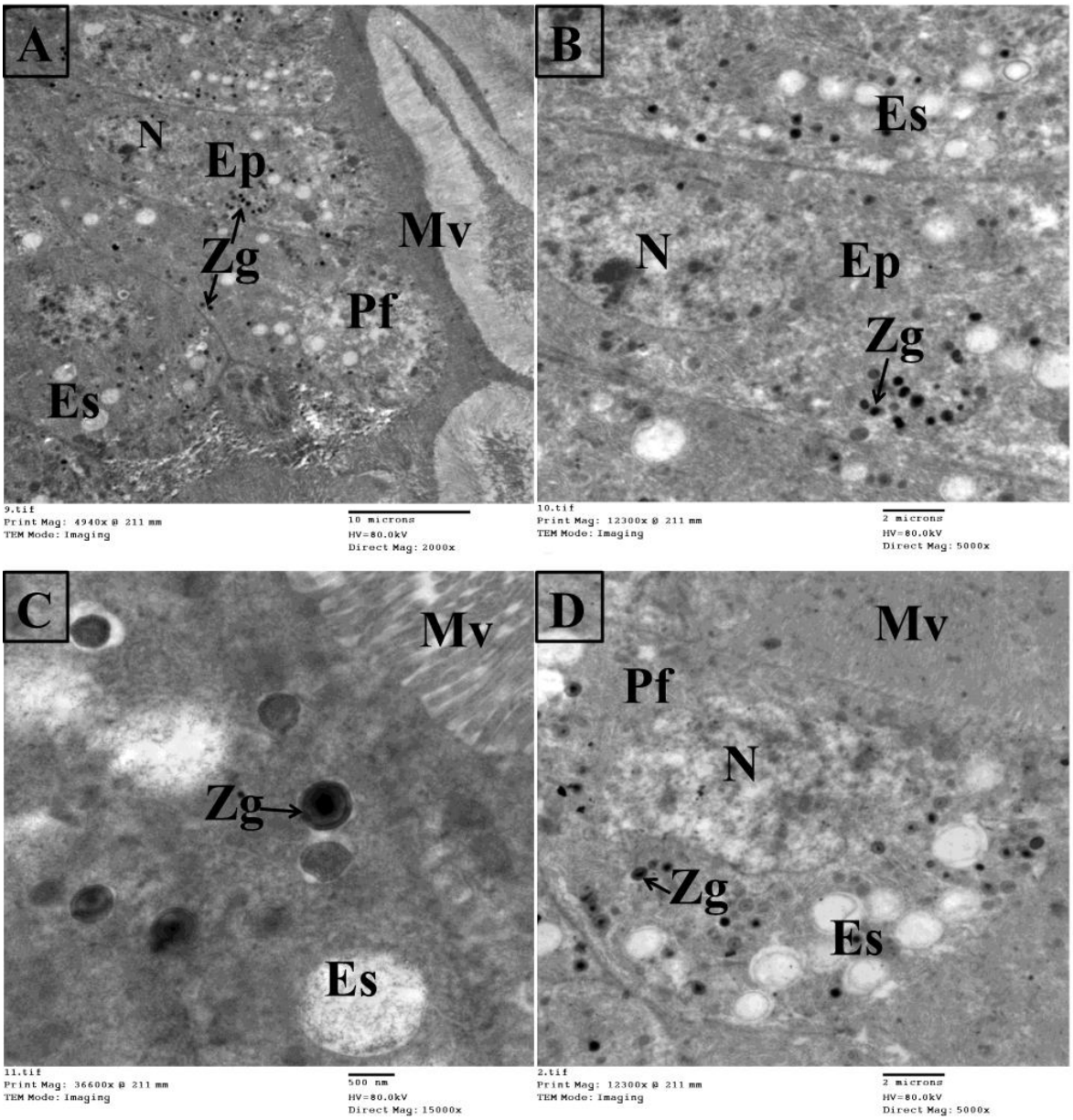

Fig. 8. (A-D) TEM micrographs of honey bee midgut epithelial cells treated with propolis (for 3 months). (Ep): Epithelial cells, (Es): Empty spores, (Mv): Microvilli, (N): Nucleus, (Pf): Proliferating small epithelium digestive cells, (Zg): Zymogen granules. (Uranyl acetate and lead citrate acetate stain).

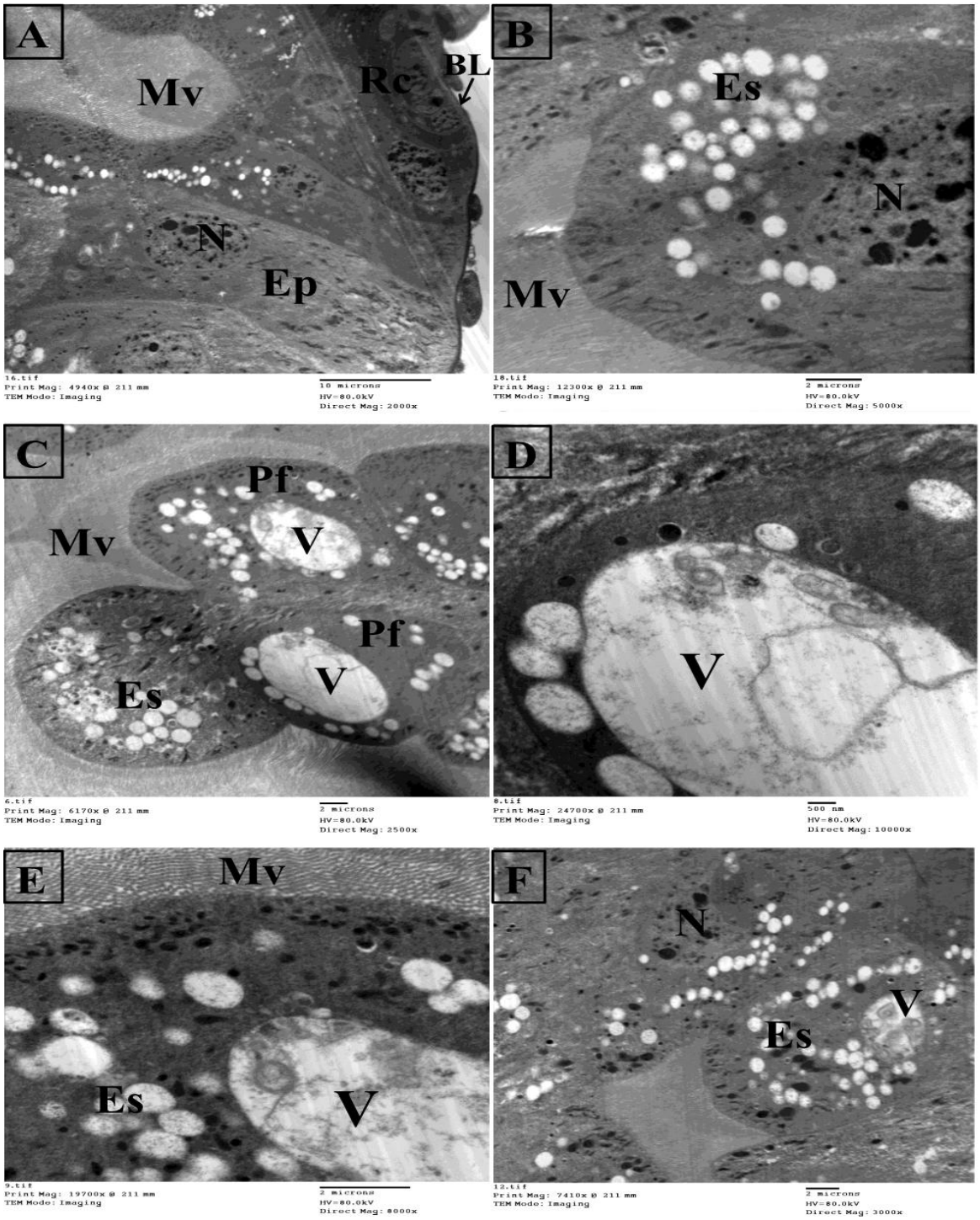

Fig. 9. (A-D) TEM micrographs of honey bee midgut epithelial cells treated with a mixture of propolis and thymol (for 3 months). (BL): Basal lamina, (Ep): Epithelial cells, (Es): Empty spores, (Mv): Microvilli, (N): Nucleus, (Pf): Proliferating small epithelium digestive cells, (Rc): Regenerative cells, (V): Vacuole. (Uranyl acetate and lead citrate acetate stain).

DISCUSSION

Our result revealed the importance of using natural products, thymol and propolis extracts, appeared through the effect of these products against *Nosema* infection, as it reduced it clearly and noticeably by calculating the expression fold of *Nosema* and also appeared in histology in improving the cells and their growth and reducing the number of

Nosema spores in the honey bee gut. Firstly, we identified *N. ceranae* using molecular technique by analyzing the small subunit rRNA gene sequences using maximum likelihood as it extremely sensitive than microscopic investigation. Ansari *et al*. (2017) conducted a study using molecular and microscopic analysis on indigenous honeybee races from eight regions in Saudi Arabia to assess the prevalence of *N. apis* and *N. ceranae* infestations. A phylogenetic study of a portion of the small subunit 16S rRNA of *N. apis* and *N. ceranae* in *Apis mellifera* colonies was conducted by Michalczyk *et al*. (2013). By analyzing the small subunit rRNA gene sequences using maximum parsimony, Chen *et al*. (2009) found that *N. ceranae* seemed to have a closer relationship to the wasp parasite *N. vespula* than with another parasite, *N. apis*, which infects the identical host.

Compared to microscopic enumeration, the sensitivity and stability of detection were enhanced when *N. ceranae* was quantified by UR-qPCR. The microscope method is constrained in its capacity to view as well as enumerate spores; however, the visible spores represent but a small portion of the microsporidia's life cycle that exists outside the host's intestinal cells, with other phases remaining invisible (Gray *et al*., 1969). As a result, minimal infection levels may be challenging to identify through microscopic examination. In molecular quantification, by contrast, the overall quantity of *Nosema* DNA in the specimen was detected and quantified according to PCR effectiveness using a standard curve. Consequently, PCR analysis with specific primers is proven effective for quantitatively determining intracellular parasites (Refardt and Ebert, 2006). This study assessed the impact of thymol and propolis extracts on the infection severity of *Apis mellifera* workers infected with *N. ceranae* by qPCR analysis. Our findings indicated that both thymol and propolis extracts significantly decreased *Nosema* infection. Besides, the mixture of both thymol and propolis extracts significantly decreased *Nosema* infection level in the honey bee colonies than thymol and propolis treatments separately. Comparable experiments were recently performed on *A. florae* bee (Suwannapong *et al*., 2010; Suwannapong *et al*., 2011; El-seedi *et al.,* 2022; Bragança Castagnino *et al.,* 2023) and on *Apis cerana* bee (Yemor *et al*., 2015), utilizing propolis obtained by *Trigona apicalis* bee. Also, Arismendi *et al*. (2018) and Naree *et al.* (2025) found that propolis had similar effects on *Nosema*-infected *A. mellifera* workers. In our bioassays, we observed that propolis significantly reduced the number of *N. ceranae* spores in bee guts, as showed earlier by (Suwannapong *et al*., 2011; Yemor *et al*., 2015; Arismendi *et al*., 2018; Suwannapong *et al*., 2018). This observation is relevant as the chemical composition of resin might vary based on its geographical origin and, subsequently, possess antibiotic properties (Lindenfelser, 1967; Wilson *et al*., 2015). According to other studies, variations in seasons may explain Group 2 (untreated samples) infection reductions (Traver *et al*., 2012). Van den Heever *et al*. (2016) revealed that thymol exhibited an efficacy of 40% in a trial involving *N. ceranae*-infected bees administered sugar syrup containing thymol under controlled laboratory circumstances. It is worth mentioning that after three years of consistent use, thymol is more effective in managing nosemosis (Yücel and Doğaroğlu, 2005; Özüiçli *et al.,* 2024); this duration significantly exceeds the three-month interval utilized in the current investigation. Thymol was reported to decrease *Nosema* sp. spores but to have negative impacts on honey bee survival (Borges *et al*., 2020; Farhadi *et al*., 2023). According to Maistrello *et al*. (2008), thymol is believed to work against *Nosema* by interacting with the spore and blocking its ability to germinate via its interference with the plasma membrane (Rice, 2001; Maistrello *et al*., 2008; El-seedi *et al.,* 2022; Bragança Castagnino *et al.,* 2023). Costa *et al*. (2010) demonstrated that bees treated with thymol had much reduced *Nosema* spore burdens, consistent with our findings. Nevertheless, two investigations have examined the impact of propolis on *N. ceranae* infections in *A. cerana* F. and *A. florea* F. Asian honey bees (Suwannapong *et al*., 2011; Yemor *et al*., 2015). In *A. florea* midgut epithelial cells, stingless bee propolis decreased the

risk of infection capacity of *N. ceranae* by inhibiting spore formation (Suwannapong *et al*., 2011). Propolis extracts, particularly the dichloromethane extract, significantly reduced *Nosema* spore levels four days post-inoculation. These extracts exhibit dose-dependent activity and demonstrate markedly higher potency compared to ethanol and methanol extracts against *N. ceranae* infections (Burnham *et al*., 2020). The good result resulting from the use of mix of thymol and propolis extract may be due to the combination of the antimicrobial properties of the two extracts. This research is considered the first study to be concerned with the use of a combination of more than one extract.

On the other hand, honeybees' digestive systems are vital to their health because that's where xenobiotics and pathogens typically make first contact (Johnson *et al*., 2009; Han *et al*., 2012). Midgut's epithelial cells detoxify the xenobiotics that have been ingested (Higes *et al*., 2013) and serve as a vital interface between the insect and its environment (Forkpah *et al*., 2014). As a result, the midgut is regarded as a critical organ for investigating toxicity because it breaks down food eaten and absorbs the nutrients consumed. Autoinfective spores and many parasitized cells indicate a high level of *Nosema* spp. infection in the gut tissue and demonstrate the parasite multiplies rapidly in *A. mellifera*, inferring that *N. ceranae* has a high parasite pathogenicity potential (Higes *et al*., 2007; Abd El-Samie *et al.*, 2021). Under a light microscope, newly formed *Nosema* spores appeared uniformly oval, highly refractive, and encircled by a dark halo. Based on these features, we think these are spores of the *N. ceranae*, not the *N. apis* (Higes *et al*., 2010b). After treatment with thymol and propolis, the midgut epithelial cells recovered more from parasite infection and the disappearance of different spores. The empty spores of *N. ceranae* are present in the treated midgut epithelial cells with thymol and propolis. The treated midgut epithelial cells with thymol and propolis showed proliferating small epithelial digestive cells containing the empty spores of the parasite. This result was agreed with (Sakla, 2021), who found that thymol did not cause any significant negative effects in the histological analyses of the worker's midgut compared to control group. The peritrophic membrane had a typical, multi-layered structure, closely like the control, and columnar cells demonstrated a slight alteration in orientation. Nonetheless, both the basement membrane and the layers of muscle were undamaged.

Conclusion

Without causing any harmful effects to honey bees, our study found that thymol and propolis extract improved the health of bees affected by *Nosema*. Using a mix of thymol and propolis extract shows better results than using them separately. This research demonstrates that a natural mixture of thymol and propolis extract can inhibit *Nosema*.

Declarations:

Ethical Approval: All experiments in this study were accepted by the Research Ethics Committee of the Faculty of Science, South Valley University, Qena governorate, Egypt. (Code No. 007/02/24).

Authors Contributions: S. I. S., Conceptualization, Methodology, Writing- original draft & editing, Formal analysis.S.A. E.: Methodology, Investigation.H. S., Methodology, Writing- original draft, Formal analysis.H. A.S. E., Methodology, Writing- original draft & editing, Investigation.

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