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Exploring Therapeutic and Prophylactic Activities of Natural Alternatives Against *Nosema* Infection

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Abstract:

Nosemosis is one of the most lethal and prevalent diseases affecting adult honey bees. It is highly encouraged to seek natural and safe alternatives to eliminate chemical and antibiotic residue issues. Bioactive ingredients from *Citrus sinensis* peel oil and Artemisia judaica (70% ethanolic extract) were extracted, and their toxicity on adult bees was assessed to determine suitable applicable concentrations. At 0.5% and 0.25% concentrations, orange peel oil and Artemisia extract exhibited toxicities of 15.3%, 3.2%, 11.5%, and 3.2%, respectively. The palatability of the tested extracts was evaluated by estimating daily food intake, revealing highly significant differences for orange peel oil and Artemisia extract. The efficacy of both extracts as therapeutic and/or prophylactic agents was assessed in vitro. Cumulative mortality rates showed significant differences for both extract trials.

Conclusion: Administration of both extracts in both trials (therapeutic and prophylactic) resulted in reductions in Nosema spore loads after 3,5, and 10 days of infection. GC/MS analysis identified 70 chemical compounds in orange peel oil (isolimonene; 12.16%). Whereas, 46 compounds were identified in the Artemisia extract,(β,7βH,10α-Eudesm-11en-1α-ol; 15.62%).

Keywords Honey bees, *Nosema ceranae*, *Citrus sinensis*, Orange peel oil, *Artemisia judaica*, GC/MS

Introduction

Among pollinators, honey bees (*Apis mellifera* L.) are most valuable, playing a critical role in enhancing the quality and increasing the quantity of crops, as well as contributing to wild flora biodiversity and the overall ecosystem. Their biological importance is well-documented **(Elhoseny *et al.* 2024)**. Additionally, numerous high-value products rely on honey bee colonies as sources, including honey, royal jelly, propolis, pollen, bee bread, venom, and wax **(Yosri *et al.* 2021; El-Seedi *et al.* 2022; El-Didamony *et al.* 2024)**. The annual economic benefit of agricultural pollination by honey bees ranges between 1.6-5.7billion$ **(Southwick and Southwick 1992; Klein *et al.* 2007)**.

A multitude of infectious and parasitic agents have contributed to the decline of honey bee populations and other pollinating insects worldwide **(WHO 2024)**. Various biological pathogens are responsible for bee colony mortality, including *Nosema* **(Marín-García *et al.* 2022)**. Nosemosis is one of the most fatal and widespread diseases affecting adult honey bees, impacting all three colony members: the queen, workers, and drones **(Alaux**

***et al.* 2011).** This fungal disease is caused by obligate intracellular microsporidia that infect the ventricular epithelium cells, *N. ceranae* (synonym *Vairimorpha ceranae*), *N. apis* (synonym *Vairimorpha apis*), and *N. neumanni* **(Higes *et al*. 2020)**. *N. ceranae* is an emerging species that was first reported in 2005 **(Higes *et al*. 2006)** and has since spread throughout Europe **(Hulaj *et al.* 2024)**, as well as in Canada, the USA, and South America **(Williams *et al.* 2008; Castelli 2012)**. *N. ceranae* is considered to be replacing *N. apis* in several regions **(Martín-Hernández *et al.* 2012)**. The Asian honey bee, *Apis cerana*, was the primary host of *N. cerane* which exhibited only mild symptoms. However, the presence of *N. ceranae* in the western honey bee, *A. mellifera*, has been increasingly reported, often with severe symptoms **(Higes *et al.* 2020).**

The infection spreads through spores via an oro-fecal transmission pathway, and trophallaxis feeding which facilitate the circulation of microsporidian spores among bees **(Fries 2010; MacInnis *et al.* 2022; Neidel *et al*. 2017)**. This disease leads to digestive issues, including difficulties in pollen digestion and diarrhea, which can result in fecal staining visible at the hive entrance. Additionally, it shortens the lifespan of bees and impairs their foraging abilities. Furthermore, nosemosis induces a range of physiological problems, such as delayed immune responses, impaired lipid synthesis, and disruptions in pheromone and hormone production **(Kurze *et al*. 2018)**. These factors contribute to significant mortality rates, a decline in the adult bee population, reduced hive productivity, and the potential for colony failure. The severity of the disease is notably exacerbated when bees are exposed to other stressors, such as pesticide treatments **(Pettis *et al*. 2012; Botías *et al*. 2013)**.

Many approaches, *including* effective beekeeping practices **(El-Seedi *et al.* 2022)**, antibiotics, and synthetic drugs such as oxytetracycline, fumagillin, and pyrethroid acaricides, are commonly employed to combat the disease **(Johnson *et al*. 2013)**. However, fumagillin has been known for over 50 years as the most effective therapy; its use is now strictly prohibited in Europe due to concerns about residue **(Huang *et al.* 2013)**. Moreover, there are the negative impacts of antibiotic use on gut bacteria, which diminishes immune function and increases vulnerability to *Nosema* infection **(Li *et al.* 2019)**. Consequently, a growing effort to find natural alternative that offer advantages such as low toxicity, safety for hive products, low resistance, and regional availability has been reported. Organic acids, plant extracts, essential oils, secondary metabolites from microorganisms, and polysaccharides have all shown promise as alternatives against *Nosema* **(Botías *et al.* 2013)**. Also, activity of several isolated compounds from natural sources was also screened, including sulfated polysaccharides derived from algae **(Roussel *et al*. 2015; Balamurugan *et al*. 2022)**.

The current study aims to investigate the presence of *N. ceranae* in apiaries located in the Sharkia Governorate in Egypt. It also evaluated the anti-microsporidian activity of *Citrus sinensis* peel oil and *Artemisia judaica* ethanolic (70%) extract. Finally, GC-MS analysis was used to identify phytochemical constituents of both extracts.

Materials and Methods:

Collecting samples:

Samples of adult worker bees were collected (from the hive entrance and hive floor) during 2022 and 2023 from various apiaries across four localities in the Sharkia Governorate: Meniet El-Kamh, Zagazig, Al Ghar, and Fakous. All samples were delivered in small cages to the Honey Bee Research Department, Plant Protection Research Institute, Agricultural Research Center, for analysis. 20 samples (50 bees per sample) were collected: seven samples from Zagazig, five samples from Minyet El-Qamh, three samples from Faqus, and five samples from El-Ghar.

DNA extraction and duplex PCR amplification:

DNA was extracted from honey bee guts according to **(Phillips and Simon 1995).** In 1.5 mL Eppendorf tubes 600 µL of extraction buffer (1.5 M NaCl, 100 mM Tris-Cl (pH 8.8), and 50 mM EDTA, 5% CTAB) was added to bee gut for DNA extraction. Bee's guts were ground with a plastic homogenizer (Eppendorf micro pestle) and 2 µL of proteinase-K solution (10 mg/mL) was added. Subsequently, tubes were incubated at 56 °C overnight with occasional shaking. After incubation, a mixture of 600 µL phenol, chloroform, and isoamyl alcohol (25: 24: 1) was added, tubes were inverted several times and centrifuged for 5 min at 6000 rpm. The upper aqueous layer was transferred carefully into new tubes containing 900 µL of water and 100 µL of (CTAB 5%, 0.4M NaCl). The mixture was spun at 6000

rpm for 15 min. The supernatants were completely removed and the obtained pellets were mixed with 300 µL of 1.2 M NaCl, shacked gently then 750 µL of absolute ethanol was added. Tubes were inverted several times and left to set at -20°C overnight for DNA precipitation. Subsequently, tubes were centrifuged at 10000 rpm for 20 min. then supernatant was removed and an aliquot of 250 µL of 70% alcohol was added then the tube was centrifuged at 10000 rpm for 5 minutes. The supernatant was completely discarded and 50 µL of Tries- EDTA buffer was added. Finally, the extracted DNA was stored at −20 °C before the execution of the polymerase chain reaction (PCR).

Extracted DNA were subjected to molecular identification by duplex PCR protocol. For the diagnosis of two *Nosema* species (*N. apis*, and *N. ceranae*), two types of primers were used. The primer sequences used to amplify the 321 bp fragment corresponding to the 16S ribosomal gene of *N. apis* were 321APIS-FOR 5′GGGGGCATGTCTTT GACGTACTATGTA-3′ and 321APIS-REV 5′- GGGGGGCGTTTAAAATG TGA AA CAACTATG-3′. The primer sequences utilized to amplify the 218 bp fragment corresponding to the 16S ribosomal gene of *N. ceranae* were 218MITOC-FOR 5′-CGGCGACGATGTGATATGAAAATATTAA-3′ and 218MITOCREV 5′-CCCGG TCATTCTCAAACAAAAAACCG-3′ **(Martín-Hernández *et al.* 2007)**. PCR thermal conditions consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 5 min. PCR products were analyzed on 2% agarose gels. Gels were stained with ethidium bromide and visualized using UV illumination according to **(Hurná *et al.* 2023)**.

Plant extraction Propagation of *Nosema ceranae* culture in laboratory

Under laboratory conditions, a stock of *N. ceranae* (GenBank accession number ON596641.1) was used to artificially infect honey bees. Continuous infection was maintained to propagate *N. ceranae* spores, allowing for the production of suitable spore suspensions to be utilized in the evaluation experiments of the plant extracts.

***Artemisia judaica* ethanolic (70%) extract**

Air-dried leaf powder of *A. judaica* (100 g) was soaked in 70% ethanol and sonicated for 15 min, then left to stand for 24 h at room temperature. The extract was percolated, and the filtrate was concentrated under vacuum using a rotary evaporator. Output was stored in the refrigerator at 4 °C until use.

Orange Peel Oil:

Orange peels from *Citrus sinensis* were collected and frozen until oil extraction. The frozen orange peels were cut into medium-sized pieces, approximately 3 cm by 3 cm. The volatile oil was extracted through hydrodistillation using a Clevenger-type apparatus for 4 h **(Clevenger 1928; Antal *et al.* 2011)**. The extracted oil was stored in a refrigerator until use.

Toxicity Assay of Plant Extracts on Honey Bees:

The toxicity of *C. sinensis* peel oil and a 70% ethanolic extract of *A. judaica* was assessed on adult honey bee workers under laboratory conditions to identify suitable concentrations for screening against *Nosema* infection. Newly emerged workers were collected and mixed to eliminate genetic bias. The bees were then grouped in perforated plastic cages, (fifty bees per group). Different concentrations of both extracts were administered in a50% sugar syrup, and the bees were allowed to feed ad libitum. All experimental groups were incubated at 32±2°C, with a relative humidity of 60±5%. The control group, which was not treated, was fed sugar syrup free of any additives.

Anti-microsporidian activity:

The therapeutic and prophylactic activities of *C. sinensis* peel oil were evaluated at two concentrations (0.25% and 0.125%). An inoculum of *N. ceranae* (21×10^6 spores/mL) was used (applied in a 50% sucrose syrup) **(Pohorecka 2004)**. The experiment comprised seven groups: two therapeutic groups (one for each concentration), two prophylactic groups (one for each concentration), a control group (non-treated and uninfected), an infected group with no treatment, and an infected group treated with Metronidazole. Three replicates per group, each replicate containing fifty newly emerged adult bees housed in cages.

The control group (non-treated and uninfected) was provided with a 50% syrup solution. In the two prophylactic groups, the bees were allowed to feed ad libitum on the tested oil concentration for the first three

days. After this period, both prophylactic groups were infected with 21 × 10^6 spores/mL. Following 24 h postinfection, the two groups were fed a 50% syrup solution until the end of the experiment.

The therapeutic, infected-untreated, and metronidazole-treated groups were initially fed ad libitum with a 50% sugar syrup mixed with 21 × 10^6 spores/mL of *N. ceranae* for 24 h. After that, the corresponding treatments commenced. The therapeutic groups received one of the oil concentrations, the infected-untreated group continued with the 50% syrup, and the metronidazole-treated group was given 50% syrup mixed with 8 µg/mL of metronidazole until the conclusion of the experiment.

Daily food intake and mortality rates were recorded. On the 3rd, 5th, and 10th days of post-infection, three bees were collected from each group to estimate spore counts using a hemocytometer, and the spore count per bee was calculated.

The prophylactic and therapeutic effects of the ethanolic (70%) extract of *A. judaica* were evaluated at two concentrations: 0.5% and 0.25%. The same procedures used for *C. sinensis* peel oil were applied.

GC-MS analysis

GC-MS analysis was estimated in the Regional Center for Food and Feed, Agricultural Research Center, Egypt. Both of the tested plant extracts were subjected to GC–MS analysis to identify their chemical compounds using Agilent Technologies 7890A gas chromatograph coupled to mass spectrometer interfaced with a massselective detector (MSD, Agilent 7000) equipped with a polar Agilent HP-5ms (5%-phenyl methyl poly siloxane as a stationary phase) capillary column (30 m length x 0.25 mm internal diameter and 0.25 µm film thickness). A volume of 1 µL of the sample was injected. Helium was used as the carrier gas with a linear velocity of 1 mL/min. The injector and detector temperatures were set at 200 ºC and 250 ºC, respectively. The mass detector process was as follows: ionization potential of 70 eV, interface temperature of 250 ºC, and acquisition mass range of 50–800 amu. The identification of the components was based on the comparison of their obtained mass spectra and retention time with those of the authentic compounds and by computer matching with NIST and WILEY GC/MS library as well as by comparison of the fragmentation pattern of the mass spectral with those reported in the literature **(Patricia *et al.* 2013)**. The compounds were expressed in percent peak area (%), calculated without any correction factor. Also, the retention time, molecular weight, and molecular formula were recorded.

Statistical analysis

Statistical analyses were performed using **Costat version 6.311 program (2005)**. Data were analyzed using One Way ANOVA applying Post hoc comparisons were made using the Tukey HSD test ($p \leq 0.05$) to compare different treatment groups.

Results:

Samples profiling

Twenty honey bee samples were collected from various apiaries across four localities in the Sharkia Governorate: Meniet El-Kamh, Zagazig, Al Ghar, and Fakous. Microscopic examination revealed the presence of *Nosema* spores in the total 20 samples. DNA was extracted from the 20 samples, and the presence of the *N. ceranae* amplicon (218 bp) was confirmed in all samples by applying duplex PCR. The *N. apis*-specific bands (amplicon size 321 bp) was not detected in any investigated samples. **Fig. (1)** exhibited the PCR amplicon product, which was visualized on a 2% agarose gel.

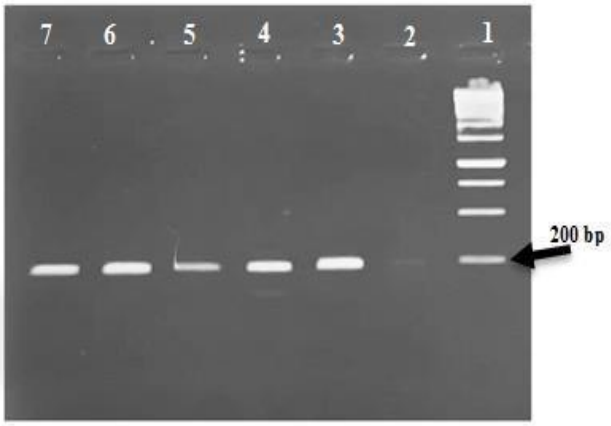

Fig. 1 Agarose gel showing some of the amplified PCR products; the presence of *N. ceranae* (218bp) was confirmed in all samples shown in lanes 2–7, lane 1: Ladder 1k bp.

Toxicity Assay on Adult Honey Bees

To evaluate suitable concentrations of the tested plant extracts that exhibit no or low toxicity on adult honey bees (worker bees), an *in vitro* study was conducted. Table 1 presents the cumulative mortality of honey bees after treatment with different concentrations of *C. sinensis* peel oil. The toxic effects of *C. sinensis* peel oil at concentrations of 1%, 0.75%, 0.5%, and 0.25% were assessed. The concentrations of *C. sinensis* peel oil demonstrated a highly significant difference ($p = 0.0002$) in cumulative mortality compared to the control group (untreated). The recorded cumulative mortalities after three days of treatment were 69.7%, 65%, 15.3%, 3.2%, and 1.01% for the 1%, 0.75%, 0.5%, 0.25% and control (0%), respectively.

Table 1 also reveals the toxicity of three concentrations (1%, 0.5%, and 0.25%) of *A. judaica* on adult honey bees. The results indicated that the toxicity of tested concentrations of *A. judaica* showed a highly significant difference, with a *p*-value of 0.0008. The cumulative mortality recorded after three days posttreatment was 71%, 11.5%, 3.2%, and 3.5% for the concentrations of 1%, 0.5%, 0.25%, and 0% (control), respectively.

Table 1: Toxicity ratio of different concentrations of *C. sinensis* peel oil and *A. judaica* ethanolic extract on adult honey bees.

Treatment / Concentrations	Cumulative mortality %	
	***C. sinensis* peel oil**	***A. judaica* extract**
1%	69.7[a]	71[a]
0.75%	65[a]	-
0.5%	15.3[b]	11.5[b]
0.25%	3.2[b]	3.2[b]
Control (0%)	1.01[b]	3.5[b]
p	0.0002***	0.0008***

Efficiency of *Citrus sinensis* peel oil and *A. judiaca* extracts as therapeutic and prophylactic agents against *N. ceranae* infection:

Efficiency of *C. sinensis* peel oil:

Three parameters were recorded as effects of *C. sinesis* peel oil treatment: cumulative mortality percentage, *Nosema* spore loads, and daily food consumption among the different experimental groups. Table 2 indicates a highly significant difference (p = 0.0004) in cumulative mortality ratios between the experimental groups. The cumulative mortality percentage of the infected-untreated group was the highest among the experimental groups at 23.7%, followed by the metronidazole treatment group, which recorded an 18.9% mortality rate. The therapeutic 0.25% treatment group exhibited the lowest mortality percentage (7.9%) among all treatment groups, in comparison to the control healthy group (uninfected-untreated), which recorded a mortality rate of 1.4%.

Table 3 presents the *Nosema* spore loads concerning different treatments at three sampling points after 3, 5, and 10 days of infection. After 3 days of infection, spore loads exhibited a moderately significant difference (p = 0.0075) among the experimental groups. The infected-untreated group recorded the highest spore load (13.1 × 10^6 spores/ bee), followed by the prophylactic treatments at 0.125% and 0.25%, which recorded spore loads of 9.5 × 10^6 and 8.7 × 10^6 spores/ bee, respectively. The lowest spore loads, 3.2 × 10^6 and 2.7 × 10^6 spores/ bee, were observed in the therapeutic treatment at 0.25% and metronidazole, respectively.

Elevations in *Nosema* spore counts across all experimental groups were confirmed, showing a moderately significant difference (p = 0.0013). Two experimental groups recorded similar spore load ranges: the prophylactic 0.25% group and the infected-untreated group, both exhibiting spore loads of 34.7 × 10^6 and 34 × 10^6 spores/ bee, respectively. The spore load for the prophylactic 0.125% group was recorded at 28.4 × 10^6 spores/ bee. In contrast, the remaining experimental groups displayed similar spore counts of 13.9 × 10^6, 10.3 × 10^6, and 9.8 × 10^6 spores/ bee for the therapeutic 0.125%, metronidazole, and therapeutic 0.25% groups, respectively.

After 10 days of *Nosema* post infection, the spore count showed a moderately significant difference. The infected-untreated group had the highest spore load, measuring 97.6 × 10^6 spores/ bee, followed by the two prophylactic groups at 0.25% and 0.125%, which recorded spore loads of 70 × 10^6 and 66.5 × 10^6 spores/ bee, respectively. In contrast, the 0.125% and 0.25% (therapeutic) groups, along with metronidazole, exhibited similar ranges of spore loads, recording 45.3 × 10^6, 33.9 × 10^6, and 28.8 × 10^6 spores/ bee, respectively.

Data in table 4, revealed that a highly significant difference (p = 0.0000) in average daily food intake among the seven tested groups. The average daily food consumptions were recorded as 11.2, 10.8, 10.4, 10.3, 10.2, 9.5, and 8.2 µL per bee for the prophylactic 0.25%, therapeutic 0.125%, prophylactic 0.125%, therapeutic 0.25%, metronidazole-treated, infected-untreated, and control (uninfected-untreated) groups, respectively.

Efficiency of *Artemisia Judaica e*xtract

Three parameters were recorded as effects of *A. Judaica* administration; cumulative mortality percentage, spore loads, and daily food intake between experimental groups. Table 2 declared a moderately significant difference in the cumulative mortality percentages between the experimental groups, the probability was recorded as p = 0.0041. Cumulative mortality percentage of the infected-untreated group was the highest among other experimental groups (19.97%). The two *A. Judaica* therapeutic groups had the same range of cumulative mortality percentages, recording 12.5% and 12.04% for 0.25% and 0.05%, respectively. Two *A. Judaica* prophylactic groups recorded lower cumulative mortality percentages than the infected-untreated group, recording 9.6% and 10.4% for 0.25 and 0.5%, respectively. The control (uninfected-untreated) group had the lowest recorded a 9.02% cumulative mortality percentage.

Table 3 declared *Nosema* spore loads concerning different treatments at three point samples after 3, 5, and 10 days of infection. Minor significant difference was indicated in spore loads (p = 0.0376) between the experimental groups after 3 days of post infection. Infected-untreated group recorded the highest spore load 16.3 ×10^6 spore/ bee. While, the metronidazole-treated group which recorded the lowest spore loads (3.2×10^6 spore/ bee), the rest of the experimental groups exhibited similar range of spore loads. The therapeutic 0.5%, 0.25%, prophylactic groups 0.5%, and 0.25% recorded spore loads 12.9×10^6, 8.4×10^6, 7.7 ×10^6 and 7.1 ×10^6 spore/ bee, respectively.

After five days of post infection, a moderately significant difference was indicated in *Nosema* spore counts between the experimental groups with probability p = 0.0087. the highest spore load was exhibited for group infected-untreated (38.1 $\times 10^6$ spore/ bee) followed by spore loads; 19 $\times 10^6$, 17.9$\times 10^6$, and 16.8 $\times 10^6$ spore/ bee for prophylactic 0.5%, and therapeutic 0.5%, and 0.25%, respectively. Prophylactic 0.25% spore load was 11.9$\times 10^6$ spore/ bee and the lowest spore load 2.8$\times 10^6$ for metronidazole-treated group.

After 10 days of *Nosema* post infection, the spore count exhibited a highly significant difference (p = 0.0001). The infected-untreated group exhibited the highest spore load 93.5$\times 10^6$ spore/ bee followed by the prophylactic group 0.25% (54.9$\times 10^6$ spore/ bee). Spore loads of 23.7$\times 10^6$, and 21.8$\times 10^6$ spore/ bee recorded for the two therapeutic groups; 0.25% and 0.5%, respectively. Group therapeutic 0.5% exhibited (16 $\times 10^6$ spore/ bee) similar level of spore loads to the metronidazole-treated (the lowest spore load; 14.5 $\times 10^6$ spore/ bee).

The average daily food intake (consumption) was reported across the experimental time. **Table 4** showed that a highly significant difference (p = 0.0000) was estimated in average daily food intake for the honey bees between the seven tested groups. The average daily food consumptions were recorded as 14.2, 12.04, 11.7, 11.5, 11.1, 8.7, and 8.6 µL/ bee for metronidazole-treated, control (uninfected-untreated), therapeutic 0.25%, therapeutic 0.5%, infected-untreated, prophylactic 0.25%, and prophylactic 0.5%, respectively.

Table 2 Cumulative mortality percentages of different treatments of *C. sinensis* peel oil and *A. judaica* ethanolic extract

Trial	Treatment	Cumulative mortality %	
		Orange peel oil	***Artemisia* ext.**
Therapeutic	0.5%	--	$12.04^{ab} \pm 6.7$
	0.25%	$7.9^{bc} \pm 5.9$	$12.5^{ab} \pm 4.1$
	0.125%	$9.3^{abc} \pm 5.9$	--
	Metronidazole	$18.9^{ab} \pm 9.8$	$9.8^{b} \pm 5.1$
Control	Infected-untreated	$23.7^{a} \pm 15.2$	$19.97^{a} \pm 5.6$
	Uninfected-untreated	$1.4^{c} \pm 1.7$	$9.02^{b} \pm 6.2$
Prophylactic	0.5%	--	$10.4^{b} \pm 3.5$
	0.25%	$11.3^{abc} \pm 5.7$	$9.6^{b} \pm 3.1$
	0.125%	$13.4^{abc} \pm 7.9$	--
p	-	0.0004***	0.0041**

Data represented as means ± SE; the same letter in the same column means non-significant at $P \leq 0.05$. (***) highly significance, (**) moderately significance

Table 3 Mean *Nosema* spore counts at different time points after treatment administration

Trial \ Treatment		Mean *Nosema* spore × 10^6 / bee					
		Orange peel oil			*Artemisia* extract		
		3 days	5 days	10 days	3 days	5 days	10 days
Therapeutic	0.5%	--	--	--	$12.9^{ab} \pm 0.7$	$17.9^{ab} \pm 3.1$	$21.8^{bc} \pm 4.8$
	0.25%	$3.2^{b} \pm 0.2$	$9.8^{b} \pm 2.4$	$33.9^{b} \pm 10.1$	$8.4^{ab} \pm 3.2$	$16.8^{ab} \pm 1.6$	$23.7^{bc} \pm 0.1$
	0.125%	$5^{ab} \pm 2.02$	$13.9^{b} \pm 0.9$	$45.3^{b} \pm 6.5$	--	--	--
	Metronidazole	$2.7^{b} \pm 0.7$	$10.3^{b} \pm 0.9$	$28.8^{b} \pm 3.03$	$3.2^{b} \pm 0.4$	$2.8^{b} \pm 0.7$	$14.5^{c} \pm 3.3$
Control	Infected-untreated	$13.1^{a} \pm 0.6$	$34^{a} \pm 6.7$	$97.6^{a} \pm 12.7$	$16.3^{a} \pm 2.5$	$38.1^{a} \pm 3.6$	$93.5^{a} \pm 16.2$
	Uninfected-untreated	--	--	--	--	--	--
Prophylactic	0.5%	--	--	--	$7.7^{ab} \pm 3.6$	$19^{ab} \pm 11.1$	$16^{c} \pm 3.2$
	0.25%	$8.7^{ab} \pm 2.1$	$34.7^{a} \pm 6.1$	$70^{ab} \pm 10.8$	$7.1^{ab} \pm 2.7$	$11.9^{b} \pm 2.5$	$54.9^{ab} \pm 9.7$
	0.125%	$9.5^{ab} \pm 3.01$	$28.4^{ab} \pm 3.3$	$66.5^{ab} \pm 7.2$	--	--	--
P		0.0075**	0.0013**	0.0013**	0.0376*	0.0087**	0.0001***

Data represented as means ± SE; the same letter in the same column means non-significant at $P \leq 0.05$. (***) highly significance, (*) low significance variance

Table 4 Average daily food consumption of different treatments and trials

Trial	Treatment	Average daily food intake µL/ bee	
		Orange peel oil	*Artemisia* ext.
Therapeutic	0.5%	--	$11.5^{b} \pm 1.1$
	0.25%	$10.3^{ab} \pm 0.9$	$11.7^{b} \pm 1.5$
	0.125%	$10.8^{ab} \pm 0.6$	--
	Metronidazole	$10.2^{ab} \pm 0.8$	$14.2^{a} \pm 1.4$
Control	Infected-untreated	$9.5^{bc} \pm 0.7$	$11.1^{b} \pm 1.2$
	Uninfected-untreated	$8.2^{c} \pm 0.07$	$12.04^{ab} \pm 1.3$
Prophylactic	0.5%	--	$8.6^{c} \pm 0.9$
	0.25%	$11.2^{a} \pm 1.008$	$8.7^{c} \pm 1.4$
	0.125%	$10.4^{ab} \pm 0.9$	--
p	-	0.0000***	0.0000***

Data represented as means ± SE; the same letter in the same column means non-significant at $P \leq 0.05$. (***) highly significance

Efficiency of *C. sinensis* peel oil and *A. judaica* ethanolic extract on reduction *N. ceranae* spore loads

Fig. 2 explored the reduction percentages of *Nosema* spores after administration of different treatments after 3 days of *Nosema*. All infection of the treatments caused reductions with different percentages. The highest reduction was recorded after administration of orange peel oil with therapeutic concentration 0.25% with a reduction percentage than the infected-untreated group followed by the therapeutic orange peel oil at concentration 0.125% which recorded a reduction percentage 61.8%. *Artemisia* prophylactic treatments (0.25, and 0.5%) recorded reduction percentages 56.4%, and 52.8%, respectively.

After 5 days of post infection, also all the treatments caused reductions in *Nosema* spore percentage except for the orange peel oil prophylactic at concentration 0.25% cause slight increase (2.1%). Also, the therapeutic orange peel oil at concentration caused the most spore reduction (71.2%) than the infected-untreated spore count followed by Artemisia prophylactic concentration 0.25% which caused a reduction percentage 68.8%.

Regarding spore count after 10 days of infection, *Artemisia* extract 0.5% (prophylactic) recorded the highest spore reduction percentage; 82.9% followed by *Artemisia* extract 0.5, and 0.25% (therapeutic groups) caused 76.7, and 74.7% spore reductions, respectively. Orange peel oil at 0.25%, 0.125% (therapeutic groups), 0.125%, and 0.25% (prophylactic groups) recorded reduction percentages 65.3%, 53.6%, 31.9%, and 28.3%, respectively.

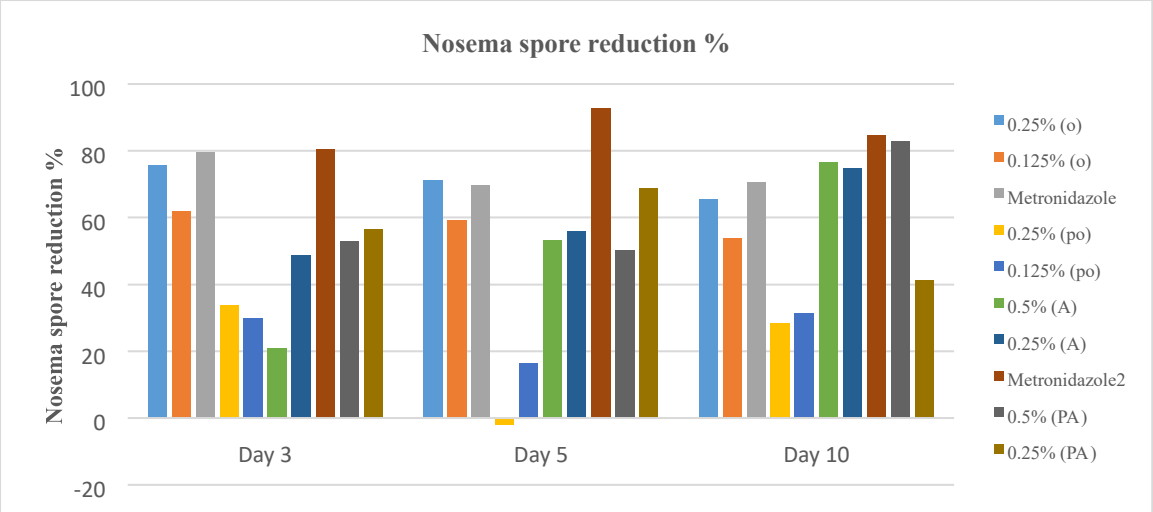

Fig. 2 Reduction percentages of *Nosema* spore loads after administration of different treatments and trials.

GC-MS profiling of *Citrus sinensis* peel oil and *Artemisia judiaca* ethanolic extract chemical constituents:

Fig. 3a exhibited the chromatogram of *C. sinensis* peel oil. The GC-MS analysis of *C. sinensis* peel oil allowed the detection of 70 chemical constituents **(Table 5)**, and out of these, 11 compounds have an average concentration (area %) of more than 2%. Isolimonene was the major compounds (12.16%) followed by 3Thujen-2-one (10.64%). Also, α-Pinene (4.82%), Isoborneol (3.74%), Citronellyl tiglate (3.52%), Quercetin 3glucoside (3.33%), Scutellarein tetramethyl ether (3.32%), β-Myrcene (2.94%), Cyperene (2.94%), βBisabolene (2.05%), and Camphor (2.02%) were revealed with area more than 2%.

Table 6 listed the forty six compounds identified in *A. judica* ethanolic extract with different percentage, **Fig. 3b** showed the chromatogram. Nine of the 46 identified compounds were exhibited area percentage more than 2%. GC-MS analysis revealed *A. judiaca* major compounds as 5β,7βH,10α-Eudesm-11-en-1α-ol (15.62%), Scutellarein tetramethyl ether (13.59%), Thiazolidine,2-methyl- (13.44%), Luteolin 6-C-glucoside (6.27%), Afromosin 7-O-glucoside (4.1%), Quercetin-3,7,3',4'-tetramethyl ether (4.06%), 2',4'-Dimethoxy-3-hydroxy-

6methylflavone (3.71%), Gossypetin 3,3',8-trimethylether (3.49%), and 5,3'-Dihydroxy-6,7,4'-trimethoxyflavone (2.26%).

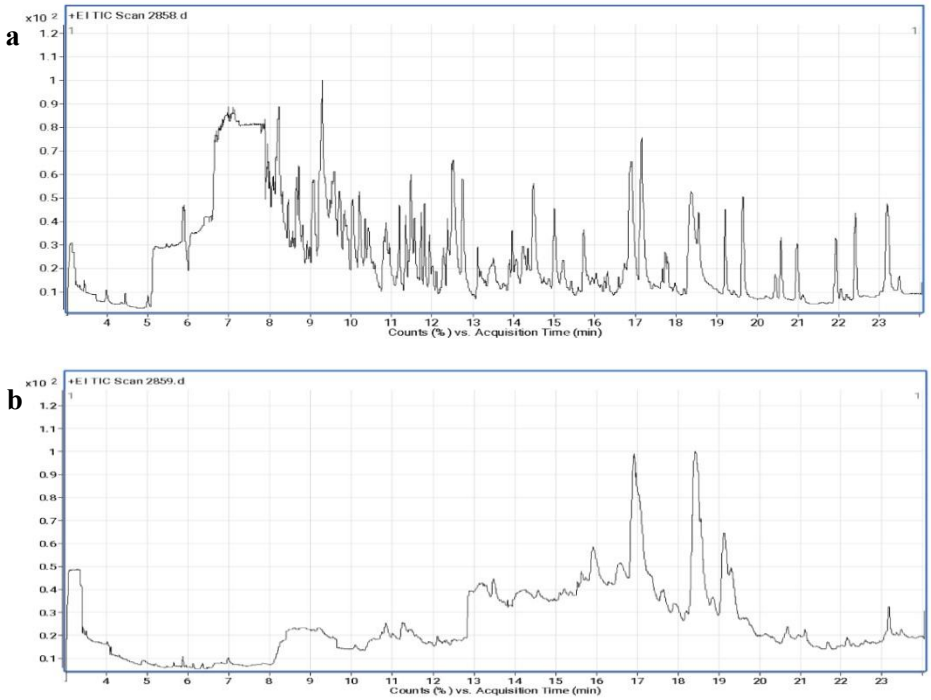

Fig. 3 Chromatograms of **a)** *C. sinensis* peels oil; b) *A. judaica* ethanolic (70%) extract.

Table 5 Bioactive compounds identified in *C. sinensis* peels oil

No	RT (min)	Compound name	MW g/mol	MF	Nature	Area %
1	3.977	7-Hydroxycoumarin-3-carboxylic acid	206.15	$C_{10}H_6O_5$	Carboxylic acid derivative	0.22
2	5.011	3-Pinanone,cis	152.23	$C_{10}H_{16}O$	Monoterpene	0.32
3	5.404	α-Pinene	136.23	$C_{10}H_{16}$	Monoterpene	4.82
4	5.88	3-Carene	136.23	$C_{10}H_{16}$	Monoterpene	1.3
5	6.13	β-Myrcene	136.23	$C_{10}H_{16}$	Monoterpene	2.94
6	6.938	Isolimonene	136.23	$C_{10}H_{16}$	Monoterpene	12.16
7	7.664	3-Thujen-2-one	150.22	$C_{10}H_{14}O$	Monoterpene	10.64
8	7.959	D-Limonene	136.23	$C_{10}H_{16}$	Monoterpene	0.5
9	8.013	Dihydrocarveol	154.25	$C_{10}H_{18}O$	Monoterpene	0.19
10	8.087	Terpinolene	136.23	$C_{10}H_{16}$	Monoterpene	0.36
11	8.23	β-Terpineol	154.25	$C_{10}H_{18}O$	Monoterpene alcohol	1.34
12	8.312	Linalool	154.25	$C_{10}H_{18}O$	Monoterpene alcohol	0.59
13	8.456	Sabinene hydrate,cis	154.25	$C_{10}H_{18}O$	Monoterpene alcohol	0.33
14	8.562	β-Terpinyl acetate	196.29	$C_{12}H_{20}O$	Monoterpene ester	0.3
15	8.661	Isopulegol	154.25	$C_{10}H_{18}O$	Monoterpene	0.5
16	8.726	4-Thujanol	154.25	$C_{10}H_{18}O$	Monoterpene alcohol	0.77
17	8.817	Citronellal	154.25	$C_{10}H_{18}O$	Monoterpene aldehyde	0.37
18	8.923	α-Terpeneol	154.25	$C_{10}H_{18}O$	Monoterpene alcohol	0.26
19	8.997	Cis-Verbenol	152.23	$C_{10}H_{16}O$	Monoterpene alcohol	0.15
20	9.075	Terpinen-4-ol	154.25	$C_{10}H_{18}O$	Monoterpene alcohol	1.49
21	9.301	Isoborneol	154.25	$C_{10}H_{18}O$	Monoterpene alcohol	3.74
22	9.582	β-Citral	152.23	$C_{10}H_{16}O$	Monoterpene aldehyde	1.54
23	9.735	Neral	152.23	$C_{10}H_{16}O$	Monoterpene aldehyde	0.85

No	RT (min)	Compound name	MW g/mol	MF	Nature	Area %
24	9.858	Cis-Geraniol	154.25	$C_{10}H_{18}O$	Monoterpene alcohol	1.24
25	10.047	Myrtanal	152.23	$C_{10}H_{16}O$	Monoterpene aldehyde	1.14
26	10.219	Ledane	206.37	$C_{15}H_{26}$	Sesquiterpene	0.98
27	10.346	Phytol	296.5	$C_{20}H_{40}O$	Diterpene alcohol	0.33
28	10.437	Perillol	152.23	$C_{10}H_{16}O$	Monoterpene alcohol	0.95
29	10.87	Shyobunone	220.35	$C_{15}H_{24}O$	Sesquiterpenoid	1.48
30	10.957	Nerolidol	222.37	$C_{15}H_{26}O$	Sesquiterpene alcohol	0.88
31	11.195	Copaene	204.35	$C_{15}H_{24}$	Sesquiterpene	0.77
32	11.351	Caryophyllene	204.35	$C_{15}H_{24}$	Sesquiterpene	0.76
33	11.482	Isomentol	156.26	$C_{10}H_{20}O$	Monoterpene	1.16
34	11.577	α-Longipinene	204.35	$C_{15}H_{24}$	Sesquiterpene	0.85
35	11.732	α-Himachalene	204.35	$C_{15}H_{24}$	Sesquiterpene	0.52
36	11.806	γ-Muurolene	204.35	$C_{15}H_{24}$	Sesquiterpene	0.83
37	11.937	β-Farnesene	204.35	$C_{15}H_{24}$	Sesquiterpene	0.98
38	12.102	Humulene	204.35	$C_{15}H_{24}$	Sesquiterpene	0.23
39	12.294	Luteolin 6-C-glucoside	610.5	$C_{27}H_{30}O_{16}$	Flavonoid glycoside	0.58
40	12.389	β-Patchoulene	204.35	$C_{15}H_{24}$	Sesquiterpene	0.63
41	12.524	Cyperene	204.35	$C_{15}H_{24}$	Sesquiterpene	2.94
42	12.749	Spinosine	327.4	$C_{19}H_{21}O_4$	Alkaloid	1.97
43	13.135	Elemol	222.37	$C_{15}H_{26}O$	Sesquiterpene	0.96
44	13.492	Hexa-hydro-farnesol	228.41	$C_{15}H_{32}O$	Alcohol	1.18
45	13.783	Germacren D	204.35	$C_{15}H_{24}$	Sesquiterpene	0.28
46	13.89	Epiglobulol	222.37	$C_{15}H_{26}O$	Sesquiterpene alcohol	0.26
47	13.988	Juniper camphor	222.37	$C_{15}H_{26}O$	Sesquiterpenoid	1.34
48	14.238	α-Eudesmol	222.37	$C_{15}H_{26}O$	Sesquiterpenoid alcohol	0.95
49	14.48	β-Bisabolene	204.35	$C_{15}H_{24}$	Sesquiterpenoid	2.05
50	14.825	Thunbergol	290.5	$C_{20}H_{34}O$	Diterpene alcohol	0.4
51	15.022	α-Bergamotene	204.35	$C_{15}H_{24}$	Sesquiterpene	1.54
52	15.391	Corymbolone	236.35	$C_{15}H_{24}O_2$	Sesquiterpenoid	0.81
53	15.739	β-Copaene	204.35	$C_{15}H_{24}$	Sesquiterpene	1.21
54	16.014	Geranyl isovalerate	238.37	$C_{15}H_{26}O_2$	Monoterpene ester	0.64
55	16.313	Rhamnazin	330.29	$C_{17}H_{14}O_7$	Flavonoid Benzopyrans	0.61
56	16.912	Scutellarein tetramethyl ether	342.34	$C_{19}H_{18}O_6$	Flavonoid	3.32
57	17.138	Quercetin 3-glucoside	463.4	$C_{21}H_{20}O_{12}$	Flavonoid	3.33
58	17.737	5β,7βH,10α-Eudesm-11-en-1α-ol	222.37	$C_{15}H_{26}O$	Sesquiterpenoid	1.12
59	17.979	Phytane	282.5	$C_{20}H_{42}$	Alkane	0.32
60	18.385	Citronellyl tiglate	238.37	$C_{15}H_{26}O_2$	Fatty alcohol esters	3.52
61	18.553	Nonacosane	408.8	$C_{29}H_{60}$	Saturated Hydrocarbon	1.96
62	19.213	Squalane	422.8	$C_{30}H_{62}$	Triterpene	1.13
63	19.656	3',4',5,6,7,8-Hexamethoxyflavone	402.4	$C_{21}H_{22}O_8$	Flavonoid	1.61
64	20.456	Heptacosane	380.7	$C_{27}H_{56}$	Alkane	0.46
65	20.587	Docosane	310.6	$C_{22}H_{46}$	Alkane	0.89
66	20.997	Octacosane	394.8	$C_{28}H_{58}$	Alkane	0.9
67	21.916	Tetratetracontane	619.2	$C_{44}H_{90}$	Alkane	0.87
68	22.408	Luteolinidin cation	271.24	$C_{15}H_{11}O_5^+$	Flavonoid	1.13
69	23.212	Camphor	152.23	$C_{10}H_{16}O$	Monoterpene	2.02
70	23.487	Crocetane	282.5	$C_{20}H_{42}$	Terpene	0.29

Major compound with area % ≥ 2% were highlighted

Table (6): Bioactive compounds identified in *A. judaica* ethanolic extract

NO	RT	Compound name	MW	MF	Nature	Area%
1	4.941	1-Pyrroline, 2-phenyl-	145.2	$C_{10}H_{11}N$	Pyrrole	0.8
2	5.655	Ferulic acid methyl ester	208.21	$C_{11}H_{12}O_4$	Cinnamate (Caffeic acid)	0.37
3	5.884	Dihydromyricetin	320.25	$C_{15}H_{12}O_8$	Flavonoid (Benzopyran)	0.34
4	5.983	3,6,2',3'-Tetramethoxyflavone	342.3	$C_{19}H_{18}O_6$	Flavonoid	0.24
5	6.126	Isoquercitin	464.4	$C_{21}H_{20}O_{12}$	Flavonoid (Benzopyran)	0.39
6	6.348	4,7-Dimethyl-3-(4-methoxyphenyl)coumarin	280.3	$C_{18}H_{16}O_3$	Flavonoid	0.31
7	6.692	Nopol	166.26	$C_{11}H_{18}O$	Monoterpene	0.5
8	6.979	β-Terpinyl acetate	196.29	$C_{12}H_{20}O$	Monoterpene ester	0.31
9	8.648	Thiazolidine,2-methyl-	103.19	C_4H_9NS	Thiazolidines	13.44
10	10.117	3,5-di-t-Butylcatechol	222.32	$C_{14}H_{22}O_2$	Phenol	0.76
11	10.859	p-Cymen-7-ol	150.22	$C_{10}H_{14}O$	Benzyl alcohol	1.84
12	11.289	Cineole (Eucalyptol)	154.25	$C_{10}H_{18}O$	Monoterpene	1.95
13	11.929	α-Ionol	194.31	$C_{13}H_{22}O$	Sesquiterpene	0.82
14	12.188	Linalool	154.25	$C_{10}H_{18}O$	Monoterpene	0.67
15	12.577	Citronellal	154.25	$C_{10}H_{18}O$	Monoterpene aldehyde	0.75
16	12.926	Quercetin-3,7,3',4'-tetramethyl ether	358.3	$C_{19}H_{18}O_7$	Flavonoid (Benzopyran)	4.06
17	13.229	2',3'-Dimethoxyflavanone	284.31	$C_{17}H_{16}O_4$	Flavonoid (Benzopyran)	1.99
18	13.508	Ledol	222.37	$C_{15}H_{26}O$	Sesquiterpenes	1.09
19	13.816	7-Methoxychromanone	178.18	$C_{10}H_{10}O_3$	carboxylic acid esters	0.27
20	14.242	7,3',4',5'-Tetramethoxyflavone	358.3	$C_{19}H_{18}O_7$	Flavonoid	1.89
21	14.583	6,2',4'-Trimethoxyflavone	312.3	$C_{18}H_{16}O_5$	Flavonoid	0.99
22	15.243	7,3'-Dimethoxy-3-hydroxyflavone	358.3	$C_{19}H_{18}O_7$	Flavonoid	1.12
23	15.551	Resveratrol	228.24	$C_{14}H_{12}O_3$	Phenol (Stilbene	0.29
24	15.657	Malvidin 3,5-diglucoside cation	655.6	$C_{29}H_{35}O_{17}^+$	Flavonoid (Anthocyanidins)	0.79
25	15.932	Gossypetin 3,3',8-trimethylether	360.3	$C_{18}H_{16}O_8$	Flavonoid	3.15
	16.338	Gossypetin 3,3',8-trimethylether	360.3	$C_{18}H_{16}O_8$	Flavonoid	0.34
26	16.605	3,2',4',5',6-Pentamethoxyflavone	372.4	$C_{20}H_{20}O_7$	Flavonoid	1.84
27	16.957	Scutellarein tetramethyl ether	342.3	$C_{19}H_{18}O_6$	Flavonoid	13.59
28	17.294	5,3'-Dihydroxy-6,7,4'-trimethoxyflavone	344.3	$C_{18}H_{16}O_7$	Flavonoid	2.26
29	17.646	Thunbergol	290.5	$C_{20}H_{34}O$	Diterpene alcohol	1.64
30	17.913	Corymbolone	236.35	$C_{15}H_{24}O_2$	Sesquiterpenoid	1.59
31	18.2	Luteolin-7,3'-di-O-glucoside	610.5	$C_{27}H_{30}O_{16}$	Flavonoid glycoside	0.38
32	18.434	5β,7βH,10α-Eudesm-11-en-1α-ol			Sesquiterpenoid	15.62

NO	RT	Compound name	MW	MF	Nature	Area%
33	18.582	Afromosin 7-O-glucoside	460.4	$C_{23}H_{24}O_{10}$	Flavonoid o-glycosides	4.1
34	18.832	3-Hydroxy-7,2',3'-trimethoxyflavone	328.3	$C_{18}H_{16}O_6$	Flavonoid	1.18
35	19.123	Luteolin 6-C-glucoside	610.5	$C_{27}H_{30}O_{16}$	Flavonoid glycoside	6.27
36	19.312	2',4'-Dimethoxy-3-hydroxy-6-methylflavone	312.3	$C_{18}H_{16}O_5$	Flavonoid	3.71
37	19.709	Caryophyllene oxide	220.35	$C_{15}H_{24}O$	Sesquiterpenes	0.82
38	20.292	β Carotene	536.9	C40H56	Terpene (Cartenoid)	1.2
39	20.677	Phytol	296.5	$C_{20}H_{40}O$	Diterpene alcohol	1.26
40	21.112	Geranyl isovalerate	238.37	$C_{15}H_{26}O_2$	carboxylic ester	1.23
41	21.695	6,7,8-Trimethoxycoumarin	236.22	$C_{12}H_{12}O_5$	Benzopyrans Coumarins	0.74
42	22.133	6,7-Dimethoxy-4-methylcoumarin	220.22	$C_{12}H_{12}O_4$	Coumarin derivatives	0.67
43	22.581	Isovitexin	432.4	$C_{21}H_{20}O_{10}$	Flavonoid	0.4
44	23.163	Camphor	152.23	$C_{10}H_{16}O$	Monoterpene	1.4
45	23.483	3-Hydroxy-6,2',3'-trimethoxyflavone	328.3	$C_{18}H_{16}O_6$	Flavonoid	0.43
46	23.93	3,7,8,2'-Tetramethoxyflavone	358.3	$C_{19}H_{18}O_7$	Flavonoid	0.2

Major compound with area % ≥ 2% were highlighted

Discussion

The current investigation reported the presence of *N. ceranae* in all the collected samples from one of the Egyptian governorates (Sharkia). This finding was similar to those reported by **Stentiford *et al.* (2016)** and **Bordin *et al.* (2022);** *N. ceranae* infection appeared to be dominant and almost completely replaced *N. apis* in countries in northern Europe and North America.

A need to evaluate and develop new strategies to combat the loss in honey bee populations, especially the depopulation caused by Nosemosis became an emergence. The present findings revealed that the orange peel oil and *A. Judaica* ethanolic extract effectively reduced *N. ceranae* spore counts in both therapeutic and prophylactic trials. Moreover, the current data validated the suitable concentrations of orange peel oil and the *A. judaica* extract that could be administered for *Nosema* infections.

The development of alternative strategies for controlling *Nosema* infections has expanded in response to concerns regarding antibiotics and chemical treatments. Most experiments involving various essential oils, such as peppermint, eucalyptus, orange, and lemon, have been conducted by beekeepers. However, the results obtained from these treatments are not scientifically substantiated **(Dumitru *et al.* 2017)**.

Plant extracts were previously screened in apiculture against various honey bee diseases and pests. The bioactivity of *Citrus paradisi* (grapefruit) and *Citrus sinensis* (sweet orange) essential oils as potential controls for *Varroa destructor* and *Paenibacillus larvae* were *in vitro* evaluated **(Fuselli *et al.* 2009)**.

Moreover, various extracts and essential oils were investigated against nosemosis **(El-Seedi *et al.* 2022a)**. *Cryptocarya alba* **(Bravo *et al.* 2017)**, *Melissa officinalis*, *Mentha piperita*, and *Coriandrum sativum* essential oils exhibited anti-microsporidia activity **(Mitrea 2017)**. Also, *Artemisia dubia* and *Aster scaber* extracted have been investigated **(Lee *et a*l. 2018)**. **Glavinic *et al.* (2021a,b)** tested anti-nosemosis activity of aqueous extracts of *Agaricus blazei* mushroom.

The present investigation used metronidazole as known effective anti-nosemosis to compare present treatments to. Nitroimidazole compounds (metronidazole, tinidazole) were previously confirmed to be highly potent (completely inhibit) against the proliferation of *N. ceranae* **(Gisder and Genersch 2015)**. However, metronidazole antibiotic residue was reported in honey **(Zhang *et al*. 2024)**.

The pharmacological and bioactivities of *C. sinensis* are attributed to the valuable secondary metabolites of this plant. *C. sinensis* is rich in various categories of chemical compounds, which have been identified in the fruits, peels, leaves, juice, and roots **(Favela-Hernández *et al.* 2016)**. Our analysis reported 70 phytochemical compounds in *C. sinensis* peel oil. The composition of the oil was similar with data explored by **Bozkurt *et al.* (2017) and Gültepe (2018)**. *C. sinensis* bioactive compounds were classified into different chemical groups; flavonoids **(Saleem *et al.* 2010; Escudero-López *et a*l. 2013)** steroids, hydroxyamides, alkanes and fatty acids **(Rani *et al.* 2009)**, coumarins **(Li *et al*. 2007)**, peptides **(Matsubara *et al*. 1991)**, carbohydrates **(Kolhed and Karlberg 2005)**, carbamates and alkylamines **(Soler *et al*. 2006)**, carotenoids **(Aschoff *et al.* 2015)** volatile compounds **(Kelebek and Selli 2011; Selli *et al*.** 2008).

Regarding the *A. judaica* extract GC-MS analysis, the current study revealed 46 phytochemical compounds in the extract. The data explored different classes of *Artemisia* extract constituents similar to the GC-MS data of different species of *Artemisia* reported by **Kumar *et al*. (2025)**. Due to the presence of these compounds in *Artemisia*, many of these species have been reported to have antimicrobial, antifungal, and anti-inflammatory activities **(Mihajilov-Krstev *et al*. 2014; Sati *et al.* 2013)**.

Conclusion:

The decline of honey bee populations worldwide is a major issue that leads to serious economic and ecological problems. Nosemosis is reported as a biotic factor that causes a significant loss in honey bee populations. The present finding indicates the therapeutic and prophylactic activities of orange peel oil and the ethanolic extract of *A. judaica* against *N. ceranae* infections. Both extracts contain highly bioactive compounds that function as effective antimicrobial treatments. This approach highlighted the application of available, low-cost, and environmentally safe alternatives in dealing with honey bee diseases and infections. Continuous research is needed to estimate the mechanism of these therapeutic and prophylactic activities on the immunity of the honey bee and evaluate the application of those natural alternatives in the apiary.

Administration of both extracts in both trials (therapeutic and prophylactic) resulted in reductions in Nosema spore loads after 3,5, and 10 days of infection. GC/MS analysis identified 70 chemical compounds in orange peel oil (isolimonene; 12.16%). Whereas, 46 compounds were identified in the Artemisia extract,(β,7βH,10α-Eudesm-11-en-1α-ol; 15.62%).

Statements & Declarations

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Competing interests

The authors declare no competing interests

Data availability all data are available in the present paper

Competing interests the authors declare no competing interests

Ethical approval Not applicable Author's contributions

B.R.A.A. carried out methodology, experimental design, and drafted the manuscript. E.H.E., and O.N.A. supervised, assisted with experimental design, and revised and edited the manuscript. G.H.I.A. assisted with experimental design, drafted and edited the manuscript, and conducted statistical analysis.

References:

1. **Alaux, C.; Folschweiller, M.; McDonnell, C.; Beslay, D.; Cousin, M.; Dussaubat, C.; Brunet, J.L. and Conte,Y., 2011.** Le Pathological effects of the microsporidium *Nosema ceranae* on honey bee queen physiology (*Apis mellifera*). J. Invertebr. Pathol. 106:380–385.
2. **Antal, T.; Figiel, A.; Kerekes, B. and Sikolya, L., 2011**. Effect of drying methods on the quality of the essential oil of spearmint leaves (Mentha spicata L.). Drying Technology: an International Journal. 29(15):1836–1844.
3. **Aschoff, J.K.; Kaufmann, S.; Kalkan, O.; Neidhart, S.; Carle, R. and Schweiggert, R.M., 2015.** In Vitro Bioaccessibility of Carotenoids, Flavonoids, and Vitamin C from Differently Processed Oranges and Orange Juices (Citrus sinensis (L.) Osbeck). J. Agric, Food Chem. 63:578–587.
4. **Balamurugan, R.; Park, J.K. and Lee, J.K., 2022.** Anti-nosemosis activity of phenolic compounds derived from Artemisia dubia and Aster scaber. J. Apic. Res. 6:519–529.
5. **Bordin, F.; Zulian, L.; Granato, A.; Caldon, M.; Colamonico, R.; Toson, M.; Trevisan, L.; Biasion, L. and Mutinelli, F., 2022.** Presence of Known and Emerging Honeybee Pathogens in Apiaries of Veneto Region (Northeast of Italy) during Spring 2020 and 2021. Appl. Sci. 12: 2134.
6. **Botías, C.; Martín-Hernández, R.; Meana, A. and Higes, M., 2013.** Screening alternative therapies to control Nosemosis type C in honey bee (Apis mellifera iberiensis) colonies. Res. Vet. Sci. 95:1041– 1045.
7. **Bozkurt, R.; Gülnaz, O. and Aka Kaçar, Y., 2017.** Chemical composition of the essential oils from some citrus species and evaluation of the antimicrobial activity. IOSR J. Environ. Sci. Toxicol. Food Technol. 11:1-8.
8. **Bravo, J.; Carbonell, V.; Sepúlveda, B.; Delporte, C.; Valdovinos, C.E.; Martín-Hernández, R. and Higes, M., 2017.** Antifungal activity of the essential oil obtained from Cryptocarya alba against infection in honey bees by Nosema ceranae. J. Invertebr. Pathol. 149:141–147.

9. **Castelli, N.L., 2012.** Prevalencia y distribución geográfica de Nosema apis y Nosema ceranae en Apis mellifera de Uruguay. Dissertation/master's thesis. Montevideo, UY: University of the Republic.

10. **Clevenger, J.F., 1928.** Apparatus for the determination of volatile oil. The Journal of the American Pharmaceutical Association (1912). 17(4):345-349.

11. **Anonymous (2005).** CoStat - Free Statistics Software. Version 6.311, Copyright (c), CoHort Software, 798 Lighthouse Ave. PMB 320, Monterey, CA, 93940, USA.

12. **Dumitru, A.; Chioveanu, G.; Ionit, A.M. and Dobre Mitrea I.L., 2017.** "In vitro" studies on using natural essential oils in treatment of Nosemosis in honey bees: Determination of the therapeutic dose. Sci. Work Ser. C Vet. Med. 63:165–170.

13. **El-Didamony, S.E.; Gouda, H.I.; Zidan, M.M. and Amer, R.I., 2024.** Bee products: An overview of sources, biological activities and advanced approaches used in apitherapy application. Biotechnology Reports. 44:e00862.https://doi.org/ 10.1016/ j.btre. 2024.e 00862

14. **Elhoseny, M.M.; El-Didamony, S.E.; Atwa, W.A.; Althoqapy, A.A. and Gouda, H.I., 2024.** New insights into changing honey bee (Apis mellifera) immunity molecules pattern and fatty acid esters, in responses to Ascosphaera apis infection. Journal of Invertebrate Pathology. 202: 108028.

15. **El-Seedi, H.R.; Eid, N.; El-Wahed, A.A.A. and Rateb, M.E., 2022[a].** Honey bee products: Preclinical and clinical studies of their anti-inflammatory and immunomodulatory properties. Front. Nutr. 8:761267–761285.

16. **El-Seedi, H.R.; El-Wahed, A.A.A.; Al Naggar, Y.; Saeed, A.; Xiao, J. and Ullah, H., 2022[b].**

    Insights into the Role of Natural Products in the Control of the Honey Bee Gut Parasite (Nosema spp.). Animals. 12:3062.

17. **Escudero-López, B.; Cerrillo, I.; Herrero-Martín, G.; Hornero-Méndez, D.; Gil-Izquierdo, A.; Medina, S.; Ferreres, F.; Berná, G.; Martín, F. and Fernández-Pachón, M.S., 2013.** Fermented orange juice: Source of higher carotenoid and flavanone contents. J. Agric. Food Chem. 61:8773–8782.

18. **Favela-Hernández, J.M.J.; González-Santiago, O.; Ramírez-Cabrera, M.A.; Esquivel-Ferriño, P.C. and Camacho-Corona, M.D.R., 2016.** Chemistry and pharmacology of Citrus sinensis. Molecules. 21(2):247.

19. **Fries, I., 2010.** Nosema ceranae in European honey bees (Apis mellifera). J. Invertebr. Pathol. 103:S73–9.

20. **Fuselli, S.R; Maggi, M.; de la Rosa, S.B.G.; Principal, J.; Eguaras, M.J. and Fritz, R., 2009.** In vitro antibacterial and antiparasitic effect of citrus fruit essential oils on the honey bee pathogen Paenibacillus larvae and the parasitic mite Varroa destructor. Journal of apicultural research. 48(1):77-

    78.

21. **Gisder, S. and Genersch, E., 2015.** Identification of Candidate Agents Active against N. ceranae Infection in Honey Bees: Establishment of a Medium Throughput Screening Assay Based on N. ceranae Infected Cultured Cells. PLoS ONE. 10:e0117200. https://doi.org/10.1371/journal.pone.0117200

22. **Glavinic, U.; Stevanovic, J.; Ristanic, M.; Rajkovic, M.; Davitkov, D. and Lakic, N., 2021[a].** Potential of Fumagillin and Agaricus blazei mushroom extract to reduce Nosema ceranae in honey bees. Insects. 12:282.

23. **Glavinic, U.; Rajkovic, M.; Vunduk, J.; Vejnovic, B.; Stevanovic, J.; Milenkovic, I. and Stanimirovic, Z., 2021b.** Effects of Agaricus bisporus mushroom extract on honey bees infected with Nosema ceranae. Insects. 12:915.

24. **Gültepe, N., 2018.** How the use of orange (Citrus sinensis) peel essential oil affected the growth performance of rainbow trout (Oncorhynchus mykiss)? Bull. Univ. Agric. Sci. Vet. Med. Cluj. Napoca. 75:16-20.

25. **Higes, M.; García-Palencia, P.; Urbieta, A.; Nanetti, A. and Martín-Hernández, R., 2020.** Nosema apis and Nosema ceranae tissue tropism in worker honey bees (Apis mellifera). Vet. Pathol. 57:132–8.

26. **Higes, M.; Martín, R. and Meana, A., 2006.** Nosema ceranae, a new microsporidian parasite in honeybees in Europe. J. Invertebr. Pathol. 92:93–5.

27. **Huang, W.F.; Solter, L.F.; Yau, P.M. and Imai, B.S., 2013.** Nosema ceranae escapes fumagillin control in honey bees. PLoS Pathog. 9: e1003185.

28. **Hulaj, B.; Granato, A.; Bordin, F.; Goga, I. Merovci, X. and Caldon, M., 2024.** Emergent and known honey bee pathogens through passive surveillance in the republic of Kosovo. Appl. Sci. 14:987.

29. **Hurná, B.; Sučik, M.; Staroň, M.; Tutka, Š.; Maková, Z.; Galajda, R. and Valenčáková, A., 2023.** Molecular Detection of Nosema spp. in Three Eco Regions of Slovakia. Current Issues in Molecular Biology. 45(6):4814-4825.

30. **Johnson, R.M.; Dahlgren, L.; Siegfried, B.D. and Ellis, M.D., 2013.** Acaricide, fungicide and drug interactions in honey bees (Apis mellifera). PLoS ONE. 8:e54092. https://doi.org/10.1371/journal.pone.0054092

31. **Kelebek, H. and Selli, S., 2011.** Determination of volatile, phenolic, organic acid and sugar components in a Turkish cv. Dortyol (Citrus sinensis L. Osbeck) orange juice. J. Sci. Food Agric. 91:1855–1862.

32. **Klein, A.M.; Vaissière, B.E.; Cane, J.H.; Steffan-Dewenter, I.; Cunningham, S.A.; Kremen, C. and Tscharntke, T., 2007.** Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. B Biol. Sci. 274:303–313.

33. **Kolhed, M. and Karlberg, B., 2005.** Capillary electrophoretic separation of sugars in fruit juices using on-line mid infrared Fourier transform detection. Analyst. 130:772–778.

34. **Kumar, B.; Wani, I.A.; Lone, J.F.; Srinivas, K. and Gairola, S., 2025.** Chemical Diversity in Essential Oils of 40 Artemisia Species from Western and Trans Himalayan Regions of India. Resources. 14(3):42.

35. **Kurze, C.; Le Conte, Y.; Kryger, P.; Lewkowski, O.; Müller, T. and Moritz, R.F.A., 2018.** Infection dynamics of Nosema ceranae in honey bee midgut and host cell apoptosis. J. Invertebr. Pathol. 154:1–4.

36. **Lee, J.K.; Kim, J.H.; Jo, M.; Rangachari, B. and Park, J.K., 2018.** Anti-nosemosis activity of Aster Scaber and Artemisia Dubia aqueous extracts. J. Apic. Sci. 62:27–38.

37. **Li, J.H.; Evans, J.D.; Li, W.F.; Zhao, Y.Z.; DeGrandi-Hoffman, G.; Huang, S.K.; Li, Z.G.; Hamilton, M. and Chen, Y.P., 2019.** New evidence showing that the destruction of gut bacteria by antibiotic treatment could increase the honey bee's vulnerability to nosema infection. PLoS ONE. 12: e0187505. https://doi.org/10.1371/journal. pone.0187505

38. **Li, S., Lambros, T.; Wang, Z.; Goodnow, R. and Ho, C.T., 2007.** Efficient and scalable method in isolation of polymethoxyflavones from orange peel extract by supercritical fluid chromatography. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 846:291–297.

39. **MacInnis, C.I.; Keddie, B.A. and Pernal, S.F., 2022.** Honey bees with a drinking problem: Potential routes of Nosema ceranae spore transmission. Parasitology. 149:573–580.

40. **Martín-Hernández, R.; Botías, C.; Bailón, E.G.; Martínez-Salvador, A.; Prieto, L. and Meana, A., 2012.** Microsporidia infecting Apis mellifera: coexistence or competition. Is Nosema ceranae replacing Nosema apis? Environ. Microbiol. 14:2127–38. https://doi.org/10.1111/j.14622920.2011.02645.x

41. **Martín-Hernández, R.; Meana, A.; Prieto, L.; Salvador, A.M.; Garrido-Bailón, E. and Higes, M., 2007.** Outcome of colonization of Apis mellifera by Nosema ceranae. Appl. Environ. Microbiol. 73(20):6331–6338.

42. **Marín-García, P.J.; Peyre, Y.; Ahuir-Baraja, A.E.; Garijo, M.M. and Llobat, L., 2022.** The Role of Nosema ceranae (Microsporidia: Nosematidae) in honey bee colony losses and current insights on treatment. Vet. Sci. 9:130.

43. **Matsubara, Y.; Yusa, T.; Sawabe, A.; Iizuka, Y.; Takekuma, S. and Yoshida, Y., 1991.** Structures of new cyclic peptides in young unshiu (Citrus unshiu Marcov.), orange (Citrus sinensis Osbeck.) and amanatsu (Citrus natsudaidai) peelings. Agric. Biol. Chem. 55:2923–2929.

44. **Mihajilov-Krstev, T.; Jovanović, B.; Jović, J.; Ilić, B.; Miladinović, D.; Matejić, J.; Rajković, J.; Đorđević, L.; Cvetković, V. and Zlatković, B., 2014.** Antimicrobial, antioxidative, and insect repellent effects of Artemisia absinthium essential oil. Planta medica. 80(18):1698-1705.

45. **Mitrea, I.L., 2017.** "In vitro" studies on using natural essential oils in treatment of Nosemosis in honey bees: Determination of the therapeutic dose. Sci. Work Ser. C Vet. Med. 63: 165–170.

46. **Neidel, V.; Steyer, C.; Schafellner, C. and Hoch, G., 2017.** Simulation of rain enhances horizontal transmission of the microsporidium Nosema lymantriae via infective feces. J. Invertebr. Pathol. 149:56–58.

47. **Patricia, M.S., Migdalia, M., Juan, A.P., Mario, S., Victor, H., Esther, P., 2013.** Gas Chromatography Mass Spectrometry Study from the Leaves Fractions Obtained of Vernonanthura patens (Kunth) H. Rob. International Journal of Organic Chemistry. 3:105-109.

48. **Pettis, J.S., Van engelsdorp, D., Johnson, J., Dively, G., 2012.** Pesticide exposure in honey bees results in increased levels of the gut pathogen Nosema. Naturwissenschaften. 99:153–158.

49. **Phillips, A.J., Simon, C., 1995.** Simple, efficient, and nondestructive DNA extraction protocol for arthropods. Annals of the Entomological Society of America. 88(3):281-283.

50. **Pohorecka, K., 2004.** Laboratory studies on the effect of standardized Artemisia absinthium L. extract on Nosema apis infection in the worker Apis mellifera. Journal of Apicultural Science. 42(2):31-136.

51. **Rani, G., Yadav, L., Kalidhar, S.B., 2009.** Chemical Examination of Citrus sinensis Flavedo Variety Pineapple. Indian J. Pharm. Sci. 71:677–679.

52. **Roussel, M., Villay, A., Delbac, F., Michaud, P., Laroche, C., Roriz, D., El Alaoui, H., Diogon, M., 2015.** Antimicrosporidian activity of sulphated polysaccharides from algae and their potential to control honeybee nosemosis. Carbohydr. Polym. 133:213–220.

53. **Saleem, M., Farooq, A., Ahmad, S., Shafiq, N., Riaz, N., Jabbar, A., Arshad, M., Malik, A., 2010.** Chemical constituents of Citrus sinensis var. Shukri from Pakistan. J. Asian Nat. Prod. Res. 12:702– 706.

54. **Sati, S.C., Sati, N., Ahluwalia, V., Walia, S., Sati, O.P., 2013.** Chemical composition and antifungal activity of Artemisia nilagirica essential oil growing in northern hilly areas of India. Nat. Prod. Res. 27(1):45–48.

55. **Selli, S., Canbas, A., Varlet, V., Kelebek, H., Prost, C., Serot, T., 2008.** Characterization of the most odor-active volatiles of orange wine made from a Turkish cv. Kozan (Citrus sinensis L. Osbeck). J. Agric. Food Chem. 56:227–234**.**

56. **Soler, C., Hamilton, B., Furey, A., James, K.J., Mañes, J., Picó, Y., 2006.** Comparison of four mass analyzers for determining carbosulfan and its metabolites in citrus by liquid chromatography/mass spectrometry. Rapid Commun Mass Spectrom. 0:2151–2164.

57. **Southwick, E.E., Southwick, L., 1992.** Estimating the economic value of honey Bees (Hymenoptera: Apidae) as agricultural pollinators in the United States. J. Econ. Entomol. 85:621–633.

58. **Stentiford, G.D., Becnel, J.J., Weiss, L.M., Keeling, P.J., Didier, E.S., Bjornson, S., et al., 2016.** Microsporidia–emergent pathogens in the global food chain. Trends in parasitology. 32(4):336-348.

59. **Williams, G.R., Shafer, A.B.A., Rogers, R.E.L., Shutler, D., Stewart, D.T., 2008.** First detection of Nosema ceranae, a microsporidian parasite of European honey bees (Apis mellifera), in Canada and Central USA. J. Invertebr. Pathol. 97:189–92.

60. **World Health Organization (WHO) 2024.** Antimicrobial resistance: global report on surveillance. Available at: https://www.who.int/publications/i/item/ 9789241 564748.

61. **Yosri, N., El-Wahed, A.A.A., Ghonaim, R., Khattab, O.M., Sabry, A., Ibrahim, M.A.A., Moustafa, M.F., Guo, Z., Zou, X., Algethami, A.F.M., et al., 2021.** Anti-viral and immunomodulatory properties of propolis: Chemical diversity, pharmacological properties, preclinical and clinical applications, and in silico potential against SARS-CoV-2. Foods. 10(8):1776.

62. **Zhang, S., Yu, S., Wang, X., Zhang, Y., Yue, Z., Li, C., Ma, Y., 2024.** A novel electrochemical sensor for the detection of metronidazole in honey using the g-C3N4/MnO2/ZnO modified electrode. Journal of Food Composition and Analysis. 27:105992.