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Impacts of Diverse Natural Products on Honey Bee Viral Loads and Health

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Featured Application: Medicines for honey bee health.

Abstract: Western honey bees (*Apis mellifera*), a cornerstone to crop pollination in the U.S., are faced with an onslaught of challenges from diseases caused by parasites, pathogens, and pests that affect this economically valuable pollinator. Natural products (NPs), produced by living organisms, including plants and microorganisms, can support health and combat disease in animals. NPs include both native extracts and individual compounds that can reduce disease impacts by supporting immunity or directly inhibiting pathogens, pests, and parasites. Herein, we describe the screening of NPs in laboratory cage studies for their effects on honey bee disease prevention and control. Depending on the expected activity of compounds, we measured varied responses, including viral levels, honey bee immune responses, and symbiotic bacteria loads. Of the NPs screened, several compounds demonstrated beneficial activities in honey bees by reducing levels of the critical honey bee virus deformed wing virus (DWV-A and-B), positively impacting the gut microbiome or stimulating honey bee immune responses. Investigations of the medicinal properties of NPs in honey bees will contribute to a better understanding of their potential to support honey bee immunity to fight off pests and pathogens and promote increased overall honey bee health. These investigations will also shed light on the ecological interactions between pollinators and specific floral food sources.

Keywords: honey bee; natural product; disease; antiviral; health

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1. Introduction

Western honey bees (*Apis mellifera*) contribute over $34B to the U.S. economy via agricultural crops [1] that are completely or largely dependent on honey bees for pollination. Colony loss and replacement can be devastating to beekeepers, and losses continue to grow, largely due to diseases caused by pathogens, parasites, and pests. These challenges are a grave concern for crop sustainability. The honey bee pathosphere [2] is a collection of parasites and pathogens, including mites, viruses, bacteria, fungi, microsporidian parasites, protozoa, and others that affect honey bee productivity, physiology, and behavior. This increases beekeeper costs due to high demands for disease and pest management, as well as the loss of marketable products and services [3].

The honey bee parasitic mite *Varroa destructor*, an external parasite that feeds on adult and developing bees, is arguably the greatest challenge for honey bee colonies. Along with direct impacts on bee physiology [4,5], this mite drives the spread of deadly bee viruses [6–11]. Honey bees face dozens of RNA viruses that can cause developmental, longevity, and behavioral symptoms [12]. Arguably the most common and important of these is the mite-vectored deformed wing virus (DWV; [13]). DWV is worldwide [12], diverse [14], and hugely impactful on bee health. Other parasites affecting bee health include *Nosema ceranae* and *Nosema apis* (gut fungal microsporidian parasites) and the trypanosome *Lotmaria passim* (unicellular parasitic flagellate protozoon) [12]. The result of these infections is lack of worker productivity at the colony level, which affects hive resources and reproduction as colony size decreases. These impacts drive searches for effective treatments for honey bee disease infection.

Many similarities exist between the classes of viruses and fungi found in humans and honey bees, and one can infer that medicines that show promise against human infection might do the same for honey bees. Natural products (NPs), gleaned from nature for their pharmacological or biological activity, offer potential new solutions for hive diseases [15] while already playing a role in the lives of bees [16]. Phytochemicals are defined as bioactive nutrient plant chemicals in natural products such as fruits, vegetables, grains, and other plant foods that may provide health benefits [17]. In addition, traditional herbal medicines and purified natural products may guide the development of novel antiviral drugs [18]. Numerous studies have suggested that natural products can directly inhibit the growth and replication of viruses and bacteria [19–22]; for example, cacao, which has broad-spectrum antimicrobial/antiviral activity when ingested in human studies [23]. Curiously, honey bees are frequently observed in nature collecting various natural products, such as resins, and applying them to the nest environment. Simone-Finstrom and Spivak [24] showed that colonies challenged with the fungal parasite *Ascosphaera apis*, the causative agent of Chalkbrood disease, responded with increased foraging for resin. Furthermore, bees showed lower pathogen levels if the colony was supplemented with resin [25,26]. Erler and Moritz reviewed the secondary metabolites of foraged hive products (alkaloids, phenolic acids and flavonoids) that may be ingested for self-medication by honey bees to decrease their parasite and pathogen load [27]. In a recent work, researchers found the antiviral activity of polypore mushrooms after feeding bees with these mushroom extracts and noticing that bees collect water from fungal sources, suggesting a possible case of self-medication [28]. Similarly, bees fed thymol (isolated from thyme) in sugar syrup tended to show lower levels of DWV [29] and below. Additional candidates include curcumin [30], a polyphenol isolated from turmeric; garlic oil, which is known prominently for its antimicrobial activity [31,32]; and cacao bean shells [33], all of which have known pharmaceutically useful properties. NPs are also currently used as controls for *Varroa* mites. Synthetic miticides, including amitraz, coumaphos, flumethrin, and fluvalinate, have proven effective but are vulnerable to increased levels of mite resistance [34]. NPs, including oxalic and formic acids, thymol (Api-Guard® and other products), and beta-acid mixes (e.g., HopGuard®, a mix of beta-acids derived from remainders from the beer brewing process), are now commercially available for reducing *Varroa* mite populations. Despite their widespread acceptance for *Varroa* mite control, NPs have not yet been registered to combat other bee diseases.

Natural products may offer many benefits aside from being directly antiviral, antibacterial, or antiparasitic, such as easing of symptoms of disease, bolstering the immune system, and speeding recovery after infection [35,36]. Consequently, numerous researchers are screening NPs for potential roles in honey bee health [15,37,38]. Interestingly, thymol has also been shown in multiple studies to decrease pathogen or parasite loads in honey bees and bumble bees [39]. In the honey bee, different infectious agents can activate the same or similar honey bee genes and pathways [40]. Therefore, a compound that generally stimulates the honey bee immune system, in response to a DWV or gut parasite infection,

may also control similar viruses and parasites. Conversely, if the compounds are found harmful to honey bees, similar compounds can likely be harmful as well.

The above evidence inspired us to test NP candidates for their effects on honey bee infections. Several candidates show promise in directly combating pathogens and/or supporting the honey bee immune system, thereby enabling the honey bee to better defend themselves against diseases. Notably, hesperidin and raw cacao lowered the levels for *Varroa destructor* virus (VDV) (500-fold, $p = 0.0208$ and 50-fold, $p = 0.0062$, respectively) and bees fed low levels of garlic oil extract showed a trend toward lower virus levels across multiple trials. We also describe the effects of natural products on honey bee gene expression and levels of symbiotic gut bacteria. *Gilliamella*, a honey bee bacterial gut symbiont, showed reduced levels in bees fed tocopherol (250-fold, $p = 0.0019$), curcumin (50-fold, $p = 0.0268$), vanillin (25-fold, $p = 0.0706$), and hesperidin (16-fold, $p = 0.1104$) compared to controls. Another gut symbiont, *Snodgrassella alvi,* showed decreased titers when bees were supplemented with beta-carotene (4-fold, $p = 0.0311$). Tocopherol and curcumin also led to lower expression of the immune effector eater (40-fold, $p = 0.0086$ and 64-fold, $p = 0.0002$, respectively). This survey helps refine promising routes for understanding honey bee floral choices [41], along with choices for new treatment strategies.

2. Materials and Methods

Our study focused on laboratory cage studies of bees fed a single compound (or extract) for a duration of six or seven days, followed by molecular assessment of infection rate, pathogen load, and immune gene transcription. While all experiments were aimed at testing natural products, trials were performed over time with significant experimental variation across trials. Hence, the treatments from one experiment were only compared to the control from that particular experiment. Primers that targeted microbes, as well as honey bee control genes, immunity, nutrition, age, and structural integrity of the gut, were described in [15].

2.1. Experimental Design

For tests using field-developed adult worker bees, these were collected from colonies at the USDA-ARS Bee Research Laboratory, Beltsville, MD, USA, by shaking outer frames of bees into a bucket and then sealing several hundred bees into individual 16 oz plastic cups. Bees were then anesthetized with CO_2 (30 s to 1 min) and counted into experimental cups [42]. To measure the effects of treatments on newly emerged bees, frames of a mature sealed brood were collected from the same research apiaries and were caged in a 34 °C incubator with high humidity overnight. Newly emerged bees from these frames were collected daily and placed in experimental cups. For either older or newly emerged bees, each treatment or control consisted of cups of 20–30 bees/cup per treatment or control. If challenged with viruses or *Nosema*, the bees were hand-fed a suspension of an infectious dose, as indicated in [15]. For all experiments, honey bees were fed either 1:1 *v/v* sugar water (control) or 1:1 *v/v* sugar water with 100 ppm of specific natural products with top feeders for six or seven days, after which all dead bees were removed and the remaining live bees were frozen at −80 °C until nucleic acid extraction. The tested compounds and concentrations are summarized in Table 1. Conditions specific to individual trials are described in the Supplementary Materials.

Table 1. Natural product sourcing and tested concentrations.

Common Name (Scientific Name)	Level of Purity	Source	Conc. (ppm)
Astragalus (*Astragalus propinquus*)	Natural	Vitamin Shoppe#VS-1637/1776483	100, 1000
Berberine chloride	98%	Sigma#B3251	100
Beta-carotene	95%	Sigma#C4582	2000
Cacao raw (*Theobroma cacao* L)	Natural	Whole raw cocoa beans (Peru)	100
Cacao roasted *(Theobroma cacao* L)	Natural	Whole roasted cocoa beans (Peru)	100
Calendula (*Calendula officinalis*)	Natural	Vitamin Shoppe # BB-1034/1083588	100, 1000
Cat's Claw (*Uncaria tomentosa*)	Natural	Vitamin Shoppe #BB-1346/1098914	100, 1000
Carvacrol	98%	Sigma#282197	100
Chrysin	97%	Sigma#C80105	100
Cinnamic acid	99%	Sigma#W228826	100
Curcumin (*Curcuma longa*)	75%	Sigma#8.20354	100
Decanoic acid	98%	Sigma #C1875	100
Echinacea (*Echinacea*)	Natural	Vitamin Shoppe #VS-1625/1776038	100, 1000
Elderberry (*Sambucus*)	Natural	Vitamin Shoppe #BB-1181/1681436	100, 1000
Garlic oil (*Allium sativum*)	Natural	Vitamin Shoppe	100, 1000, 10,000
Ginger (*Zingiber officinale*)	Natural	Sigma#W252204	100, 1000
Hesperidin	80%	Sigma#H5254	100
Licorice (*Glycyrrhiza glabra*)	Natural	Vitamin Shoppe #BB-1101/1087241	100, 1000
Limonene	97%	Sigma#183164	100
Olive leaf (*Olea europaea*)	Natural	Vitamin Shoppe	100, 1000
Oil of oregano (*Origanum vulgare*)	Natural	Vitamin Shoppe #VS-1782/1484956	100, 1000
Thymol	99%	Sigma#16254	10, 100
Tocopherol	FCG	Sigma#W530066	100
Tyrosine low, high	98%	Sigma#T3754	10, 100
Vanillin (*Vanilla planifolia*)	99%	Sigma#V1104	100

2.2. RNA Extraction, cDNA Synthesis, and qPCR

RNA was extracted following the bulk total RNA extraction, first-strand cDNA synthesis, and real-time qPCR protocols outlined in [43]. Alternatively, total RNA was extracted from individual bees using TRIzol™ and then followed the protocol outlined in the BEEBOOK for cDNA synthesis and qPCR [43]. For a subset of the samples, pooled bees were extracted using a modified RNAswift protocol [44] followed by DNase treatment, cDNA synthesis, and qPCR, as in [15]. The individual experimental details for RNA extraction, cDNA synthesis, and qPCR are described in [15] and all primers used in this study are described in the Supplemental Materials Table S1.

2.3. Data Analysis

Transcript abundance is expressed as the change in the cycle threshold (ΔCq). Data are presented as ΔCq, which indicates the relative gene expression of a target after normalization by a stable reference gene (RpS5, AmActin, or Arp1), with a higher number indicative of a higher level of transcripts for that gene. Various host-, pathogen-, and symbiont-related qPCR primer pairs were used [15] to detect the level of infection or to evaluate bee health. Reactions producing no amplification or failing melt temperature confirmation (+/−1 °C from desired melt) were recorded as Cq of 50 (the last cycle) and the ΔCq was determined. The ΔCq was imported into the statistical software JMP (version 15 for MacOS) or R. ΔΔCq was calculated using a control group that was only fed sucrose water solution.

3. Results

3.1. Chrysin, Curcumin, Hesperidin, Vanillin, and Tocopherol

For these compounds, there were no significant differences in DWV compared to the control for any treatment (DF = 6, $p = 0.6336$), (Figure 1). The only significant difference in VDV levels when the bees were fed each of these treatments was seen when bees were fed hesperidin (mean control = −5.302 +- 2.6289 (SE); mean hesperidin = −14.230 +- 2.6289 (SE), $n = 8$, $p = 0.0208$), where a ~9-cycle difference indicates a 500-fold decrease in VDV when bees are fed hesperidin (Figure 1). There were no significant differences in the virus LSV compared to the control for any treatment (DF = 6, $p = 0.2751$). Although all treatments had generally higher levels of trypanosomes when compared to the controls, there was no significant difference for any of the treatments. Finally, there were no significant differences in *Nosema ceranae* compared to the control for any treatment (DF = 6, $p = 0.6101$).

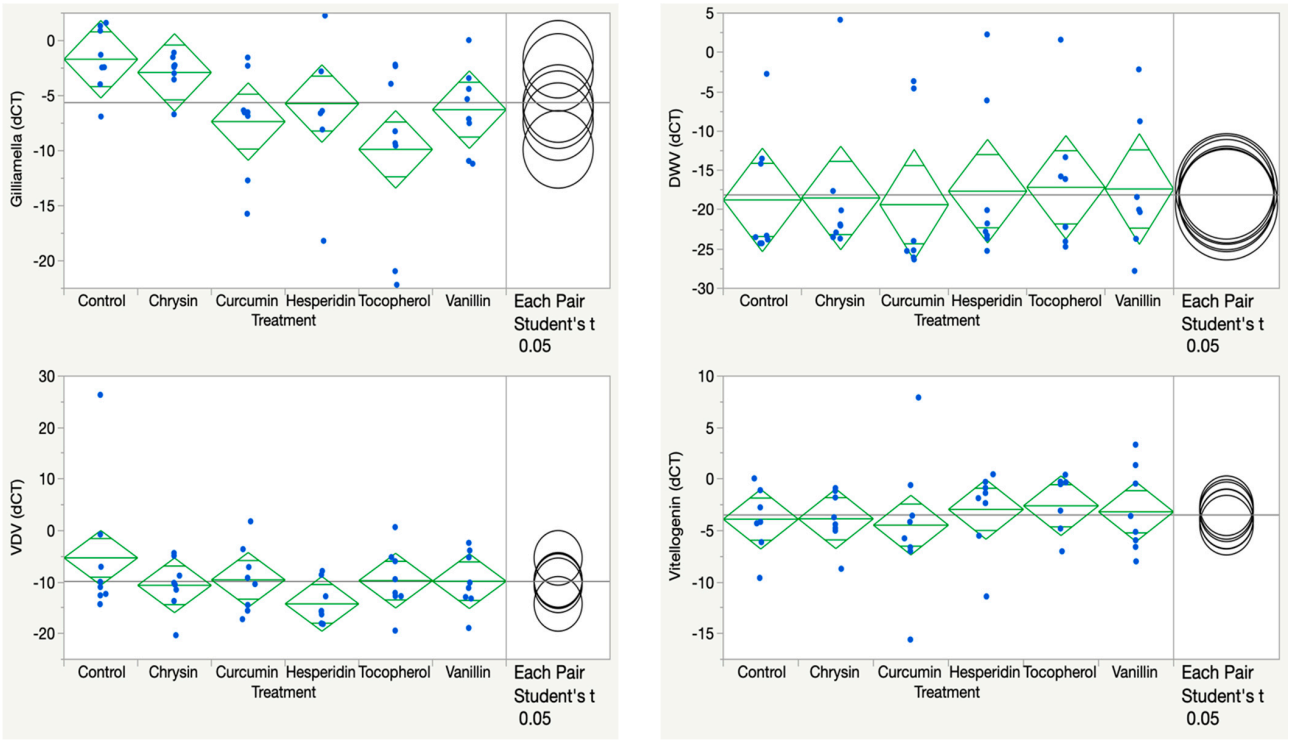

Figure 1. One-way analysis of viral/bacterial loads and vitellogenin by treatment. Normalized Cq values of two honey bee viruses, DWV and VDV, the honey bee gut bacterial symbiont, *Gilliamella*, and transcripts for the storage protein vitellogenin. Treatments were supplemented at 100 ppm in sugar syrup. The diamond tips represent the 95% confidence intervals for the ANOVA, with the mean designated with the middle horizontal line across each diamond. Comparison circles shown the means, where circles that overlap are not significantly different, whereas circles that do not overlap are significantly different.

Gilliamella was lower in all treatments tested, some significantly so (ANOVA DF = 6, $p = 0.0210$), such as tocopherol with an ~8 cycle, 2^7-2^8-fold difference compared to the control (mean control = −1.6958 +- 1.7447 (SE); mean tocopherol = −9.8808 +- 1.7623 (SE), $n = 8$, $p = 0.0019$). Vanillin showed a ~6-cycle difference (mean control = −1.6958 +- 1.7447 (SE); mean vanillin = −6.2737 +- 1.76447 (SE), $n = 8$, $p = 0.0706$), curcumin a ~7-cycle difference (mean control = −1.6958 +- 1.7447 (SE); mean curcumin = −7.3573 +- 1.7447 (SE), $n = 8$, $p = 0.0268$), hesperidin a ~5-cycle difference (mean control = −1.6958 +- 1.7447(SE); mean hesperidin = −5.7193 +- 1.7447 (SE), $n = 8$, $p = 0.1104$), and chrysin a ~3 cycle difference (mean control = −1.6958 +- 1.7447 (SE); mean chrysin = −2.8945 +- 1.7447 (SE), $n = 8$), with lower levels of *Gilliamella* detected, but not significantly different ($p = 0.629$) (Figure 1).

There are significant differences in the eater expression between some of the treatments and the control (DF = 5, $p = 0.0002$). Curcumin and tocopherol led to lower expression levels of the immune effector eater compared to the control (mean control = −13.938 +- 1.3716 (SE); mean curcumin = −19.284 +- 1.3716 (SE), $n = 8$, $p = 0.0086$ and mean control = −13.938 +- 1.3716 (SE); mean tocopherol = −17.306 +- 1.3716 (SE), $n = 8$, $p = 0.0.0899$). Curcumin showed a significant ~6-cycle reduction in Cq values, reflecting a 40-fold reduction in expression with this supplement (Figure 2). Chrysin, hesperidin, and vanillin remained consistent with the expression levels of the control group. With the exception of hesperidin (mean control = 2.6212 + 1.9925 (SE); mean hesperidin = −3.1320 + 1.9925 (SE), $n = 8$, $p = 0.0475$), which had a ~6 cycle difference, reflecting 64 times more expression than in the control, there were no significant differences in the expression of hymenoptaecin in any other treatments tested when compared to the control (Figure 2). There were no differences in the expression of vitellogenin in any treatment when compared to the control (DF = 5, $p = 0.9428$) (data not shown).

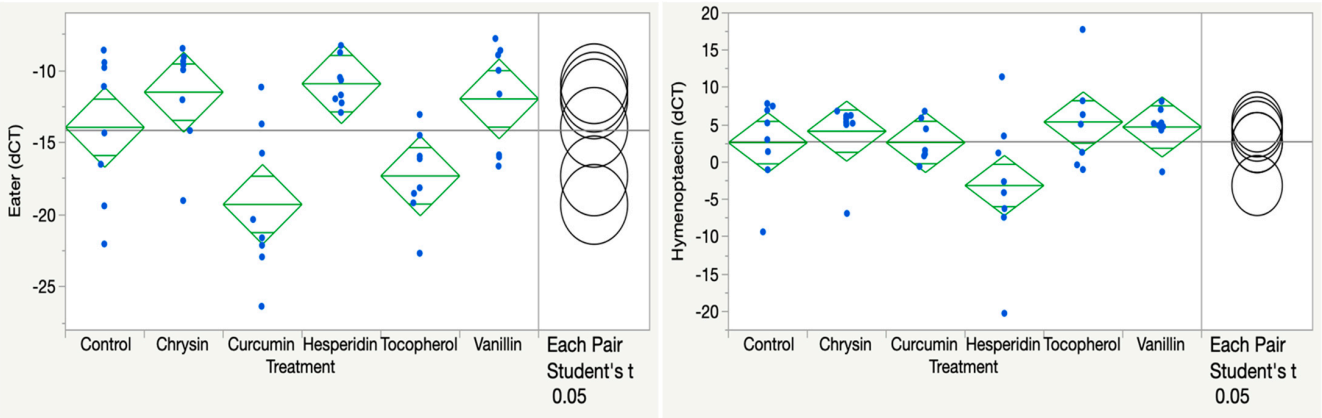

Figure 2. Analysis of honey bee immune genes by treatment. Normalized ΔCq values of two honey bee immune genes, eater and hymenoptaecin. Cup study, 8 replicates/cup per treatment or control. The control is sugar water. Treatments were supplemented with sugar water at 100 ppm. A larger value indicates a higher expression of the honey bee gene. The diamond tips represent the 95% confidence intervals for the ANOVA, with the mean designated with the middle horizontal line across each diamond. Comparison circles shown means, where circles that overlap are not significantly different, whereas circles that do not overlap are significantly different.

3.2. Raw Cacao, Roasted Cacao, Limonene, and Tyrosine

When adult honey bees were supplemented with 100 ppm raw cacao (fermented dried unroasted cacao bean shells), subsequently referenced to as raw cacao, there was a significant reduction in VDV compared to the control (mean control = −5.1170 +- 1.1099 (SE); mean raw cacao = −10.744 +- 1.1865 (SE), $n = 7$, $p = 0.00613$) (Figure 3). The other treatments, roasted cacao (fermented dried roasted cacao bean shells), subsequently referred to as roasted cacao, limonene, and tyrosine had VDV loads consistent with the control. All treatments had DWV levels similar to that of the control ($p = 0.2910$), with the exception of raw cacao, which appeared to increase the DWV loads (mean control = −4.3237 +- 1.6417 (SE);

mean raw cacao = 0.9717 +- 1.7621 (SE), $n = 8$, $p = 0.0307$), indicating a ~30-fold *increase* in DWV (Figure 3).

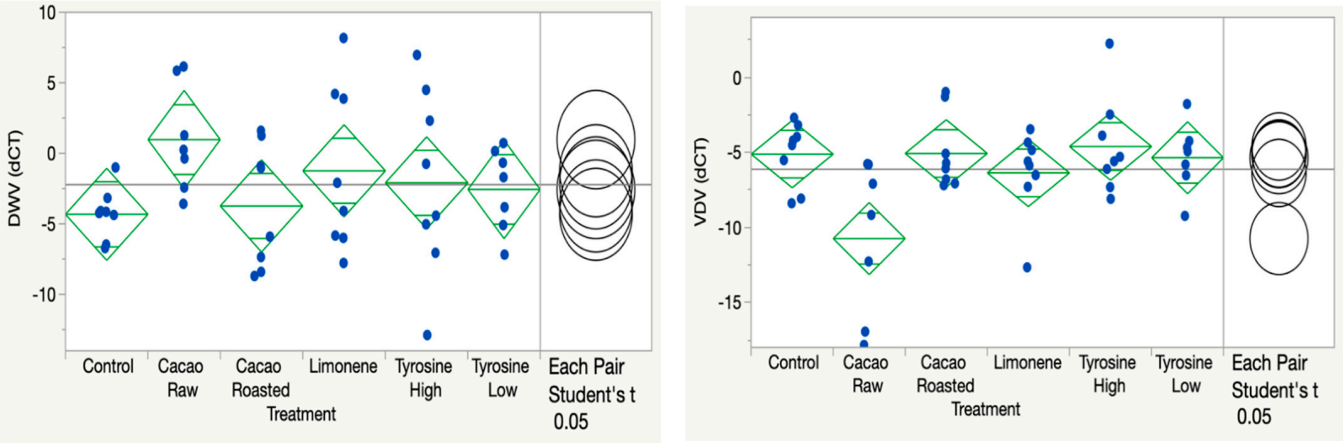

Figure 3. One-way analysis of DWV and VDV by treatment. The control was fed sugar water only. Treatments were dissolved in sugar water as described in the Methods section. The diamond tips represent the 95% confidence intervals for the ANOVA, with the mean designated with the middle horizontal line across each diamond. Increased values indicate an increased viral load. Student's *t*-test. Comparison circles shown means, where circles that overlap are not significantly different, whereas circles that do not overlap are significantly different.

3.3. Cat's Claw, Garlic Oil, Olive Leaf, Oregano Oil, and Elderberry

Although not significant, the DWV levels trended lower when young bees were orally inoculated with DWV and then fed garlic oil (ΔΔCq DWV-fed control = −6.6170 +- 2.4050 (SE); ΔΔCq DWV- and garlic-fed = −9.3700 +- 2.4050 (SE), $p = 0.4288$, $n = 10$) (Figure 4). This experiment followed a smaller, preliminary experiment, where DWV- and garlic-fed (effect not seen with simultaneous testing of oregano oil) young honey bees also showed a lower ΔΔCq compared to the respective control (ΔΔCq DWV-fed control = −4.2800 +- 2.3144 (SE); ΔΔCq DWV- and garlic-fed = −7.8870 +- 2.3114 (SE), $p = 0.2844$, $n = 10$) (Figure 4). This trial with garlic was also not statistically significant, despite the increase in dose from 1000 to 10,000 ppm. In both initial experiments, DWV infection was apparent, and a there was a consistent ~3-cycle (10-fold) decrease in DWV in garlic oil-fed bees vs. the controls in both experiments. These compounds were also tested by feeding field-collected adult honey bees of variable age, not inoculated with DWV, which showed no statistically significant ΔΔCq changes for garlic compared to the control, as well as for cat's claw, olive leaf, oregano oil, and elderberry (data not shown, ANOVA DF = 5, $p = 0.8800$).

3.4. Beta-Carotene

Supplementation by beta-carotene led to no difference in VDV loads between beta-carotene-fed bees and the control (ANOVA $n = 6$, DF = 1, $p = 0.6531$) or DWV ($n = 6$, DF = 1, $p = 0.6862$). The levels of the honey bee symbiont *S. alvi* were lower in the treatment samples versus the controls (mean control = 2.6885 +- 0.5713 (SE), $n = 10$; mean beta-carotene = 0.4533 +- 0.7376 (SE), $n = 6$, $p = 0.0311$) (Figure 5). There was also no difference in the immune-related genes hymenoptaecin ($n = 6$, DF = 1, $p = 0.8622$), abaecin ($n = 6$, DF = 1, $p = 0.3702$), apidaecin ($n = 6$, DF = 1, $p = 0.8347$), and eater ($n = 6$, DF = 1, $p = 0.5425$) between bees supplemented with beta-carotene and the controls. Similarly, a suite of physiological genes did not show differences between treatments and controls: Cytochrome p450 ($p = 0.1735$), defensin ($p = 0.8176$), Pgrp-lc ($p = 0.1353$), Vg ($p = 0.1391$), Mrjp1 ($p = 0.7023$), endochitinase ($p = 0.5428$), glucosidase ($p = 0.6669$), trehalase ($p = 0.2995$), and peritriphin, ($p = 0.5006$).

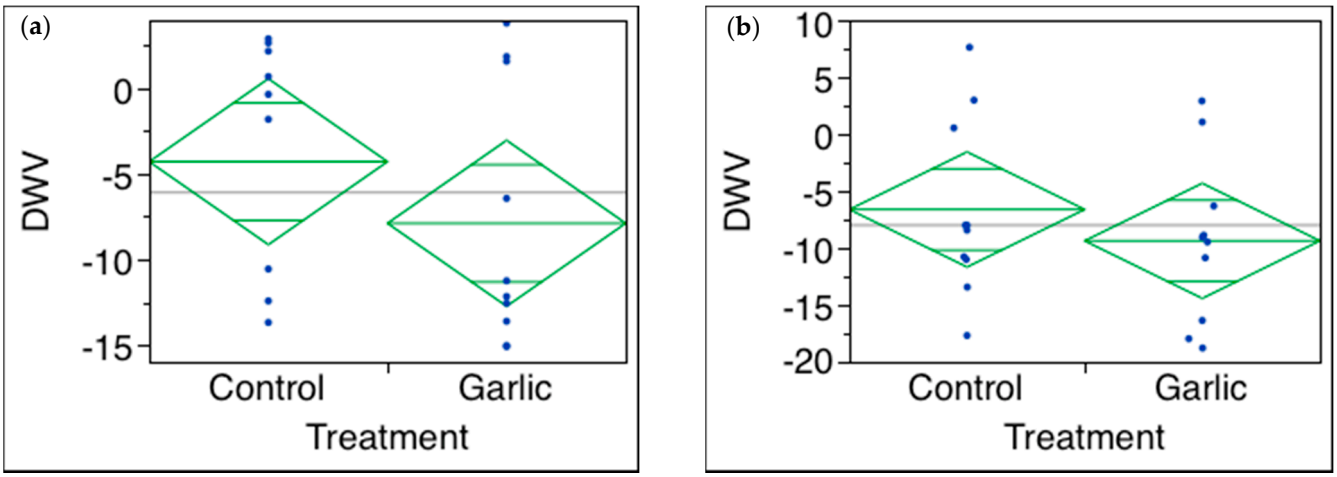

Figure 4. One-way analysis of DWV by treatment. Cup study with newly emerged bees, 10 replicate cups/treatment or control. The control is DWV-inoculated sugar-fed control. GAR was DWV-inoculated with garlic oil supplemented with sugar water at 1000 ppm (**a**) and 10,000 ppm (**b**). The Y-axis shows ΔCq. The diamond tips represent the 95% confidence intervals for the ANOVA, with the mean designated with the middle horizontal line across each diamond. Increased values indicate an increased DWV load. (**a**) Mean DWV-fed control = −4.2800 +- 2.3144 (SE) (mean DWV- and garlic-fed = −7.8870 +- 2.3114 (SE), $p = 0.2844$, $n = 10$). (**b**) Mean DWV-fed control = −6.6170 +- 2.4050 (SE); (mean DWV- and garlic-fed = −9.3700 +- 2.4050 (SE), $p = 0.4288$, $n = 10$).

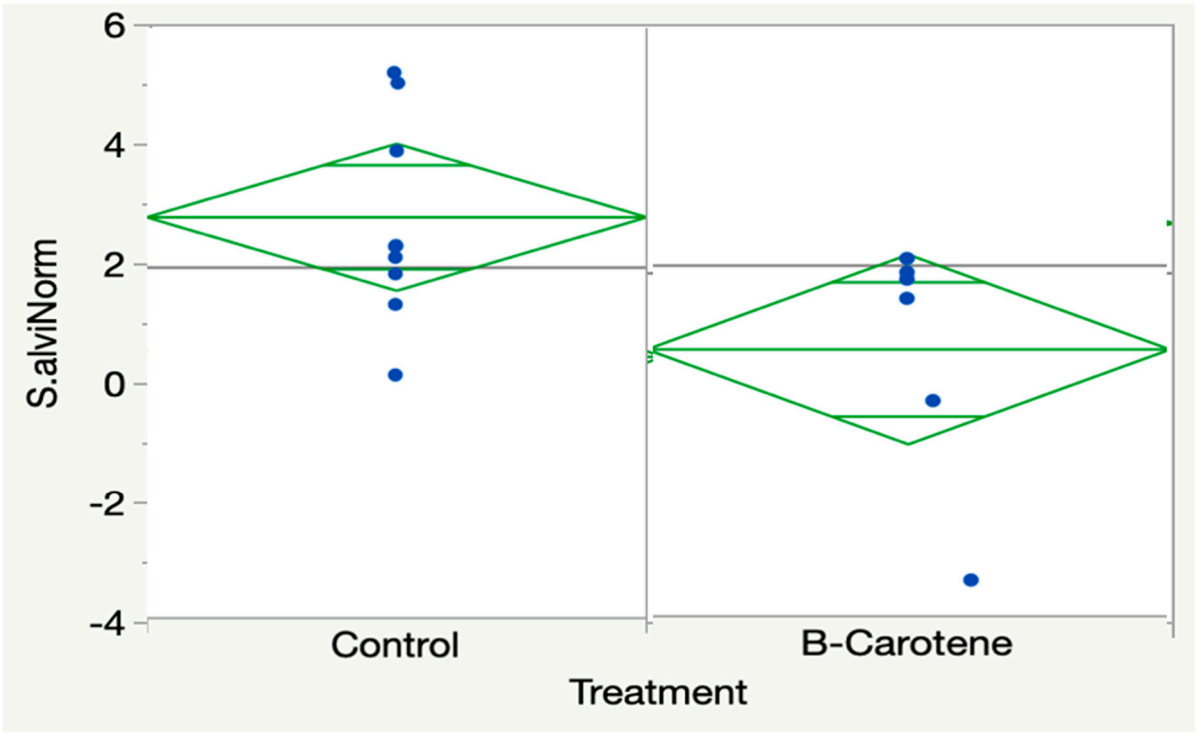

Figure 5. One-way analysis of a honey bee gut symbiont in bees fed beta-carotene vs. the control. Normalized Cq values of a cup study with 6 replicates/cup per treatment or 10 replicates/cup for the control. Treatments were supplemented with sugar water at 2000 ppm. A larger value indicates increased detection of the honey bee gut symbiont *S. alvi*. The diamond tips represent the 95% confidence intervals for the ANOVA, with the mean designated with the middle horizontal line across each diamond.

3.5. Carvacrol, Cinnamic Acid, and Ginger

When honey bees were supplemented with carvacrol, cinnamic acid, or ginger, there were no differences in DWV loads compared to the control (DF = 3, p = 0.9651), no differences in vitellogenin expression compared to the control (DF = 3, p = 0.3915), and no differences in eater expression compared to the control (DF = 3, p = 0.7326). However, hymenoptaecin expression was significantly lower when bees were supplemented with cinnamic acid (mean control = −0.6965 +- 1.3806 (SE); mean cinnamic acid = −4.8787 +- 1.3806 (SE), n = 10, p = 0.0392). When bees were supplemented with carvacrol (mean control = −0.6965 +− 1.3806 (SE); mean carvacrol = −3.4426 +- 1.4553 (SE), n = 9, p = 0.1797) or ginger (mean control = −0.6965 +- 1.3806 (SE); mean ginger = −2.1924 +- 1.3806 (SE), n = 10, p = 0.4487), there was no difference in hymenoptaecin expression compared to the control (data not shown). No significant or trending differences in viral load were observed for astragalus, berberine (toxic to bees at 100 ppm), calendula, echinacea, ginger, licorice, or limonene.

4. Discussion

A main priority of this study was to explore additional natural products for their activities against honey bee viruses, namely, DWV and VDV. While we did not find consistent results across all products tested, we did find some that affected viral loads. Intriguingly, raw cacao had significant effects on the level of both DWV and VDV when bees were supplemented with these NPs. The tropical rainforest tree *Theobroma cacao L* produces cacao beans that must be fermented, dried, and roasted to make chocolate. Cacao beans have a broad range of bioactive chemical compounds, such as polyphenols, flavonols, and procyanidins [23,45]. These compounds are found in the nib (used to make chocolate) and the seed coat (a waste product). Many of these chemical compounds are in higher concentrations [46] prior to roasting, which results in losses of up to 71% for total phenolic compounds and 53–77% for antioxidants [47,48]. For this reason, unroasted and roasted cacao bean shells were used in this study. Interestingly, the DWV levels increased (p = 0.0307), whereas the VDV levels decreased (p = 0.0013) with similar 30-fold changes in viral loads, albeit in opposite directions. Hesperidin is a major flavonoid found in citrus fruits such as lemons and sweet oranges, as well as in some other fruits and vegetables [49]. Hesperidin has been linked to a range of health benefits, including antioxidant, analgesic, anti-carcinogenic, anti-hypertensive, anti-viral, and anti-inflammatory effects [50]. Hesperidin also significantly lowered the VDV levels (p = 0.0208). Generally, of those treatments showing effects, most trends exhibited a lowering of viral loads that were not statistically significant (i.e., chrysin, curcumin, limonene, tocopherol, tyrosine, and vanillin (Table 2)). Others, despite showing promising properties in the literature, showed little or no effect in our experiments. For example, carvacrol is a phenolic monoterpenoid found in essential oils of oregano, thyme (with current applications fighting bee pests), pepperwort, and other plants. While carvacrol possesses a wide range of biological activities, including antibacterial, antifungal, antioxidant, and anticancer effects [51] we were unable to achieve differences in disease loads for bees fed carvacrol.

Another potential avenue to study is the effect of natural products on eukaryotic parasites afflicting honey bees. Herein, we looked at levels of *Nosema ceranae* (microsporidian parasite) and trypanosomes (parasitic protozoon). During the course of this experiment, we found no *Nosema* infections in the honey bees tested in either the control or treated groups, and we were therefore unable to test the efficacy. We found no significant differences in the trypanosome levels in the treatments tested (chrysin, curcumin, hesperdin, tocopherol, and vanillin). We also looked at the effects of compounds on the honey bee core microbiota, specifically *Snodgrasella alvi* and *Gilliamella apicola. G. apicola* showed significant decreases when bees were fed diets supplemented with tocopherol, giving rise to a 28-fold difference in expression (p = 0.0019). Tocopherol, vitamin E, has been reported in the prevention of oxidative damage or the modulation of signal transduction and gene expression in antioxidant and non-antioxidant manners [52]. Interestingly, almonds have the highest amount of

tocopherol of all nuts [53] and are frequented by honey bees in yearly almond pollination services. Likewise, albeit less significant, vanillin (selected for its potential antimicrobial properties [54]), curcumin, hesperidin, and chrysin treatments also led to lower levels of *Gilliamella*. A shift in the honey bee microbiota, in any direction, should be interpreted carefully. For example, *Gilliamella* is a honey bee gut symbiont that is responsible for the breakdown of potentially harmful/toxic sugars produced by pollen digestion [55]; this argues that more of this bacteria is better for bees. Alternatively, a higher abundance of *Gilliamella* could also infer potential negative aspects, as bees reported as having high amounts of this taxon (i.e., in honey bees, after feeding them with *Snodgrassella*, resulting in an increase in *Gilliamella*) also have higher levels of infection with the gut parasitic trypanosome, *L. passim* [2]. When beta-carotene, a bioactive carotenoid with potent antioxidant activity [56], was supplemented to honey bees, levels of *S. alvi*, another core member of the honey bee gut microbial community, were significantly decreased ($p = 0.0311$).

Table 2. Overview of natural product or compound tested and observed effects. Gene expression (up- or downregulated) of a target gene or virus/microbe detection was relative to the sugar water control for that experiment. NS = no significant change; grey = $p < 0.1$; green = $p < 0.05$; red = $p < 0.01$.

Compound	Honey Bee Immune Gene	Virus/Microbe
Beta-carotene 2000 ppm	Abaecin NS ($p = 0.3702$) Hymenoptaecin NS ($p = 0.8662$) Apidaecin NS ($p = 0.8347$) PGRP-LC NS ($p = 0.1352$) Vitellogenin NS ($p = 0.1391$) Eater NS ($p = 0.5425$) MRJP1 NS ($p = 0.7023$) CytochromeP450 NS ($p = 0.1735$) Defensin1 NS ($p = 0.8176$) Endochitinase NS ($p = 0.5428$) Glucosidase NS ($p = 0.6669$) Trehalase NS ($p = 0.2995$) Peritrophin NS ($p = 0.5006$)	DWV NS ($p = 0.6862$) VDV NS ($p = 0.6531$) *S. alvi*lower (mean control = 2.6885 +- 0.5713 (SE) $n = 10$; mean beta-carotene = 0.4533 +— 0.7376 (SE), $n = 6$, ($p = 0.0311$)
Cacao (raw) 100 ppm		DWV increased (B control vs. A cacao raw, $p = 0.0307$) VDV decrease (A control vs. B cacao raw, $p = 0.0013$)
Cacao (roasted) 100 ppm		DWV NS (B control vs. AB cacao roasted, $p = 0.7970$) VDV NS (A control vs. A cacao roasted, $p = 0.9769$)
Carvacrol 100 ppm	Eater NS ($p = 0.3931$) Hymenoptaecin lower (A control vs. AB Carvacrol $p = 0.1797$) Vitellogenin NS ($p = 0.9502$)	DWV NS ($p = 0.8588$)
Cat's Claw Jill2 1000 ppm		DWV NS ($p = 0.5532$)
Chrysin 100 ppm	Eater lower (AB control vs. A Chrysin, $p = 0.2149$) Hymenoptaecin NS ($p = 0.5896$) Vitellogenin NS ($p = 0.9854$)	DWV NS ($p = 0.9571$) Trypanosome NS ($p = 0.1276$) VDV lower (A control vs. AB chrysin $p = 0.1592$) *Gilliamella* lower (A control vs. AB chrysin $p = 0.6296$)
Cinnamic acid 100 ppm	Eater NS, $p = 0.8382$ Hymenoptaecin lower (A control vs. B cinnamic acid, $p = 0.0392$) Vitellogenin NS ($p = 0.1909$)	DWV NS, $p = 0.8911$
Curcumin 100 ppm	Eater lower (AB control vs. C curcumin, $p = 0.0086$) Hymenoptaecin NS ($p = 0.9952$) Vitellogenin NS ($p = 0.7768$)	DWV NS ($p = 0.9001$) Trypanosome NS ($p = 0.1776$) VDV lower (A control vs. AB curcumin $p = 0.2573$) *Gilliamella*lower (A control vs. BC curcumin $p = 0.0268$)
Elderberry Jill2 1000 ppm		DWV NS ($p = 0.5940$)
Ginger 100 ppm	Eater NS $p = 0.7334$ Hymenoptaecin lower (A control vs. AB ginger $p = 0.4487$) Vitellogenin NS ($p = 0.2233$)	DWV NS ($p = 0.7434$)

Table 2. *Cont.*

Compound	Honey Bee Immune Gene	Virus/Microbe
Garlic oil 1000 (A&B) or 10,000 ppm (C)		DWV NS (p = 0.2780) (Supplemental File experiment A) DWV NS (p = 0.6484) (B) DWV NS (p = 0.4288) (C)
Hesperidin 100 ppm	Eater lower (AB control vs. A hesperidin, p = 0.1260) Hymenoptaecin lower (A vs. B P = 0.0475) Vitellogenin NS (p = 0.6422)	DWV NS (p = 0.8099) Trypanosome NS (p = 0.3059) VDV lower (A control vs. B hesperidin p = 0.0208) *Gilliamella* lower (A control vs. ABC hesperidin, p = 0.1104)
Limonene 100 ppm		DWV NS (B control vs. AB limonene, p = 0.1844) VDV NS (A control vs. A limonene, p = 0.4293)
Olive leaf 1000 ppm		DWV NS (p = 0.2295)-(B)
Oregano oil 1000 ppm		DWV NS (p = 0.3750)-(A) DWV NS (p = 0.5759)-(B)
Tocopherol 100 ppm	Eater lower (AB control vs. BC tocopherol, p = 0.0899) Hymenoptaecin NS (p = 0.3320) Vitellogenin NS (p = 0.5248)	DWV NS (p = 0.7300) Trypanosome NS (p = 0.4107) VDV lower (A control vs. AB tocopherol p = 0.2414) *Gilliamella*lower (A control vs. C tocopherol, p = 0.0019)
Tyrosine (high) 100 ppm		DWV NS (B control vs. AB tyrosine (high), p = 0.3345) VDV NS (A control vs. A tyrosine (high), p = 0.7490)
Tyrosine (low) 10 ppm		DWV NS (B control vs. AB tyrosine (low), p = 0.4598) VDV NS (A control vs. tyrosine (low), p = 0.8813)
Vanillin 100 ppm	Eater lower (AB control vs. A vanillin, p = 0.3416) Hymenoptaecin NS (p = 0.4622) Vitellogenin NS (p = 0.7257)	DWV NS (p = 0.7720) Trypanosome NS (p = 0.1548) VDV lower (A control vs. AB vanillin p = 0.2301) *Gilliamella*lower (A control vs. ABC vanillin, p = 0.0706)

Monitoring immune gene expression, along with viruses and microbes, offers additional information. The protein Eater is involved in cellular immunity, Apidaecin and Hymenoptaecin are antimicrobial peptides involved in humoral immunity [40], and Vitellogenin is a marker of general honey bee health [57]. By measuring the responses of these genes, we could determine the effect of the compounds on the immune response of the honey bee. There is significant downregulation in eater gene expression between some of the treatments and the control in curcumin and tocopherol treatments (p = 0.0086 and p = 0.089, respectively) with up to a 40-fold reduction in gene expression. Curcumin is a flavonoid from the rhizome of *Curcuma longa* with well-recognized antioxidant, anti-inflammatory, antimicrobial, and antiviral properties. Curcumin has been linked to a range of health benefits, including potential protection against cancer, neurodegenerative disorders, and metabolic disorders in humans [58–60]. Hymenoptaecin only showed altered expression in treatment with one natural product, hesperidin, with significantly lower levels in treatment vs. the control (p = 0.0475). Cinnamic acid is the first molecule in the phenylpropanoid pathway and is the source of most hydroxycinnamic acid derivatives such as coumaric, caffeic, ferulic, and sinapic acids normally present in plant material in either ester or glyosidic forms [61]. Cinnamic acid is well known for its antioxidant, antitumor, antimicrobial, and antimycobacterial properties [62]. Cinnamic acid and hesperidin both also lowered hymenoptaecin levels (p = 0.0392 and p = 0.0475, respectively), whereas apidaecin and vitellogenin showed no significant changes with the natural products tested here. Together with the effect on viruses, pathogenic agents, or beneficial microbes, we can see that NPs have a range of effects on honey bee physiology, and further research on the mechanisms behind such effects could be of great value to further understand the many potential uses of these potent compounds.

Whereas some extracts show potential promise in their use as prophylaxis or treatment of disease, our success rate in finding compounds with significant impacts was low. It is also important to note that some compounds were eliminated from further study, as initial tests showed toxicity (i.e., decanoic acid and berberine) to honey bees (all life stages must be

considered), highlighting the importance of standardized and thorough testing of products. One way to overcome adverse effects is via delivery methods. For example, miticide oxalic acid is vaporized as a means of colony treatment, rather than being introduced via feeding bees. Moreover, chemical modifications of NP structures using computer-based docking simulations may also increase their potency or selectivity [18]. Nevertheless, we feel it is important to first assess the dosing, uptake by bees in sugar water, acute toxicity (or related sublethal effects) and effects on the honey bee gut microbiome, proteomics, and metabolomics. For promising candidates, it will be important to next assess impacts at the colony and apiary levels, measuring both disease loads and, ultimately, colony growth and survival.

On the market today are numerous honey bee nutritional supplements for which a natural product is the purported active ingredient. None of these are approved as drugs by the U.S. Food and Drug Administration. To be approved as a drug, the chemical must (1) be safe and (2) effective in honey bees; (3) have an acceptable environmental impact; (4) be manufactured with good practices; and (5) be safe for people to consume a small amount in honey. Few chemicals satisfy all five of these criteria and it can take years and millions of dollars to demonstrate the safety and efficacy of a successful candidate. One promise of natural products, as compared to synthetic chemicals, is a richer, more complex, and more diverse space of structure. Moreover, the acceptable daily intake, toxicity, manufacturability, and environmental impact of many natural products are already known. This prior information may substantially reduce the time and cost of conducting regulatory studies.

One source of potential natural product drugs is the U.S. Food and Drug Administration (FDA) catalog of over 450 compounds, plant extracts, and classes of plant-based molecules, which it considers "generally regarded as safe" (GRAS) for human consumption [63] when consumed at a reasonable level. A compound derived from this list that is safe and effective in honey bees could be more likely to receive regulatory approval. Low-cost regulatory approval would benefit beekeepers with a product retail price that does not need to subsidize millions of dollars of regulatory studies. This consideration is especially important for honey bees because hive products make their way into the lives of humans as wax and royal jelly in cosmetics, pollen and honey as food, and bee venom in apitherapy.

The chemicals studied here are only a glimpse into the diversity and depth of natural products. The use of natural products is favored by beekeepers over synthetic chemicals or antibiotics in general, as they do not want to add toxic chemicals to their bees or the environment. The methods described here and by others [27,37] can be used to vet hundreds of these compounds to identify those with favorable health impacts. We believe that the screening of natural products, along with computational drug discovery and the repurposing of existing drugs, will be critical for curing diseases in honey bees.

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