Egypt. J. Chem. **Vol. 66,** No. 3 pp. 351 - 358 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Antibacterial Activity of some Honey Bee Products Treated in Controlling the American Foulbrood, Paenibacillus larva

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Abstract

This study aimed to evaluate the effectiveness of some honey bee products treated with compounds, including organic acids, antibiotics, and extract oils, as antibacterial agents for controlling the American foulbrood disease (AFB). The effectiveness of aqueous and ethanolic pollen extract, honey, and royal jelly at 5%, 10%, and 15% concentrations in controlling AFB was assessed in combination with formic acid, oxalic acid, lactic acid, tylosin, and carnation treatments. The treatment with formic acid positively impacted the antibacterial activity of the aqueous pollen extract (APE) and honey (8.22, 10.71) respectively. However, it decreased the activity of the ethanolic pollen extract (EPE) and exhibited an insignificant effect on royal jelly (8.21). The carnation oil only enhanced the activity of ethanolic pollen extract (7.10). The Oxalic acid, lactic acid, and tylosin either decreased the activity of the tested products or had an insignificant impact. We conclude that although antibiotics, extract oils, and organic acids are valuable in controlling pathogenic microorganisms, there are still concerns regarding their residual effects on honeybee products. Thus, further studies are required to find reliable control alternatives with insignificant residual effects either through screening other existing substances or developing new ones.

Keywords: American foulbrood (AFB), Bee pollen, Honey, Residue, Royal jelly

1. Introduction

Bee infectious diseases contaminating derived products are commonly controlled with antibiotics and chemicals. The World Health Organization (WHO) currently recognises multidrug resistance as a serious developing issue of global significance due to the rapid increase in multidrug resistance caused by widespread use of antibiotics [1]. The contamination of bee products can originate from numerous sources; acaricides (e.g., organic acids and components of essential oils), antibiotics used for control of bee brood diseases (mainly tetracyclines, sulfonamides, streptomycin, and chloramphenicol), and dichlorobenzene, which is used for the control of hives diseases [2,3,4,5]. Environmentally harmless substances such as methyl salicylate, clove oil, formic acid, sulfur, acetic acid, and basil oil are harmful to bee brood and human health [6, 7]. [8] reported the high toxicity of oxalic acid to bees. In contrast, menthol and cinnamon oil exhibited no difference in mortality compared with the controls fed on the plain sugar syrup. Although most studies have concerned residues in honey, royal jelly can be contaminated by antibiotics [9,10]. Formic and oxalic acids are among the most common compounds naturally occurring in honey; they are widely used as agents for controlling the *Varro destructor*. In particular, oxalic acid was reported to have low acute toxicity on honey bees and high acute toxicity on mites [11].Oxalic acid and clove oil are natural substances to treat the American foulbrood (AFB) disease and control the ectoparasite, *V. Jacobsoni* [12,13]. *Paenibacillus larvae* infect old larvae [14], and young pupae are digested by certain enzymes secreted by the bacteria inside the colony [15, 16]. Control with antibiotics can be effective but may reduce the life span of bees and lead to the emergence of resistant strains. Control with acaricides and other chemotherapeutics may lead to residues in honey, affecting its quality and limiting its use for human consumption [17, 18]. Another type of antibiotic used to control AFB is Oxytetracycline. However, this can also be a source of contamination for honey [19]. In general, the use of antibiotics and

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Receive Date: 05 May 2022, Revise Date: 08 July 2022, Accept Date: 19 July 2022

DOI: 10.21608/EJCHEM.2022.132516.6040

sulfonamides is the cause of residues in beehive products and constitutes a threat to food safety[20]. Honey is one of the well-known compounds with tremendous health benefits. Its effectiveness is associated with its high osmolality, low pH, and hydrogen peroxide content, for which it is also referred to as a natural source of inhibition. Non-peroxide antibacterial substances such as aromatic acids, phenolic, and flavonoids are groups of the antibacterial present in honey [21, 22]. [23] assessed the antibacterial activity of trade honey samples and naturally occurring Iraqi honeys (Sidr, Eucalyptus) against MRSA Staphylococcus heamolyticus that was isolated from burn patients in Alsader Hospital-Misan City. Royal jelly is another useful bee product. Its benefits cannot be attributed to any individual components. Numerous studies have demonstrated a wide range of medicinal properties in royal jelly, including antimicrobial effects [24, 25]. These antibacterial effects might be related to particular fatty acids in the ether-soluble fraction of royal jelly known as trans-10-hydroxy decanoic acid [26]. The low molecular weight of royal jelly proteins and peptides seems to constitute a host-defence function against honeybee pathogens [14]. Moreover, a potent antibacterial protein in royal jelly called defensin-1 (formerly known as royalisin) is active against various Gram-positive bacteria at low concentrations but not against Gram-negative bacteria [25]. The therapeutically properties of pollen, essential oils, and organic acids also been widely studied. Bee pollen is consumed for its api-therapeutical, nutritional, and medicinal properties [27, 28]. Its effectiveness is attributed to its chemical composition, mainly comprising carotenoids, fatty acids, vitamins, minerals, essential amino acids, and phenolic [29]. Numerous essential oil products demonstrated advantages such as high toxicity to mites, low toxicity to bees, few residues in the derived products, and no promotion of microbial resistance. They are valuable alternatives to acaricides and traditional pesticides [30, 13]. [31] determined residue contamination in honey and observed that the levels of thymol and formic acid residues in the experimental groups significantly exceeded those observed in the control group. Considering the problems associated with conventional disease control in apiculture, finding new solutions is essential for avoiding harming bees or affecting their products and the food safety for consumers.

The objective of this study is to evaluate the effectiveness of several honey bee products processed with certain organic acids, antibiotics, and essential oils as antibacterial agents for controlling AFB disease by comparing pre-and post-treatment for bee colonies.

2. Materials and Methods

2.1. Experiment time

The experiments were performed in the Department of Apiculture, Plant Protection Research Institute, Agriculture Research Center, Dokki, Giza, Egypt, during spring, summer and autumn seasons in (2019–2020).

2.2. Preparing honey bee colonies

Fifteen experimental honey bee colonies (Carniolan hybrid F1) were chosen for this study, and divided into five groups, with three replicates colonies per group. Three colonies were assigned for each treatment (5 treatments) with one of the following compounds used to control honey bee diseases and parasites: carnation oil, oxalic acid, formic acid, lactic acid, and tylosin over 16 days.

2.2.1. Treatments and dosages

First group: Carnation oil used as a natural substance in controlling AFB, and *Varroa destructor* was used as a suspension by mixing 3 mL of crude oil with 3 mL of Tween 80 (emulsifier) and adding it to 500 mL of a sugar solution (1:1). This was used to feed the honey bee colonies every 4 days overa period of16 days.

Second group: Oxalic acid was used at a concentration of 3% by adding 3.0 g of oxalic acid to 97 mL of water and spraying it between the bee combs. The treatment was repeated every 4 days over a period of 16 days.

Third group: Lactic acid was effective only against *Varroa destructor* mites present on the adult bees and came into contact with droplets This treatment involved removing each frame and spraying a 15% solution on each side. The treatment was repeated every 4 days over a period of 16 days [32].

Fourth group: Formic acid treatment was used as an evaporator for *Varroa destructor* control. It consisted of a plastic bottle filled with a formic acid solution (60% concentration), a wick, and a patented evaporation system that allowed the acid solution to slow release. The most favorable performance (without harming the bees) with this evaporator was achieved at a temperature of 8℃–35℃. The treatment was over a period of 16 days [33].

Fifth group: Tylosin was effective only against AFB.The active ingredient of tylosin was used as a suspension by mixing 1 ml of the antibiotic (1% concentration) with 99 mL of sugar solution (1:1) as feed, which was done once per week and repeated three times [34].

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2.2.2. Collection of honey, royal jelly, and pollen samples

Honey samples were aseptically collected before and after the treatments in sterile screwed cups and stored overnight in a dark, dry place at room

temperature .The honey bee queens were removed from the bee hives, and the royal jelly was collected from their cells on the third day and stored at -18℃..

Pollen grains were collected as pellets sing pollen traps attached to the hives and stored at −20℃.

2.3. Preparation and isolation of bacteria

According to a diagnostic test by [35] *Paenibacillus larvae* (previously *Bacillus larvae*) bacteria were obtained from an infected brood comb by collecting bacterial spores from the dried remains of infected bees and inoculating them. Using sterile cotton swabs suspended in 9 mL sterile distilled water in a screw-capped container, streaks were taken. The suspension was freeze-shocked at −80℃ for 10 min to kill any non-spore-forming bacteria. Then, 0.2 ml of the stock suspension was used for a bioassay [36]. Following the isolation of *P. larval* bacteria, the bacteria were grown in Columbia sheep blood agar and cultured for 3 days at 37°C to prepare the inoculation. The bacterial culture was then transferred to a liquid medium (brain heart infusion broth, Oxoid ltd) and cultured at 37°C for 48 hours. Then, until needed, 1 mL of the aqueous suspension was frozen at −70°C.*P. larvae* were verified using a catalase test with Columbia sheep blood agar slants [37] and then injected with 40 mL autoclaved brain heart infusion broth and 1 mL defrosted bacterial suspension. The suspension was heat-shocked at 77°C for 10 minutes before being incubated at 37°C for 48 hours, resulting in an optical extinction of 0.22–0.23.

2.4. Sampling and assay

The obtained honey bee products were extracted and tested for their antibacterial activities at the Microbial Genetic Department, Genetic Engineering and Biotechnology Division, National Research Center, Giza, Egypt.

2.4.1. Honey samples preparation

Honey solutions were prepared immediately before testing by diluting honey samples with various quantities of sterile distilled water to reach the required concentrations of 5%, 10%, and 15% of honey per the final volume. All samples were then incubated for 30 min at 37℃ in a shaking water bath. Incubation was performed in the dark [38].

2.4.2. Preparation of royal jelly suspension for microbiological tests

The suspensions containing 5%, 10%, and 15% royal jelly in sterile distilled water were prepared in clean, sterile test tubes. They were stored in a refrigerator at 5℃–7℃.

2.4.3. Preparation of ethanolic extracts from pollen grains

A total of 20 g of pollen grain samples were milled, homogenized, and extracted separately using 100 mL of 70% ethanol solution at 5%, 10%, and 15% as the extraction solvent. The solid residue was re-extracted after the supernatant was separated. The pollen grains were then mixed with ethanol extracts and kept at 5°C. All of the samples were extracted twice.

2.4.4. Preparation of aqueous extracts from pollen grains

A total of 20 g of pollen grain samples were milled, homogenized, and extracted separately using 100 mL distilled water as the extraction solvent at 5%, 10%, and 15%. The solid residue was re-extracted after the supernatant was separated. The pollen water extracts were then mixed and kept at 5°C.

2.4.5. Determination of inhibition zone diameters

The antimicrobial activity of honey bee products was determined using the paper disk plate method described by Anon (1982) [39].

2.5. Statistical analysis

The statistical analysis data of all treatments were analyzed in a randomized complete block design analysis of variance by MSTAT-C version 1.41 [40]. All means were compared using Duncan's multiple range tests at a level of 0.05.

3. Results and discussion

3.1. Antibacterial activity of pollen collected from colonies treated with organic acid, carnation oil, and the antibiotics,tylosin.

From the data recorded in **Fig. 1** and **Table 1**, the mean inhibition zone at all concentrations (5%, 10%, and 15%) of aqueous pollen extract (APE) exposed to the residue of oxalic acid was 10-15 mm pre-treatment and 8.08 mm post-treatment, the lactic acid residue (10-45 mm pre-treatment and 7.67mm post-treatment), tylosin residue (9.07mm, 7.80 mm) and carnation residue (8.62 mm, 7.10 mm), which had a negative effect on the antibacterial activity against the AFB bacteria form. In contrast, a formic acid residue demonstrated a positive effect (7.63 mm, 8.22 mm).

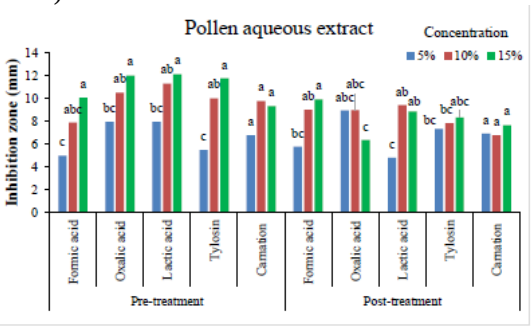

Fig.1 Effect of aqueous pollen extract treated on inhibition zone (mm) of American foulbrood

Also, as shown in **Fig. 2** and **Table 1**, the mean inhibition zone at all concentrations (5%, 10%, and 15%) of ethanol pollen extract (EPE) exposed to formic acid was 7.87 mm pre-treatment and 6.64 mm post-treatment. Moreover, the oxalic acid residue detected 8.28 mm pre-treatment and 8.18 mm post-treatment. They had negatively affected the antibacterial activity of EPE against the AFB bacteria form. In contrast, lactic acid (8.97 mm, 9.12 mm) and

tylosin (9.95 mm, 9.62 mm) residues did not have any effect, but the carnation residue (9.59 mm, 10.84mm) had a significant positive difference.

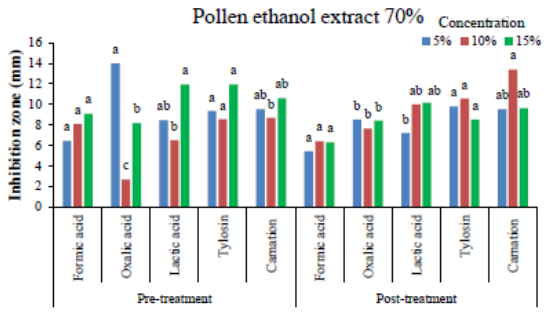

Fig. 2 Effect of pollen ethanol extract treated on inhibition zone (mm) of American foulbrood

3.2. Antibacterial activity of honey collected from colonies treated with organic acid, carnation oil, and tylosin

Likewise in **Fig. 3** and **Table 1**, the mean inhibition zone at all concentrations (5%, 10%, and 15%) of honey exposed to the formic acid residue (7.59 mm, 10.71 mm) had a significant positive effect on the antibacterial activity of honey against the AFB bacteria form. In contrast, another treatment had a significant adverse effect on the honey quality

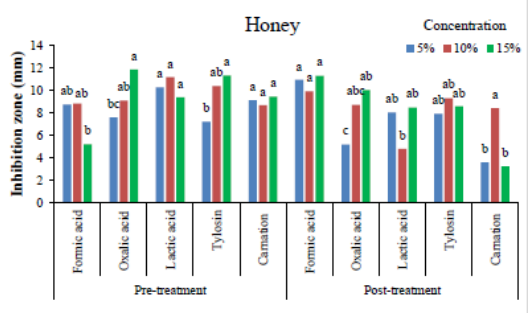

Fig. 3 Effect of honey treated on inhibition zone(mm) of American foulbrood

3.3. Antibacterial activity of royal jelly collected from colonies treated with organic acid, carnation oil, and tylosin

As shown in **Fig. 4** and **Table 1**, the mean inhibition zone at all concentrations (5%, 10%, and 15%) of royal jelly exposed to tylosin residue was 6.79mm pretreatment and 4.17 mm post-treatment, which negatively affected the antibacterial activity of royal jelly against the AFB bacterial. In contrast, other treatments did not detect affected royal jelly quality. On the other hand, there were significant differences among all concentrations (5%, 10%, and 15%) of all products pre or post-treatment against the AFB bacteria.

Although numerous studies proved the effectiveness of antibiotics, essential oils, and organic acids in controlling pathogenic microorganisms and mites, some reports showed concerns regarding their residual effects on honey bees and derived products.

The present study revealed a significantly varied effect of all tested products on the AFB bacteria before and after each treatment. Several studies found that oxalic acid treatments, whether single or repeated, did not cause residues' accumulation in honey [21, 41, 42].

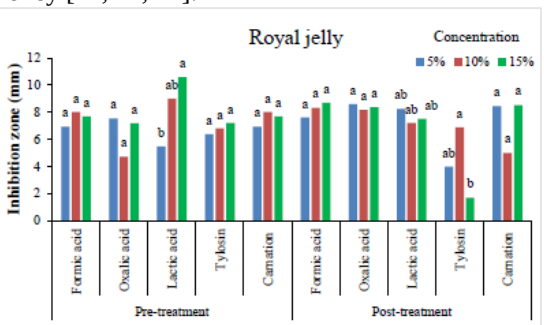

Fig. 4 Effect of royal jelly treated on inhibition zone (mm) of American foulbrood

Also, formic acid is a natural component of honey and demonstrates a strong acaricidal effect [43]. It is inexpensive]and does not leave any residues above the naturally present levels in honey [21, 44]. Tylosin was also demonstrated to effectively control the AFB in laboratory and field studies [34].

In our study, tylosin treatment negatively affected the quality of aqueous pollen extract, honey, and royal jelly but did not affect the EPE. [45,46] reported that although tylosin seemed effective in treating AFB, antibiotics were not recommended and even prohibited in some countries because they were considered a significant contaminant to bee products, mainly honey and royal jelly**.**

The results are consistent with those of [47], indicating that pollen grains' antibacterial activity against *Paenibaciillus larvae*, perhaps due to other biological properties, correlated with their pollen ethanol aqueous concentrations but primarily to their chemical composition, which could be a variable vegetal source. [48] indicated that propolis, pollen, bee wax, and honey showed antibacterial properties against Gram-positive and Gram-negative bacteria and demonstrated that pollen exhibited the highest antibacterial activity compared with the other bee products against all tested organisms. *Staphylococcus aureus* (Gram-positive bacteria) exhibited the highest sensitivity to ethanolic pollen extract among the tested bacteria. [49] evaluated venom of Egyptian honey bee in vitro and in vivo for its antibacterial effects as natural antibacterial products after its safety and effectiveness against *S. aureus* (Gram-positive bacteria). It is demonstrated that Egyptian honey bee has antibacterial activity. Different sensitivity patterns to the pollen loads were attributed to distinct phenolic compounds in pollen [50, 29].

Table I. Antibacterial activity of honeybee products (at 5%, 10%, and 15% concentration) against *P. larvae*, with the mean diameter of inhibition zone (mm) before and after treatments.

Materials		Pretreatment			Mean	Post-treatment			Mean	General mean
		5%	10%	15%		5%	10%	15%		
Pollen extract aqueous	Formic	4.97^{c}	7.87^{abc}	10.06^{a}	7.63^{c}	5.75^{bc}	9.00 ^{ab}	9.90 ^{a}	8.22^{bc}	7.93
	Oxalic	7.95 ^{bc}	10.50 ^{ab}	12.00 ^{a}	10.15^{ab}	8.93 ^{abc}	8.97 ^{abc}	6.33 ^{c}	8.08^{bc}	9.11
	Lactic acid	7.93 ^{bc}	11.29 ^{ab}	12.12 ^{a}	10.45^{a}	4.77 ^{c}	9.40 ^{ab}	8.84 ^{ab}	7.67^{c}	9.06
	Tylosin	5.45 ^{c}	10.00 ^{ab}	11.75 ^{a}	9.07^{abc}	7.30 ^{bc}	7.80 ^{bc}	8.30 ^{abc}	7.80^{c}	8.43
	Carnation	6.77 ^{a}	9.77 ^{a}	9.33 ^{a}	8.62^{abc}	6.90 ^{a}	6.75 ^{a}	7.65 ^{a}	7.10^{c}	7.86
Mean		6.61^{d}	9.88^{ab}	11.05^{a}	9.18	6.73^{cd}	8.38^{bc}	8.20^{bcd}	7.77	8.48
Pollen extract 70% ethanol	Formic	6.43 ^{a}	8.07 ^{a}	9.10 ^{a}	7.87^{bc}	5.43 ^{a}	6.40 ^{a}	6.30 ^{a}	6.04^{c}	6.96^{c}
	Oxalic	14.0 ^{a}	2.67 ^{c}	8.16^{b}	8.28^{b}	8.50 ^{b}	7.63 ^{b}	8.40 ^{b}	8.18^{bc}	8.23^{bc}
	Lactic acid	8.45 ^{ab}	6.50 ^{b}	11.95 ^{a}	8.97^{ab}	7.20^{b}	10.00 ^{ab}	10.17 ^{ab}	9.12^{ab}	9.04^{ab}
	Tylosin	9.35 ^{a}	8.55 ^{a}	11.95 ^{a}	9.95^{ab}	9.80 ^{a}	10.57 ^{a}	8.50 ^{a}	9.62^{ab}	9.79^{ab}
	Carnation	9.50 ^{ab}	8.67 ^{b}	10.60 ^{ab}	9.59^{ab}	9.50 ^{ab}	13.40 ^{a}	9.63 ^{ab}	10.84^{a}	10.22^{a}
Mean		9.55^{ab}	6.89^{c}	10.35^{a}	8.93	8.09^{bc}	9.60^{ab}	8.60^{bc}	8.76	8.85
Honey	Formic	8.73 ^{ab}	8.83^{ab}	5.20 ^{b}	7.59^{bc}	10.93 ^{a}	9.90 ^{a}	11.30 ^{a}	10.71^{a}	9.15^{a}
	Oxalic	7.57^{bc}	9.07 ^{ab}	11.83 ^{a}	9.49^{ab}	5.17^{c}	8.70 ^{abc}	10.03^{ab}	7.97^{bc}	8.73^{a}
	Lactic acid	10.27 ^{a}	11.17 ^{a}	9.37 ^{a}	10.27^{a}	8.03 ^{ab}	4.80^{b}	8.47 ^{ab}	7.10^{cd}	8.68^{a}
	Tylosin	7.20^{b}	10.37^{ab}	11.33 ^{a}	9.63^{ab}	7.90 ^{ab}	9.30 ^{ab}	8.57 ^{ab}	8.59^{abc}	9.11^{a}
	Carnation	9.10 ^{a}	8.67 ^{a}	9.43 ^{a}	9.07^{abc}	3.57 ^{b}	8.40 ^{a}	3.23^{b}	5.07^{d}	7.07^{b}
Mean		8.57^{ab}	9.62^{a}	9.43^{a}	9.18	7.12^{b}	8.22^{ab}	8.32^{ab}	7.89	8.55
Royal jelly	Formic	6.93 ^{a}	8.00 ^{a}	7.70 ^{a}	7.54^{a}	7.60 ^{a}	8.33 ^{a}	8.70 ^{a}	8.21^{a}	7.88^{a}
	Oxalic	7.53 ^{a}	4.70 ^{a}	7.17 ^{a}	6.47^{a}	8.60 ^{a}	8.17 ^{a}	8.37 ^{a}	8.38^{a}	7.42^{a}
	Lactic acid	5.47 ^{b}	9.00 ^{ab}	10.60 ^{a}	8.36^{a}	8.23 ^{ab}	7.20 ^{ab}	7.50 ^{ab}	7.64^{a}	8.00^{a}
	Tylosin	6.37 ^{a}	6.80 ^{a}	7.20 ^{a}	6.79^{a}	3.97 ^{ab}	6.87 ^{a}	1.67 ^{b}	4.17^{b}	5.42^{b}
	Carnation	6.93 ^{a}	8.00 ^{a}	7.70 ^{a}	7.54^{a}	8.43 ^{a}	5.00 ^{a}	8.53 ^{a}	7.32^{a}	7.43^{a}
Mean		6.65 ^{a}	7.30 ^{a}	8.07 ^{a}	7.34	7.37 ^{a}	7.11 ^{a}	6.95 ^{a}	7.14 ^{a}	7.24
General mean		7.85^{bc}	8.42^{b}	9.73^{a}	8.71	7.33^{c}	8.33^{b}	8.02^{bc}	7.89	8.30

Means with different letters in each row are significantly different (P≤0.05).
Means with different letters in each column are significantly different (P≤0.05).

Also, the results of the EPE pre-and post-treatment showed an insignificant effect of the lactic acid and tylosin on the size of the inhibition zone of the AFB, while a significant increase was observed in post-treatment with the carnation oil. However, formic and oxalic acids had negatively affected the antibacterial activity of the EPE on the AFB.

The results also showed varying levels of the antibacterial activity of the honey depending on the concentrations. The formic acid was the only compound that significantly increased the activity of honey. Our findings are consistent with those of [51, 52], who examined the effect of different types of honey on *Paenibaciillus larvae with mention to their* inhibitory activity against *Paenibaciillus* larvae.

In addition, our results are in agreement with those of [53], who studied the antimicrobial effect of honey collected from honey bee colonies treated with marjoram oil. They found insignificant differences between the inhibition zone of all microbes at 50% concentration. In contrast, at 100% concentration, significant variations were found between *Escherichia coli* and all microbes, *Bacillus subtilis*, and all microbes, but no significant variations between *Streptomyces somaliensis*, *Salmonella bongori*, and *Paenibaciillus larvae* regarding the inhibition zones.

These results are consistent with [54], who determined six Libyan honey varieties (with different floral sources) and studied their antimicrobial effect on nine pathogenic microorganisms. The growth of *E. coli* was inhibited by all tested honey samples with varying degrees, whereas *Bacteroids* spp. and *Klebsiella pneumonia* showed strong resistance against most honey samples. Our data also revealed an insignificant difference among the means of the inhibition zones of all tested concentrations of royal

Jelly on the AFB bacteria pre-and post-treatment, except for tylosin, which had a negative impact on royal Jelly activity against the AFB bacteria.

Our data also revealed an insignificant difference among the means of the inhibition zones of all tested concentrations of royal jelly on the AFB bacteria pre- and post-treatment, except for tylosin, which had a negative impact on royal jelly activity against the AFB bacteria. Our results are consistent with [53], who examined the antimicrobial effect of royal jelly collected from colonies treated with marjoram oil. On the other hand, the antimicrobial activity of royal jelly from colonies treated with oxalic acid showed significant differences between inhibition zones among the most microbes under different concentrations. [55, 56] demonstrated the variation of the inhibitory effects of royal jelly samples against four different types of bacteria.

4. Conclusion

The bactericidal activity of the aqueous pollen extract (APE) and honey was enhanced by the treatment with formic acid. The ethanolic pollen extract's (EPE) activity was reduced, although the impact on RJ was minimal. The activity of EPE was only increased by the carnation oil Oxalic acid, lactic acid, and tylosin either reduced the activity of the studied products or significantly reduced it. Tylosin treatment reduced the quality of aqueous pollen extract, honey, and RJ but had no effect on EPE. Additionally, the results of the EPE pre- and post-treatment demonstrated that the size of the AFB's inhibitory zone was not significantly affected by the lactic acid or tylosin treatments, but was significantly increased by the carnation oil post-treatment. However, the EPE's antibacterial action on the AFB had been adversely impacted by formic and oxalic acids.

Acknowledgment

The authors want to thank the technicians of the Apiculture Department, Plant Protection Research Institute, Agricultural Research Centre and Microbial Genetic Department, Genetic Engineering and Biotechnology Division, and National Research Center, Giza, Egypt, for their assistance.

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