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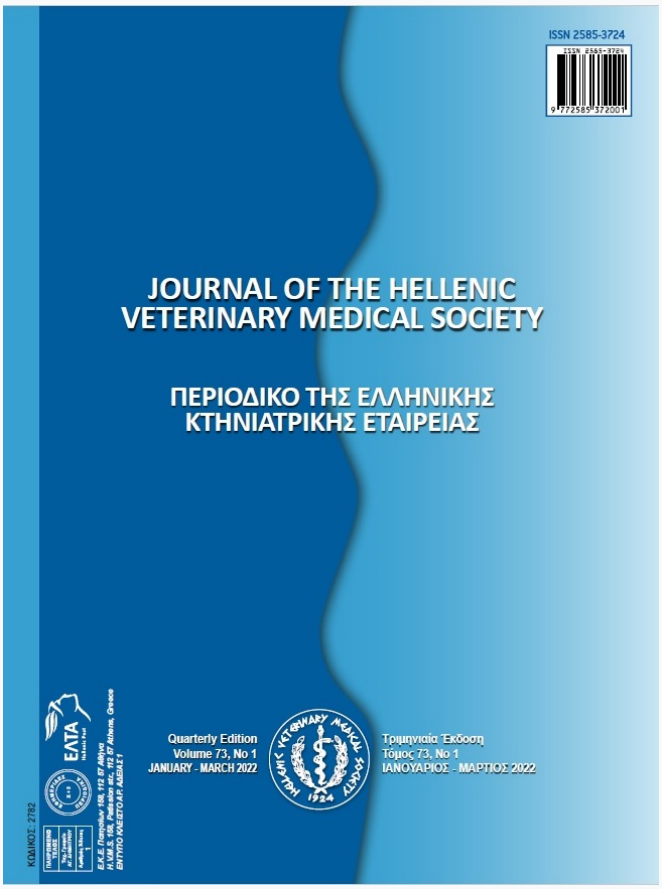

Influence of lavender essential oil (Lavandula angustifolia) and oxytetracycline in nutrition of honey bees, prevention of American foulbrood and overall welfare

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Research article
Ερευνητικό άρθρο

The influence of single lavender essential oil in honey bee prevention of American foulbrood

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ABSTRACT: Recently, there has been an increasing demand for natural, healthy, and safe products without residual antibiotics for human consumption, particularly bee products. Beekeepers have been struggling with this problem many years, having in mind often occurrence of American foulbrood (AFB), which is one of the most severe honey bee brood diseases, and in the past have been successfully eradicated with heavy usage of antibiotics. Such controlled, or mostly uncontrolled usage of antibiotics in fighting against American foulbrood lead to a residual quantity of antibiotic in honey. To overcome this problem, this research aimed to investigate the influence of single essential oil (*Lavandula angustifolia*) in protecting bees against AFB compared to the antibiotic oxytetracycline. Totally three treatments were formed artificially infected with *P. larvae* spore suspension, at concentration 2×10^9 spore/ml. The course of the disease was regularly monitored. Treatment one (T1) did not receive antibiotic therapy. Treatment two (T2) was given lavender essential oil at a concentration of 0.1% of sugar syrup. The treatment was applied for 30 days, at 48h intervals. Treatment three (T3) received antibiotics in the sugar syrup at a concentration of 0.1%, respectively. Clinical and laboratory examinations were performed on days 10, 20, 40 and 60, respectively. Besides, *L. angustifolia* essential oil rich in Ethanol, 2- (2-ethoxyethoxy) - (13.05%), linalool (10.71%), α-Terpinyl acetate (10.93%) and linalool acetate (9.60%), showed its positive effects against antibiotics in combat of American foulbrood, further research with a specifically designed qualitative and quantitative mixture of essential oils are more than necessary because single essential oil is not enough and didn't show expected results.

Keywords: Essential oils, bees, honey, nutrition, medicinal plants, lavender, welfare

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INTRODUCTION

Honey, bee bread, bee venom, bee pollen, propolis, and royal jelly are the products produced by honey bee colonies which prolonging, sustaining, and retaining the health of their consumers (Easton-Calabria et al., 2019). Nowadays, it is seen an increased interest in bee products, both traditional and contemporary ones (Durand & Fournier, 2017; Ignjatijević et al., 2019; Jenkins, 2016; Živanović et al., 2019). At the moment, modern science performs investigations which have the aim to analyze and discover the exact mode of action and directed health benefits and pharmacological properties of bee products (López-Romero et al., 2018; Srivastava et al., 2019). Today, honey and other honey bee products are conceptualized as a functional food with the ability to promote better physiological or psychological health compared to traditional remediated and nutritional food (Ignjatijević et al., 2019; Kevan & Menzel, 2012; Prodanović et al., 2019).

The main obstacle in the increased production of healthy honey bee products with functional and beneficial properties for humans is diseases of honey bee colonies and usage of antibiotics, which lead to final products with a high concentration of residual antibiotics (de Graaf et al., 2013).

The most often disease which makes serious healthy and economic problems to bees and producers is American Foulbrood (Hansen & Brødsgaard, 1999). Even after more than a century of American Foulbrood research, this fatal brood infection is still among the most harmful bee diseases (Genersch, 2008). Its etiological agent is the Gram-positive, spore-forming bacterium *Paenibacillus larvae* (Genersch, 2007). Enormous progress has been made, especially in the last thirty years, in the understanding of the disease and of the underlying host-pathogen interactions (Yoshiyama & Kimura, 2009). As a severe disease that affects the larval stage of honey bees, antibiotics have been wildly used to control this disease (Antúnez et al., 2011). Twenty years ago, the only drugs utilized for this purpose were sodium sulfathiazole and oxytetracycline hydrochloride, the latter also employed for the treatment of European foulbrood (Forsgren, 2010; Thompson et al., 2005).

Honey is a complex product that has always been considered as natural and healthy food (Eteraf-Oskouei & Najafi, 2013). Substances that could be present in non-negligible quantities in honey may be authorized or prohibited veterinary substances, such as various drugs and environmental pollutants such as pesticides and heavy metals (Al-Waili et al., 2012; Bogdanov, 2006). Because of all this reason modern science is trying to find an ecologically safe way to treat diseases of food animals (Copping & Duke, 2007; Puvača et al., 2020), as well as honey bees with natural substances (Fuselli et al., 2006; Kuzyšinová et al., 2016) without any adverse effects on bees themselves as well to their products intended for human consumption, especially to children (Sinha, 1997; Tagboto & Townson, 2001).

In that way, naturally, existing plants such as *Achillea millefolium*, *Thymus vulgaris*, *Ocimum basilicum, Taraxacum officinale,* and commercial phytotherapeutic product such as Protofil® have been reported as a possible solution in combat of honey bee diseases (Cristina et al., 2020).

Worldwide, in the last decade, the allopathic drugs against various honey bees' diseases were restricted to a few active substances and, finally, to only a single synthesis fumidil, an antibiotic obtained from *Aspergillus fumigatus* and is permitted only in third countries and in Canada as well (Cristina et al., 2020; van den Heever et al., 2014). Unfortunately, although an efficient product, due to the risk of residues, EMA has excluded this product from use in Europe in February 2016 (Özkök & Akyol, 2017; Puvača et al., 2013; van den Heever et al., 2014). In the given circumstances in the disease's treatment the ecologic phytotherapy, with the usage of whole medicinal plants or their parts and essential oils rich in bioactive substances, with recognized antiprotozoal activity currently being viewed as a great opportunity (Carson & Riley, 1995; Puvača et al., 2019; Puvača et al., 2020).

Recent findings show that the use of essential oils such as lavender and cinnamon can improve the health of bee colonies (Nicoleta & Silvia, 2020). Essential oils include a series of antimicrobial, antibacterial, antifungal, antiparasitic compounds from plants that play a role in reducing bacterial resistance. Natural antibiotics based on essential oils can be alternatives to chemically synthesized antibiotics, as they do not contaminate the bee products (Nicoleta & Silvia, 2020). Research performed by several researchers (Sammataro et al., 2009), using dietary essential oils, in honey bee colonies feeding, recorded changes in the size of the bee population, improvement of the queen fertility. Essential oils used *in vitro* and *in vivo* have shown to be very useful in the protection of honey bee colonies from pathogenic bacteria (Ebert et al.,

2007). Corresponding to the experiments carried out by (Roussenova, 2011), essential oils derived from different plants can play an unconventional role in controlling honey bee diseases, without any drug residues in bee products (Nicoleta & Silvia, 2020).

Roussenova (2011) aimed to determine the *in vitro* activity of various essential oils to field strains (isolated from apiaries in Bulgaria), and a reference *Paenibacillus larvae* strain concerning their utilization as alternative means for prevention and control of American foulbrood without antibiotics. Essential oils from thyme, cloves, cinnamon, marjoram, tea tree, sage, peppermint, oregano, grapefruit, lemongrass, and mandarin, but no lavender essential oils were used in this research. Obtained results from investigation have shown that essential oils exhibited a strong inhibitory effect against all tested *P. larvae* strains (Roussenova, 2011). The usage of essential oils for control of bacterial, fungal and parasitic honey bee colonies diseases takes more than a few benefits over standard methods. In the literature, resistance of bacteria to essential oils has not been recorded in the last forty years from the early beginnings (Hitokoto et al., 1980). Besides, ecological ingredients in bee products decompose quickly, their amount in honey is low, and they do not have a harmful impact on the health of honey purchasers (Nozal et al., 2002; Tasić, 2018; Vapa-Tankosić et al., 2020). It has been proven when essential oil of lavender is used in honey bee nutrition during the summer or winter period, it also expresses analgesic, mood stabilizers, anticonvulsant, curative, neuroprotective, and carminative properties (Koulivand et al., 2013).

Giving the *in vitro* investigation on the antibacterial potential of lavender essential oil, it has been shown that it inhibits the growth of infected areas between 8 and 30 mm in size, with amounts of lavender oil between 1 and 20 μL (Roller et al., 2009).

Having in mind, this research aimed to investigate the influence of lavender (*Lavandula angustifolia*) essential oil in honey bee production protecting bees against AFB compared to the antibiotic oxytetracycline (OTC).

MATERIALS AND METHODS

Biological experiment with honey bees was performed following the EU legislation and principle of the Three Rs within Directive 2010/63/EU.

The experiment was performed in field conditions, in the hives outside, in the northern part of Serbia, the Autonomous Province of Vojvodina. The study included 15 honey bee colonies distributed in three treatments. Each honey bee colony consisted of about 1500 young worker bees and a young breeder queen. All treatments in the experiment were infected with *P. larvae* spore suspension, at concentration 2×10^9 spore/ml. The course of the disease was regularly monitored. Treatment one (T1) did not receive oxytetracycline therapy. Treatment two (T2) was given lavender essential oil at a concentration of 0.1% of sugar syrup. The therapy was applied for 30 days, at 24h intervals. Treatment three (T3) received oxytetracycline in the sugar syrup at a concentration of 0.1%, respectively. Clinical and laboratory examinations were performed on days 10, 20, 40, and 60. Experimental design with honey bees is presented in Table 1.

In this experiment honey bee colonies free from American foulbrood were chosen. Examination of the honey from the investigated hives revealed the absence of *P. larvae* spores. In purpose of establishing a high hygiene instinct in bees "Pin-test" was used (Facchini et al., 2019; Spivak & Downey, 1998). Artificial infection was performed using referent strains of *P. larvae* (5875 CAMP, Czech Republic). A particular nutritive culture medium J-agar was used, consisting of 20 g agar, 5 g tryptone, 15 g yeast extract, 3 g K_2HPO_4, 2 g glucose, and one liter of demineralized water. Media was used as a part of the suspension for bees.

Table 1. Experimental design with the application of essential oil and oxytetracycline in artificially infected honey bees with American Foulbrood

Parameter	Treatments		
	T1	T2	T3
Hives	5	5	5
Honey bee colony	1500 bees Young breeder queen	1500 bees Young breeder queen	1500 bees Young breeder queen
Sugar syrup and supplements	10 mL/day syrup	10 mL/day syrup 0.1% Lavender essential oil	10 mL/day syrup 0.1% Oxytetracycline

The microscopic examination method was used to observe the morphology of *P. larvae* to distinguish the American Foulbrood from other diseases. A preliminary check to the inspected diseased larvae in a direct way by using suitable spores stain such as 0.2% carbon fuchsin. About two drops of water were mixed with the larvae. The suspension was then transferred by loop and smeared on a glass slide as a thin film. The slide was then stained. After washing and drying the slide, it was examined under the microscope for *P. larvae* spores. A smear of suspected larvae colony was taken on a slide, Gram stained, the slide was flooded with crystal violet for 1 min, washed then flooded again with iodine solution for the same time, the decolorizing agent was used as ethanol for 5 sec, the final steps involved applying safranin, after each step the slide was rinsed with water for 5 sec. The prepared slide was then examined under the microscope. The bacterium was identified as *P. larvae* with gram positive rods 0.5-0.6 μm wide and 1.5-6 μm long (Nizar et al., 2015).

A bacteriological method such as bacterial cultivation and isolation to obtain pure cultures. Several media for cultivating *P. larvae* were examined previously to choose the appropriate one. The media used, such as sheep blood was approved by OIE (World Organisation for Animal Health, 2008) and described by other investigators as a good quality nurturing media for *P. larvae*. Ram blood agar with 8% defibrinated blood, nutritive medium with 2% NaCl, and nutritive medium with 5% NaCl, nutritive broth adjusted at 6.8 pH, was used in our research, respectively.

Antibiotic containing 55 mg oxytetracycline / 1 g of powder for oral application purchased from local veterinary medical supply was used in artificial honey bee infection.

Lavender (*Lavandula angustifolia*) essential oil analysis was performed by Gas chromatography (GC) and gas chromatography-mass spectrometric (GC-MS) on used thyme essential oil using an Agilent 7890A GC equipped with an inert 5975C XL EI/CI mass spectrometer detector (MSD) and flame ionization detector (FID) connected by capillary flow technology 2-way splitter with make-up. An HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) was used. The GC oven temperature was programmed from 60 to 300 °C at a rate of 3 °C min^{-1} and held for 15 min. Helium was used as the carrier gas at 16.255 psi (constant pressure mode). An auto-injection system (Agilent 7683B Series Injector) was employed to inject 1 μL of the sample. The sample was analyzed in the splitless mode. The injector temperature was 300 °C and the detector temperature 300 °C. MS data were acquired in the EI mode with scan range 30-550 m/z, source temperature 230 °C, and quadrupole temperature 150 °C; the solvent delay was 3 min (Puvača et al., 2020).

RESULTS AND DISCUSSION

On the tenth day, microscopic examination showed the presence of vegetative rods of *P. larvae* in two colonies of the treatment T1. Clinical testing on day 20 confirmed symptoms of American foulbrood in the same treatment, with *patchy* comb appearance (Figure 2), and dark colorcappings (Figure 1) with brown colored larvae, elastic and sticky (Figure 3). Bacteriological examination revealed vegetative rods of *P. larvae* (Figure 4).

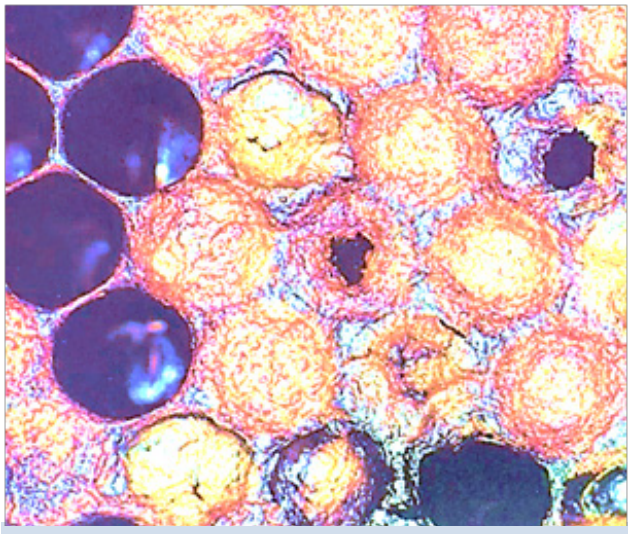

Figure 1. The characteristic appearance of the capping

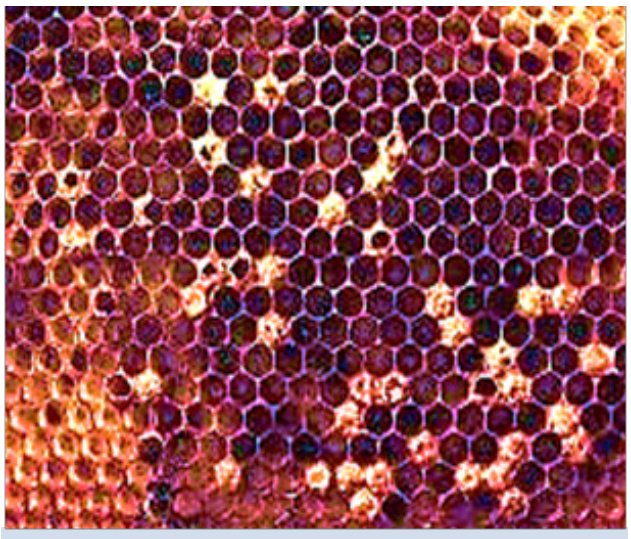

Figure 2. Patchy comb

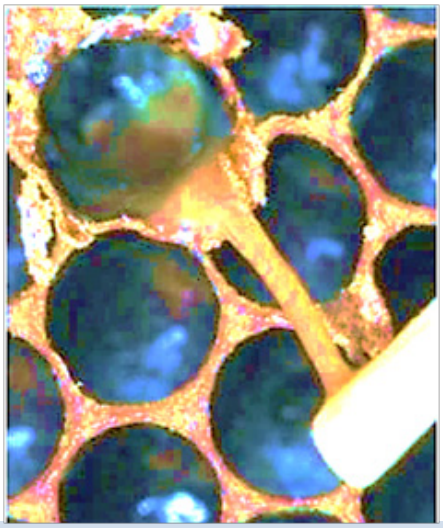

Figure 3. Rotten dead larva

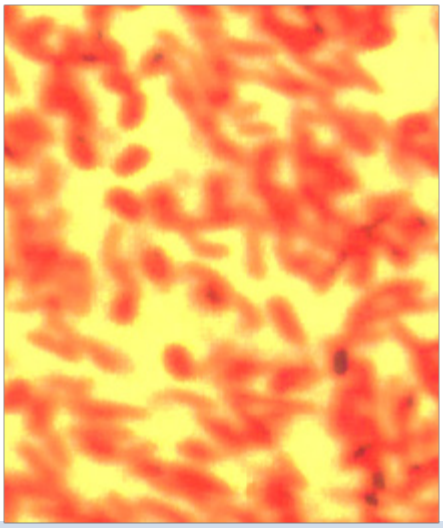

Figure 4. *P. larvae* (spores and rods)

In treatments T2 and T3 clinical signs of the disease were not expected; however, bacteriological analyses showed the presence of bacterial rods. Clinical and bacteriological analyses on day 40 showed the American foulbrood in all treatments. In the treatments, T1 and T2 two and three honey bee colonies were alive, respectively. In the treatment T3 with applications of oxytetracycline, all honey bee colonies were alive. On day 60, there were not left any alive honey bee colony in treatment T1, while only one alive colony was recorded in treatment T2 with the application of lavender essential oil, but with pronounced clinical signs of the disease. In treatment, T3 with the addition of oxytetracycline two alive colonies was seen with significant symptoms of the American Foulbrood and positive bacteriological findings (Table 2).

American foulbrood is a lethal disease of the honey bee brood and the infection often appears in closed broods. The susceptible larvae are essential for the infection, but also the development of the disease is dictated by the number of *P. larvae* spores. The number of spores varies depending on different factors. Sturtevant and Revell (Sturtevant & Revell, 1953) in the middle fifties induced artificial infection with American foulbrood with 5×10^7 spores of *P. larvae*/g honey, while forty years later was observed that feeding bee colonies with honey containing 2×10^9 spore/g necessarily caused the outbreak of the disease. Frequent feeding of larvae in the first weeks of life may introduce a large number of*P. larvae* spores into the larvae itself and cause an outbreak of disease. Some investigations have shown that only ten spores are enough for one-day-old larva, and millions of them are needed to induce the infection in 4 to 5-days-old larvae. Research of Plavša et al. (2011) indicated that in the artificial infection with *P. larvae* showed first clinical symptoms of American foulbrood and the first spores were detected after 25 days post-infection, while the honey bees treated with oxytetracycline in sugar syrup early signs of the disease have shown on 47 days post-infection. Khan et al. (2019) have highlighted that communicable diseases are not only the past but also the present problem in developing as well as developed countries.

The medicinal plants and nano-silver have been used against the pathogenic microbes. Herbal medicines are generally used for healthcare because they have a low price and a rich source of antimicrobial properties. Like medicinal plants, silver nanoparticles also have new applications in biomedical fields due to their inherent therapeutic performance. As in other species, as well in honey bees' medicinal plants and silver nanoparticles show their antiviral, bactericidal, and fungicidal properties. The applications of medicinal plants against honey bee pathogen such as fungi (*Ascosphaera apis*), mites (*Varroa* spp. and *Tropilaelaps* sp.), bacteria (*Melissococcus plutonius Paenibacillus larvae*), and microsporidia (*Nosema apis* and *Nosema ceranae*), could be a possible natural solution against synthetic drugs which have been used over the years disputes their harmful effect on bees and decrease quality and safety of honey. Wiese et al. (2018) have tested six major plant terpenes and their corresponding acetates, characterizing six natural *Thymus vulgaris* chemotypes, for their antimicrobial activity on bacteria associated with European

Table 2. Clinical signs of honey bee colonies with the application of essential oil and oxytetracycline artificially infected with American Foulbroo

Control periods	Treatments		
	T1	T2	T3
Day 10			
Descriptive control	Comb appearance without the changes	Comb appearance without the changes	Comb appearance without the changes
Bacteriological control	Two colonies positive on bacterial rods Three colonies positive on *P. larvae* spores	Negative	Negative
Day 20			
Descriptive control	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color	Compact and convex cappings, one colony cappings are in a dark color	Compact and convex cappings
Bacteriological control	Two colonies positive on bacterial rods Three colonies positive on *P. larvae* spores	Two colonies positive on bacterial rods One colony positive on *P. larvae* spores	Negative
Day 40			
Descriptive control	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color in three colonies
Bacteriological control	Two colonies positive on *P. larvae* spores	One colony positive on bacterial rods Three colonies positive on *P. larvae* spores	Two colonies positive on bacterial rods Three colonies positive on *P. larvae* spores
Day 60			
Descriptive control	-	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color in two colonies
Bacteriological control	-	One colony positive on *P. larvae* spores	Two colonies positive on *P. larvae* spores

foulbrood. The same group of authors (Wiese et al., 2018) has concluded that bee-forageable thyme product terpenes (mainly from pollen) yield effective oxytetracycline activity by reducing the growth of bee disease-associated bacteria and can be detected with different response levels by the honey bees' antennae. Kuzyšinová et al. (2016) have given insights into the use of antibiotic therapy in countries that permit this therapy is disputable regarding its low effectiveness, development of resistant bacterial strains, and residues in honey bee products. Because of all the harmful effects of antibiotics, alternative methods of prevention or therapy of American foulbrood have been considered. They are based mostly on substances of natural origin that neither adversely affect the honey bee products nor put some load on the environment. Such substances include for example probiotics, prebiotics, fatty acids, plant essential oils, and other plant materials. These substances are commonly used in the prevention or treatment of a whole range of diseases of farm and pet animals and have also recently been used in beekeeping. This "green" and healthy approach is especially important having in mind an interest in substances of natural origin which increasing constantly for many years and recently discovered are of great interest to the researchers. This interest also applies to bee products because of their extensive nutritional and therapeutic properties (Kieliszek et al., 2018).

The results are presented in Table 3, which reveals the most dominant subgroup of the phenolic compound of the investigated essential oil. The conducted analyses showed that *L. angustifolia* essential oil is the richest in Ethanol, 2- (2-ethoxyethoxy) - (13.05%). The results of our research emphasized that the lavender essential oil was also rich in linalool (10.71%), α-Terpinyl acetate (10.93%) and linalool acetate (9.60%). Ethanol, 2- (2-ethoxyethoxy) - (CH-

$_3CH_2OCH_2CH_2OCH_2CH_2OH$) is known as a solvent for dyes, nitrocellulose, paints, inks, and resins. It is a component of wood stains for wood, for setting the twist and conditioning yarns and cloth, in textile printing, textile soaps, lacquers, penetration enhancer in cosmetics, drying varnishes, and enamels, and brake fluids (Da Porto et al., 2009). It used to determine the saponification values of oils and as a neutral solvent for mineral oil-soap and mineral oil-sulfated oil mixtures. Linalool is a monoterpenoid that is octa-1, 6-diene substituted by methyl groups at positions 3 and 7 and a hydroxy group at position 3. It has been isolated from plants like *Ocimum canum*. It has a role as a plant metabolite, a volatile oil component, an antimicrobial agent, and a fragrance (González-Rivera et al., 2016). It is tertiary alcohol and a monoterpenoid. While α-Terpinyl acetate is found in cardamom and it is often used as a flavoring agent.

Several findings were introduced, testing the ability of different essential oils to inhibit the growth of *P. larvae* (Kuzyšinová et al., 2016). The highest activity against *P. larvae* in essential oils of lemongrass, thyme, and chamomile were noticed. Like our study with lavender essential oil, peppermint and Andean thyme showed inhibitory activity against the causative agent of American foulbrood (Fuselli et al., 2006), respectively.

Table 3. Identified phenolic compounds of the Lavender (***Lavandula angustifolia***) essential oil, %

Compound	RT [2]	RI [3]	*L.angustifolia* Composition ± SD
α-Thujene	5.632	923	0.05±0.00
α-Pinene	5.816	930	0.72±0.01
Camphene	6.229	944	0.25±0.00
Sabinene	6.928	970	0.12±0.01
β-Pinene	7.033	974	0.60±0.02
Myrcene	7.428	988	0.56±0.01
Ethanol, 2- (2-ethoxyethoxy) -	7.863	1003	13.05±0.04
Hexyl acetate	8.146	1011	0.13±0.00
α-Terpinene	8.297	1015	0.41±0.01
p-Cymene	8.570	1022	0.87±0.00
Limonene	8.713	1026	2.23±0.06
1, 8-Cineole	8.805	1029	5.55±0.03
β- (Z) -Ocimene	9.033	1035	0.06±0.00
γ-Terpinene	9.828	1056	0.05±0.00
Terpinolene	10.985	1088	0.04±0.01
Linalool	11.405	1110	10.71±0.01
Camphor	13.267	1143	3.72±0.00
Isoborneol	13.787	1154	1.04±0.02
Borneol	14.173	1163	0.46±0.02
Isononyl acetate	14.530	1171	3.45±0.00
Terpinen-4-ol	14.696	1175	0.90±0.01
α-Terpineol	15.577	1188	2.00±0.03
Citronellol	16.923	1226	2.50±0.04
Geraniol	18.110	1254	1.28±0.01
Linalool acetate	18.194	1255	9.60±0.02
Bornyl acetate	19.562	1285	0.21±0.00
α-Terpinyl acetate	22.374	1349	10.93±0.04
Neryl acetate	23.038	1364	0.44±0.00
Geranyl acetate	23.898	1364	0.80±0.00
Caryophyllene (E-)	25.443	1420	1.80±0.01
NI [1]			25.10
Total peak area			98030240

[1] Not identified; [2] Retention time; [3] Experimental retention indices based on n-alkane series under identical experimental conditions and comparison was done with the mass spectra library search NIST; SD—standard deviation calculated for n (n = 3) gas chromatography-mass spectrometric (GC-MS) analysis.

In most cases usually, the essential oils from oregano, thyme, and clove with strong inhibition of *P. larvae*, or essential oils from chamomile, rosemary and fennel with weak antibacterial activity were investigated while the investigation with essential oil of lavender in inhibition of *P. larvae*, performed just a few (Laird & Phillips, 2012).

It was recorded that among the very useful extracts of medicinal plants belongs those of Indian lilac (*Melia azadirachta*) with MIC equal to 10-800 μg/ml, Ceylon cinnamon with MIC 25-100 μg/ml and lemon grass with MIC 50-100 μg/ml (Ács et al., 2018).

Even when investigating the inhibitory activity of essential oils against pathogens, one must not forget to consider their potential toxic effect on honey bees. Many investigations have shown that essential oils can be used in honey bees because their toxicity to them is none or minimal. Large number of essential oils inhibit the growth of *P. larvae* and also presents LD50 values of the respective extracts for honey bees. Still, for example, peppermint oil as entirely nontoxic and LD50 values of thymol reached 100 mg/Kg, of cinnamon oil 50 mg/Kg and clove oil 200 mg/Kg (Albo et al., 2003).

CONCLUSIONS

Based on the obtained results, lavender essential oil rich in Ethanol, 2- (2-ethoxyethoxy) -, linalool, α-Terpinyl acetate, and linalool acetate, showed its positive effects in this experiment, indicating that lavender essential oil can be useful for the prevention or slow down the course of the disease (comparing with control group T1). Still, it is not enough for the healing process of American foulbrood. Further research with a specially designed qualitative and quantitative mixture of essential oils is more than necessary because single essential oil is not enough and didn't show expected results. Consumers search for the highest quality products, preferably with health benefits, rich in vitamins, valuable bio elements, and nutrients. Therefore, honey that is rich in beneficial ingredients has proved to fulfill these expectations, but further research in fighting the bee's diseases without any adverse effect on honey bees' products is essential very soon.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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