Antibacterial Activity of Different Plant Extracts and Phenolic Phytochemicals Tested on *Paenibacillus Larvae* Bacteria

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Abstract
Paenibacillus larvae, a Gram-positive and spore-forming bacterium is responsible for American foulbrood disease in bees. The antimicrobial activity of different plant extracts and phenolic phytochemical was evaluated on *Paenibacillus larvae* bacteria. In addition possible correlation with antioxidant activity of the same plant extracts was studied. Extracts of the following plants were utilized: *Achillea millefolium* (yarrow), *Ocimum basilicum* (basil), *Thymus vulgaris* (thyme) and *Urtica dioica* (nettle). The extracts that showed antimicrobial activity were later tested to determine the Minimal Inhibitory Concentration (MIC). Although nettle present the lowest polyphenolic content compared with the other plant extracts, exhibit the highest antimicrobial activity, measured as the inhibition zone using Mueller-Hinton agar plates. Basil presented both polyphenolic content and antimicrobial activity at higher levels, while thyme had the lowest antimicrobial activity, even it present high amount of polyphenols.

Keywords: bacterium, basil, *Paenibacillus larvae*, phenolic pyhtochemicals, nettle, thyme, yarrow.

1. Introduction

American foulbrood (AFB) is an infectious disease of honey bee larvae. The etiological factor of the disease is a *Paenibacillus larvae* bacterium. AFB is difficult to manage for beekeepers because the pathogen produces a spore form – endospore, highly virulent and very resistant to external factors (heat, desiccation and chemical disinfectants) [1].
The source of infection is the diseased brood and bee products: honey, pollen, wax and all the apiary equipments in contact with infected colonies [2].
Due to the fact that the use of antibiotics is forbidden, because of the accumulation in bee products, it is developing more and more the use of natural antimicrobials present in bee products [3, 4] or in different medicinal plants [5, 6, 7, 8, 9, 10]. Medicinal plants represent a rich source of antimicrobial agents, being used in different countries as a source of many potent and powerful drugs [11, 12]. The different parts of medicinal plants include root, stem, leaves, flowers, and fruits. Some of them are harvested in small quantities, but many of them are collected in larger quantities and traded for raw material in drug industry. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with side effects and have great therapeutic potential to heal many infectious diseases [13].
Taking into consideration the large potential of plants as sources for antimicrobial drugs, many systematic investigations were taken into consideration and screened in different parts of the world [5, 6, 7, 8, 9, 10].

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The aim of this study is to evaluate the effect of natural compounds from different plant macerates against one strain of *Paenibacillus larvae*, an the evaluation of phenolic profile in order to find a correlation between the chemical composition of the tested plant extracts and their antibacterial activity.

2. Materials and methods

The plant materials used in this study were mature leaves and flowers of the following species:
Yarrow (*Achillea millefolium*) belongs to the family Asteraceae. Is a herbaceous perennial plant that produces one to several stems and has a rhizomatous growth form. It has diaphoretic, astringent, tonic [14], anti-inflamatorry properties. Chemical composition comprises isovaleric and salycilic acid, sterols, flavonoids, bitters, tannins, coumarins and proazulenes [15, 16].
Basil (*Ocimum basilicum*) belongs to the family Lamiaceae. Chemical composition shows the presence of essential oils (linalool, estragol and eugenol), tannins and flavonoids [15]. Scientific studies have demonstrated that compounds in basil have potent antioxidant, anticancer, antiviral and antimicrobial properties [17, 18, 19].
Nettle (*Urtica dioica*) belongs to the family *Urticaceae*, are mostly herbaceous perrenial plants, most of the speciaes having hairs on the stems and leaves. Chemical composition shows the presence of acetylcholine, histamine, serotonine, formic acid [20, 21].
Thyme (*Thymus vulgaris*) belongs to the family Lamiaceae, and has as principal components of chemical composition essential oils (thymol and carvacol), flavonoids, tannins and triterpenes [15]. Due to the high content of thymol(20-54% from the essential oil) it is used as antiseptic, antibiotic or antifungus.
Ethanolic macerates of the above-described plants were obtained from 5 g dried plant and 100 ml 70% ethanol. After 14 days of maceration at room temperature in the dark, stirring the content daily, extracts were filtered on filter paper and the final content was brought up to 100 ml with ethanol. The polyphenol content (mg/g) of the ethanol extracts was carried out according to Singleton et al.(1999) [22] using a modified Folin-Ciocalteu assay, which is sensitive to phenol and polyphenol entities as well as to other electro-donating antioxidants.
Briefly, 0.5 ml of the extracts were mixed with 2.5 ml Folin–Ciocâlteu 0.2N reagent, for 5 minutes and than 2 ml of 75 $g \cdot l^{-1}$ sodium carbonate solutions was added. Samples were incubated at room temperature and in the dark for 2 hours, and the absorbance of the mixture was read at 760 nm, against a blank consisting of methanol – Folin reagent and sodium carbonate.
For the calibration curve, a stock solution of 1 mg/ml Gallic acid was used, and successive dilutions, following the same protocol as described above, were read at 760 nm.
The total polyphenol content was calculated using the following linear equation, based on the calibration curve: y = 8.03727x-0.06356, r^2=0.9990.
The total flavonoid content was determined using a method adapted by Arvonet-Grand et al.(1994) [23], using a standard curve of Quercetin (0,5 – 0,01 mg/ml)(y=11.11546x-0.00664; r^2=0.9988)
Briefly, 2 ml of extract was placed in a glass tube and 2 ml of aluminium chloride solution (2%) was added and mixed thoroughly and absorbtion readings at 415 nm using a Pharmaspech UV-1700, Shimadzu Spectrophotometer were taken against a blank sample. The flavonoid content was expressed as mg of quercetin equivalents (QE)/g, mean of three readings ± standard deviation.
The High Performance Liquid Chromatography (HPLC) method, adapted after Bunea et al.(2008) [24], was used for the phenolic acid and flavonoids determination, using a Shimadzu LC-10AD *VP* system, equipped with degasser, LC-10 AD *SP* pumps, SIL-10 AF autosampler and SCL-10 A *VP* system controller with DAD SPD-M20*A* detector. Chromatographic separation was made on a Supelcosil LC-18 column (Ø 4.6 mm, 250 mm length, 5 μm particle size) and Supelguard LC-18 2CM KIT guard column. Mobile phase: methanol:acetic acid:water (80:2:8)(solvent A) and methanol:acetic acid:water (10:3:73), flow rate 0.8 ml.min. Phenolic acids and flavonoid standards were dissolved in HPLC grade methanol (1 mg/ml solution), and diluted to perform the calibration curve on HPLC. Each standard was injected separately, to register the retention time and than in mixture, to see if all standards were baseline separated. Quantification was obtained by peak integration in comparison with standards. Results were expressed as mg/g dry plant.
For „in vitro" testing of plant alcoholic macerates, an isolated strain of *Paenibacillus*

larvae from a bee family contaminated with American foulbrood was used. In the first step, direct testing of antibacterial activity was carried out using the agar well diffusion method [25]. Sterile Petri plates of 9 mm diameter with Brain Heart Infusion (BHI) broth with B1 vitamin supplementation were used for antibacterian activity against Paenibacillus larvae. Every dish contains approximately 15 ml BHI broth, in order to obtain a height of 5 mm in the plate.

Wells (diameter of 5 mm) were punched in the afar following a radial model. The Petri plates were flooded with a bacterial suspension of *Paenibacillus larvae* adjusted to a turbidity of 0.5 MacFarland standard scale (10^8CFU/ml). With an automatic pipette, in every well 20 µl of analyzed sample were placed and thermostatated at 37°C for 48-72 hours. If the germ (*Paenibacillus larvae* bacterium) is sensitive to one of the tested extracts, an inhibition zone of variable dimension will be developed from the edges of the well. If the extract does not posses antibacterial activity, the bacterium will be multiplied until the edges of the well.

In the second stage of the experiment, minimal inhibitory concentration was determined following the successive dilutions method (broth micro dilution method). Sterile 96 wells micro titration plates, with 200 µl well capacity, were used, and 100 µl of the analyzed product (plant extract) were placed in the well, mixing with 100 µl of Mueller-Hinton broth. 100µl of every well is aspirated with the multichannel pipette and placed in the second column of the plate, adding another 100 µl of broth. Successive dilutions are made in this way until the plate is full. For the last column 100 µl mixture is discharged. Each well is seeded in the end with 24 hours culture of *Paenibacillus larvae* and incubated for 48-72 hours at 37°C.

Minimal inhibitory concentration is given by the lowest dilution from the analyzed product, in which the development of the bacterium strain is inhibited (broth remain clear).

3. Results and discussion

Plant phenolics constitute one of the major groups of compounds responsible for antioxidant behavior, as well as for antimicrobial effects. Flavonoids, this diverse and widespread group of natural compounds are the most important natural phenolics. They possess a broad spectrum of biological activities, including radical scavenging properties and antibacterial effect. Therefore, total phenol and flavonoid content in basil, nettle, thyme and yarrow was registered in ethanolic extracts (*Figure 1*). The content of phenolic compounds (mg GAE/g DW plant sample) was determined from regression equation of calibration curve and varied between 8.4 and 44.0 mg/g. The highest amount was registered in basil, followed by thyme and yarrow extract. Ethanolic extract of nettle present the smaller quantity of polyphenols.

The content of flavonoids (mgQE/g DW sample), determined from regression equation of calibration curve with quercetin, varied from 4.5 to 7.5 mg/g. The highest amounts of flavonoids were found in nettle and basil extracts. Lower quantities were registered in extracts of thymus and yarrow.

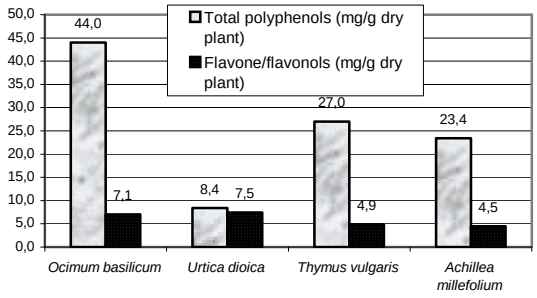

Figure 1. Total phenolic and flavone/flavonols content in plant macerates

HPLC determinations of phenolic acid and flavonoid profile revealed the presence of phenolic acids in higher quantities than flavonoid aglicones (*Table 1*). It is well known that plant extracts exhibit high quantities of flavonoid glycosides, and as our research was carried out on alcoholic macerates without hydrolization, only rutin was identified and quantified from the extracts.

From basil macerate we could identify and quantify a high quantity of rosmarinic acid (29.4 mg/g dry plant), as in nettle extract (29.2 mg/g). These two macerates present also a high amount of ferulic acid (3.17 and 3.09 mg/g respectively) and rutin (3.77 and 3.16 mg/g). In basil extract was quantified also caffeic acid (1.20 mg/g), same as in nettle extract (1.23 mg/g). Small quantity of chlorogenic acid was also quantified in nettle extract (0.71 mg/g).

Table 1. Identified and quantified plenolic acids and flavonoids in plant macerates (mg/g dry plant)

Vegetal species	Clorogenic acid	Caffeic acid	Ferulic acid	Rutin	Rosmarinic acid
Basil (*Ocimum basilicum*)	0.00	1.20	3.17	3.77	29.40
Nettle (*Urtica dioica*)	0.71	1.23	3.09	3.16	29.20
Thyme (*Thymus vulgaris*)	1.90	1.30	0.00	10.10	9.20
Yarrow (*Achillea millefolium*)	0.01	0.46	0.00	7.36	7.96

Thyme and yarrow extracts present smaller quantities of rosmarinic acid (9.2 and 7.96 mg/g respectively) and no ferulic acid. Rutin was quantified in the highest amount in thyme extract (10.1 mg/g, followed by yarrow extract (7.36 mg/g) and the already named basil and nettle extracts. Thyme extract exhibit chlorogenic and caffeic acid (1.9 and 1.3 mg/ml respectively), meanwhile yarrow extract present smaller quantities of chlorogenic (0.01 mg/g) and caffeic acid (0.46 mg/g).

All plants assigned in this study are used as medicinal plants in Romania for human consumption, but also as adjuvants in bee feeding. Previous studies [26, 27], showed that nettle extract (*Urtica dioica*) may have potential in bee disease prevention and colony development. The antibacterial activity of specific concentrations of alcoholic extracts for the studied plants is shown in Table 2.

Table 2. Inhibition growth of different plant macerates and minimal inhibitory concentration (MIC) against *Paenibacillus larvae* bacterium

Vegetal species	Inhibition area[1] (mm)		MIC[3] (ppm)
	Control[2]	Plant macerates	
Basil (*Ocimum basilicum*)	0	17	0.195
Nettle (*Urtica dioica*)	0	21	0.195
Thyme (*Thymus vulgaris*)	0	11	1.562
Yarrow (*Achillea millefolium*)	0	11	0.781

[1]Numbers represent the average diameter (in mm) of the inhibition zone
[2]Ethanol 70% was used as negative control
[3]Minimal inhibitory concentration of plant extracts

Using agar well diffusion method, highest antibacterial activity was registered for nettle extract (21 mm diameter inhibition zone)(Figure 2), followed by basil extract (17 mm inhibition zone). Thyme and yarrow macerates show weak inhibition activity with only 11 mm diameter of the inhibition zone. Regarding the minimum inhibitory concentration (MIC), again basil and nettle macerates exhibit better results, growth inhibition of the bacterium Paenibacillus larvae was observed until a concentration of 0.195 ppm, thyme extract needs a higher concentration for inhibition of the bacterium (1.562 ppm) and yarrow extract 0.781 ppm. Even though, the chosen plants are good antibacterians against Paenibacillus larvae development.

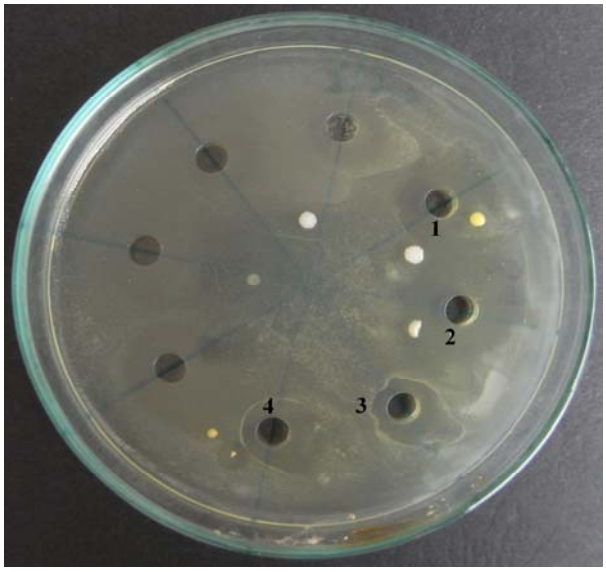

Figure 2. Inhibition zone for the plant macerates using difuzimetric method for Paenibacillus larvae; 1-Ocimum basilicum extract; 2-Urtica dioica extract; 3-Thymus vulgaris extract; 4-Achillea millefolium extract

4. Conclusions

Plants are very important sources of potential useful raw materials for the new development of natural chemotherapeutic agents. Many reports are available on the antiviral, antibacterial, antifungal or anti-inflamatory properties of plants [5, 6, 7, 8, 9, 10, 28, 29, 30, 31 32]. These studies have helped in identifying the active principles

responsible for the mentioned activities and some of the studied plants are used as ingredients in different drugs used in human and animal cure.

Our study indicate that the plants we took into consideration (basil, nettle, thyme and yarrow) have the potential to copntrol the growth of *Paenibacillus larvae in vitro* at the concentrations established by the microbiological methods. The use of the biologically active compounds from these plants could represent a natural alternatiove to antibiotic treatments on honeybees, in controlling American foulbrood disease, produced by *Paenibacillus larvae* bacterium.

Beside their inhibitory effect, the plant macerates also have low toxicity to bees, acting like nutritive supplements in bee colony development.

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