

## ***In vitro* Antimicrobial Activity of Four Slovak Medicinal Plants against Different Strains of Bacteria**

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### **Abstract**

Medicinal plants are traditionally used for the treatment in human medicine. The antibacterial activity of ethanol extract of four plant species (*Achillea millefolium* L., *Agrimonia eupatoria*, *Melissa officinalis* and *Tilia platyphyllos*) applied in the traditional medicine in Slovakia were tested. Extracts of certain parts of these plants were tested *in vitro* against six bacterial species (*Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Lactobacillus brevis*, *Lactobacillus hilgardii* and *Serratia rubidea*) strains using the disc diffusion method and microbroth dilution method. Minimum inhibitory concentrations for the extracts against all the microorganisms were determined by serial dilutions. All the extracts demonstrated antibacterial activity against Gram-positive bacteria and partially against Gram-negative bacteria.

**Keywords:** Antimicrobial activity, disc diffusion method, ethanolic extract, minimum inhibitory concentration (MIC), traditional medicinal plants

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### **1. Introduction**

Medicinal plants have been used extensively by a large proportion of the world population for their health care and remedy of diseases during the 2000 years. Achieved effect of medicinal plant application revealed a high degree of correlation between home-made preparation of traditional medicinal plants and laboratory produced [1]. Phytotherapy is based on the use of biological active components contained in plants [2]. The most interesting area of application of medicinal plant extracts is the inhibition of growth and reduction in numbers of the

pathogens responsible about human and animal diseases [3, 4].

*Achillea millefolium* L., commonly known as yarrow, belongs to *Asteraceae* family and it is very common in mountain meadows, pathways, crop fields and home gardens. Its infusion or alcohol extract is widely used in Europe to treat digestive problems, diabetes, hepato-biliary diseases and amenorrhea. Plant extract poses also antitumour, antimicrobial, anti-inflammatory and antioxidant properties [5-12].

*Agrimonia eupatoria* (Rose Family: *Rosaceae*) is a further interest of medicinal plants. The plant is known as agrimony and it is used in folkloric medicine to treat a wide range of ailments; for instance, eye infections, diarrhea and disorders of gall bladder, liver and kidneys [13]. Despite this, the recent studies have demonstrated several other biological and pharmaceutical potentials; for instance, anti-mutagenic and anti-tumor [14],

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hepatoprotective [15], anti-viral [16], anti-bacterial [17] and anti-oxidant and anti-inflammatory [18] effects.

*Melissa officinalis* is a medicinal plant, usually is taken as infusion, with a variety of beneficial effects, i.e., anti-depressive, anxiolytic, antitumoral, neurobiological and it has also been involved in the regulation of lipidemic disorders and in the prevention of oxidative damage [19-22]. The microorganisms mentioned above pose also antimicrobial activity and the present study was designed to determine the potential antibacterial activity of ethanolic extracts from *Achillea millefolium* L., *Agrimonia eupatoria*, *Melissa officinalis* and *Tilia platyphyllos* against *Bacillus cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Lactobacillus brevis*, *Lactobacillus hilgardii* and *Serratia rubidea*.

## 2. Materials and methods

### 2.1 Plant materials

The plant materials used in this experiment consisted of leaves of *Achillea millefolium* L., *Agrimonia eupatoria*, *Melissa officinalis* and *Tilia platyphyllos*. The plants were collected in Slovakia. The material was initially dried at the room temperature in the dark.

### 2.2 Microbial strains

Six strains of microorganisms were tested in this study, including two Gram-negative bacteria (*Escherichia coli* CCM 3988, *Serratia rubidea* CCM 4684, four Gram-positive bacteria (*Bacillus cereus* CCM 98, *Enterococcus faecalis* CCM 1875, *Lactobacillus brevis* CCM 1815, *Lactobacillus hilgardii* CCM 7701). All tested strains were collected from the Czech Collection of microorganisms. The bacterial suspensions were cultured in the nutrient broth (Imuna, Slovakia) at 37 °C.

### 2.3. Preparation of plant extracts

After drying, the plant materials were crushed, weighed out to 50 g and soaked separately in 300 mL of ethanol p.a. (99.9%, Sigma, Germany) during two weeks at room temperature. Then, ethanolic plant extracts were filtered through the Whatman No. 1 filter paper. The obtained extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove the ethanol

(Stuart RE300DB rotary evaporator, Bibby Scientific Limited, UK, and vacuum pump KNFN838.1.2KT.45.18, KNF, Germany). For the antimicrobial assays, the crude plant extracts were dissolved in dimethylsulfoxid (DMSO) (Penta, Czech Republic) to 102.4 mg/mL as stock solution, while for chemical analysis ethanol was used as solvent. Stock solutions of plant extracts were stored at -16 °C in refrigerator until the experiments were initiated.

### 2.4 Disc diffusion method

Antimicrobial activity of each plant extract was determined by a disc diffusion method. Briefly, 100 µl of the test bacteria were grown in 10 ml of fresh media until they reached a count of approximately  $10^5$  cells.mL<sup>-1</sup>. Then 100 µl of the microbial suspension was spread onto Mueller Hinton agar plates. The extracts were tested using 6 mm sterilized filter paper discs. The diameters of the inhibition zones were measured in millimeters. All measurements were to the closest whole millimeter. Each antimicrobial assay was performed in at least triplicate. Filter discs impregnated with 10 µl of distilled water were used as a negative control.

### 2.5 Microbroth dilution method

MICs were determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute recommendation [23] in Mueller Hinton broth (Biolife, Italy). Briefly, the DMSO plant extracts solutions were prepared as serial two-fold dilutions obtaining a final concentration ranging between 0.5-512 µg.mL<sup>-1</sup>. After that each well was inoculated with microbial suspension at the final density of 0.5 McFarland. After 24 h of incubation at 37 °C, the inhibition of microbial growth was evaluated by measuring the well absorbance at 450 nm in an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The 96 microwell plates were measured before and after experiment. Differences between both measurements were evaluated as growth. Measurement error was established for 0.05 values of absorbance. Wells without plant extracts were used as negative controls of growth. Pure DMSO was used as negative control. This experiment was done in eight-replicates for a higher accuracy of the MICs of used medical plant extracts.

## 2.6 Statistical analysis

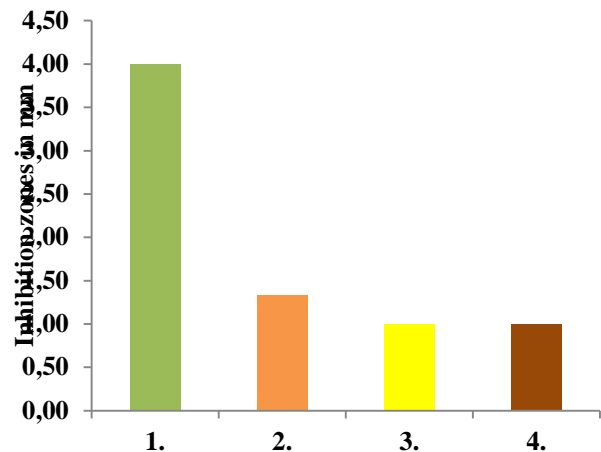
Differences in absorbance between the measurements before and after the analysis were expressed as a set of binary values. These values were assigned to exact concentrations. The following formula was created for this specific experiment: value 1 (inhibitory effect) was assigned to absorbance values lower than 0.05, while value 0 (no effect or stimulant effect) was assigned to absorbance values higher than 0.05. For this assigned to absorbance values higher than 0.05. For this statistical evaluation the probit analysis in Statgraphics software was used.

## 3. Results and discussion

In the last few years, there has been target interest in biologically active compounds of plants origin. These substances are exhibiting antimicrobial effect and not raising resistance concerns as in case of antibiotics [24]. Our results of antibacterial testing with disc diffusion method (fig. 1-6) showed that *Bacillus cereus* and *Enterococcus faecium* were the most sensitive to *Achillea millefolium* (4 mm and 4.33 mm, respectively), while *Escherichia coli*, *Lactobacillus brevis* and *Lactobacillus hilgardii* was the most sensitive to *Tilia platyphyllos* (2.33, 2.33 and 1.33 mm, accordingly). *Serratia rubidea* was sensitive to all plant extract tested - *Agrimonia eupatoria*, *Tilia platyphyllos*, *Melissa officinalis* with inhibition zone of 1.33 mm.

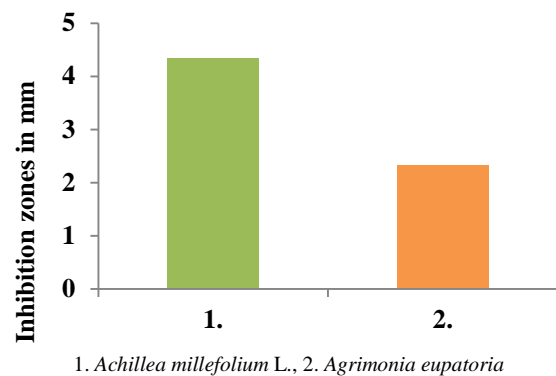
Results of Mazandarani et al. [25] demonstrate that the oil of *A. millefolium* L. may become alternative to antimicrobial drugs in controlling of certain Gram-positive and Gram-negative pathogens.

The antibacterial activity of some extracts of *A. eupatoria* (aqueous and ethanolic) against pathogenic bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*) and their activity on wound healing in rats were confirmed. Also the presence of some active compounds in both aqueous and ethanolic extracts were confirmed, showing that *A. eupatoria* may exhibit antimicrobial activity. The results of the present study showed that the ethanolic extract was more effective in inhibition of tested bacteria than the aqueous extract in the present study.



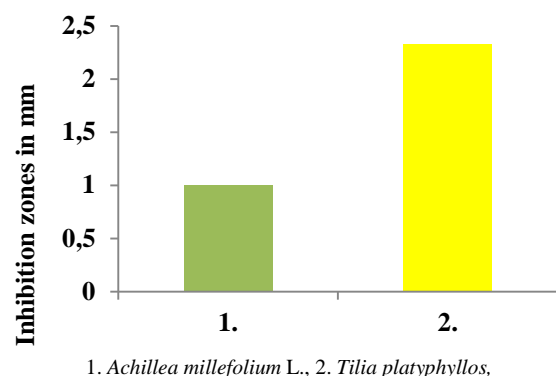
1. *Achillea millefolium* L., 2. *Agrimonia eupatoria*, 3. *Tilia platyphyllos*, 4. *Melissa officinalis*

**Figure 1.** Antimicrobial activity of medicinal plants against *Bacillus cereus*



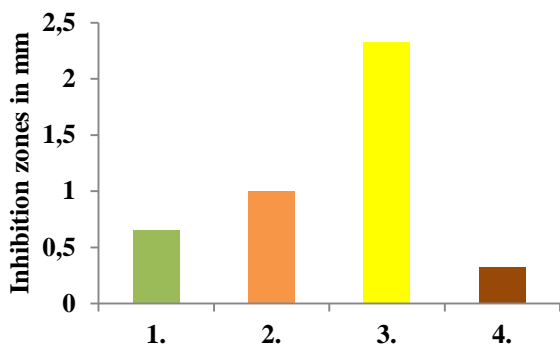
1. *Achillea millefolium* L., 2. *Agrimonia eupatoria*

**Figure 2.** Antimicrobial activity of medicinal plants against *Enterococcus faecalis*



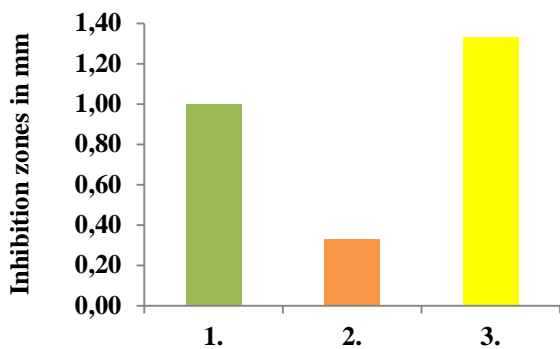
1. *Achillea millefolium* L., 2. *Tilia platyphyllos*,

**Figure 3.** Antimicrobial activity of medicinal plants against *Escherichia coli*



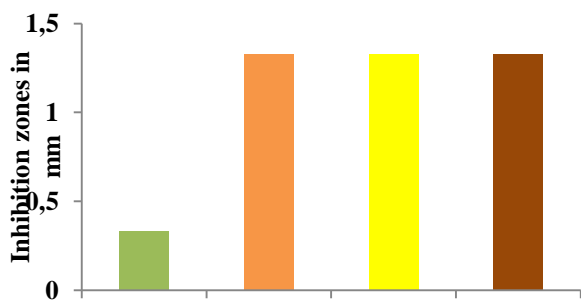
1. Achillea millefolium L., 2. Agrimonia eupatoria, 3. Tilia platyphyllos, 4. Melissa officinalis

Figure 4. Antimicrobial activity of medicinal plants against *Lactobacillus brevis*



1. Achillea millefolium L., 2. Agrimonia eupatoria, 3. Tilia platyphyllos

Figure 5. Antimicrobial activity of medicinal plants against *Lactobacillus hilgardii*



1. Achillea millefolium L., 2. Agrimonia eupatoria, 3. Tilia platyphyllos, 4. Melissa officinalis

Figure 6. Antimicrobial activity of medicinal plants against *Serratia rubidea*

*P. aeruginosa* was the most resistant to action of ethanolic extract, while the most susceptible was *E. coli* with the highest zone of inhibition of 20

mm. There was a moderate activity against *S. aureus* with inhibition zone of 15 mm after application of ethanolic extract ( $10 \text{ mg.ml}^{-1}$ ) [26]. Considering the antimicrobial activity of *M. officinalis* oil, Romeo et al. [27] and Hussain et al. [28] reported its antibacterial effect against *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus pumilis*, *Pseudomonas aeruginosa*, *Salmonella poona*, *Escherichia coli* and *Listeria innocua*. Despite this, *M. officinalis* didn't exhibited antimicrobial activity again all microorganisms were tested, and the extract was active against *S. rubidea*, *B. cereus* and *L. brevis*, but not against *L. hilgardii*, *E. coli* and *E. faecalis*. The antimicrobial activity (expressed as  $\mu\text{g.ml}^{-1}$ ) of four ethanolic extracts from *Achillea millefolium* L., *Agrimonia eupatoria*, *Melissa officinalis* and *Tilia platyphyllos* against various strains of Gram-positive and Gram-negative bacteria are summarized in Table 1.

The organism *B. cereus* was found to be more susceptible to *A. eupatoria* extract with MIC50 value of  $0.80 \mu\text{g.ml}^{-1}$ . *L. brevis* was less susceptible to *A. eupatoria* with MIC50 value of  $1.48 \mu\text{g.ml}^{-1}$ .

The organisms *L. hilgardii*, *S. rubidea*, *E. faecalis* and *E. coli* were less susceptible to *A. eupatoria* extract and MIC50 values were higher (MIC  $2.56\text{--}17.06 \mu\text{g.ml}^{-1}$ ). *L. hilgardii* was found to be more susceptible to the *A. millefolium* L. extract with a MIC50 value of  $0.53 \mu\text{g.ml}^{-1}$ , but *S. rubidea* was found more susceptible to *T. platyphyllos* extract with MIC50 value of  $1.06 \mu\text{g.ml}^{-1}$ . *L. brevis* was more susceptible to *M. officinalis* extract with MIC50 value of  $6.39 \mu\text{g.ml}^{-1}$ . Furthermore, activity of *A. millefolium* against both Gram-positive and Gram-negative bacteria was in contrast with previous reports, there antibacterial activity was limited to Gram-positive bacteria [29].

The antibacterial activity inhibited by the plant extract in the present study indicate the presence of some compounds with antibacterial activity, and many antibacterial agents could be derived from medicinal herbs [29]. The essential oil antibacterial activity of *M. officinalis* was reported in some papers [30-33].

**Table 1.** The antimicrobial activity of medicinal plant extracts (MIC,  $\mu\text{g}\cdot\text{ml}^{-1}$ )

Bacteria	<i>Agrimonia eupatoria</i>		<i>Achillea millefolium</i> L.		<i>Tilia platyphyllos</i>		<i>Melissa officinalis</i>	
	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90
<i>B. cereus</i>	0.80	0.85	0.80	0.85	6.40	6.82	17.06	19.08
<i>E. faecalis</i>	5.75	19.84	1.60	1.71	1.60	1.71	25.57	27.19
<i>S. rubidea</i>	5.16	13.33	1.06	1.19	1.06	1.19	17.06	19.08
<i>E. coli</i>	17.06	19.04	12.78	13.59	12.78	13.59	17.06	19.08
<i>L. brevis</i>	1.48	7.24	0.80	0.85	2.13	2.38	6.39	6.82
<i>L. hilgardii</i>	2.56	5.72	0.53	0.59	4.26	4.76	8.52	9.52

#### 4. Conclusions

This study also report that the medicinal plants (*Achillea millefolium* L., *Agrimonia eupatoria*, *Melissa officinalis* and *Tilia platyphyllos*) may possess antimicrobial activity against the Gram-positive and Gram-negative bacteria. These studies must be continued to include toxicity testing, isolate active compounds, elucidate the structures, and also evaluate the plant extracts against wider range of bacterial and fungal strains with the goal to find new therapeutic principles.

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