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Research Article

Antibacterial Activity of *Melissa officinalis* L., *Mentha piperita* L., *Origanum vulgare* L. and *Malva mauritiana* against Bacterial Microflora Isolated from Fish

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Abstract
The aim of the present study was to detect the antibacterial activity of medicinal plants against fish microflora. A total of 4 ethanolic extracts of 6 plant species were collected from local environments of Slovakia and screened for antibacterial activity against bacterial microflora. Extracts of *Melissa officinalis* L., *Mentha piperita* L., *Origanum vulgare* L. and *Malva mauritiana* were used. Bacterial strains were isolated from common bleak (*Alburnus alburnus*) and common rudd (*Scardinius erythrophthalmus*) of Latvian origin. All bacterial strains were identified with the Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS). Among fish microflora, *Acinetobacter pittii*, *A. baumannii*, *Buttiauxella agrestis*, *Delftia acidovorans*, *Enterobacter cloacae*, *Serratia liquefaciens*, *Pseudomonas alcaligenes*, *Ps. oryzihabitans*, *Staphylococcus epidermidis*, *St. caprae*, *Pantoea agglomerans*, *Lelliottia amnigena*, *Providencia rettgeri*, *Escherichia coli* and *Rahnella aquatilis* were identified. It has been shown that all plant extracts exhibit different degrees of antimicrobial activity against the tested bacteria. All bacterial strains in the present study were moderate sensitive to all extracts applied. The strongest antimicrobial effect of *Malva mauritiana* and *Melissa officinalis* L. against *Pseudomonas oryzihabitans* (6.67±1.53 resp. 9.67±0.58 mm) were found. The best antimicrobial activity of *Mentha piperita* L. was against *Staphylococcus epidermis* (7.33±0.58 mm) and strongest antimicrobial effect of *Origanum vulgare* L. was same against two bacterial strains *Enterobacter cloacae* and *Serratia liquefaciens* (9.67±0.58 mm).

Key words: *Alburnus alburnus*, *Scardinius erythrophthalmus,* MALDI-TOF MS identification, medicinal plants, disc diffusion method.

Introduction

Water is the natural habitat of fish, where fish are exposed to permanent contamination with the microorganisms present in surrounding aquatic environment. The microflora consists not only from heterotrophic but also from the contaminants from municipal and rural run-off and industrial discharges of sewage/faeces [1]. The aquatic microflora influences the composition of microorganisms on fish skin, including the gills. Microorganisms can reach the intestinal tract with water and feed with subsequent colonization. Colonization of fish may start at the egg and larval stage and continue alongside the fish development [2].

Fish microflora was studied intensively and a large variety of the fish inhabiting microorganisms was recognized [3].

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Traditional herbal medicines are a source of novel antibacterial drugs. Since they are considered as safe for human use, plant based medicines are widely recognized and used for primary health care in developed and developing countries [4, 5]. According to the WHO, 60–80% of the world population applies the traditional treatment [6]. Plants have also been used to get a natural extracts for developing new antimicrobial drugs because of their antimicrobial effect on opportunistic pathogenic and pathogenic microflora. This antimicrobial effect has been shown in food industry on extension of shelf-life of foods [7]. Antimicrobial effect of the medicinal plants is attributable to the content and composition of the plant essential oils (EO), which consist of a large number of secondary metabolites - alkaloids, tannins, and flavonoids [8,9]. Since the fish microflora may facilitate fish spoilage and contain opportunistic pathogens and pathogens, the application of the plant extracts can be useful tool to delay the fish microflora development.

The aims of present study were: i) identify the fish microflora with MALDI-TOF MS Biotyper and ii) to test the extracts of medicinal plants against the isolated and identified fish microflora.

Material and Methods

Sample collection

For microbiological analyses, three sample of common bleak (*Alburnus alburnus*) and three samples of common rudd (*Scardinius erythrophthalmus*) of Latvian origin were used. Samples were bought on the retail market, placed on ice and transported to the laboratory for the investigations.

Microbiological analyses

Skin and muscle tissues were used for microbiological testing. Skin samples were taken with sterile swabs from skin surface, but muscle samples were obtained after incision of skin and separation of muscle tissues. The primary dilution was done by adding the peptone buffered water to sample with 1:10 ratio (sample: peptone buffered water). Primary dilution was used for preparation of tenfold dilution.

The total viable count (TVC) and *Enterobacteriaceae* were detected in samples. TVCs was determined with the pour plate method in plate count agar (PCA, Biolife, Italy) and inoculated plates were incubated at 30 °C for 48-72 h. on plate count agar, the number of was determined on violet red bile glucose agar (VRBGA), McConkey and Endo agars (Biolife, Italy) were used for detection of *Enterobacteriaceae*. Inoculated agars were incubated at 37 °C for 24-48 h.

After evaluation of bacterial growth, 2-5 colonies from each agar were selected for confirmation with MALDI-TOF MS Biotyper. Bacterial colonies were subcultured on the nutrition agar at 37°C for 18-24 h for identification.

Sample preparation and MALDI-TOF MS measurement

One colony of each bacterial isolate was transferred into an Eppendorf vial with 300 µL of sterile water. Later, the ethanol (900 µL) was added to the suspension and mixed well. The suspension was centrifuged (13 000 g, 2 min), the supernatant was removed and the pellets were dried at room temperature until dry or at least for 5 min. The bacterial pellet was resuspended in 20–50 µL of formic acid (70 % in water) and the same amount of acetonitrile. After centrifugation (2 min at 13 000 g), an l µL of the supernatant was spotted onto a sample position of a polished steel MALDI target plate and dried at room temperature. Subsequently, 1 µl MALDI matrix (solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50 % acetonitrile/2.5 % trifluoro-acetic acid) was added to the spot and dried again. The MALDI target plate was introduced into the MALDI-TOF mass spectrometer for automated measurement and data interpretation. MALDI-TOF profile mass spectra were imported into the MALDI Biotyper 3.0 software and processed automatically after measurement. The logarithm of the score (log[score]) was displayed as the matching result. The MALDI Biotyper output was a log(score) between 0 and 3.0, which was calculated from a comparison of the peak list from an unknown isolate with the reference MSP in the database. A log(score) ≥1.7 indicated identification at the genus level, while a log(score) ≥2.0 was set as the threshold for a match at the species level. Isolates with ≥2.0 were accepted as a correct identification.

Preparation of plant extracts

The plant materials used in this experiment consisted of leaves of *Melissa officinalis* L., *Mentha piperita* L., *Origanum vulgare* L. and *Malva mauritiana.* The plants were collected in Slovakia. The material was initially dried at the room temperature in the dark. After drying, the plant materials were crushed and an amount of 10 g of leaves of each plant was soaked separately in 100 mL of ethanol (99.9%, Sigma, Germany) for 14 days at room temperature. Then, the ethanolic plant extracts were filtered through the Whatman No. 1 filter paper. After filtration, the obtained extracts were evaporated 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 plant extracts were dissolved in dimethylsulfoxid (DMSO) (Penta, Czech Republic). Stock solutions of plant extracts were stored at -16 °C in refrigerator until the experiments were initiated. For experiment, 6 mm sterilized filter paper discs were impregnated with plant extract.

Detection of antimicrobial activity

Antimicrobial activity of each plant extract was determined by a disc diffusion method. Briefly, a quantity of 100 µL of suspension of the test bacteria were grown in 10 ml of fresh media until the concentration of 10^5 CFU/mL was reached. Then, an amount of 100 µL of the microbial suspension was spread onto Mueller Hinton agar (Biolife, Italy) and impregnated discs were placed onto surface of inoculated agar. After incubation at 37 for 24 h, the diameters of the inhibition zones were measured. All measurements were to the closest whole millimeter. Each antimicrobial assay was performed in, at least, triplicate. Filter discs impregnated with a 10 µl of distilled water were used as a negative control.

Experiments were run triplicate, one colony for each of the three samples of each fish.

Statistical analyses

For each medicinal plants and pathogenic microorganisms the mean values and the standard deviation were calculated.

Results and Discussions

Altogether, 15 bacterial species were recovered from fish samples. All the mass spectrometry results indicated good identification on the species with score value from 2.123 to 2.407 for *Pantoea agglomerans* and *Staphylococcus caprae*, respectively. All isolated species were identified with ≥2.0 score and this score confirm very good identification. Isolated bacterial species and their MALDI-TOF scores are shown in Table 1.

Identified bacterial species belonged to five different families: *Comamonadaceae*, *Enterobacteriaceae*, *Moraxellaceae*, *Pseudomonadaceae* and *Staphylococcaceae* (Table 2). The most abundant bacterial family was *Enterobacteriaceae* with eight bacterial species isolated but the less abundant - *Comamonadaceae* with one bacterial species isolated. Bacterial strains *Acinetobacter pittii, Acinetobacter baumannii, Buttiauxella agrestis, Delftia acidovorans, Enterobacter cloacae, Staphylococcus epidermidis, Staphylococcus caprae* were isolated from skin of fish and *Escherichia coli, Lelliottia amnigena, Pantoea agglomerans, Pseudomonas alcaligenes, Pseudomonas oryzihabitans, Providencia rettgeri, Rahnella aquatilis, Serratia liquefaciens* were isolated from muscles of fish.

Table 1
MALDI-TOF scores of isolated microorganisms

Microorganisms	Score value
Acinetobacter pittii	2.351
Acinetobacter baumannii	2.125
Buttiauxella agrestis	2.225
Delftia acidovorans	2.128
Enterobacter cloacae	2.178
Escherichia coli	2.156
Lelliottia amnigena	2.246
Pantoea agglomerans	2.123
Pseudomonas alcaligenes	2.229
Pseudomonas oryzihabitans	2.269
Providencia rettgeri	2.219
Rahnella aquatilis	2.355
Serratia liquefaciens	2.258
Staphylococcus epidermidis	2.177
Staphylococcus caprae	2.407

Table 2
Family of the identified bacterial species

Microorganisms	Family
Acinetobacter pittii	*Moraxellaceae*
Acinetobacter baumannii	*Moraxellaceae*
Buttiauxella agrestis	*Enterobacteriaceae*
Delftia acidovorans	*Comamonadaceae*
Enterobacter cloacae	*Enterobacteriaceae*
Escherichia coli	*Enterobacteriaceae*
Lelliottia amnigena	*Enterobacteriaceae*
Pantoea agglomerans	*Enterobacteriaceae*
Pseudomonas alcaligenes	*Pseudomonadaceae*
Pseudomonas oryzihabitans	*Pseudomonadaceae*
Providencia rettgeri	*Enterobacteriaceae*
Rahnella aquatilis	*Enterobacteriaceae*
Serratia liquefaciens	*Enterobacteriaceae*
Staphylococcus epidermidis	*Staphylococcaceae*
Staphylococcus caprae	*Staphylococcaceae*

The present study revealed the abundance of *Enterobacteriaceae* and *Pseudomonaceae* in fish microflora and our finding is in accordance with previously reported [3]. The bacterial microflora of freshwater fish skin have been reported to include *Acinetobacter johnsonii* [10], aeromonads (notably *Aeromonas hydrophila, A. bestiarum, A. caviae, A. jandaei*, *A. schubertii*, and *A. veronii* biovar sobria [11]), *Alcaligenes piechaudii, Enterobacter aerogenes, Escherichia coli, Flavobacterium* [12], *Flexibacter* spp., *Micrococcus luteus, Moraxella* spp., *Pseudomonas fluorescens*, *Psychrobacter* [10] and *Vibrio fluvialis* [13,14,15]. Our study supports the previous observation with isolation of *Escherichia*, *Acinetobacter*, *Pseudomonas* spp. and other bacterial species.

Composition of microflora and occurrence of specific microorganisms may reflect the contamination of fish from surrounding environment [12]. Fish in the present study were bought at the retail market, therefore the occurrence of *Escherichia* and *Staphylococcus* may indicate the human presence then the fish could be contaminated during the unhygienic handling on market. In general, the composition of the fish microflora indicates that the fish are prone to spoilage caused by *Enterobacteriaceae* – *Rahnella aquatilis*, *Pantoea agglomerans* and *Providencia rettgeri* and *Pseudomonaceae*.

Isolation of *Acinetobacter baumanii* from fish samples is an issue of concern. Despite the environment, including water and soil, is a well-known habitat of *Acinetobacter* spp, the microorganisms has been confirmed as an important source of nosocomial infections with extra-hospital reservoirs. Isolation of those opportunistic pathogens from fish shows that *A. baumanii* could spread also with fish.

All the plant extracts tested in the present study were found to show the inhibitory against fish microflora. In our study, the best antimicrobial activity of *Melissa officinalis* L. was found against *Pseudomonas oryzihabitans* (9.67±0.58), *Serratia liquefaciens* (7.67±0.58) and *Pseudomonas alcaligenes* (6.67±0.58) (Table 3). *Melissa officinalis* L. (fam. *Lamiaceae*) is a perennial, aromatic herb native to southern Europe. *M. officinalis* is used widely in food and cosmetics because of the intense lemon aroma and flavor of leaves. *M. officinalis* leaf extract is known to exhibit the antiviral and antioxidant activity but the essential oil possesses the antibacterial, antifungal and antihistaminic effect [16]. The antibacterial activity of *M. officinalis* has been reported in previously [17,18]. In this study, the plant ethanol extract was used. Other researchers showed that the ethanol extract of *M. officinalis* can possess an antioxidant [19] and antinociceptive effect [20].

Table 3

Antimicrobial activity of medicinal plants extract against bacteria isolated from fish

Microorganisms/extract	***Malva mauritiana***	***Melissa officinalis* L.**	***Mentha piperita* L.**	***Origanum vulgare* L.**
Acinetobacter pittii	2.67±0.58	1.67±1.54	4.67±0.58	2.33±0.58
Acinetobacter baumannii	2.33±0.58	3.00±1.00	2.00±1.00	1.33±1.53
Buttiauxella agrestis	2.00±1.00	2.00±1.00	3.33±0.58	2.33±0.58
Delftia acidovorans	3.33±0.58	4.33±1.15	1.67±0.58	2.00±1.00
Enterobacter cloacae	1.67±0.58	5.00±1.00	6.67±1.53	9.67±0.58
Escherichia coli	2.67±0.58	2.33±0.58	5.33±0.58	6.67±1.58
Lelliottia amnigena	3.33±1.53	1.33±1.53	2.00±1.00	4.67±0.58
Pantoea agglomerans	2.33±1.53	2.33±0.58	1.33±0.58	3.00±0.71
Pseudomonas alcaligenes	4.67±0.58	6.67±0.58	5.33±0.58	5.67±0.58
Pseudomonas oryzihabitans	6.67±1.53	9.67±0.58	5.67±0.58	7.33±0.58
Providencia rettgeri	1.67±0.58	2.33±0.58	3.33±0.58	1.67±1.15
Rahnella aquatilis	2.00±0.00	3.33±1.53	2.67±0.58	3.00±1.00
Serratia liquefaciens	5.00±1.00	7.67±0.58	5.67±0.58	9.67±0.58
Staphylococcus epidermidis	4.33±1.15	5.33±0.58	7.33±0.58	4.33±1.15
Staphylococcus caprae	5.00±1.00	3.67±0.58	5.67±0.58	5.33±0.58

Antimicrobial activity against fish microflora has been shown by *Malva mauritiana.* The best antimicrobial activity against *Pseudomonas oryzihabitans* (6.67±1.53), *Staphylococcus carpae* (5.00±1.15) and *Serratia liquefaciens* (5.00±1.00). *Malva* sp., widely known as common mallow, is native to Europe. Application of mallow in traditional medicine has long-time practice, however, little clinical evidence is available. Different parts of plant including roots, leaves, flowers, fruits, and seeds were used for preparation of infusions, decoctions, liniments, lotions, baths and gargles [21,22,23,24,25,26]. Both flowers and leaves were found to show antimicrobial but not antifungal activity [27].

The antimicrobial activity was exhibited by *Mentha piperita* as well. The best results were found against *Staphylococcus epidermidis* (7.33±0.58), *Enterobacter cloacae* 6.67±1.53, *Pseudomonas oryzihabitans, Serratia liquefaciens* and *Staphylococcus caprae* (all 5.67±0.58).

Mentha piperita is a perennial plant with world-wide distribution, which is cultivated and wild. *Mentha piperita* is used for preparation of tea, tinctures, oils or extracts, can be included in rubs or liniments because of it astringent, antiseptic, antipyretic, antispasmodic, anticatarrhal, antimicrobial, rubefacient, stimulant, emmenagogue and antiaging properties [28]. It has been reported, that the leaf extracts were found to be very active against the bacterial strains. Antimicrobial activity was possessed by stem and root extracts as well. Antibacterial properties of leaf and stem extracts were suggested for application in wound healing and septicemia [29,30].

The best antimicrobial activity of *Origanum vulgare* was found against *Enterobacter cloacae*, *Serratia liquefaciens* (both 9.67±0.58) and *Pseudomonas oryzihabitans* (7.33±0.58).

Oregano (*Origanum vulgare* subsp. *hirtum*) is a herb of the *Labiatae* family and has been used widely in cooking and traditional medicine. Antimicrobial activity of oregano is related to the the presence of several ingredients, most of which possess antioxidant and antimicrobial properties [31]. Carvacrol and thymol, which comprise about 78–85% of oregano EOs, are mainly responsible for the antimicrobial activity [32]. Oregano has been reported an effective in modern medicine for inhibition of pathogenic microorganisms and for treatment of infectious diseases [33]. In general, all the medicinal plant extract exhibited the antimicrobial activity indicating the possible application of the extract for prevention of proliferation of microorganisms in fish.

Conclusions

The present study revealed that the extract from leaves from *Melissa officinalis* L., *Mentha piperita* L., *Origanum vulgare* L. and *Malva mauritiana* exhibited the antibacterial activity against fish microflora. *Pseudomonas oryzihabitans*, *Serratia liquefaciens, Enterobacter cloacae* and *Staphylococcus* spp. were among the most susceptible bacterial species to the action of plant extracts. In conclusion we recommend use *Malvia mauritiana* and *Melissa officinalis* L. extract against *Pseudomonas oryzihabitans*, *Mentha piperita* L. against *Staphylococcus epidermis* and *Origanum vulgare* L. against two bacterial strains *Enterobacter cloacae* and *Serratia liquefaciens*.

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