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ANTIBACTERIAL ACTIVITY OF ETHANOLIC LEAF EXTRACTS OBTAINED FROM VARIOUS *FICUS* SPECIES (MORACEAE) AGAINST THE FISH PATHOGEN, *CITROBACTER FREUNDII*

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Abstract

The diversity of culturable bacteria inhabiting the Baltic sea surface waters was more divergent at a polluted location than at clean areas. The most important members of the family Enterobacteriaceae that are pathogenic to fish are the enteric redmouth disease agent, *Yersinia ruckeri* and two species of *Edwardsiella*, *E. tarda* and *E. ictaluri*, *Serratia*, *Proteus* and *Citrobacter* have all been implicated as potential fish pathogens. The use of pharmaceutical substances is rather limited in fish compared to mammalian therapeutics. Medicinal herbs play an alternative role to antibiotic therapy in aquaculture. *Ficus* species (Moraceae) leaves possess great medicinal potential for the therapy of bacterial and fungal infections and may be used as a natural antiseptic and antimicrobial agent in veterinary. Accordingly, these products can be used in aquaculture as therapeutic and prophylactic agents against fish pathogens, with antimicrobial properties. Present study aimed to investigate the *in vitro* antimicrobial activity of the ethanolic leaf extracts of various *Ficus* species against fish pathogen, *Citrobacter freundii*. The antimicrobial susceptibility testing was done on Muller-Hinton agar by disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol). Our results demonstrate that various species of *Ficus* had mild antibacterial *in vitro* activity against *C. freundii* isolated locally from infected eel (*Anguilla anguilla* L.). The results proved that the extracts from *F. drupacea*, *F. septica*, *F. deltoidea* as well as *F. hispida*, *F. mucuso*, *F. pumila*, *F. craterostoma* exhibit a favorable antibacterial activity against *C. freundii*. These validate scientifically their inhibitory capacity attributed by their common use in folk medicine and contribute towards the development of new treatment options in aquaculture based on natural products. The chemical analysis of the aforementioned plant extracts should be performed to determinate their chemical composition and identify the exact phytocompounds responsible for antimicrobial activity against *C. freundii*. In addition, they should be subjected to pharmacological evaluations with the aim of assessing their *in vivo* efficacy, toxicity, potential adverse effects, interactions and contraindications. Given the increasing rate of resistance development in bacterial pathogens in aquaculture environments, medicinal plants with antibacterial properties are very important as natural resources for new active compounds.

Key words: antibacterial activity, *Citrobacter freundii*, infected eel (*Anguilla anguilla*), *Ficus* spp., ethanolic extracts, zone of growth inhibition

INTRODUCTION

The sewage effluent causes contamination of marine wildlife along coastal lines (Al-Bahry et al. 2009). Indeed, inadequately purified wastewater is regularly disposed into coastal waters is resulted in a negative influence on marine ecosystems and its inhabitants (Rinawati et al. 2012). Other anthropogenic activities such as capture fisheries and aquaculture also affect benthic communities as well as local fish communities and their environment (Dsikowitzky et al. 2011, Hennersdorf et al. 2016). Numbers of stored contaminants varied between species which is probably related to differences in microhabitat and feeding mode (Dsikowitzky et al. 2011). The consequences of these factors on the microbiome of fish and the possible implications on fish health are yet unknown (Hennersdorf et al. 2016). It can be assumed that the environmental conditions, parasite infections and viral or bacterial disease outbreaks are linked and influence each other (Hennersdorf et al. 2016).

Nearly half the seabed of the Baltic Proper is incapable of supporting life of higher organisms as a consequence of oxygen depletion resulting from eutrophication. However, these areas are actually teeming with microbial life (Edlund et al. 2006). Moreover, diversity of culturable bacteria inhabiting the Baltic Sea surface waters was also studied by Hantula and collegues (1996). Rarefaction analysis revealed that the bacterial community was more divergent at a polluted location than at clean areas. Also the most common operational taxonomic units were different in clean locations compared to the polluted site suggesting that both diversity and species composition of the bacterial community is greatly affected by pollution (Hantula et al. 1996).

The use of pharmaceutical substances is rather limited in fish compared to mammalian therapeutics. It is basically restricted to anaesthetic agents and anti-infective agents for parasitic and microbial diseases. The anti-infective agents are used for controlling diseases and the choice of drug depends on efficacy, ease of application, human safety, target animal safety including stress to the fish, environmental impact, regulatory approval, costs, and implications for marketing the fish (Burka et al. 1997). In fish aquaculture, disinfectants are used against bacterial and protozoal infections. These compounds cause oxidative stress that may stimulate the generation of reactive oxygen species, and subsequently the alteration in antioxidant systems of exposed organisms (Stara et al. 2014).

Antibiotics are widely used in fish farms to prophylactically treat bacterial infections and as a growth promoter. Despite its widespread use, there is no regulation on this drug class in fish (Rigos and Troisi 2005). It is desirable that antibiotic use in fish cultures be reduced and replaced by natural medicines to prevent the emergence of bacterial resistance in aquatic animals and its environment. Therefore, use of plant extracts can be more effective for preventive and therapeutically aims in aquaculture. Ability of herbs to inhibit growth of bacteria having potential interest as fish pathogens has been well documented. The antimicrobial activity of various plants has been tested against many Gram positive and Gram negative bacteria by different research groups worldwide highlighting the importance of herbs as alternative therapeutic agents in aquaculture. Herbal immunostimulants as an alternative to drugs, chemicals and antibiotics have been recommended to control fish diseases in fish culture (Haniffa and Kavitha 2012). Abdul Kader Mydeen and Haniffa (2011) have suggested that heavy antibiotics in aquaculture should be reduced and replaced with alternative agents in fish diseases to avoid the emergence of antibiotic resistance in pathogenic and environmental bacteria.

The preliminary screening assays have indicated that some plants with antibacterial properties may offer alternative therapeutic agents against bacterial infections in aquaculture industry. Antimicrobial properties of medicinal herbs are being increasingly reported from different parts of the world. Furthermore, the use of plant extracts by the traditional extraction method in aquaculture is considered to be the eco-friendly approach and cost-effectiveness method, which does not cause side effects (Thanigaivel et al. 2015). Thus, the use of medicinal plants is an alternative to antibiotics in fish health management.

Medicinal plants involved in traditional medicine are potential sources of antimicrobial compounds. Recently, there has been increasing interest in *Ficus* spp. (*Moraceae*) due to its chemical composition and the potential health benefits. *Ficus* spp. have been extensively used in traditional medicine for a wide range of ailments of the central nervous system, endocrine system, gastrointestinal tract, reproductive system, respiratory system and infectious disorders (Kumar and Augusti 1989, Cherian and Augusti 1993, Kirana et al. 2009, 2011, Usman et al. 2009, Ahmad et al. 2011, Ilyanie et al. 2011, Dangarembizi et al. 2012, Arunachalam and Parimelazhagan 2013, Gul-e-Rana et al. 2013, Farsi et al. 2014). However, although many species within the genus *Ficus* have been encompassed by phytochemical and pharmacological investigations in previous years, there are many species that have not been studied and whose ethnobotanical relevance is yet to be investigated. The aim of present study was to evaluate the antibacterial capacity of ethanolic extracts obtained from leaves of various *Ficus* species against *C. freundii* in order to validate scientifically the inhibitory activity attributed by their popular use and to propose new sources of antimicrobial agents in aquaculture for prevention and treatment of fish disease caused by these bacteria.

MATERIALS AND METHODS

Collection of Plant Material. The leaves of *F. aspera* G. Forst, *F. benghalensis* L., *F. benjamina* L., *F. benjamina* 'Reginald', *F. binnendijkii* (Miq.) Miq., *F. binnendijkii* 'Amstel Gold', *F. binnendijkii* 'Amstel King', *F. carica* L., *F. craterostoma*

Mildbr. & Burret, *F. cyathistipula* Warb., *F. deltoidea* Jack, *F. drupacea* Thunb., *F. drupacea* 'Black Velvet', *F. elastica* Roxb., *F. elastica* 'Variegata', *F. erecta* Thunb., *F. erecta* var. *sieboldii* (Miq.) King, *F. hispida* L.f., *F. luschanthiana* (Miq.) Miq., *F. lyrata* Warb., *F. macrophylla* Desf. ex Pers., *F. mucuso* Welw. ex Ficalho, *F. natalensis* Hochst. subsp. *natalensis*, *F. natalensis* Hochst. subsp. *leprieurii* (Miq.) C.C. Berg, *F. palmeri* S.Watson, *F. platypoda* (Miq.) A. Cunn. ex Miq., *F. pumila* L., *F. religiosa* L., *F. rubiginosa* Desf. ex Vent., *F. sagittata* J. Koenig ex Vahl, *F. septica* Burm. f., *F. sur* Forssk., *F. sycomorus* L., *F. vasta* Forssk., *F. villosa* Blume were collected in M. Gryshko National Botanical Garden (Kyiv, Ukraine). The whole collections of tropical and subtropical plants at NBG (including *Ficus* spp. plants) have the status of a National Heritage Collections of Ukraine. The species author abbreviations were followed by Brummitt and Powell (Authors of plant names... 1992) (Davis and Schmidt 1996, Jones et al. 1997).

Preparing Plant Extracts. The sampled leaves of *Ficus* spp. were brought into the laboratory for antimicrobial studies. Freshly crushed leaves were washed, weighted, and homogenized in 96% ethanol (in proportion 1:10) at room temperature.

Method of Culturing Pathological Sample and identification Method of the Bacteria. *Citrobacter freundii* isolated locally from gill of eel (*Anguilla anguilla* L.) with clinical features of disease. Fish with clinical signs (hatcheries eels, *Anquilla anquilla*) of disease were euthanized with Propiscin (IRŚ, Poland), anesthetic designed for fish, in immersion using 2 ml per liter of water. Samples of internal organs (kidney, spleen, liver) weighting 2 g were taken and homogenized before preincubation in TSB broth (Tripticase Soya Broth, Oxoid) for 24 hrs. After preincubation, bacterial culture was transferred to two different cultivation mediums: TSA (Tripticase Soya Agar, Oxoid) and BHIA (Brain Heart Infusion Agar, Oxoid) supplemented with 5% of sheep blood (OIE Fish Diseases Commission 2016, www.oie. int/animal-health-in-the-world/oie-listed-diseases-2016/ access on 03/08/2016). After 48 h of incubation in 27°C, characteristic round, smooth and conver colonies were selected for further examination. They were not pigment and did not induce haemolysis on blood agar. Bacterial species were identified with the use of the oxidase test and API E test kit (Biomerieux, France). The results of test were interpreted in accordance with the manufacturer's protocol, after 24 h of incubation at 37°C.

Bacterial Growth Inhibition Test of Plant Extracts by the Disk Diffusion Method. Strain tested was plated on TSA medium (Tryptone Soya Agar) and incubated for 24 hrs at 25°C. Then the suspension of microorganisms were suspended in sterile PBS and the turbidity adjusted equivalent to that of a 0.5 McFarland standard. Antimicrobial activity of extracts was evaluated by using agar well diffusion method (Bauer et al. 1966). Muller-Hinton agar plates were inoculated with 200 µl of standardized inoculum (10^8 CFU/mL) of bacterium and spread with sterile swabs.
Sterile filter paper discs impregnated by extract were applied over each of the culture plates, 15 min after bacteria suspension was placed. The antimicrobial susceptibility testing was done on Muller-Hinton agar by disc diffusion method (Kirby-

Bauer disk diffusion susceptibility test protocol). The *Citrobacter freundii* isolates were individually tested against 4 antibiotics. The results were determined using the disk diffusion method. The tested antibiotics were as follows: gentamicin, tetracycline, enrofloxacin, and sulfonamide. A negative control disc impregnated by sterile ethanol was used in each experiment. The sensitivity of strain was also studied to the commercial preparation with extracts of garlic (in dilution 1:10, 1:100 and 1:1000). After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 hrs at 25°C. The assessment of antimicrobial activity was based on measurement of the diameter of the inhibition zone formed around the disks.
The diameters of the inhibition zones were measured in millimeters, and compared with those of the control and standard susceptibility disks. Activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated six times. The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (S) ≥ 15 mm, Intermediate (I) = 11-14 mm, and Resistant (R) ≤ 10 mm (Okoth et al. 2013).

RESULTS

The results of screening study of antimicrobial activity of ethanolic extracts obtained from *Ficus* spp. leaves are presented in Table 1, Figs 1-5. A comparison of susceptibility categories, i.e., intermediate and resistant, for the disk diffusion method is shown in Table 1.

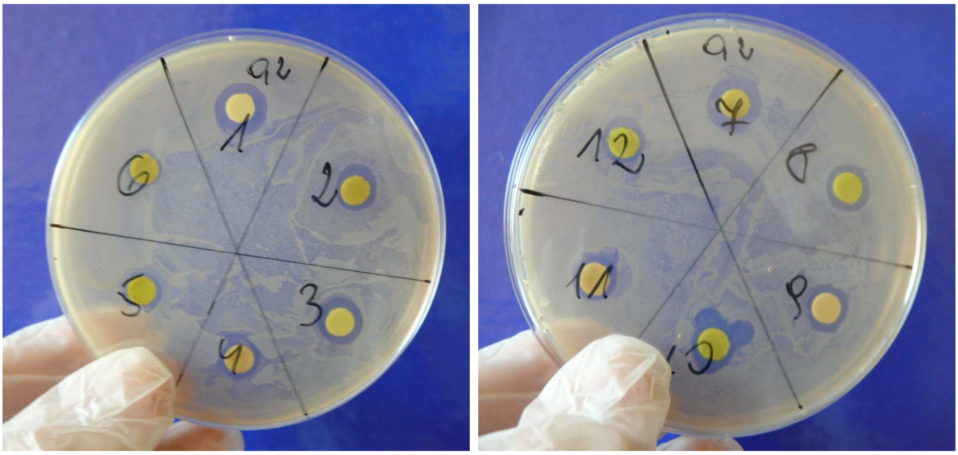
Table 1

Susceptibility or resistance of *Citrobacter freundii* against ethanolic extracts obtained from *Ficus* spp. leaves

Ficus spp.	Susceptibility or resistance of *Citrobacter freundii*	
	Intermediate (I) = 11-14 mm	Resistant (R) ≤ 10 mm
F. aspera		+
F. benghalensis		+
F. benjamina		+
F. benjamina 'Reginald'		+
F. binnendijkii		+
F. binnendijkii 'Amstel Gold'		+
F. binnendijkii 'Amstel King'		+
F. carica		+
F. craterostoma	+	
F. cyathistipula		+
F. deltoidea	+	
F. drupacea	+	

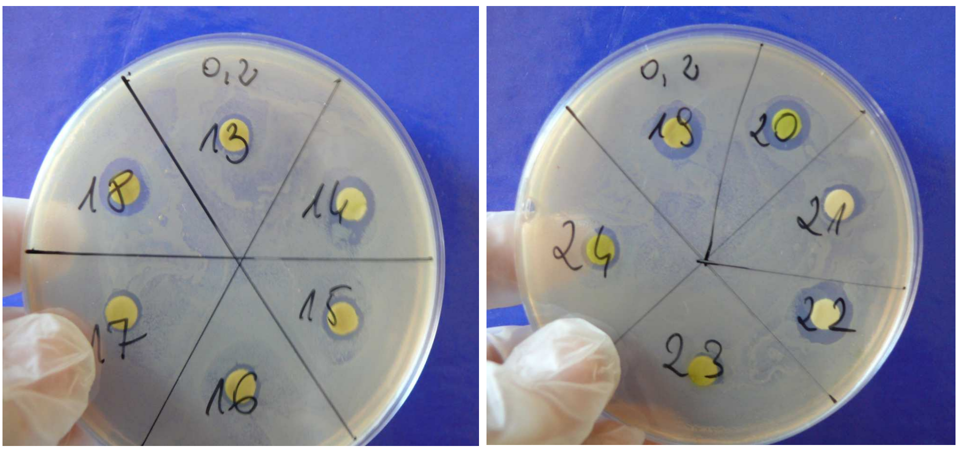
Ficus spp.	Susceptibility or resistance of *Citrobacter freundii*	
	Intermediate (I) = 11-14 mm	Resistant (R) ≤ 10 mm
F. drupacea 'Black Velvet'		+
F. elastica		+
F. elastica 'Variegata'		+
F. erecta		+
F. erecta var. *sieboldii*		+
F. hispida	+	
F. luschanthiana		+
F. lyrata		+
F. macrophylla		+
F. mucuso	+	
F. natalensis subsp. *leprieurii*		+
F. natalensis subsp. *natalensis*		+
F. palmeri		+
F. platypoda		+
F. pumila	+	
F. religiosa		+
F. rubiginosa		+
F. sagittata		+
F. septica	+	
F. sur		+
F. sycomorus		+
F. vasta		+
F. villosa		+

Our results demonstrated that the *C. freundii* (200 µl of standardized inoculum) revealed intermediate susceptibility (diameter of inhibition zone) concerning to ethanolic extracts obtained from *F. drupacea*, *F. septica*, *F. deltoidea* (mean of inhibition zone diameters was 12 mm), *F. hispida*, *F. mucuso*, *F. pumila*, *F. craterostoma* (11 mm). *C. freundii* was resistant against ethanolic extracts from *F. aspera*, *F. benghalensis*, *F. benjamina*, *F. benjamina* 'Reginald', *F. binnendijkii*, *F. binnendijkii* 'Amstel Gold', *F. binnendijkii* 'Amstel King', *F. binnendijkii* 'Amstel Gold', *F. carica*, *F. cyathistipula*, *F. deltoidea*, *F. drupacea*, *F. drupacea* 'Black Velvet', *F. elastica*, *F. elastica* 'Variegata', *F. erecta*, *F. erecta* var. *sieboldii*, *F. luschanthiana*, *F. lyrata*, *F. macrophylla*, *F. natalensis* subsp. *natalensis*, *F. natalensis* subsp. *leprieurii*, *F. palmeri*, *F. platypoda*, *F. religiosa*, *F. rubiginosa*, *F. sagittata*, *F. sur*, *F. sycomorus*, *F. vasta*, *F. villosa* (Table 1).



A B

Fig. 1. Antimicrobial activity of ethanolic extracts obtained from *F. hispida* (1), *F. villosa* (2), *F. mucuso* (3), *F. benghalensis* (4) (A), *F. cyathistipula* (7), *F. macrophylla* (8), *F. sycomorus* (9), *F. luschanthiana* (10) (B) against *C. freundii*

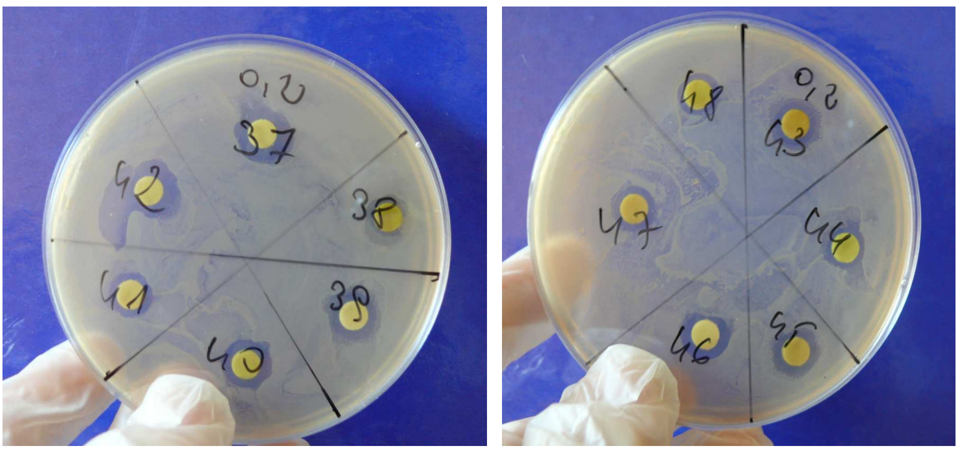


A B

Fig. 2. Antimicrobial activity of ethanolic extracts obtained from *F. binnendijkii* (18) (A), *F. elastica* (19), *F. benjamina* 'Reginald' (20), *F. pumila* (22) (B) against *C. freundii*

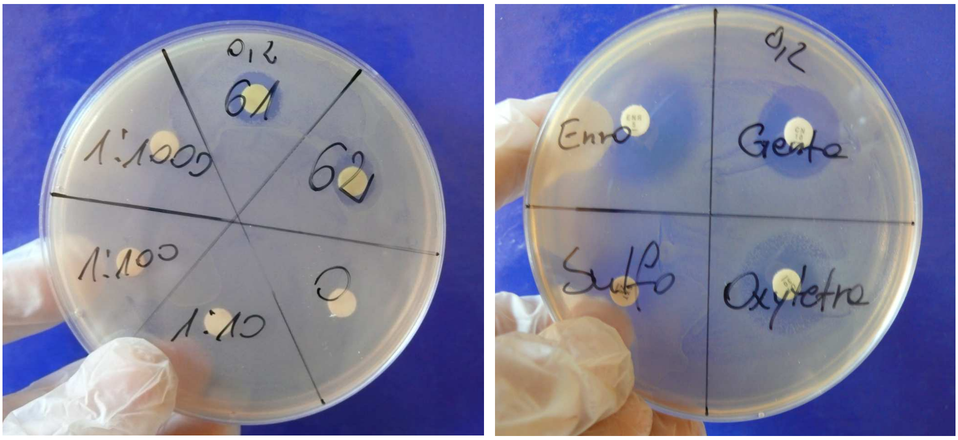
A B

Fig. 3. Antimicrobial activity of ethanolic extracts obtained from *F. craterostoma* (27), *F. drupacea* 'Black Velvet' (28), *F. drupacea* (30) (A), *F. septica* (31), *F. natalensis* subsp. *leprieurii* (34), *F. binnendijkii* 'Amstel Gold' (35), *F. deltoidea* (36) (B) against *C. freundii*



A B

Fig. 4. Antimicrobial activity of ethanolic extracts obtained from *F. erecta* var. *sieboldii* (37), *F. rubiginosa* (40), *F. sagittata* (42) (A), *F. lyrata* (43), *F. vasta* (48) (B) against *C. freundii*



A B

Fig. 5. Antimicrobial activity of different concentrations of garlic (*Allium sativum*) extract (A), and tested antibiotics (B) against *C. freundii*

DISCUSSION

In line with the growing interest in the antibacterial potential of various plants, the antimicrobial properties of the ethanolic extract prepared from leaves of various *Ficus* species were assessed against *Citrobacter freundii*. It is a member of the family Enterobacteriaceae and is often the cause of significant opportunistic infections. It may cause fatal septicemia in humans and animals (Fernández et al. 2011). *C. freundii* has also been associated with neonatal meningitis and brain abscess (Badger et al. 1999). *C. freundii* is an enterobacterium usually isolated from soil, water, sewage, and food as well as from different organs of diseased and healthy animals, including mammals, birds, reptiles, and amphibians, in which it is considered to be an opportunistic or secondary pathogen (Toranzo et al. 1994). The study of Chen and co-workers (2002) has demonstrated the importance of appropriate antimicrobial therapy to the successful outcome of *C. freundii* bacteremia. The resistance of *C. freundii* to most third-generation cephalosporins and broad-spectrum penicillins increased in both nosocomial and community-acquired *C. freundii* bacteremia, and the strategy of using a combination of antimicrobial agents to treat *C. freundii* infection was effective (Chen et al. 2002).

There are only a few reports suggesting that other enterics may be pathogenic for fish. However, recent reports suggest that they may represent an emerging problem. The most important members of the family Enterobacteriaceae that are pathogenic to fish are the enteric redmouth disease agent, *Yersinia ruckeri* and two species of *Edwardsiella*, *E. tarda* and *E. ictaluri*. *Serratia, Proteus* and *Citrobacter* have all been implicated as potential fish pathogens. These bacteria share some common characteristics with other members of the Enterobacteriaceae: they are small, Gram-negative rods, catalasepositive and oxidase-negative and produce acid from glucose by oxidative and fermentative metabolism. Motile species have peritrichous flagella and most reduce nitrate to nitrite. Further biochemical characterization can be carried out using the various commercial test strips that are available (Daly and Aoki 2011).

C. freundii have been suggested as possible fish pathogens, on the basis of isolations from infections and mortalities (Sato et al. 1982, Baya et al. 1990, Sanz 1991, Karunasagar et al. 1992). Toranzo and co-workers (1994) found that nine fish isolates were of low virulence (LD_{50} of greater than 5×10^7) but could establish a carrier state within the fish. As enterobacteria are ubiquitous in the environment, it is not surprising that some should be opportunistic pathogens of fish. They would present a particular risk in warm water ponds that frequently are exposed to faecal material, such as silver carp ponds fertilized regularly with poultry manure (Bejerano et al. 1979) or other polluted waters. A case in point is the recent report of a *C. freundii* infection of carp fingerlings. This may have resulted from a contaminated water supply after the onset of the monsoon (Karunasagar et al. 1992). Baya and co-workers (1992) have reported that fish living in river water that receives city sewage carry a significantly higher number of bacterial species in comparison with fish from less polluted waters. Nawaz and co-workers (2008) have revealed that citrobacters from catfish could serve as reservoirs of tetracycline-resistance determinants. Fifty-two tetracycline-resistant *Citrobacter* spp. strains were isolated from farm-raised catfish. Morphological and biochemical characteristics indicated that 38 of the 52 citrobacters were *C. freundii*, 7 were *C. amalonaticus* and 7 were *C. braakii*. All isolates were resistant to multiple antibiotics (Nawaz et al. 2008).

Pathoanatomical examination of Jeremic and co-workers (2003) has identified lesions typical of hemorrhagic septicemia caused by *C. freundii*. An increase in the quantity of mucous mass on the surface of the skin and gills, erosion and dropping off of scales on the skin, diffuse bleeding on the skin and fins, bilateral exophthalmus and bleeding in the eyes, diffuse bleeding on the ventral part of the abdomen were recorded in fish infected by *C. freundii*. The anal orifice was bloody. The gills were pale due to anemia, exhibited petechial hemorrhages, and were edematous and with necrosis of the tips of the gill filaments in some specimens. All internal organs and the wall of the swim bladder were edematous. A small quantity of reddish fluid was present in the abdominal cavity. Bleeding was recorded on the internal organs, primarily on the inner wall of the swim bladder and on the gonads, intestine, muscles, kidneys, and liver. The spleen was enlarged and unevenly colored. The wall of the intestine was edematous, the lumen expanded, lacking food, and filled with a bloody fluid. Petechial and diffuse hemorrhaging was present in the wall (Jeremic et al. 2003).

As noted by Tukmechi and co-workers (2010), some fish bacterial pathogens are also associated with diseases in humans, making the aquaculture products as a potential risk to the customers. Consequently, one of the overriding considerations is how disease may be controlled. On the other hand, resistance against antibiotics of many bacteria is accumulating. Therefore, searches for new substances with antimicrobial activity have become an urgent necessity.

Our results indicated that extracts offer a promising alternative to the use of antibiotics in controlling of infection caused by *C. freundii*. In our study, most ethanolic extracts obtained from *Ficus* spp. proved effective against *C. freundii* tested, with 10-12 mm zones of inhibition being observed. *C. freundii* demonstrated the highest susceptibility against *F. drupacea*, *F. septica*, *F. deltoidea* (Table 1, Fig. 3). Among various species of *Ficus* with moderate activity against *C. freundii*, the highest antibacterial activity for *F. hispida*, *F. mucuso*, *F. pumila*, *F. craterostoma* was noted (Table 1, Figs 1-3).

The comprehensive review of usefulness of some medicinal plants (herbal drugs) against fish diseases has been presented by Govind Pandey and coauthors (2012). On the basis of several studies, Yin and coauthors (2008) reported that oral administration of ginger (*Zingiber officinale*) extract increases the phagocytic capability of cells in rainbow trout, while the extracts of 4 Chinese herbs (*Rheum officinale*, *Andrographis paniculata*, *Isatis tinctoria* and *Lonicera japonica*) increased the phagocytosis of white blood cells of carp.

The ginger (*Zingiber officinale*) and guava (*Psidium guajava* L.) represent a promising food additives for carps in aquaculture. The evaluation of the effects of ginger as a feeding supplement on the growth, skin mucus immune parameters, and cytokine-related gene expression of *Labeo rohita*, and its susceptibility to *Aeromonas hydrophila* infection was studied by Sukumaran and collegues (2016). Dietary supplements of ginger (at 0.8%) can promote growth performance, skin mucus immune parameters, and strengthen immunity of *L. rohita*. The expression of genes encoding pro-inflammatory cytokines (IL-1β, TNF-α), signaling molecules Kelch-like-ECH-associated protein 1 (Keap1), and nuclear factor kappa B p65 (NF-κBp65) were down-regulated in treatment groups. Moreover, fish fed a 0.8% ginger supplemented diet exhibited significantly higher relative post-challenge survival (65.52%) against *A. hydrophila* infection. Dietary supplementation with guava leaves (at 0.5% concentration) could also promote growth performance and strengthen immunity of *L. rohita* (Giri et al. 2015). The effects of *Psidium guajava* L. (guava) leaves (0% (basal diet), 0.1%, 0.5%, 1%, and 1.5%) on the growth and immune response of the fish species *L. rohita* and its susceptibility to *A. hydrophila* infection. The lysozyme levels, leukocyte phagocytic activity, and alternative complement pathway activity were significantly high in guava fed group. Moreover, fish fed the guava diet exhibited a significantly higher post-challenge survival rate (66.7%) (Giri et al. 2015).

From ancient times neem (*Azadirachta indica*) leaves containing nimbin, azadirachtin and meliantroil have been reported to possess a variety of properties, including antimicrobial, insecticidal and antiviral (Pandey et al. 2012). Neem leaf aqueous extract was found to be effective in the treatment of infection caused by *C. freundii* in tilapia (Thanigaivel et al. 2015). According to Winkaler and co-workers (2007), *A. indica* extract can be used successfully in aquaculture to control fish predators. Sutili and co-workers (2015) also investigated the *in vitro* antibacterial activity of the essential oils of *Hesperozygis ringens*, popularly known as 'espanta-pulga' and two different species of basil, *Ocimum gratissimum* and *Ocimum americanum*, as well as the potential of these products used in silver catfish (*Rhamdia quelen*) infected with *A. hydrophila*. All tested essential oils showed *in vitro* antibacterial properties against *A. hydrophila*. *H. ringens* and *O. americanum* showed potential to be used in the treatment of infected fish (Sutili et al. 2015). Al Laham and Al Fadel (2014) have investigated the anti-bacterial activity of the extracts prepared from different parts of *Olea europaea*, *Myrtus communis*, *Thymus vulgaris*, *Rosmarinus officinalis*, and *Achillea falcata* occurring in Syria against *A. hydrophila*. They have revealed that ethanolic extracts of the studied plants had different antibacterial effects against antibiotic-resistant *A. hydrophila*. *T. vulgaris* had the highest activity, *R. officinalis* had the second, and *M. communis* and *A. falcata* were in the third place, while the *O. europea* had the weakest antibacterial activity (Al Laham and Al Fadel 2014). Anusha and co-workers (2014) also screened *in vitro* antibacterial and immunostimulant activity of *Ixora coccinea*, *Pergularia daemia* and *Tridax procumbens* against the freshwater fish pathogen *A. hydrophila*. Initial screening results revealed that ethyl acetate extracts and its purified fraction of *I. coccinea* were able to suppress the *A. hydrophila* strains at more than 15 mm of zone of inhibition and exhibited positive immunostimulant activity (Anusha et al. 2014). Likewise, the essential oils from *Origanum vulgare* and *Rosmarinus officinalis* led to a significant decrease in *A. hydrophila* viability after 24 h of exposure (Azerêdo et al. 2012).

In line with these general findings, there are copious evidences that various species of genus *Ficus* exhibit antimicrobial properties against broad spectrum of microorganisms. The scientific research on *Ficus* spp. indicated that these plants have received increasing interest in recent years. It is well documented that various *Ficus* spp. have been used against Gram-positive and Gram-negative bacteria (Salem et al. 2013). For instance, Yessoufou and co-workers (2015) have investigated the antimicrobial (fungi and bacteria) and antiproliferative activities of crude extracts of the stem bark and isolated compounds from *F. drupaceae*. It stem barks contain several compounds that have antimicrobial activities against diverse human pathogenic, food and agricultural microbes as well as anticancer activities against human cancer cells of HeLa, MCF-7, Jurkat, HT-29 and T24. Seven biochemical compounds from stem bark extracts including β-amyrin (1), β-sitosterol-3-O-β-D-glucopyranoside (2), 5-O-methyllatifolin (3), oleanolic acid (4), epifriedelanol (5), friedelin (6) and epilupeol acetate (7) were isolated and identified. Of all the seven compounds, the compounds 3 and 7 exhibited the highest antifungal and antibacterial activities against screened microorganisms. *Aspergillus versicolor* and *A. ochraceus* were the most sensitive microorganisms to the isolated compounds whereas *Candida albicans* was the most resistant fungus. Compounds 4, 5, and 6 did not exhibit much variation in their antibacterial activities except against *Staphylococcus aureus* and *Escherichia coli*. The most sensitive bacterium to isolated compounds was *Bacillus cereus* whereas the most resistant one was *Enterobacter cloacae*. However, compounds 4, 6 and 7 exhibited the highest antiproliferative activities against most cancer cells (Yessoufou et al. 2015).

F. septica has been used in folk medicine to treat colds, fevers, headaches, gastralgia, and fungal and bacterial diseases, and has been reported to contain phenanthroindolizine-type alkaloids, triterpenoids, and phenolic compounds. Among these components, the phenanthroindolizine-type alkaloids have important biological effects, including anti-inflammatory, antitumor, antifungal, and antibacterial activities (Kubo et al. 2016). The alkaloids in various parts of *F. septica* exhibited potent anticancer effects. It has been reported that the anticancer compounds from *F. septica* leaves are phenanthroindolizidine alkaloids, namely, ficuseptine, (+)-tylophorine, and a mixture of (+)-tylocrebrine and (+)-isotylocrebrine which showed a potent anticancer effect on two human cancer cell lines (Wu et al. 2002). Similar correlations between the secondary metabolites content and anticancer properties of *F. septica* plants have been demonstrated by Ueda and co-workers (2009). These researchers have also reported that other alkaloids from methanolic extract of *F. septica* leaves possess cytotoxic effects, such as ficuseptamines A and B (aminocaprophenone alkaloids) and ficuseptamine C (pyrrolidine alkaloid) (Ueda et al. 2009). Phenanthroindolizidine alkaloids could be isolated from both roots and stems of *F. septica*, and showed potent cytotoxic effects on gastric adenocarcinoma and nasopharyngeal carcinoma cell lines (Damu et al. 2005, 2009). Similarly, Nugroho and co-workers (2012) have revealed that the combination of ethyl acetate soluble fraction of *F. septica* leaves and doxorubicin synergically increases the cytotoxic effect on T47D breast cancer cell line through cell cycle arrest and apoptosis induction. The methanolic extract from the twigs of *F. septica* was shown to have potent antimalarial activity (Kubo et al. 2016). Bioassay-guided fractionation of a methanol extract of the twigs of *F. septica* led to the isolation of a new seco-phenanthroindolizine alkaloid and three known phenanthroindolizine alkaloids. All isolated compounds were tested against *Plasmodium falciparum*. Compounds 2-4 displayed antimalarial activity against the 3D7 strain of *P. falciparum*, whereas a new compound 1 exhibited a moderate antimalarial activity (Kubo et al. 2016). Numerous studies have been made on antimicrobial activity of alkaloids. Alkaloids are a large and structurally diverse group of compounds that have served as scaffolds for important antibacterial drugs such as metronidazole and the quinolones (Cushnie et al. 2014).

F. deltoidea has been reported to have beneficial pharmaceutical uses as an antidiabetic, anti-inflammatory, antinociceptive, antimelanogenic, antiphotoaging, antioxidant, antiulcerogenic, and antibacterial agent with antioxidant, antihyperglycemic, anti-inflammatory, antiulcerogenic and antinociceptive activity (Bunawan et al. 2014, Dzolin et al. 2015). As noted by Adam and co-workers (2009), almost all of the parts of *F. deltoidea* plant including the root, bark, leaf and fig have their own medicinal properties. Abdsamah and co-workers (2012) studied the *in vitro* antimicrobial activity of the chloroform, methanol and aqueous extracts of *F. deltoidea* against 2 Gram positive (*Staphylococcus aureus* (IMR S-277), *Bacillus subtilis* (IMR K-1)), 2 Gram negative (*Escherichia coli* (IMR E-940), *Pseudomonas aeroginosa* (IMR P-84)) and 1 fungal strain, *Candida albicans* (IMR C-44). All the extracts showed inhibitory activity on the fungus, Gram-positive and Gram-negative bacteria strains tested except for the chloroform and aqueous extracts on *B. subtilis*, *E. coli*, and *P. aeroginosa*. The methanolic extract exhibited good antibacterial and antifungal activities against the test organisms and significantly inhibited the growth of *S. aureus*. *B. subtilis* was the least sensitive to the chloroform extract. Methanolic extracts of whole *F. deltoidea* exhibited antibacterial activity against S. *aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* and antifungal activity towards *C. albicans*. Antimicrobial activity of *F. deltoidea in vitro* further justifies its utility in folkleric medicines for the treatment of infections of microbial origin (Abdsamah et al. 2012). Experimental research performed by Uyub and co-workers (2010) found that *F. deltoidea* leaf extract has antimicrobial activity towards *Helicobacter pylori*, the major agent in chronic gastritis and gastric ulcers. Furthermore, methanol extracts of

F. deltoidea showed highest activity against *H. pylori*. Another study found that ethanolic and methanolic extracts of *F. deltoidea* leaves inhibited growth of *B. subtilis* (Bunawan et al. 2014, Dzolin et al. 2015). In line with these studies, in our investigation, ethanolic extract obtained from *F. deltoidea* leaves showed mild antibacterial efficiency against *C. freundii* with 12 mm of zone of growth inhibition observed (Fig. 3).

Moreover, in our previous study the in vitro antimicrobial activity of ethanolic extracts from leaves of various *Ficus* species (Moraceae) against the bacterial strain of *A. hydrophila* isolated locally from infected rainbow trout (*Oncorhynchus mykiss* Walbaum) was determined (Tkachenko et al. 2016 a). Our results indicated that extracts offer a promising alternative to the use of antibiotics in controlling *A. hydrophila* growth. In our study, most ethanolic extracts obtained from *Ficus* spp. proved effective against the bacterial strain of Gram-negative *A. hydrophila* tested, with 10-12 mm zones of inhibition being observed. *A. hydrophila* demonstrated the highest susceptibility against *F. pumila*. Among various species of Ficus with moderate activity against *A. hydrophila*, the highest antibacterial activity for *F. benghalensis*, *F. benjamina*, *F. deltoidea*, *F. hispida*, *F. lyrata* was displayed. Thus, *Ficus* spp. leaves possess great medicinal potential for the therapy of bacterial and fungal infections and may be used as a natural antiseptic and antimicrobial agent in veterinary (Tkachenko et al. 2016 a).

The results of the present study reinforce the importance of the analyzed plants as a source of bioactive compounds for the treatment of *C. freundii* related infectious diseases. In agreement with the results obtained from the present study, our previous studies found that various *Ficus* species have noticeable antibacterial activity against bacterial strains (Tkachenko et al. 2016 a, b). Data obtained from other studies demonstrated positive results for these plants as well.

Antibacterial activity of various *Ficus* species can be explain due to the presence of secondary metabolites that are probably responsible for the tests organism susceptibility to them. Indeed, the photochemical screening of leaves and stem bark extracts of various *Ficus* species revealed the presence of alkaloids, balsams, carbohydrates, flavonoids, free anthraquinones, tanins, glycosides, terpenes, resins, sterols and saponins. The presence of alkaloids and flavonoids both reveals its activity against pathogenic bacteria and suggests a role in the limitation of fungal infection, given that many flavonoids exhibit antifungal activity (Cushnie and Lamb 2005). Furthermore, it is interesting that antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Cowan 1999). The B ring of the flavonoids may intercalate or form hydrogen bond with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria (Mori et al. 1987). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes (Cowan 1999).

Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity (Cushnie and Lamb 2005). In addition, several high-quality investigations have examined the relationship between flavonoid structure and antibacterial activity and these are in close agreement. The activity of quercetin, for example, has been at least partially attributed to inhibition of DNA gyrase. It has also been proposed that sophoraflavone G and (-)-epigallocatechin gallate inhibit cytoplasmic membrane function, and that licochalcones A and C inhibit energy metabolism (Cushnie and Lamb 2005). Moreover, the crude extracts of plants are pharmacologically more active than their isolated active principles due to the synergistic effects of various components present in the whole extract (Padmanabhan and Jangle. 2012).

CONCLUSIONS

The findings presented in this paper indicate that the ethanolic extracts obtained from the leaves of *Ficus drupacea*, *F. septica*, *F. deltoidea* as well as *F. hispida*, *F. mucuso*, *F. pumila*, *F. craterostoma* exhibit a favorable antibacterial activity against *C. freundii*. On the basis of these results, it is concluded that the studied plants extracts might be potential sources of new antimicrobial drug. These data validate scientifically their inhibitory capacity attributed by their common use in folk medicine and contribute towards the development of new treatment options in aquaculture based on natural products. The chemical analysis of the aforementioned plant extracts should be performed to determinate their chemical composition and identify the exact phytocompounds responsible for antimicrobial activity against *C. freundii*. In addition, they should be subjected to pharmacological evaluations with the aim of assessing their *in vivo* efficacy, toxicity, potential adverse effects, interactions and contraindications.

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AKTYWNOŚĆ ANTYBAKTERYJNA ETANOLOWYCH EKSTRAKTÓW UZYSKANYCH Z LIŚCI RÓŻNYCH GATUNKÓW FIKUSÓW W STOSUNKU DO PATOGENU RYB, *CITROBACTER FREUNDII*

Streszczenie

Zioła lecznicze odgrywają rolę alternatywnej terapii antybiotykowej w akwakulturze. Substancje biologicznie aktywne pozyskiwane z roślin mają równie dobre, a nawet bardziej skuteczne działanie w porównaniu z tradycyjnymi lekami syntetycznymi, w dodatku nie wywołują oporności drobnoustrojów, która często pojawia się podczas antybiotykoterapii. W różnych regionach świata tradycyjnie w celach medycznych stosowane są różne gatunki roślin tropikalnych, m.in. fikusy (Moraceae). Głównym celem badań była ocena wrażliwości patogenu ryb *Citrobacter freundii* na etanolowe ekstrakty z liści wybranych gatunków fikusów. Przeciwbakteryjne działanie ekstraktów oceniano *in vitro*, stosując metodę dyfuzyjno-krążkową. Nasze wyniki wskazują, że różne gatunki *Ficus* wykazują *in vitro* łagodne działanie przeciwbakteryjne przeciwko *C. freundii* izolowanego lokalnie z zainfekowanego wę-

gorza (*Anguilla anguilla* L.). Przeprowadzone badania wykazały, że największą aktywność przeciwbakteryjną wobec szczepu *C. freundii* wykazały etanolowe wyciągi uzyskane z liści gatunków fikusów: *F. drupacea*, *F. septica*, *F. deltoidea*, *F. hispida*, *F. mucuso*, *F. pumila*, *F. craterostoma*. Wstępne badania screeningowe wskazują zatem, że wyciągi z liści niektórych gatunków fikusów o właściwościach antybakteryjnych mogą stanowić alternatywne środki terapeutyczne przeciwko infekcjom bakteryjnym w akwakulturze. Produkty te mogą być stosowane jako środki terapeutyczne i profilaktyczne, niemniej jednak powinni jeszcze zostać przeprowadzone bardzo intensywne badania dotyczące ich właściwości przeciwbakteryjnych.