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RESEARCH ARTICLE

Preliminary *in vitro* screening of the antibacterial activity of leaf extracts from various *Ficus* species (Moraceae) against *Yersinia ruckeri*

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Abstract. Remarkable progress in the field of antibacterial herbal therapy has been made in recent decades in response to the development of drug-resistant pathogens in aquaculture. Studies have focused on the *in vitro* antimicrobial activity screening of ethanolic extracts of various plants belonging to the genus *Ficus*. The aim of the present study was to evaluate the antibacterial efficacy of ethanolic extracts of various *Ficus* species against *Yersinia ruckeri*. *In vitro* tests for antibacterial activity revealed that ethanolic leaf extracts of various *Ficus* species and their cultivars offer a promising alternative to antibiotics and chemotherapeutics for controlling the growth of *Y. ruckeri*. In our study, ethanolic extracts obtained from leaves of *F. natalensis* subsp. *leprieurii* and *F. macrophylla* proved effective against a bacterial strain at a dose of 400 µl standardized inoculum (10^8 CFU ml^{-1}). It should be noted that *Y. ruckeri* demonstrated an intermediate susceptibility to more extracts derived from the leaves of *Ficus* species. Our investigation showed that among the various *Ficus* species, ethanolic leaf extracts of ten *Ficus* species against *Y. ruckeri* were the most effective. The effect of the leaf extracts that expressed the highest antimicrobial activity (*F. macrophylla*, *F. natalensis* subsp. *leprieurii*) against *Y. ruckeri* was comparable to that of gentamicin. Therefore, preliminary screening indicated that the ethanolic leaf extracts of some *Ficus* species with antibacterial properties can be used in aquaculture as therapeutic and prophylactic agents against fish pathogens, including *Y. ruckeri*.

Keywords: antibacterial activity, Kirby-Bauer disk diffusion technique, ethanolic extracts, fish pathogens, susceptibility, resistance

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Introduction

Yersinia ruckeri is a ubiquitous pathogen of finfish capable of causing major mortalities in farmed fish stocks (Ghosh et al. 2016). This bacterium is an etiological agent of enteric redmouth disease (ERM) of farmed salmonids (Ormsby et al. 2016). The

causative agent, a Gram-negative enteric bacterium, which was first isolated in the Hagerman Valley, Idaho, USA, in the early 1950s, is described fully by Ross et al. (1966), and it was identified as the new species, *Y. ruckeri*, in 1978 (Ewing et al. 1978). *Y. ruckeri* is a member of the family *Enterobacteriaceae* within the gamma-proteobacteria subdivision. Generally of coccoid-rod cell morphology, *Y. ruckeri* cells are slightly curved, 1.0 μm in diameter, and 2–3 μm in length, although culture for 48 h or longer results in long filamentous cells (Barnes 2011). *Y. ruckeri* has a wide host range, a broad geographical distribution, and causes significant economic losses in the fish aquaculture industry (Kumar et al. 2015).

Y. ruckeri can be transmitted vertically from parent to progeny as well as horizontally in the water column from both clinically infected fish and asymptomatic carriers and is consequently capable of infecting fish at early stages of development (Ghosh et al. 2016). The disease takes its name from the subcutaneous hemorrhages it can cause at the corners of the mouth and in the gums and tongue. Other clinical signs include exophthalmia, darkening of the skin, splenomegaly, and inflammation of the lower intestine with an accumulation of thick yellow fluid. The bacterium enters the fish through the secondary gill lamellae and from there it spreads to the blood and internal organs (Kumar et al. 2015). Infected fish and asymptomatic carriers are the main sources of infection, and the bacteria are spread by feces. Gills are regarded as the entry route of *Y. ruckeri* rods, but the likelihood of the disease depends on the virulence of a given strain. Characteristic clinical signs of yersiniosis, such as hemorrhages around the oral cavity, are caused by extracellular products of *Y. ruckeri* (Pękala and Antychowicz 2010). Antibiotics are frequently used in aquaculture to prevent the occurrence and to control these pathogens (Romero et al. 2012, Caruso 2016).

Antibiotic resistance has prompted research into developing novel strategies that can prevent bacterial growth (Abouzeed et al. 2013, Khameneh et al. 2015). Growing numbers of studies are focusing on identifying alternatives to antibiotics with similar antimicrobial and growth-promoting effects without inducing bacterial resistance and potential side effects to animals (Romero et al. 2012, Yang et al. 2015). Natural products from higher plants have traditionally been regarded as important sources of antimicrobial agents and have attracted extensive attention in fundamental research and clinic applications (Cheng et al. 2014). Numerous scientific reports have shown that plants have a high potential to synthesize different antimicrobial substances that play multiple essential roles in plant physiology and act as plant defense mechanisms and protect them against abiotic (UV radiation, drought, high or low temperatures, excessive soil salinity) and biotic stresses (i.e., microorganisms, insects, and herbivores) (Daglia 2012, Nabavi et al. 2015). It is assumed that phytogenic compounds play an important role in mediating interactions between plants and the environment (Yang et al. 2015). Natural products, especially those obtained from plants (phenolics and polyphenolics, terpenoids, alkaloids, lectins, polypeptides, and polyacetylenes), have proven to be outstanding compounds with unique properties, making them perfect candidates for these much-needed therapeutics (Borges et al. 2016). These secondary metabolites have potentially healthy properties for the human body mainly as antioxidants and anti-allergen, anti-inflammatory, anticancer, antihypertensive, and antimicrobial agents (Daglia 2012). The search for the healing potential of natural products is an idea from ancient times that is once again being pursued (Nabavi et al. 2015).

In addition to their medicinal use in humans, medicinal plants are used as chemotherapeutics and food additives in aquaculture because of their ability to enhance fish immune systems (Van Hai 2015). Currently, herbs are used widely in commercial aquaculture as growth-promoting substances, antibiotics, antimicrobial agents, nutrient sources, etc. Many herbal medicines have been found to be effective against fish pathogens (Birinci Yıldırım and 2018). Some studies report on the antimicrobial activities of essential oils on aquatic animal diseases (Al Laham et al. 2014). Treatments with medicinal plants that have antibacterial properties is a potentially beneficial alternative in aquaculture (Madhuri et al. 2012, Turker and Yıldırım 2015, Birinci

Yıldırım and Turker 2018). Phytogenic feed additives offer one way of improving fish health. Therefore, it is reasonable to assume that natural plant products can be a valuable source of antibacterial properties against multi-drug-resistant pathogens, including in aquaculture.

Botanical gardens play a fundamental role in the ex-situ conservation and exploration of global plant biodiversity (Cibrian-Jaramillo et al. 2013). Thus, it is assumed that living collections of tropical plants maintained in botanical gardens are an underutilized worldwide resource both for biodiversity conservation and practical uses. Therefore, given that many groups of plants are valuable sources of diverse compounds, possessing a broad spectrum of biological activity, the inter-institutional project between the Institute of Biology and Environmental Protection, Pomeranian University in Słupsk (Poland) and two botanical gardens in Ukraine, has recently begun assessing unexploited properties of various plant species derived from tropical biomass, and of *Ficus* species plants in particular (Tkachenko et al. 2016).

The genus *Ficus* L. (Moraceae), one of the most species-rich and ecologically important plant genera in lowland tropical rainforests, was chosen for an evaluation of antimicrobial activity, in particular, that of leaf extracts. In preparation for this study, ethnobotanical literature was reviewed regarding the traditional medicinal uses of various species of this large genus (Ali and Chaudhary 2011, Majumder and Paridhavi 2013).

Along with being an object of extreme interest for researchers over the last two centuries, *Ficus* has a long history of use by humans as a food source, in medicine, planting, and other industries and fields of human activity, partly owing to its great diversity and wide distribution range. Popular ethnomedicinal uses of *Ficus* include treatments of skin damage, disorders of the digestive system and related organs, and parasitic infections. In addition, the range of healing targets of particular *Ficus* species compiled from local medicines can be competitive with that of broad-spectrum traditional remedies (Lansky and Paavilainen 2011). A number of *Ficus* species are used as food and for medicinal properties in Ayurvedic and traditional Chinese medicine especially by the people inhabiting areas where these species grow. They are used widely to treat various diseases. Recent pharmacological investigations have reported diverse medicinal properties of the plants belonging to the genus *Ficus*, e.g., anti-diabetic, cognitive enhancer, wound healing, anticonvulsant, anti-inflammatory, analgesic, antimicrobial, antiviral, hypolipidemic, antioxidant, immunomodulatory, anti-asthmatic, parasympathetic modulatory, estrogenic, antitumor, anti-ulcer, antianxiety, antihelminthic, endothelin receptor antagonistic, apoptosis inducer, and hypotensive activity, which have been validated in numerous scientific studies on various species of *Ficus* genus (Dangarembizi et al. 2012, Salem et al. 2013, Badgujar et al. 2014, Bunawan et al. 2014).

However, although many species within the genus *Ficus* have been the subjects of phytochemical and pharmacological investigations, there are many species that have not yet been studied and whose ethnobotanical relevance has yet to be investigated. Consequently, an attempt was made to study the *in vitro* antimicrobial activity of ethanolic extracts of various plants belonging to the genus *Ficus*. Several important *Ficus* species were chosen for an evaluation of their antimicrobial efficacy against locally isolated *Y. ruckeri*. Therefore, the aim of the present study was to evaluate the antibacterial efficacy of ethanolic extracts of various plants belonging to the genus *Ficus* against *Y. ruckeri* in order to validate scientifically the inhibitory activity attributed to their common use and to propose new sources of antimicrobial agents in aquaculture for the prevention and treatment of disease caused by these bacteria.

Materials and methods

Collection of plant material and plant extract preparation

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* Warb. ex Mildbr. & Burret; *F. cyathistipula* Warb.; *F. deltoidea* Jack; *F. drupacea* Thunb.; *F. elastica* Roxb.; *F. elastic*a "Variegata"; *F. erecta* Thunb.; *F. hispida* L.f.; *F. luschnathiana* (Miq.) Miq.; *F. lyrata* Warb.; *F. macrophylla* Desf. ex Pers.; *F. mucuso* Welw. ex Ficalho; *F. natalensis* Hochst; *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. sagittat*a Vahl; *F. septica* Burm. f.; *F. sur* Forssk.; *F. sycomorus* L.; *F. vasta* Forssk.; and *F. villosa* Blume were sampled at the M. M. Gryshko National Botanical Garden (NBG, Kyiv, Ukraine) and the Botanical Garden of Ivan Franko Lviv National University (Lviv, Ukraine). The entire collections of tropical and subtropical plants at both the NBG and the Botanical Garden of Ivan Franko Lviv National University, including the *Ficus* spp. plants, have the status of the National Heritage Collection of Ukraine. The species author abbreviations were taken from Brummitt and Powell (1992). It is well known that the lack of the standardization of species names can result in mismatched observations leading to erroneous scientific conclusions (Bortolus, 2008). Therefore, in our investigation close attention was paid to the correct identification of plant species names and the appropriate use of botanical nomenclature. The taxonomic identification of the *Ficus* plant species that were used in the investigation was confirmed by Sosnovskiy (2014). The authors of this paper used the authoritative digitized global taxonomy source of plant names.

Leaves sampled from various *Ficus* species were brought into the laboratory for antimicrobial studies. Freshly crushed leaves were washed, weighed, and homogenized in 96% ethanol (in proportions of 1:10, w/w) at room temperature, and centrifuged at 3,000 g for 5 minutes. Supernatants were stored at -20°C in bottles protected with laminated paper until analyses.

Methods for culturing pathological samples and for identifying the *Y. ruckeri* strain

The *Y. ruckeri* strain was collected from clinically healthy fish and fish with clinical symptoms of yersiniosis. Internal tissues (predominantly pronephros and gills) and intestinal swabs were sampled. Tissue samples were homogenized and inoculated on nutritional agar with 5% blood (Columbia Blood Agar, Oxoid®). Following a 24 h incubation period at 25 ±2°C, distinctive colonies were transferred onto TSA. Round, elevated, shining, and whitish colonies without hemolytic properties were considered typical of *Y. ruckeri.* After 24h-incubation at 25 ± 2°C, an oxidase test and Gram-staining were performed. Gram-negative and oxidase-negative isolates were cultured on TSA medium and incubated for 24 h at 25±2°C. The strain was obtained from the Diagnostics Laboratory of Fish and Crayfish Diseases, Department of Veterinary Hygiene, Provincial Veterinary Inspectorate in Olsztyn (Poland).

The *Y. ruckeri* strain used for the study was initially identified using the morphological assessment and staining method (Gram-stained and then morphologically evaluated) (Kocwowa 1981, Whitman and MacNair 2004). Oxidase activity was determined for biochemical identification (Oxidase test, Merck Inc.) and the API 20E system (BioMérieux, France). Tests were performed according to the manufacturer's instructions. The *Y. ruckeri* strain according to API test 4104100 showed no movement.

Bacterial growth inhibition test of plant extracts by the disk diffusion method

The strain tested was plated on TSA medium (Tryptone Soy Agar) and incubated for 24 h at 25°C. Subsequently, the microorganisms were suspended in sterile PBS, and the turbidity was adjusted equivalent to that of the 0.5 McFarland standard. The antimicrobial susceptibility testing was done on Muller-Hinton agar with the disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol) (Bauer et al. 1966). Muller-Hinton agar plates

were inoculated with 200 and 400 µl standardized inoculum (10^8 CFU ml^{-1}) of the bacterium and spread with sterile swabs.

Sterile 6 mm filter paper discs with the plant extracts (200 µl) were applied over each of the inoculated plates, 15 min after the bacterial suspension was placed on them. A negative control disc impregnated with sterile ethanol was used in each experiment. The sensitivity of the strain to a commercial preparation with garlic extracts was also studied (Alligastran, BIOfaktor, Poland) at dilutions of 1:10, 1:100, and 1:1000. The *Y. ruckeri* isolates were individually tested against four antibiotics, as follows: oxytetracycline (30 µg); enrofloxacin (5 µg); gentamicin (10 µg); sulphamethoxazole/trimethoprim (25 µg).

The plates were incubated at 25°C for 24 hrs. Antibacterial activities were evaluated by measuring the diameters of inhibition zones in mm against the test organism and compared with those of the control and standard susceptibility disks. Activity was evidenced by the presence of an inhibition zone surrounding the paper discs. The plates were then observed for the inhibition zone produced by the antibacterial activity of various ethanolic extracts obtained from leaves of *Ficus* species. At the end of the period, the inhibition zones formed were measured in mm using a vernier. Six replicates were assayed for each extract. The plates were examined and photographs were taken. Zone diameters were determined and averaged.

Statistical analysis

Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of the extracts tested. The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (S) ≥ 15 mm, Intermediate (I) = 10-15 mm, and Resistant (R) ≤ 10 mm (Okoth et al. 2013).

Results

The data of the screening study of antimicrobial activity of ethanolic leaf extracts obtained from various *Ficus* species plants against the *Y. ruckeri* strain (plates with 200 and 400 µl standardized inoculum) are presented in Tables 1 and 2 and in Figs. 1 and 2. The comparison of susceptibility categories, i.e., high and intermediate susceptibility and resistant, for the disk diffusion technique is shown in Tables 1 and 2. Our results demonstrated that the *Y. ruckeri* strain (200 µL standardized inoculum) revealed

Table 1
Inhibition zone diameters (IZD) of *Y. ruckeri* growth (200 µL inoculum) induced by leaf ethanolic extracts obtained from various *Ficus* species (n=6)

Ficus species	Inhibition zone diameters (mm)
Intermediate susceptibility, IZD = 10-15 mm	
F. benjamina	12.25 ± 0.73
F. deltoidea	12.17 ± 0.95
F. erecta	14.25 ± 0.48
F. hispida	13.67 ± 0.49
F. lyrata	11.67 ± 0.61
F. macrophylla	12.17 ± 0.95
F. natalensis subsp. *leprieurii*	13.08 ± 0.90
F. natalensis	14.17 ± 0.31
F. palmeri	14.08 ± 0.66
F. platypoda	11.67 ± 0.56
F. pumila	12.67 ± 0.71
F. sagittata	14.33 ± 1.23
F. sycomorus	13.83 ± 0.79
Resistant, IZD ≤ 10 mm	
F. aspera	8.58 ± 0.33
F. benghalensis	8.83 ± 0.33
F. benjamina 'Reginald'	8.83 ± 0.34
F. binnendijkii	8.75 ± 0.31
F. binnendijkii 'Amstel Gold'	8.58 ± 0.33
F. binnendijkii 'Amstel King'	8.92 ± 0.45
F. carica	9.25 ± 0.38
F. craterostoma	8.92 ± 0.37
F. cyathistipula	8.50 ± 0.26
F. drupacea	8.58 ± 0.51
F. elastica	8.42 ± 0.20
F. elastica 'Variegata'	8.92 ± 0.27
F. luschnathiana	8.75 ± 0.50
F. mucuso	8.92 ± 0.27
F. religiosa	8.83 ± 0.38
F. rubiginosa	8.50 ± 0.43
F. septica	8.75 ± 0.31
F. sur	8.50 ± 0.34
F. vasta	8.75 ± 0.50
F. villosa	8.83 ± 0.28

Table 2
Inhibition zone diameters (IZD) of *Y. ruckeri* growth (400 µL inoculum) induced by leaf ethanolic extracts obtained from various *Ficus* species (n=6)

Ficus species	Inhibition zone diameters (mm)
High susceptibility, IZD > 15 mm	
F. macrophylla	15.33 ± 0.76
F. natalensis subsp. leprieurii	15.50 ± 0.43
Intermediate susceptibility, IZD = 10-15 mm	
F. benghalensis	13.17 ± 0.79
F. benjamina	12.50 ± 0.76
F. benjamina 'Reginald'	12.75 ± 0.72
F. binnendijkii	13.25 ± 0.48
F. binnendijkii 'Amstel King'	11.67 ± 0.79
F. cyathistipula	13.75 ± 0.31
F. drupacea	14.42 ± 0.66
F. elastica	12.67 ± 0.67
F. erecta	12.50 ± 0.76
F. luschnathiana	12.50 ± 0.76
F. palmeri	11.75 ± 0.44
F. pumila	14.83 ± 0.95
F. religiosa	13.42 ± 0.37
F. sagittata	11.33 ± 0.44
F. septica	12.50 ± 0.76
Resistant, IZD ≤ 10 mm	
F. aspera	8.50 ± 0.50
F. binnendijkii 'Amstel Gold'	8.17 ± 0.31
F. carica	8.5 ± 0.50
F. craterostoma	8.51 ± 0.34
F. deltoidea	8.75 ± 0.31
F. elastica 'Variegata'	8.75 ± 0.36
F. hispida	8.72 ± 0.31
F. lyrata	8.92 ± 0.37
F. mucuso	8.25 ± 0.40
F. natalensis	8.83 ± 0.65
F. platypoda	8.92 ± 0.66
F. rubiginosa	8.50 ± 0.45
F. sur	8.42 ± 0.20
F. sycomorus	8.58 ± 0.21
F. vasta	8.50 ± 0.34
F. villosa	8.58 ± 0.42

intermediate susceptibility (according to the inhibition zone diameter) to the ethanolic extracts derived from *F. benjamina*, *F. deltoidea*, *F. erecta, F. hispida*, *F. lyrata*, *F. macrophylla*, *F. natalensis* subsp. *leprieurii*, *F. natalensis*, *F. palmeri*, *F. platypoda*, *F. pumila*, *F. sagittata*, and *F. sycomorus* (the mean of inhibition zone diameters ranged from10 to 15 mm). The highest value of inhibition zone diameter was noted for *F. sagittata* (14.33 ± 1.23 mm), *F. erecta* (14.25 ± 0.48 mm), *F. natalensis* (14.17 ± 0.31 mm), and *F. palmeri* (14.08 ± 0.66 mm). On the other hand, the *Y. ruckeri* strain was resistant to ethanolic extracts obtained from the leaves of *F. aspera*, *F. benghalensis*, *F. benjamina* "Reginald," *F. binnendijkii* and its cultivars ("Amstel Gold," "Amstel King"), *F. carica*, *F. craterostoma*, *F. cyathistipula*, *F. drupacea*, *F. elastica*, *F. elastica* "Variegata," *F. luschnathiana*, *F. mucuso*, *F. religiosa*, *F. rubiginosa*, *F. septica*, *F. sur*, *F. vasta*, and *F. villosa* (the mean inhibition zone diameters were less than 10 mm; Table 1).

The *Y. ruckeri* strain applied to the plates in doses of 400 µl standardized inoculum revealed a high level of susceptibility to ethanolic extracts obtained from the leaves of *F. natalensis* subsp. *leprieurii* and *F. macrophylla* (the mean values of inhibition zone diameters were 15.50 ± 0.43 mm and 15.33 ± 0.76 mm, respectively; Table 2). Moreover, high inhibition zone diameter values were noted for *F. pumila* (14.83 ± 0.95 mm) and *F. drupacea* (14.42 ± 0.66 mm) from a group of *Ficus* species with intermediate susceptibility against *Y. ruckeri*. The inhibition zone diameters for *F. aspera*, *F. binnendijkii* "Amstel Gold," *F. carica*, *F. craterostoma*, *F. deltoidea*, *F. elastica* "Variegata," *F. hispida*, *F. lyrata*, *F. mucuso*, *F. natalensis*, *F. platypoda*, *F. rubiginosa*, *F. sur*, *F. sycomorus*, *F. vasta*, and *F. villosa* were in a range of less than 10 mm (Table 2). Data on the antimicrobial activity of ethanolic extracts obtained from *F. religiosa*, *F. cyathistipula*, *F. lyrata*, *F. benghalensis*, and *F. binnendijkii* against the *Y. ruckeri* strain applied to the plates in doses of 200 and 400 µl standardized inoculum and *Y. ruckeri* strain susceptibility against oxytetracycline (30 µg), enrofloxacin (5 µg), gentamicin (10 µg), and sulphamethoxazole/trimethoprim (25 µg) are presented in Figs. 1 and 2.

As is evident from Fig. 2, the *Y. ruckeri* strain was found susceptible to the antibiotics studied. Specifically, our results also revealed that the *Y. ruckeri* strain applied to plates in doses of 200 µl exhibited a high level of susceptibility to enrofloxacin (the inhibition zone diameter was 40.5 ± 0.62 mm), sulphamethoxazole (30.0 ± 0.58 mm), oxytetracycline (25.17 ± 0.40 mm), and gentamicin (15.0 ± 0.26 mm) (Fig. 2A). On plates with the *Y. ruckeri*

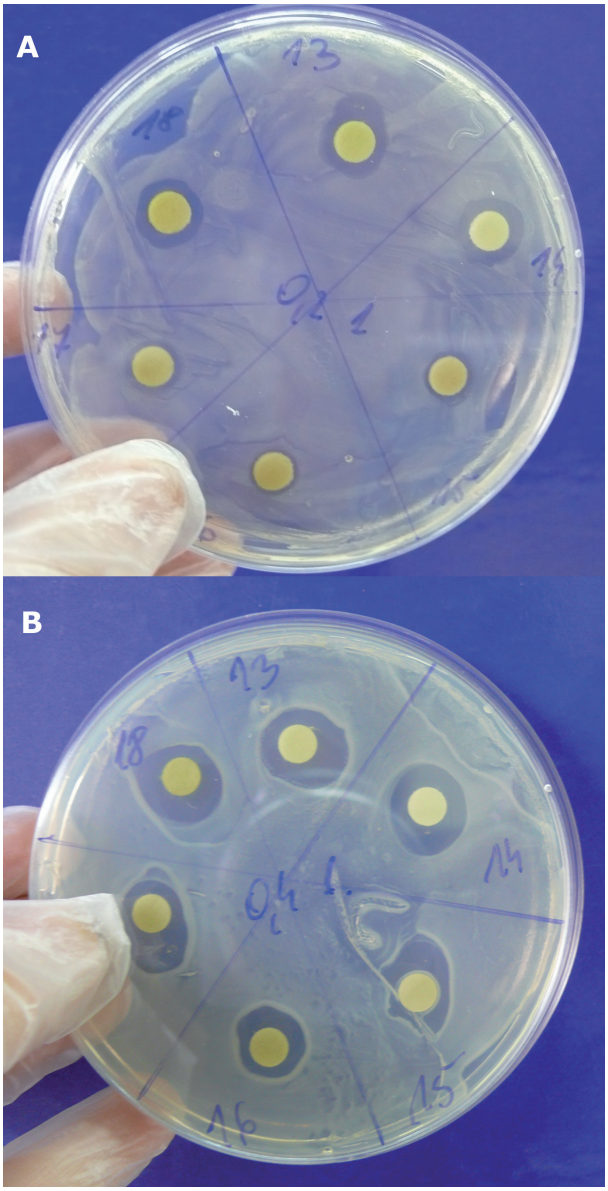

Figure 1. Antimicrobial activity of ethanolic extracts obtained from *F. religiosa* (13), *F. cyathistipula* (14), *F. lyrata* (15), *F. benghalensis* (16, 17), and *F. binnendijkii* (18) against the *Y. ruckeri* strain applied to plates in doses of 200 µL (A) and 400 µL standardized inoculum (B).

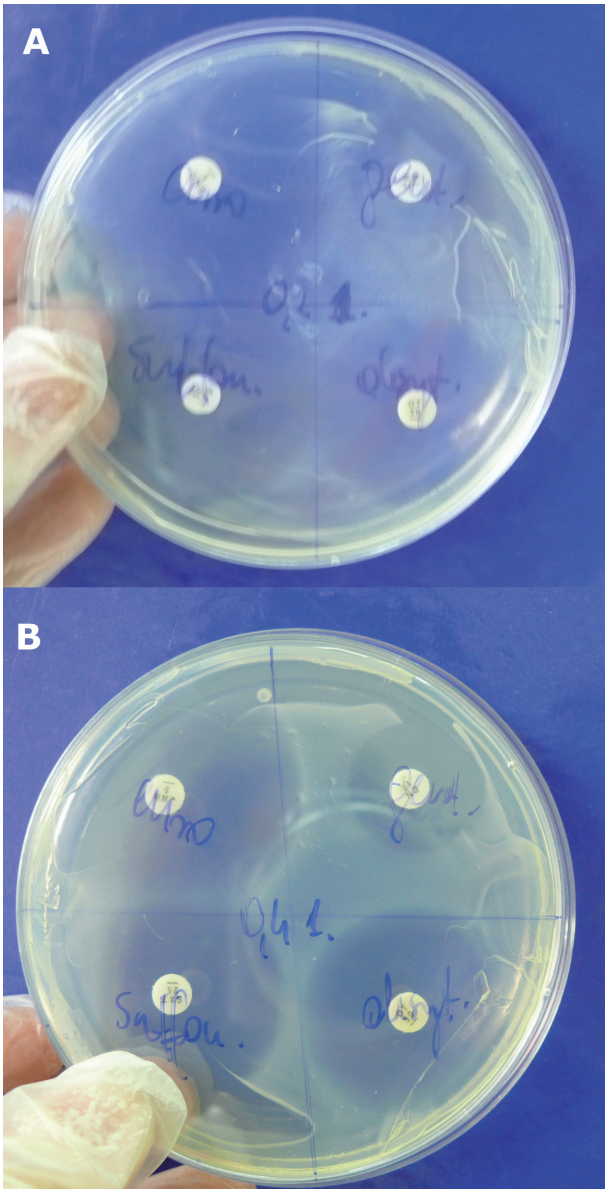

Figure 2. Susceptibility of the *Y. ruckeri* strain applied to plates in doses 200 µL (A) and 400 µL standardized inoculum (B) against oxytetracycline (30 µg), enrofloxacin (5 µg), gentamicin (10 µg), and sulphamethoxazole/trimethoprim (25 µg).

strain applied in doses of 400 µl, the inhibition zone diameters were 35.5 ± 0.43 mm for enrofloxacin, 30.17 ± 0.54 mm for sulphamethoxazole, 25.0±0.58 mm for oxytetracycline, and 21.33 ± 0.80 mm for gentamicin (Fig. 2B). The largest inhibition zones against the bacterial strain were observed in positive controls (reference antibiotics), while there was no inhibition zone in the negative control (ethanol and preparation with garlic extracts in dilutions of 1:10, 1:100, and 1:1000).

Discussion

Pursuant to the increased interest in the study of the antibacterial potential of different tropical plants, we

examined the antibacterial efficacy of 35 extracts obtained from *Ficus* species and their cultivars against the fish pathogen *Y. ruckeri*. The results from the screening study performed by the disc diffusion technique are shown in Tables 1 and 2 and Fig. 1. These results showed that 22 of 35 plant extracts exhibited no activity against the *Y. ruckeri* strain (200 µL inoculum), specifically the ethanolic extracts of *F. aspera*, *F. benghalensis*, *F. benjamina* "Reginald," *F. binnendijkii* and its cultivars ("Amstel Gold," "Amstel King"), *F. carica, F. craterostoma*, *F. cyathistipula*, *F. drupacea*, *F. elastica*, *F. elastica* "Variegata," *F. erecta*, *F. luschnathiana*, *F. mucuso*, *F. religiosa*, *F. rubiginosa*, *F. septica*, *F. sur*, *F. vasta*, and *F. villosa*. The largest inhibition zones were noted for the ethanolic extracts of *F. sagittata*, *F. erecta*, *F. natalensis*, and *F. palmeri*. These extracts showed similar intermediate activity to that of gentamicin against *Y. ruckeri* (Table 1).

The extracts that showed the broadest antibacterial potential against *Y. ruckeri* applied in doses of 400 µl standardized inoculum were the ethanolic extracts of *F. natalensis* subsp. *leprieurii* and *F. macrophylla*. Fifteen extracts showed intermediate activity against the bacterial strain tested in this study (*F. benghalensis*, *F. benjamina*, *F. benjamina* "Reginald," *F. binnendijkii*, *F. binnendijkii* "Amstel King," *F. cyathistipula*, *F. drupacea*, *F. elastica*, *F. erecta*, *F. luschnathiana, F. palmeri*, *F. pumila*, *F. religiosa*, *F. sagittata*, and *F. septica*). The weakest antibacterial activity was recorded for 16 species (*F. aspera*, *F. binnendijkii* "Amstel Gold," *F. carica*, *F. craterostoma*, *F. deltoidea*, *F. elastica* "Variegata," *F. hispida*, *F. lyrata*, *F. mucuso*, *F. natalensis*, *F. platypoda*, *F. rubiginosa*, *F. sur*, *F. sycomorus*, *F. vasta*, *F. villosa*) (Table 2).

This investigation concurs with our previous work that revealed the great potential of *Ficus* species as plants with potent antimicrobial properties. In our previous study, we evaluated the *in vitro* antimicrobial activity of ethanolic leaf extracts of various *Ficus* species against *Aeromonas hydrophila*, *Citrobacter freundii*, and *Pseudomonas fluorescens* (Tkachenko et al. 2016, 2017). Similarly to these general findings, there is copious evidence that various species of the genus *Ficus* possess antimicrobial activity against a broad spectrum of microorganisms. *Ficus* species have been the focus of increasing scientific interest in recent years. Consequently, it is well documented that various *Ficus* species have been used against Gram-positive and Gram-negative bacteria (Salem et al. 2013). For instance, Mousa et al. (1994) tested chloroform extract from the fruit of four *Ficus* species (*F. benghalensis*, *F. benjamina*, *F. religiosa*, and *F. sycomorus*) for antimicrobial activity against 22 pathogenic bacterial and fungal strains. The extracts had significant antibacterial activity but no antifungal activity. *F. benjamina* extracts were generally the most active against bacteria, while those of *F. religiosa* were the least active. The strain of *P. aeruginosa* HAMBI 25, which was generally weakly susceptible among the organisms tested, was weakly inhibited by *F. benghalensis*, *F. benjamina*, and *F. sycomorus* extracts (inhibition zone diameters of 16–19 mm) (Mousa et al. 1994).

Nair and Chanda (2006) have screened aqueous and ethanol extracts from 20 plant species, among which were four species of *Ficus* (*F. benghalensis*, *F. racemosa*, *F. religiosa*, and *F. tisela*), against seven Gram-negative (*Pseudomonas aeruginosa* ATCC27853, *Pseudomonas testosteroni* NCIM5098, *Proteus mirabilis* NCIM2241, *Proteus vulgaris* NCTC8313, *Enterobacter aerogenes* ATCC10240, *Escherichia coli* ATCC25922, and *Citrobacter freundii* ATCC10787) and five Gram-positive (*Staphylococcus epidermidis* ATCC12228, *Bacillus cereus* ATCC11778, *Streptococcus fecalis* ATCC29212, *Streptococcus cremoris* NCIM2179, and *Streptococcus agalactiae* NCIM2401) bacterial strains. Aqueous extracts generally showed less activity than ethanol extracts, and Gram-positive bacteria were generally more affected than Gram-negative ones. The *Ficus* species examined, of which bark extracts were used, showed low inhibition activity in general. Only their methanolic extracts affected *P. aeruginosa* with a small inhibition zone diameter of 3 mm for *F. tisela*, 2.5 mm for *F. racemosa*, and 2 mm for *F. benghalensis* (Nair and Chanda 2006).

Likewise, further studies (Nair and Chanda 2007) tested aqueous and ethanol extracts from ten Indian plant species, including the same species of *Ficus* (*F. benghalensis*, *F. racemosa*, *F. religiosa*, and *F. tisela*), against several medically important bacterial strains (*Alcaligenes faecalis* ATCC 8750, *Bacillus cereus* ATCC 11778, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* NCIM 2241, *Salmonella typhimurium* ATCC 23564, and *Staphylococcus aureus* ATCC 25923). The ethanol extracts were more potent than aqueous extracts of all the plants studied. Almost all *Ficus* bark extracts showed activity against each of the bacteria tested, although the strength of inhibition varied. *P. aeruginosa* was among the most resistant bacteria tested. *Ficus* species demonstrated low inhibition of *P. aeruginosa*, with inhibition zone diameters of 3 mm (*F. benghalensis* and *F. tisela* ethanol extracts) and 2 mm (*F. racemosa* ethanol extract). Aqueous extracts of all *Ficus* species and both extracts of *F. religiosa* were inactive against *P. aeruginosa* (Nair and Chanda 2007).

Atindehou et al. (2002) tested crude ethanol extracts from 115 plant species against Gram-negative bacteria (*E. coli* and *P. aeruginosa*), Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*), and fungi (*Candida albicans* and *Cladosporium cucumerinum*). Three *Ficus* species, namely *F. exasperata*, *F. mucuso*, and *F. sur*, were among the plants tested. The Gram-negative bacteria appeared unaffected by any plant extract tested, whereas the Gram-positive bacteria and fungi were inhibited by at least several plant species. Among the *Ficus* species tested, *F. exasperata* and *F. mucuso* had no significant effect on any of the microorganisms, while *F. sur* was one of the most active plant species against Gram-positive bacteria (Atindehou et al. 2002).

Similarly to the results of the present study, previous studies found that *F. septica* extract possessed moderate activity against bacteria and fungi and high effectiveness against protozoans. Vital et al. (2010) tested the leaf ethanol extract from *F. septica* Burm. and *Sterculia foetida* L. on a number of microorganisms such as bacteria (*Bacillus cereus* UPCC 1281, *E. coli* UPCC 1195, *Pseudomonas aeruginosa* UPCC 1244, and *Staphylococcus aureus* UPCC 1143), fungi (*Candida albicans* UPCC 2168), and protozoans (*Entamoeba histolytica* HK-9 and *Trichomonas vaginalis* DSHC 2021) with disc diffusion assays (for bacteria and fungi), growth curve determinations, and antiprotozoal and cytotoxicity assays (for protozoans). Their study showed moderate activity of both plant species extracts against bacteria and fungi and high effectiveness against protozoans. The extract from *F. septica* inhibited the growth of only *E. coli* and *S. aureus* among the bacteria tested, and its effectiveness was similar for both pathogens (inhibition zone diameter of 13.0 and 13.83 mm, respectively. Although Vital et al. (2010) present results of phytochemical analysis, listing the chemical classes found (such as alkaloids, quaternary base, tannins, 2-deoxysugars, and benzopyrone nucleus), no suggestions or speculations are provided regarding any possible antimicrobial activities of particular chemicals.

Previous reports have demonstrated that the various *Ficus* species possess potent antimicrobial activity against pathogenic bacterial and fungal strains, and these effects can be explained due to the presence of secondary metabolites that are probably responsible for microorganism susceptibility to them. According to Salem et al. (2013), the phytochemical screening of leaves and stem bark extracts of various *Ficus* species revealed the presence of alkaloids, balsams, carbohydrates, flavonoids, free anthraquinones, tannins, glycosides, terpenes, resins, sterols, and saponins.

Numerous investigations have shown that plant extracts contain natural compounds, such as phenolic compounds, polysaccharides, proteoglycans, and flavonoids that are able to stimulate fish immune systems, and, therefore, they may play major roles in the prevention or control of infectious microbes (Reverter et al. 2014). The presence of alkaloids and flavonoids both reveals 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 have multiple cellular targets,

rather than one specific site of action. One of their molecular actions is to form complexes with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, and by covalent bond formation (Cowan 1999). The B ring of the flavonoids may intercalate or form a hydrogen bond with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria. 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). Among polyphenols, flavan-3-ols, flavonols, and tannins received the most attention because of their wide spectrum and higher antimicrobial activity in comparison with other polyphenols and because most of them are able to suppress a number of microbial virulence factors (such as the inhibition of biofilm formation, the reduction of host ligands adhesion, and the neutralization of bacterial toxins) and show synergism with antibiotics (Daglia 2012). Moreover, crude plant extracts are pharmacologically more active than their isolated active principles because of the synergistic effects of various components present in whole extracts (Padmanabhan et al. 2012).

Thus, both a review of the literature and the authors' own previous findings have shown that disease control in aquaculture is an active research field, and the application of plant extracts are promising alternatives to antibiotic treatments. Moreover, taking into account the numerous hazards to public health associated with the use of antimicrobials in aquaculture, e.g., the development and spread of antimicrobial-resistant bacteria, resistance genes, and the presence of antimicrobial residues in aquaculture products and the environment (Romero et al. 2012, Yang et al. 2015, Caruso 2016), such considerations make the search for plant-derived antimicrobial agents as eco-friendly alternatives to antibiotics especially urgent.

Conclusions

Our results revealed that the ethanolic leaf extracts of various *Ficus* species and their cultivars that were tested are promising alternatives to the use of antibiotics and chemotherapeutics in controlling *Y. ruckeri* growth. In our study, ethanolic extracts obtained from leaves of *F. natalensis* subsp. *leprieurii* and *F. macrophylla* proved effective against bacterial strains in doses of 400 µl standardized inoculum (10^8 CFU ml^{-1}) with mean inhibition zone diameters of 15.50 and 15.33 mm, respectively. It should be noted that *Y. ruckeri* demonstrated intermediate susceptibility to more extracts derived from the leaves of *Ficus* species. Therefore, these results can be considered for further investigations aimed at identifying novel natural antimicrobial compounds in leaf extracts of some *Ficus* species that can be used in the aquaculture industry as therapeutic and prophylactic agents against fish pathogens, including *Y. ruckeri*.

Nevertheless, more extensive studies should be conducted prior to the development of novel antimicrobial pharmaceuticals based on *Ficus* species and their cultivars. The potential antimicrobial compounds comprising the extracts of various *Ficus* species tested should be isolated, purified, and examined further. Finally, the mechanisms of action of these potential active compounds should also be assessed. Further studies are needed to evaluate the effectiveness of the screened extracts and the potential impact of these substances on fish and on the environment.

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