FISHERIES & AQUATIC LIFE (2022) 30: 217 - 231
Archives of Polish Fisheries
DOI 10.2478/aopf-2022-0021

RESEARCH ARTICLE

A comparative assessment of the antibacterial activity of extracts derived from leaves of various *Ficus* species (Moraceae) against fish pathogens

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Received – 04 July 2022/Accepted – 13 December 2022. Published online: 31 December 2022; ©Inland Fisheries Institute in Olsztyn, Poland
Citation: Tkachenko, H., Pękala-Safińska, A., Buyun, L., Kurhaluk, N. (2022). A comparative assessment of the antibacterial activity of extracts derived from leaves of various *Ficus species* (Moraceae) against fish pathogens . Fisheries & Aquatic Life 30, 217-231.

Abstract. The aim of the study was to compare the *in vitro* antibacterial activity of leaf extracts obtained from various *Ficus* species against four bacterial strains of fish pathogens (*Serratia liquefaciens*, *Yersinia ruckeri*, *Pseudomonas fluorescens*, *Shewanella putrefaciens*). *In vitro* tests for antibacterial activity assessment revealed that various *Ficus* species have notable antibacterial efficacy. The six most effective *Ficus* plants against *S. liquefaciens* that caused a zone of inhibition of at least 15 mm were *F. lingua*, *F. erecta*, *F. rubiginosa*, *F. tinctoria*, *F. sur*, and *F. aspera*. The maximum antimicrobial activity against the *Y. ruckeri* strain was observed for ethanolic extracts of *F. hederacea*, *F. formosana*, and *F. hispida*. Our results also demonstrated that the *Pseudomonas fluorescens* strain exhibited high susceptibility to ethanolic extracts derived from 20 plants (the mean value of inhibition zone diameters (IZD) was more than 15 mm). *F. erecta*, *F. sur*, and *F. virens* extracts were more effective against *P. fluorescens*. The *Shewanella putrefaciens* strain revealed a high level of susceptibility to ethanolic extracts derived from the leaves of 32 species (the highest value of the IZDs was observed for *F. erecta*). The data presented in the current study indicated that ethanolic extracts derived from leaves of various *Ficus* species are a promising alternative to the use of antibiotics and chemotherapeutics in controlling infections caused by the *Serratia liquefaciens*, *Yersinia ruckeri*, *Pseudomonas fluorescens*, and *Shewanella putrefaciens* strains.

Keywords: antimicrobial efficacy, Kirby-Bauer disk diffusion technique, ethanolic extracts, fish pathogens, susceptibility, resistance

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Introduction

The global aquaculture industry is an important source of food that provides livelihoods for hundreds of millions of people around the world, and it is one of the fastest-growing animal protein sectors (FAO 2016). The use of antibiotics in aquaculture can generate antibiotic-resistant bacteria in the environments in which it is applied (Pepi and Focardi 2021, Rangel-López et al. 2022). The application of antibiotics and chemotherapeutics used extensively as growth and immunity enhancers and for treating bacterial-induced diseases in fish have negative

consequences for aquaculture, including the risk of increasing the numbers of resistant pathogens, problems of antibiotic residues accumulating in treated fish, and unfavorable impacts on the environment (McPhearson et al. 1991).

Antibiotics are biologically active compounds that can exert toxic effects on aquatic environments. These pharmaceuticals are considered contaminants of increasing concern, based on their widespread presence in aquaculture systems and the lack of specific regulations for monitoring them (Pepi and Focardi 2021). The use of antimicrobial drugs in aquaculture could lead to the emergence of resistance in pathogenic microorganisms. Moreover, the major problem associated with their use in aquaculture is the potential of facilitating the development of a pool of antimicrobial resistance genes (ARGs) that could eventually be transferred to clinically relevant bacteria (Miranda et al. 2012). In order to prevent major economic losses from diseases, various medications are used for the treatment and prevention of infections.

It is estimated that approximately 80% of antimicrobials used in aquaculture enter the environment and could pose potential health consequences for fishes, terrestrial animals, human beings, and the environment in general (Marshall and Levy 2011, Cobello et al. 2013). Horizontal gene transfer between aquatic and human pathogens is an important phenomenon involving antibiotic-resistant genes (Bello-López et al. 2019). Therefore, quite reasonably, it is suggested that good management strategies in aquaculture be based on minimizing the use of antimicrobials in fishes in order to prevent or, at least, delay the emergence and spread of antimicrobial resistance in aquaculture environments, and medicinal plants are one potential option (Reverter et al. 2014, 2017, Jana et al. 2018). Consequently, over the past two decades there has been considerable interest in the use of medicinal plants in aquaculture with a view to providing safe, ecologically friendly compounds to replace antibiotics and other chemotherapeutic drugs and to enhance the immune status of fishes and to control fish diseases (Gabriel and González-Redondo 2019). Plants are rich in secondary metabolites and phytochemical compounds that affect viral, bacterial, and parasitic diseases in fishes. Their main advantage is their natural origin and most of these plants do not pose threats to human health, fishes, or the environment (Stratev et al. 2018).

The antibacterial properties of medicinal plants are by far the best-studied of biological activities, with numerous *in vitro* studies reporting antibacterial activity in many plants against both Gram-positive and Gram-negative marine bacteria (Castro et al. 2008, Roomiani et al. 2013). Plants that can potentially be used as antimicrobials to enhance survival and immune competence are those from the genus *Ficus*. Recently, researchers have reported promising effects from many species of the genus *Ficus* for treating parasitic diseases, and they also exhibit broad activity against bacteria and fungi (Salem et al. 2013). *Ficus* trees have a number of uses in various industries and fields of human activity. Virtually all parts of them are utilized in ethnomedicine to cure disorders of the digestive and respiratory systems, skin diseases, parasitic infections, etc. Additionally, some species are reported to have analgesic, tonic, and ecbolic effects (Lansky and Paavilainen 2011).

Jana et al. (2018) compiled a list of medicinal herbs that can be used as therapeutics in aquaculture; however, when we reviewed the list we found no mention of *Ficus* species. The genus *Ficus* (Moraceae) constitutes one of the largest genera of angiosperms with over 1,000 species consisting of trees, shrubs, and epiphytes (Wagner et al. 1999). Plants in the genus are all woody, ranging from life forms of trees and shrubs to climbers. A number of studies have reported on the various biological activities of *Ficus* plants (Lansky and Paavilainen 2011).

Ficus plants contain secondary metabolites including flavonoids, tannins, alkaloids, terpenoids, essential oils, glycosides, etc. (Mousa et al 1994, Rao et al. 2008, 2008, Dangarembizi et al. 2012, Salem et al. 2013, Mohan and Nishanthini, 2015, Awolola et al. 2017). There is, however, not much information on the antibacterial effect of leaf extracts obtained from various *Ficus* species against fish pathogens. Therefore, we hypothesized that the ethanolic

extracts derived from leaves of various *Ficus* species might provide protection against fish pathogens and inhibit bacterial growth in vitro. Thus, the aim of the current study was to evaluate the antimicrobial potential of leaf extracts derived from various *Ficus* species against four bacterial strains (*Serratia liquefaciens*, *Yersinia ruckeri*, *Pseudomonas fluorescens*, *Shewanella putrefaciens*).

The current study was conducted as a part of an ongoing project between the Institute of Biology and Earth Sciences (Pomeranian University in Słupsk, Poland), the Faculty of Veterinary Medicine and Animal Sciences, University of Life Sciences (Poznań, Poland), M.M. Gryshko National Botanic Gardens of the National Academy of Sciences of Ukraine (Kyiv, Ukraine), and Ivan Franko National University in Lviv (Lviv, Ukraine) undertaken within the framework of program of cooperation aimed at assessing the medicinal properties of tropical plants cultivated ex-situ.

Materials and Methods

Collecting plant material and preparing plant extracts

Leaves were collected from the following plants at the M.M. Gryshko National Botanic Garden (NBG, Kyiv, Ukraine) and the Botanic Garden of Ivan Franko Lviv National University (Lviv, Ukraine): *F. aspera* G. Forst.; *F. barteri* Sprague; *F. benghalensis* L.; *F. benjamina* L.; *F. binnendijkii* (Miq.) Miq.; *F. carica* L.; *F. craterostoma* Warb. ex Mildbr. & Burret; *F. cyathistipula* Warb.; *F. deltoidea* Jack; *F. drupacea* Thunb.; *F. elastica* Roxb. ex Hornem.; *F. erecta* Thunb.; *F. formosana* Maxim.; *F. hederacea* Roxb.; *F. hispida* L.f.; *F. johannis* subsp. *afghanistanica* (Warb.) Browicz; *F. lingua* Warb. ex De Wild. & T.Durand; *F. luschnathiana* Miq.; *F. lyrata* Warb.; *F. lyrata* 'Bambino;' *F. macrophylla* Pers.; *F. malayana* C.C.Berg & Chantaras.; *F. microcarpa* L.f.; *F. mucuso* Welw. ex Ficalho; *F. natalensis* Hochst.; *F. natalensis* Hochst. subsp. *leprieurii* (Miq.) C.C. Berg; *F. petiolaris* Kunth; *F. platypoda* A. Cunn. ex Miq.; *F. pumila* L.; *F. religiosa* L.; *F. retusa* L.; *F. rubiginosa* Desf. ex Vent;, *F. sagittata* Vahl; *F. sarmentosa* var. *henryi* (King ex D.Oliv.) Corner; *F. septica* Burm. f.; *F. sur* Forssk.; *F. sycomorus* L.; *F. tinctoria* G.Forst.; *F. vasta* Forssk.; *F. villosa* Blume; *F. virens* Aiton.

The entire collections of tropical and subtropical plants at the M.M. Gryshko National Botanic Gardens of the National Academy of Sciences of Ukraine (Kyiv, Ukraine) and the Botanical Garden of Ivan Franko National University in Lviv (including *Ficus* spp. plants) have the status of National Heritage Collection of Ukraine and are supported through State funding. The author's abbreviations of the species are from Brummitt and Powell (1992). The taxonomic identity of *Ficus* plant species that were used in the investigation was confirmed by Dr. Yevhen V. Sosnovskiy. The authors of the paper consulted the authoritative digitized global taxonomy source for plant names (WFO, The World Flora Online, http://www.worldfloraonline.org/).

Samples of leaves of various *Ficus* species were placed in labeled paper bags and transported to the laboratory. The fresh leaves were washed, weighed, crushed, and homogenized in 96% ethanol (at a proportion of 1:9, 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 required for antimicrobial studies.

Bacteria isolation

Bacteria were isolated from visibly healthy rainbow trout, *Oncorhynchus mykiss* (Walbaum) and individuals exhibiting clinical signs of disease following procedures developed at the Department of Fish Disease of the National Veterinary Research Institute in Poland (Pękala et al. 2015). The following isolates were used in the study: *Serratia liquefaciens*, *Yersinia ruckeri*, *Pseudomonas fluorescens*, *Shewanella putrefaciens*. The bacteria collected were morphologically, physiologically, and biochemically characterized with conventional methods (Austin and Austin 2016). All isolates were preliminarily identified using the API

system (bioMérieux, France) according to the manufacturer's instructions, except for the incubation temperature, which was 27 ± 2°C. The results were interpreted using apiweb™ (bioMérieux). To confirm the correctness of biochemical identification, sequencing was performed according to procedures described previously (Pękala et al. 2015).

For susceptibility to antimicrobial agents, the following chemotherapeutics (Oxoid, UK) from different groups of drugs were used: sulfonamides consisted of compound sulfonamides (S3, 300 μg) and sulfamethoxazole with trimethroprim (SXT, 25 μg); quinolones were flumequine (UB, 30 μg) and enrofloxacin (ENR, 5 μg); oxytetracycline (OT, 30 μg) was the only tetracycline; florfenicol (FFC, 30 μg) was the only amphenicol. After media plate inoculation and placing the appropriate antimicrobial discs (five discs per plate), the plates were incubated at 28 ± 2°C for 24 h (Pękala-Safińska et al. 2021).

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

The sensitivity of bacteria to selected plants extracts was determined by the disk diffusion method (Bauer et al. 1966), a standard procedure for testing the susceptibility of bacterial isolates that was adapted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2006, Miller et al. 2014) with some of our own modifications. Each inoculum of a particular bacteria species at a density of 0.5 McFarland was cultured on Mueller-Hinton agar. After the inoculation of bacteria, a maximum of five wells per Petri dish with a diameter of 6 mm each was cut into the medium, and plant extracts were added to them. Plates were incubated for 24 h at 28 ± 2°C, and the zones of inhibition were measured for each well. Eight replicates were assayed for each extract. The plates were observed and photographs were taken. Zone diameters were measured and averaged. Ethanol 96% (POCH, Poland) was used to prepare the extracts and also as the negative control for the microbiological study.

Statistical analysis

Statistical analysis of the data obtained was performed with mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of the extracts tested. To assign bacterial susceptibility or resistance to the extracts tested, inhibition zone diameters (IZD) of susceptibility testing results were categorized as sensitive (susceptible), intermediate, or resistant based on the following criteria: Susceptible (S) ≥ 15 mm, Intermediate (I) = 10–15 mm, and Resistant (R) ≤ 10 mm (Okoth et al. 2013).

Results

In response to growing interest in antibacterial efficacy assessment of various plants, ethanolic extracts of 40 plants belonging to the genus *Ficus* were tested against four bacterial strains (*Serratia liquefaciens*, *Yersinia ruckeri*, *Pseudomonas fluorescens*, *Shewanella putrefaciens*). The results from the antimicrobial screening study performed with the disc diffusion method are presented in Tables 1–4. The values of the zones of inhibition were also recorded (Tables 1–4).

The results of the disc diffusion screening indicated that extracts derived from the leaves of various *Ficus* species clearly possessed antibacterial properties against the four bacterial strains tested. As summarized in Tables 1–4, the six most effective plants that caused inhibition zones of more than 15 mm against *S. liquefaciens* were *F. lingua* (20.75 ± 1.41 mm), *F. erecta* (17.75 ± 0.94 mm), *F. rubiginosa* (17.25 ± 0.67 mm), *F. tinctoria* (15.25 ± 0.86 mm), *F. sur* (15.19 ± 0.65 mm), and *F. aspera* (15.13 ± 1.33 mm). The data also indicated that 12 extracts displayed an intermediate degree of activity against the *S. liquefaciens* strain (Table 1). The remaining 22 plants (of 40 plants) had the lowest antibacterial

Table 1
Inhibition zone diameters of *Serratia liquefaciens* growth (1000 µL inoculum) induced by ethanolic extracts obtained from leaves of various *Ficus* species. Data are presented as mean ± standard error, n = 8

Ficus species	Inhibition zone diameters (IZD), mm
F. aspera	15.13 ± 1.33
F. erecta	17.75 ± 0.94
F. lingua	20.75 ± 1.41
F. rubiginosa	17.25 ± 0.67
F. sur	15.19 ± 0.65
F. tinctoria	15.25 ± 0.86
F. barteri	14.38 ± 0.63
F. benghalensis	12.38 ± 0.78
F. hederacea	14.63 ± 0.80
F. hispida	11.38 ± 0.53
F. jahannis subsp. *afghanistanica*	12.25 ± 0.94
F. malayana	13.25 ± 0.86
F. natalensis	12.50 ± 0.71
F. petiolaris	12.13 ± 0.90
F. pumila	13.13 ± 0.99
F. sagittata	12.38 ± 0.82
F. septica	13.63 ± 0.75
F. virens	14.25 ± 0.77
F. benjamina	9.50 ± 0.50
F. binnendijkii	9.38 ± 0.72
F. carica	9.63 ± 0.65
F. craterostoma	9.25 ± 0.53
F. cyathistipula	9.38 ± 0.92
F. deltoidea	9.63 ± 0.72
F. drupacea	9.75 ± 0.53
F. elastica	9.38 ± 0.63
F. formosana	9.5 ± 0.42
F. luschnathiana	9.25 ± 0.56
F. lyrata	9.50 ± 0.50
F. macrophylla	9.13 ± 0.67
F. microcarpa	9.0 ± 0.33
F. mucuso	9.75 ± 1.10
F. natalensis subsp. *leprieurii*	9.38 ± 0.50
F. platypoda	9.25 ± 0.65
F. religiosa	9.13 ± 0.61
F. retusa	9.25 ± 0.59
F. sarmentosa var. *henryi*	9.50 ± 0.46
F. sycomorus	9.13 ± 0.55
F. vasta	9.63 ± 0.56
F. villosa	9.25 ± 0.56

Table 2
Inhibition zone diameters of *Yersinia ruckeri* growth (1000 µL inoculum) induced by ethanolic extracts obtained from leaves of various *Ficus* species. Data are presented as mean ± standard error, n = 8

Ficus species	Inhibition zone diameters (IZD), mm
F. aspera	21.88 ± 1.33
F. barteri	22.25 ± 0.99
F. erecta	17.50 ± 0.73
F. formosana	25.50 ± 1.44
F. hederacea	27.75 ± 1.15
F. hispida	25.50 ± 1.21
F. jahannis subsp. *afghanistanica*	17.25 ± 1.10
F. lingua	17.38 ± 0.63
F. malayana	16.88 ± 0.77
F. pumila	15.25 ± 0.56
F. religiosa	17.25 ± 0.49
F. sarmentosa var. *henryi*	18.50 ± 0.94
F. septica	15.63 ± 0.68
F. sur	20.13 ± 1.11
F. virens	18.88 ± 0.93
F. benghalensis	11.25 ± 0.49
F. drupacea	11.38 ± 0.71
F. elastica	13.38 ± 1.55
F. luschnathiana	12.13 ± 0.40
F. natalensis subsp. *leprieurii*	11.25 ± 0.65
F. natalensis	11.63 ± 0.56
F. petiolaris	14.25 ± 0.59
F. sagittata	14.38 ± 0.56
F. tinctoria	15.13 ± 0.78
F. benjamina	9.25 ± 0.80
F. binnendijkii	9.50 ± 0.53
F. carica	9.25 ± 0.37
F. craterostoma	9.13 ± 0.40
F. cyathistipula	9.50 ± 0.88
F. deltoidea	9.38 ± 0.38
F. lyrata	9.25 ± 0.59
F. macrophylla	9.38 ± 0.42
F. microcarpa	9.00 ± 0.27
F. mucuso	9.50 ± 0.60
F. platypoda	9.13 ± 0.48
F. retusa	9.38 ± 0.53
F. rubiginosa	9.38 ± 0.38
F. sycomorus	9.25 ± 0.31
F. vasta	9.63 ± 0.56
F. villosa	9.38 ± 0.63

activity against the *S. liquefaciens* strain. Ethanol, when used as a negative control, showed a minimum inhibition zone (8.86 ± 0.40 mm).

Among all the extracts screened, the maximum antimicrobial activity was observed with the ethanolic extracts of *F. hederacea*, *F. formosana*, and *F. hispida* against the *Y. ruckeri* strain with IZDs of 27.75 ± 1.15, 25.50 ± 1.44, and 25.50 ± 1.21 mm, respectively. The *Y. ruckeri* strain displayed high susceptibility to the leaf extracts of 15 *Ficus* species, while the same bacterial isolate exhibited an intermediate susceptibility to leaf extracts of nine *Ficus*

Table 3
Inhibition zone diameters of *Pseudomonas fluorescens* growth (1000 μL inoculum) induced by ethanolic extracts obtained from leaves of various *Ficus* species. Data are presented as mean ± standard error, n = 8.

Ficus species	Inhibition zone diameters (IZD), mm
F. aspera	15.50 ± 0.85
F. barteri	17.88 ± 0.90
F. benghalensis	20.63 ± 0.75
F. carica	17.50 ± 0.57
F. craterostoma	19.13 ± 0.40
F. deltoidea	17.75 ± 0.82
F. drupacea	18.13 ± 0.67
F. erecta	25.88 ± 0.74
F. formosana	24.50 ± 1.27
F. hederacea	23.50 ± 1.04
F. lingua	16.63 ± 0.56
F. malayana	15.75 ± 0.53
F. mucuso	20.88 ± 0.67
F. natalensis subsp. *leprieurii*	17.50 ± 0.71
F. petiolaris	17.63 ± 0.75
F. religiosa	21.38 ± 0.89
F. rubiginosa	16.00 ± 1.02
F. septica	15.50 ± 0.71
F. sur	25.38 ± 0.75
F. virens	25.38 ± 1.31
F. benjamina	12.75 ± 0.67
F. binnendijkii	12.38 ± 0.53
F. elastica	13.88 ± 1.01
F. hispida	14.75 ± 0.96
F. jahannis subsp. *afghanistanica*	13.13 ± 0.95
F. luschnathiana	13.25 ± 0.65
F. lyrata	10.25 ± 0.56
F. natalensis	11.88 ± 0.44
F. pumila	12.38 ± 0.56
F. sagittata	12.63 ± 0.75
F. sarmentosa var. *henryi*	14.25 ± 0.80
F. tinctoria	14.38 ± 0.82
F. vasta	10.75 ± 0.67
F. villosa	12.13 ± 0.40
F. cyathistipula	9.63 ± 0.84
F. macrophylla	9.25 ± 0.62
F. microcarpa	9.50 ± 0.68
F. platypoda	9.00 ± 0.53
F. retusa	9.38 ± 0.53
F. sycomorus	9.38 ± 0.50

Table 4
Inhibition zone diameters of *Shewanella putrefaciens* growth (1000 μL inoculum) induced by ethanolic extracts obtained from leaves of various *Ficus* species. Data are presented as mean ± standard error, n = 8.

Ficus species	Inhibition zone diameters (IZD), mm
F. aspera	25.75 ± 1.00
F. benghalensis	25.75 ± 1.82
F. benjamina	25.38 ± 0.86
F. binnendijkii	20.25 ± 0.98
F. craterostoma	25.25 ± 0.80
F. cyathistipula	23.13 ± 0.83
F. drupacea	20.63 ± 0.96
F. elastica	20.50 ± 0.60
F. erecta	30.13 ± 0.97
F. formosana	17.38 ± 0.42
F. hederacea	20.63 ± 0.80
F. hispida	25.13 ± 0.83
F. jahannis subsp. *afghanistanica*	20.38 ± 0.50
F. luschnathiana	25.25 ± 0.96
F. lyrata	20.63 ± 0.71
F. macrophylla	20.63 ± 1.03
F. malayana	25.63 ± 0.71
F. mucuso	20.63 ± 0.63
F. natalensis subsp. *leprieurii*	25.38 ± 1.43
F. natalensis	18.25 ± 0.65
F. petiolaris	20.13 ± 0.67
F. platypoda	20.75 ± 0.70
F. pumila	25.50 ± 1.05
F. religiosa	20.50 ± 0.68
F. retusa	20.00 ± 0.87
F. rubiginosa	20.38 ± 0.98
F. sarmentosa var. *henryi*	20.38 ± 1.25
F. septica	25.63 ± 0.75
F. sur	25.75 ± 0.67
F. tinctoria	20.63 ± 0.75
F. villosa	20.38 ± 0.86
F. virens	17.63 ± 0.65
F. carica	12.38 ± 0.73
F. lingua	12.13 ± 0.67
F. sagittata	12.13 ± 0.69
F. sycomorus	13.25 ± 0.45
F. vasta	14.38 ± 0.56
F. barteri	9.25 ± 0.37
F. deltoidea	9.25 ± 0.53
F. microcarpa	9.13 ± 0.35

species with IZDs from 10 to 15 mm. The remaining 16 extracts screened had the lowest anti-*Y. ruckeri* activity (Table 2).

Our results also demonstrated that the *Pseudomonas fluorescens* strain revealed high susceptibility (according to the IZDs) to ethanolic extracts derived from 20 plants (the mean of IZD was more than 15 mm) (Table 3). Further, *F. erecta*, *F. sur*, and *F. virens* extracts were effective against *P. fluorescens* with IZDs of 25.88±0.74 mm, 25.38 ± 0.75 mm, and 25.38 ± 1.31 mm, respectively. In the group of species that exhibited intermediate activity against the *P.*

Table 5
Antibiotic susceptibility results of the bacteria tested. Data are presented as mean ± standard error, n = 8

	Inhibition zone diameters (IZD)					
Bacteria strains/Antibiotics	S3	SXT	OT	UB	ENR	FFC
Serratia liquefaciens (Pt521)	6.57±0.30	6.43±0.20	22.43±0.72	25.29±0.84	22.14±0.59	20.29±0.57
Yersinia ruckeri (UP 2)	6.29±0.29	32.43±0.90	25.29±0.42	23.14±0.51	28.29±0.97	33.43±0.57
Pseudomonas fluorescens (Pt 433)	6.43±0.20	6.29±0.18	17.00±0.82	17.29±0.57	22.14±0.67	6.14±0.14
Shewanella putrefaciens (St15)	6.29±0.18	25.43±0.37	21.29±0.42	30.14±0.51	30.29±0.52	35.14±0.40

Antibiotics used (μg $disc^{-1}$): S3 – Sulfonamides, 300 μg; SXT – sulfonamides potentiated with trimethoprim, 25 μg; OT – oxytetracycline, 30 μg; UB – Flumequine, 30 μg; ENR – enrofloxacin, 5 μg; FFC – florfenicol, 30 μg

fluorescens strain, the highest antibacterial activity was noted for *F. hispida* (14.75 ± 0.96 mm), *F. tinctoria* (14.38 ± 0.82 mm), and *F. sarmentosa* var. *henryi* (14.25 ± 0.80 mm). On the other hand, the *P. fluorescens* strain was resistant to ethanolic extracts obtained from six *Ficus* species (14.6%) (the IZD means were less than 10 mm) (Table 3).

The *Shewanella putrefaciens* strain revealed a high level of susceptibility to ethanolic extracts obtained from the leaves of 32 species (IZD means were more than 15 mm) (Table 4). Moreover, high IZD values were noted for *F. erecta* (30.13 ± 0.97 mm) in a group with a high susceptibility against *Sh. putrefaciens*. *F. vasta* extract exhibited the highest anti-*Sh. putrefaciens* activity in the group with intermediate susceptibility (IZD of 14.38 ± 0.56 mm). The IZDs for three species (7.3%) were less than 10 mm (Table 4).

The antibacterial efficacy of ethanolic extracts derived from leaves of various *Ficus* species was compared with selected antibiotics commonly used in aquaculture. The results are presented in Table 5. The antibiotic profile of each bacterium was determined using specified antibiotic discs (Table 5). *S. liquefaciens*, *Y. ruckeri*, *P. fluorescens*, and *Sh. putrefaciens* were resistant to sulfonamides. Among the four strains, *P. fluorescens* was resistant to sulfonamides potentiated with trimethoprim and florfenicol. Thus, all bacterial strains were susceptible to oxytetracycline (30 μg), flumequine (30 μg), and enrofloxacin (5 μg) (Table 5).

Discussion

Determining the *in vivo* effectiveness of alternative agents that are natural, reliable, antimicrobial, and do not have harmful impacts on ecosystems is urgently needed in aquaculture today (Pepi and Focardi 2021, Rangel-López et al. 2022). Plant-derived products or phytobiotics with antibacterial properties are studied widely with potential applications in aquaculture systems (Bulfon et al. 2013, Devi et al. 2016, Wang et al. 2017). Nevertheless, although the properties of medicinal plants are well documented and used in human herbal medicines around the world, very few commercial agents are currently available for use in large-scale aquaculture globally (Devi et al. 2016).

In our study, forty plant extracts from mature leaves of various *Ficus* species extracted in ethanol were used to test the growth of *Serratia liquefaciens*. Six different extracts derived from leaves of *F. lingua*, *F. erecta*, *F. rubiginosa*, *F. tinctoria*, *F. sur*, and *F. aspera* exhibited a high degree of growth inhibition against *S. liquefaciens* with IZDs of more than 15 mm (Table 1). The highest IZD value was noted for the *F. lingua* extract (20.75 ± 1.41) mm. The highest antibacterial activity among the *Ficus* species that had IZDs from 10 to 15 mm (intermediate susceptibility) was noted with *F. hederacea* (14.63 ± 0.80 mm), *F. barteri* (14.38 ± 0.63 mm), and *F. virens* (14.25 ± 0.77 mm). The *Serratia liquefaciens* strain was resistant to twenty-two of the plant species of the genus *Ficus* tested (Table 1).

Of the 40 *Ficus* species tested, the only plants that controlled all the pathogens were *F. sur* and *F. erecta*. In particular, the growth of *S. liquefaciens*, *Y. ruckeri*, *P. fluorescens*, and *Sh. putrefaciens* strains was inhibited by *F. sur* extract with IZDs of 15.19 ± 0.65 mm, 20.13 ± 1.11 mm, 25.38 ± 0.75 mm, and 25.75 ± 0.67 mm, respectively. The *F. erecta* extract showed significant in vitro antimicrobial activity against *S. liquefaciens* (17.75 ± 0.94 mm), *Y. ruckeri* (17.50 ± 0.73 mm), *P. fluorescens* (25.88 ± 0.74 mm), and *Sh. putrefaciens* (30.13 ± 0.97 mm). The *F. lingua* extract was effective against *S. liquefaciens* with IZDs of 20.75 ± 1.41 mm, *Y. ruckeri* – 17.38 ± 0.63 mm, and *P. fluorescens* – 16.63 ± 0.56 mm, while the *F. rubiginosa* extract was effective against *S. liquefaciens* with IZDs of 17.25 ± 0.67 mm, *P. fluorescens* – 16.0 ± 1.02 mm, and *Sh. putrefaciens* – (20.38 ± 0.98) mm (Tables 1–4).

Serratia liquefaciens and *S. plymuthica* are associated with bacterial septicemia and mortalities in salmonid fishes. *S. liquefaciens* was the predominant isolate recovered from dead and dying Atlantic salmon in Scottish marine cages (McIntosh and Austin 1990). The internal organs were affected, particularly the kidney, spleen, and liver, but there were no external signs of infection. Starliper (2001) described *S. liquefaciens* as a pathogen of laboratory-reared Arctic charr *Salvelinus alpinus* (L.). The only external sign of the disease was redness and swelling of the anus, but internally the tissues were severely hemorrhagic. Aydin et al. (2001) reported that diseased rainbow trout lost scales, had bloody, swollen kidneys, hemorrhagic spots on the gills, and bloody exudates in the intestine. The initial diagnosis of the Atlantic salmon isolate was difficult because of its anomalous oxidase reaction, and the arrangement of the flagella suggested that it was an aeromonad (McIntosh and Austin 1990). When injected into Atlantic salmon, the isolate caused pronounced damage to the musculature at the site of injection and rapid mortalities (72 h for an infective dose of bacteria). Rainbow trout also showed muscle and internal organ necrosis when injected with bacteria, but mortalities did not occur (McIntosh and Austin 1990). Vigneulle and Baudin-Laurencin (1995) described *S. liquefaciens* as a turbot pathogen that caused low but continuous mortality in marine cages in France. Externally, there were no clinical signs, except for the hyperpigmentation of the skin, while internally there was abundant ascites and both the kidney and spleen were swollen.

Shewanella putrefaciens is a heterogeneous group of microorganisms of the family Alteromonadaceae. Bacteria of the *S. putrefaciens* group are Gram-negative and rod-shaped measuring 0.5–1.0 μm × 1.5–2.0 μm. The bacteria are motile thanks to a single polar flagellum (Paździor 2016). Recently, *S. putrefaciens* has come to be associated with serious diseases in freshwater fishes. Therefore, it is described as a new etiologic agent of the disease named shewanellosis (Paździor 2016). *S. putrefaciens* is an opportunistic bacterial pathogen that can cause disease in fish under stressful conditions (Kozinska and Pekala 2004). The first isolation of *S. putrefaciens* from fish, more specifically from cultured rabbit fish, was in 1987 by Saeed et al. (1987). The most interesting fact about *S. putrefaciens*, commonly known as halophytic bacteria, is that it can adapt to freshwater environments (Paździor 2016). It is reported that, in healthy fish, *S. putrefaciens* colonizes the gills (Al-Harbi and Uddin 2008), and similarly to other opportunistic bacteria (*Aeromonas* spp., *Pseudomonas* spp.), it is part of the physiological flora of marine and freshwater fish species. The intensity of shewanellosis usually depends on the kind of bacterial isolate. The disease is generally manifested by ulcerative and necrotic lesions on the skin. Clinical symptoms can include lethargy, dark skin discoloration, swollen anus, and gill necrosis (Pękala et al. 2015, Paździor 2016).

Pseudomonas fluorescens is a Gram-negative bacterium and a common pathogen in a wide range of farmed fishes. *P. fluorescens*-associated infection is widely distributed in the aquaculture industry and is considered one of the primary causes of bacterial hemorrhagic septicemia in fish, and it appears to be a stress-related disease of freshwater and salt-water fishes throughout the world (Austin and Austin 1999). *P. fluorescens* is normally found in water, soil, and fish bodies. These infections are often prominent

under stressful conditions that alter the natural defenses of fishes (Kumar and Day 1992). *P. fluorescens* also causes severe economic losses and decreases the efficiency of fish farms especially under poor culture conditions such as overcrowding, low temperatures, inappropriate handling and transportation, and secondary pathogens of damaged fish tissues (Fayed et al. 1997, El-Barbary and Hal 2017). Austin and Austin (1999) suggested the reason for the widespread incidence of *Pseudomonas* sp. in aquatic environments could be due to its transmission through water, which is a major vector of infection.

Infections in humans caused by these bacterial pathogens transmitted through fishes or aquatic environments are common (Kumar and Day 1992). *Yersinia ruckeri* is a ubiquitous pathogen of finfish capable of causing major mortalities in farmed fish stocks (Ghosh et al. 2016). This bacterium is the etiological agent of enteric redmouth disease (ERM) of farmed salmonids (Ormsby et al. 2016). The causative agent, a Gram-negative enteric bacterium, was first isolated in the early 1950s and it was identified as a new species, *Y. ruckeri*, in 1978 (Ewing et al. 1978). *Y. ruckeri* is a member of the family Enterobacteriaceae within the γ-proteobacteria subdivision. *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 the 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).

Extracts from different plants belonging to the *Ficus* genus are reported to possess diverse medicinal activities, i.e., antioxidant (Mohan et al. 2015), antiplasmodial (Muregi et al. 2003), anticancer (Mbosso et al. 2016), antimicrobial (Salem et al. 2013, Awolola et al. 2017), antidiarrheal (Mandal and Kumar 2002), anti-pyretic (Rao et al. 2002), and gastroprotective (Rao et al. 2008). Extracts from various *Ficus* plants are active against human and animal pathogens (McPhearson et al. 1991, Dangarembizi et al. 2012). Remarkable progress has been made recently in the field of antibacterial herbal therapy in aquaculture to control the development of drug-resistant pathogens. Today, tropical ecosystems are suffering from climate change much more than temperate ecosystems (Sheldon 2019). Therefore, a good alternative to native tropical plants with strong antibacterial properties can be *ex-situ* cultivated plants and particularly species of the genus *Ficus* (Tkachenko et al. 2016a, 2016b, 2016c, 2016d, 2016e, 2017a, 2017b, 2019). Consequently, this study is the continuation of a series of our previous studies revealing the great potential of *Ficus* species as plants that have considerable antimicrobial properties against fish pathogens. Regarding the excellent functional properties of *Ficus* plants, the antibacterial efficacy of ethanolic extracts of various *Ficus* species was evaluated against *Citrobacter freundii* (Tkachenko et al. 2016a, 2017a), *Pseudomonas fluorescens* (Tkachenko et al. 2016a, c, f), *Aeromonas* spp. (Tkachenko et al. 2016d, Pękala-Safińska et al. 2021), and *Yersinia ruckeri* strains isolated locally from the internal organs of infected fish (Tkachenko et al. 2019). Studies on the inhibitory properties of leaf ethanolic extracts obtained from *Ficus* species against *Aeromonas* spp. strains were conducted previously by our scientific team (Pękala-Safińska et al. 2021). In our previous study, the variable antimicrobial activity of the 41 tested ethanolic extracts against selected *Aeromonas* strains was observed. The *A. sobria* strain was susceptible to 14 (34.2%) of 41 extracts tested, while both *A. hydrophila* and *A. salmonicida* subsp. *salmonicida* were susceptible to 10 (24.4%) and 13 (31.7%), respectively (Pękala-Safińska et al. 2021).

Several *Ficus* plants reported here were studied by other researchers. According to preliminary studies by other researchers, a number of *Ficus* plant extracts prepared in different solvents showed antibacterial activity against pathogenic isolates and

antibiotic-resistant bacteria (Atindehou et al. 2002, Eldeen et al. 2005, Solomon-Wisdom et al. 2011). For example, Mousa et al. (1994) tested chloroform extract of fruits from four *Ficus* species (*F. benghalensis*, *F. benjamina*, *F. religiosa*, and *F. sycomorus*) for toxicity (against *Artemia salina*), antitumor activity (against *Agrobacterium tumefaciens*), and 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 *S. aureus* HAMBI 66, being low to moderately susceptible among tested organisms in general, was most strongly inhibited by *F. benjamina* extract (IZD 17–20 mm), while *F. benghalensis* and *F. sycomorus* showed weaker activity (16–19 mm), and *F. religiosa* was inactive (Mousa et al. 1994).

Medicinal plants possess immunomodulatory and antioxidant properties that lead to antibacterial activities. They are known to have versatile immunomodulatory activity by stimulating both non-specific and specific immunity. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. Over the past two decades, there has been an increase in the number of studies with general conclusions that medicinal plant-derived extracts can potentially replace synthetic chemicals such as antibiotics and other chemotherapeutic agents in aquaculture systems (Pepi and Focardi 2021, Rangel-López et al. 2022). Moreover, in the last few years, a number of studies have been conducted in different countries to prove such efficacy. Many plants are used because of their antimicrobial traits, which stem from the compounds synthesized in the secondary metabolism of the plant (Salem et al. 2013, Amenu 2014).

Recently, many researchers have focused on the investigation of plant extracts, and essential oils as potential antimicrobial agents against fish pathogens (Reverter et al. 2014, 2017, Turker and Yıldırım 2015). In order to identify the most commonly studied plants and their activities, an extensive literature review was done of published studies on plant use in aquaculture by Reverter et al. (2014, 2017). They concluded that thousands of plant-derived compounds have been screened, and their inhibitory effects against many types of fish pathogens have been confirmed (Reverter et al. 2017). Plants are reported to decrease stress, promote growth, stimulate appetite, stimulate immunity, act as an aphrodisiac, and have antipathogen properties in aquaculture thanks to their various active compounds such as alkaloids, terpenoids, tannins, saponins, and flavonoids (Citarasu 2010, Chakraborty and Hancz 2011). This comprehensive review of plant species used in aquaculture cites just two papers that highlight the biological properties of *Ficus* species, namely *F. benghalensis* as an immunostimulant (Olusola et al. 2013) and *F. septica* as an antimicrobial agent (Caruso et al. 2013).

The plant species that display the highest potential for use in aquaculture are garlic (*Allium sativum* L.), pomegranate (*Punica granatum* L.), bermudagrass (*Cynodon dactylon* L. (Pers.)), Indian ginseng (*Withania somnifera* (L.) Dunal), and ginger (*Zingiber officinale* Roscoe) (Mwitari et al. 2013). The antimicrobial activity of *Terminalia arjuna* (Roxb. ex DC.) Wight & Arn., *Centella asiatica* (L.) Urb., *Ziziphus jujuba* Mill. (syn. *Z. mauritiana* Lam.), *Murraya koenigii* (L.) Spreng., and *Ocimum tenuifolium* L. (syn. *O. sanctum* L.) was screened to evaluate the antibacterial activity of their extracts against pathogenic bacteria of aquatic animals, namely, *Aeromonas hydrophila*, *Aeromonas sobria*, and *Vibrio paraharmolyticus* (Banerjee et al. 2011).

A positive antibacterial interaction was evident between quercetin and selected polyphenolic acids present in plants. Prasad et al. (2014) evaluated combinations of quercetin with gallic acid, p-anisic acid, and cinnamic acid *in vitro* for synergistic activity against common Gram-negative bacterial pathogens of fish viz., *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Edwardsiella tarda*. Quercetin and other polyphenolic compounds exhibited antibacterial action against the selected fish pathogens. It was observed that fractional inhibitory concentration indices for the combination of quercetin with gallic

acid, p-anisic acid, or cinnamic acid against *A. salmonicida* were less than 0.5, indicating a synergistic interaction. However, the above combinations produced additive antimicrobial activity against *A. hydrophila* and *E. tarda* (Prasad et al. 2014).

Understanding mechanisms of the antimicrobial action of medicinal plant extracts is the first step in the optimal utilization of these extracts as natural antimicrobial agents (Gonelimali et al. 2018). To enhance the understanding of the antimicrobial activity mechanism of plant extracts, the changes in internal pH (pHint), and membrane potential were measured in *Staphylococcus aureus* and *Escherichia coli* cells after exposure to the plant extracts. The results indicated that the plant extracts significantly affected the cell membrane of Gram-positive and Gram-negative bacteria, as demonstrated by the decline in pHint as well as cell membrane hyperpolarization (Gonelimali et al. 2018). To evaluate the action mechanisms of extracts of medicinal plants traditionally used in Kenya to treat microbial infections and cancer (*Withania somnifera*, *Warburgia ugandensis* Sprague, *Prunus africana* (Hook. f.) Kalkman, and *Plectranthus barbatus* Andrews), Mwitari et al. (2013) employed the IEC-6 cells and RT-PCR technique. These researchers suggested that the activity mechanisms of plant extracts can largely be attributed to cytotoxicity, gene silencing, and immunopotentiation. Further elucidation of the molecular mechanisms underlying the activity of these chemicals is critical to evaluate the possibility of using plant extracts in future drug development.

To conclude, further investigations should be conducted to examine the influence of plants on fish health (including physiological and histological parameters) as a preliminary step for using them on large scales in aquaculture (Awad and Awaad 2017).

Conclusions

In vitro tests of antibacterial activity revealed that various *Ficus* species have notable antibacterial activity. Our study found that the six most effective plants that caused a zone of inhibition of at least 15 mm against *S. liquefaciens* were *F. lingua*, *F. erecta*, *F. rubiginosa*, *F. tinctoria*, *F. sur*, and *F. aspera*. The maximum antimicrobial activity against the *Y. ruckeri* strain was observed for the ethanolic extracts of *F. hederacea*, *F. formosana*, and *F. hispida*. Our results also revealed that the *Pseudomonas fluorescens* strain exhibited high susceptibility (according to the IZD) to ethanolic extracts derived from 20 plants (the mean value of the IZDs was more than 15 mm). Extracts derived from the leaves of *F. erecta*, *F. sur*, and *F. virens* were more effective against *P. fluorescens*. The *Shewanella putrefaciens* strain revealed a high level of susceptibility to ethanolic extracts derived from the leaves of 32 *Ficus* species (the highest IZD value was noted for *F. erecta*).

Overall, our findings support conducting further chemical analyses of the aforementioned plant extracts to determine their chemical composition and identify the exact phytocompounds responsible for antimicrobial activity. HPLC profiling of extracts to characterize and isolate the active antibacterial constituents of various *Ficus* species to target fish pathogens is in progress. Additionally, plant extracts should be subjected to pharmacological evaluations to assess the *in vivo* efficacy, toxicity, potential adverse effects, interactions, and contraindications.

Conflict of Interests Statement: The authors declare that there are no conflicts of interest regarding the publication of this article.

Animal Rights Statement: None required.

Author contributions. H.T., L.B., A.P.-S., N.K. – ideas, formulation, evolution of overarching research goals and aims; H.T., A.P.-S., N.K. – development, methodology design; H.T., L.B., A.P.-S., N.K. – validation and data curation; H.T., N.K. – formal analysis; H.T., L.B., N.K. – responsibility for implementing the research, including writing the first draft; H.T., L.B., A.P.-S., N.K. – writing, reviewing, and editing.

All authors have read and agreed to the published version of the manuscript, as well as agreed to be accountable for all aspects of the work in ensuring that

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