

In vitro antimicrobial activity of ethanolic extracts obtained from *Ficus* spp. leaves against the fish pathogen *Aeromonas hydrophila*

Halyna Tkachenko, Lyudmyla Buyun, Elżbieta Terech-Majewska, Zbigniew Osadowski

Received – 01 August 2016/Accepted – 18 November 2016. Published online: 31 December 2016; ©Inland Fisheries Institute in Olsztyn, Poland

Citation: Tkachenko H., Buyun L., Terech-Majewska E., Osadowski Z. 2016 – *In vitro* antimicrobial activity of ethanolic extracts obtained from *Ficus* spp. leaves against the fish pathogen *Aeromonas hydrophila* – Arch. Pol. Fish. 24: 219-229.

Abstract. The main goal of this study was to determine *in vitro* antimicrobial activity of ethanolic extracts obtained from the leaves of various *Ficus* species against *Aeromonas hydrophila* isolated locally from infected rainbow trout (*Oncorhynchus mykiss* Walbaum) with the aim of providing scientific rationale for the use of the plant in the treatment of bacterial infections induced by *Aeromonas* spp. in fish. Antimicrobial susceptibility testing was done on Muller-Hinton agar with the disc diffusion method. In the present study, most ethanolic extracts proved effective against the *A. hydrophila* tested, with 10-12 mm inhibition zones observed. *A. hydrophila* demonstrated the highest susceptibility to *F. pumila*. Among various species of *Ficus* with moderate activity against *A. hydrophila*, the highest antibacterial activities were noted for *F. benghalensis*, *F. benjamina*, *F. deltoidea*, *F. hispida*, and *F. lyrata*. Thus, *Ficus* can be used as a natural antiseptic and antimicrobial agent in veterinary practice. Further investigations need to be conducted to isolate and identify the bioactive compounds

that can then be subjected to detailed pharmacological studies and the development of clinical applications. The alarming rate of increasing resistance in bacterial pathogens in aquaculture environments means that medicinal plants with antibacterial properties are very important as natural resources of new active compounds.

Keywords: antibacterial activity, *Aeromonas hydrophila*, infected rainbow trout, *Ficus* spp., ethanolic extracts, growth inhibition zone

Introduction

Aeromonas hydrophila, a Gram-negative facultative anaerobe, is an autochthonous species in freshwater environments and a component of the normal microflora of fish intestinal tracts (Cipriano 2001). *Aeromonas* bacteria are also the etiologic agents responsible for a variety of infections in both immunocompetent and immunocompromised humans (Austin and Austin 2007). The severity of disease it causes is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress exerted on a fish population, and the resistance and physiological condition of the host. Pathologic conditions attributed to members of the *A. hydrophila* complex include dermal ulceration, hemorrhagic septicemia, red sore disease,

H. Tkachenko [✉], Z. Osadowski
Institute of Biology and Environmental Protection
Pomeranian University in Slupsk, Poland
Arciszewskiego 22B, 76-200 Slupsk, Poland
e-mail: tkachenko@apsl.edu.pl, biology.apsl@gmail.com

L. Buyun
M. Gryshko National Botanical Garden, National Academy of Science
of Ukraine, Kyiv, Ukraine

E. Terech-Majewska
Department of Epizootiology, University of Warmia and Mazury in
Olsztyn, Poland

red rot disease, and scale protrusion disease (Cipriano 2001). In salmonids, *A. salmonicida* causes furunculosis, a disease characterized by skin ulcers and septicemia. Moreover, other *Aeromonas* species are involved in similar pathological diseases (Austin and Austin 2007). Zepeda-Velázquez et al. (2015) confirm that the *Aeromonas* species *A. hydrophila*, *A. salmonicida*, and *A. veronii* are associated with septicemia and dermal lesions in rainbow trout. Furthermore, some fish bacterial pathogens are also associated with diseases in humans, which suggests that aquaculture products pose a potential risk to consumers (Tukmechi et al. 2010).

One of the overriding considerations is how disease can be controlled. While vaccine development has been focused less on motile aeromonads than on other pathogens, current research addresses the use of probiotics, immunostimulants, and medicinal plants, some of which stimulate immune memory, to control them (Cipriano and Austin 2011). The benefits of medicinal plants have been the focus of many Asian studies on controlling infections caused by *Aeromonas* (Cipriano and Austin 2011). Preliminary screening assays indicate that some plants with antibacterial properties can be used as alternative therapeutic agents against bacterial infections in the aquaculture industry. Increasing numbers of studies on the antimicrobial properties of medicinal herbs are being reported from different parts of the world. For instance, two Chinese medicinal herbs (*Astragalus membranaceus* and *Lonicera japonica*) administered at doses of 0.1% in combination with 0.5% boron for four weeks led to greatly reduced mortality in Nile tilapia, *Oreochromis niloticus* (L.), after they were challenged with *A. hydrophila*, and there was also evidence of immunostimulation (Ardo et al. 2008). An aqueous leaf extract of the Indian medicinal herb *Azadirachta indica* was tested against *A. hydrophila* infection in common carp, *Cyprinus carpio* L., and the results indicate *in vitro* inhibitory activity against this bacterium (Harikrishnan and Balasundaram 2005). The benefit of using 5 g of mango (*Mangifera indica*) kernel per kg of feed administered orally for 60 days to carp

fingerlings was highlighted by Sahu et al. (2007), who report almost complete survival (98% survival compared with 50% survival of the controls) after a challenge with *A. hydrophila* (Cipriano and Austin 2011).

Ginger (*Zingiber officinale* Roscoe) and guava (*Psidium guajava* L.) are promising food additives that can be used in carp 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* (Hamilton), and its susceptibility to *A. hydrophila* infection was studied by Sukumaran et al. (2016). Ginger dietary supplements (at 0.8%) can promote growth performance, skin mucus immune parameters, and strengthen immunity in *L. rohita*. The expression of gene encoding pro-inflammatory cytokines (IL-1 β , TNF- α), signaling molecules Kelch-like-ECH-associated protein 1 (Keap1), and nuclear factor kappa B p65 (NF- κ Bp65) were down-regulated in treatment groups. Moreover, fish fed a diet supplemented with 0.8% ginger exhibited significantly higher relative post-challenge survival (65.52%) against *A. hydrophila* infection. Dietary supplementation with guava leaves (at 0.5% concentration) could also improve the growth performance and strengthen the immunity of *L. rohita* (Giri et al. 2015). Lysozyme levels, leukocyte phagocytic activity, and alternative complement pathway activity were significantly higher in the group fed guava. Moreover, the fish fed the guava diet exhibited a significantly higher post-challenge survival rate (66.7%) (Giri et al. 2015).

Bacterial resistance to antibiotics is increasing, and the threats posed by the resistance of bacterial strains that have disseminated widely in aquaculture environments have never been greater. In recent years, there has been a growing interest in researching and developing new antimicrobial agents from various plants to overcome microbial resistance. Therefore, greater attention is being paid to screening antimicrobial activity and evaluating methods. Consequently, a number of medicinal plants have been screened for antimicrobial activity against Gram-positive and Gram-negative bacteria in recent

years. The active compounds of herbs possess characteristics that could be useful in fish and shrimp culture, and various herbs stimulate growth and appetite, enhance immune system responses, and exhibit broad-spectrum antimicrobial activity (Friedman et al. 2002). In particular, there has been increasing interest in *Ficus* spp. (*Moraceae*) because of its chemical composition and potential health benefits. *Ficus* spp. have been used extensively 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 (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 from the genus *Ficus* have been subjected to phytochemical and pharmacological investigations, there are many species that have not been studied and the ethnobotanical relevance of them has yet to be investigated. Therefore, an attempt was made to determine the *in vitro* antimicrobial activity of ethanolic extracts from the leaves of various *Ficus* species against the bacterial strain of *A. hydrophila* isolated locally from infected rainbow trout (*Oncorhynchus mykiss* Walbaum) with the aim of providing scientific rationale for using these plants in the treatment of bacterial infections induced by *Aeromonas* spp. in fish.

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. binnendijkii* "Amstel Gold", *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. mucoso* 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 the M. Gryshko National Botanical Garden (NBG) (Kyiv, Ukraine) during March and September of 2015. The entire collection of tropical and subtropical plants at NBG (including *Ficus* spp. plants) is classified as being from the National Heritage Collection of Ukraine. The species author abbreviations were taken from Brummitt and Powell (1992), Davis and Schmidt (1996), Jones et al. (1997).

Preparation of plant extracts

The *Ficus* spp. leaves sampled were brought into the laboratory for antimicrobial studies. Freshly crushed leaves were washed, weighed, and homogenized in 96% ethanol (in a proportion of 1:10) 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.

Identification method of the bacteria

A. hydrophila (strain E 2/7/15) isolated locally from the gills of rainbow trout (*Oncorhynchus mykiss* Walbaum) with clinical symptoms of furunculosis (the kidneys were gray, the liver was pale and fragile, the enlarged was spleen with exudate was noted in the body cavity) was used as the test organism. Samples of internal organs (kidneys, spleen, liver) weighing 2 g were taken and homogenized before preincubation in Trypticase Soya Broth (TSB; Oxoid) for 24 h. After preincubation, the bacterial culture was transferred to two different cultivation media: Trypticase Soya Agar (TSA; Oxoid) and Brain Heart Infusion Agar (BHIA; Oxoid) supplemented with 5% sheep blood (OIE 2000). After 48 h of incubation at

27°C, characteristic pink colonies were selected for further examination. The bacteria species were identified using an oxidase test and an API E test kit (Biomerieux, France). The results of the test were interpreted according to the manufacturer's protocol after 24 h of incubation at 27°C. Codes ++V-V---+V+++---+VV+ in the API E test were identified as *A. hydrophila*. The strain was obtained from the Diagnostics Laboratory of Fish and Crayfish Diseases, Department of Veterinary Hygiene, Provincial Veterinary Inspectorate in Olsztyn (Poland).

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

The strain tested was plated on TSA medium and incubated for 24 h at 25°C. Then the microorganism suspensions were suspended in sterile PBS, and turbidity was adjusted to the equivalent of the 0.5 McFarland standard. The antimicrobial activity of the extracts was evaluated with the agar well diffusion method (Bauer et al. 1966). Muller-Hinton agar plates were inoculated with 400 µl of standardized inoculum (10^8 CFU ml⁻¹) of bacterium and spread with sterile swabs.

Sterile filter paper discs impregnated with extract were applied to each of the culture plates 15 min after the bacteria suspension had been applied. The antimicrobial susceptibility testing was done on Muller-Hinton agar with the disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol). The *A. hydrophila* isolates were tested individually against four antibiotics. The antibiotics tested were oxytetracycline (30 µg), enrofloxacin (5 µg), gentamicin (10 µg), and sulphamethoxazole/trimethoprim (25 µg). A negative control disc impregnated with sterile ethanol was used in each experiment. The sensitivity of the strain to a commercial preparation with garlic extract (in dilution 1:10, 1:100, and 1:1000) was also studied. After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 h at 25°C. 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 inhibition zones surrounding the well. Each test was repeated six times, and the average values of antimicrobial activity were calculated.

The following zone diameter criteria were used to assign bacterial susceptibility or resistance to the phytochemicals tested: Susceptible (S) ≥ 15 mm, Intermediate (I) = 11-14 mm, and Resistant (R) ≤ 10 mm (Okoth et al. 2013).

Results

The data on the antimicrobial potential of ethanolic extracts obtained from *Ficus* spp. leaves are presented in Table 1 and Figs. 1-3. A comparison of susceptibility categories, i.e., susceptible, intermediate, and resistant, for the disk diffusion method is shown in Table 1.

The results indicated that the *A. hydrophila* (400 µl of standardized inoculum) revealed intermediate susceptibility to ethanolic extracts obtained from *F. benghalensis*, *F. benjamina*, *F. benjamina* "Reginald", *F. binnendijkii*, *F. carica*, *F. cyathistipula*, *F. deltoidea*, *F. drupacea*, *F. drupacea* "Black Velvet", *F. elastica*, *F. erecta*, *F. erecta* var. *sieboldii*, *F. hispida*, *F. lyrata*, *F. macrophylla*, *F. natalensis* subsp. *leprieurii*, *F. natalensis* subsp. *natalensis*, *F. palmeri*, *F. religiosa*, *F. rubiginosa*, *F. septica*, and *F. sycomorus*. *A. hydrophila* possessed resistance against ethanolic extracts from *F. aspera*, *F. binnendijkii* "Amstel Gold", *F. binnendijkii* "Amstel King", *F. craterostoma*, *F. elastica* "Variegata", *F. luschanthiana*, *F. mucoso*, *F. platypoda*, *F. sagittata*, *F. sur*, *F. vasta*, and *F. villosa*. The highest susceptibility of *A. hydrophila* was observed to *F. pumila* (the mean diameter of the inhibition zone was 17.1 mm) (Table 1).

Discussion

The results indicated that extracts offer a promising alternative to the use of antibiotics in controlling the growth of *A. hydrophila*. In this study, most ethanolic

Table 1

Susceptibility or resistance of *A. hydrophila* against ethanolic extracts obtained from *Ficus* spp. leaves

| <i>Ficus</i> spp. | Susceptibility or resistance of <i>A. hydrophila</i> | | |
|---|--|-----------------------------|----------------------------|
| | Susceptible (S) \geq 15 mm | Intermediate (I) = 11-14 mm | Resistant (R) \leq 10 mm |
| <i>F. aspera</i> | | | + |
| <i>F. benghalensis</i> | | + | |
| <i>F. benjamina</i> | | + | |
| <i>F. benjamina</i> "Reginald" | | + | |
| <i>F. binnendijkii</i> | | + | |
| <i>F. binnendijkii</i> "Amstel Gold" | | | + |
| <i>F. binnendijkii</i> "Amstel King" | | | + |
| <i>F. carica</i> | | + | |
| <i>F. craterostoma</i> | | | + |
| <i>F. cyathistipula</i> | | + | |
| <i>F. deltoidea</i> | | + | |
| <i>F. drupacea</i> | | + | |
| <i>F. drupacea</i> "Black Velvet" | | + | |
| <i>F. elastica</i> | | + | |
| <i>F. elastica</i> "Variegata" | | | + |
| <i>F. erecta</i> | | + | |
| <i>F. erecta</i> var. <i>sieboldii</i> | | + | |
| <i>F. hispida</i> | | + | |
| <i>F. luschanthiana</i> | | | + |
| <i>F. lyrata</i> | | + | |
| <i>F. macrophylla</i> | | + | |
| <i>F. mucoso</i> | | | + |
| <i>F. natalensis</i> subsp. <i>leprieurii</i> | | + | |
| <i>F. natalensis</i> subsp. <i>natalensis</i> | | + | |
| <i>F. palmeri</i> | | + | |
| <i>F. platypoda</i> | | | + |
| <i>F. pumila</i> | + | | |
| <i>F. religiosa</i> | | + | |
| <i>F. rubiginosa</i> | | + | |
| <i>F. sagittata</i> | | | + |
| <i>F. septica</i> | | + | |
| <i>F. sur</i> | | | + |
| <i>F. sycomorus</i> | | + | |
| <i>F. vasta</i> | | | + |
| <i>F. villosa</i> | | | + |

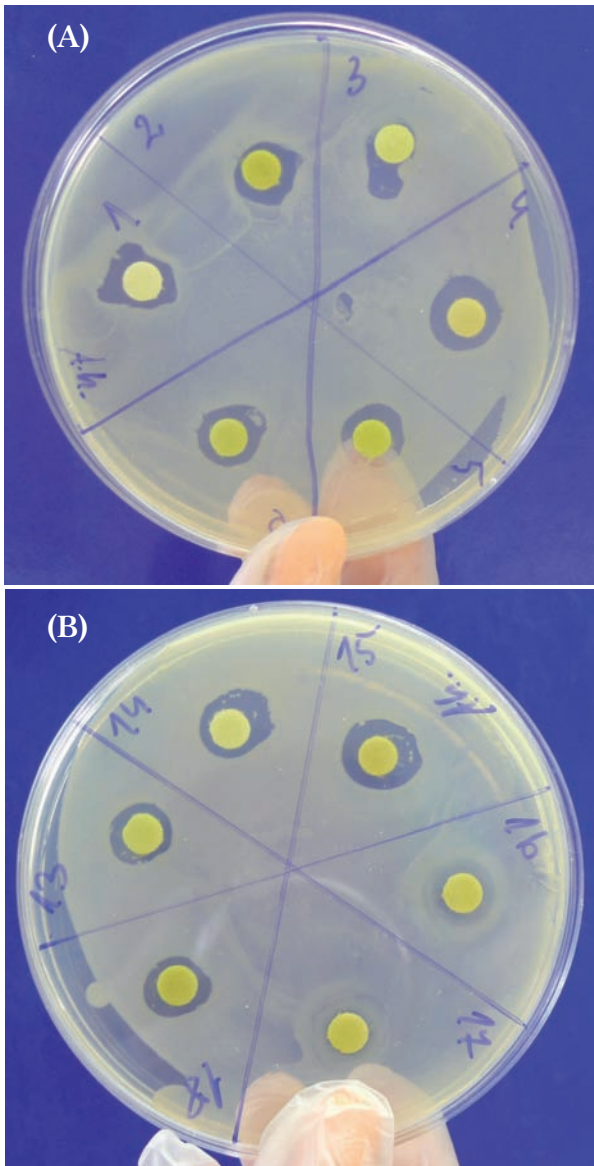


Figure 1. Antimicrobial activity of ethanolic extracts obtained from *F. benghalensis* (4), *F. benamina* (5) (A), *F. cyathistipula* (14), and *F. lyrata* (15) against *A. hydrophila* (B) measured as inhibition zone diameter.

extracts obtained from *Ficus* spp. proved effective against the bacterial strain of Gram-negative *A. hydrophila* tested with 10–12 mm inhibition zones observed. *A. hydrophila* demonstrated the highest susceptibility to *F. pumila* (Table 1, Fig. 3).

It is well documented that various *Ficus* spp. have been used against Gram-positive and Gram-negative bacteria (Salem et al. 2013). The scientific research on

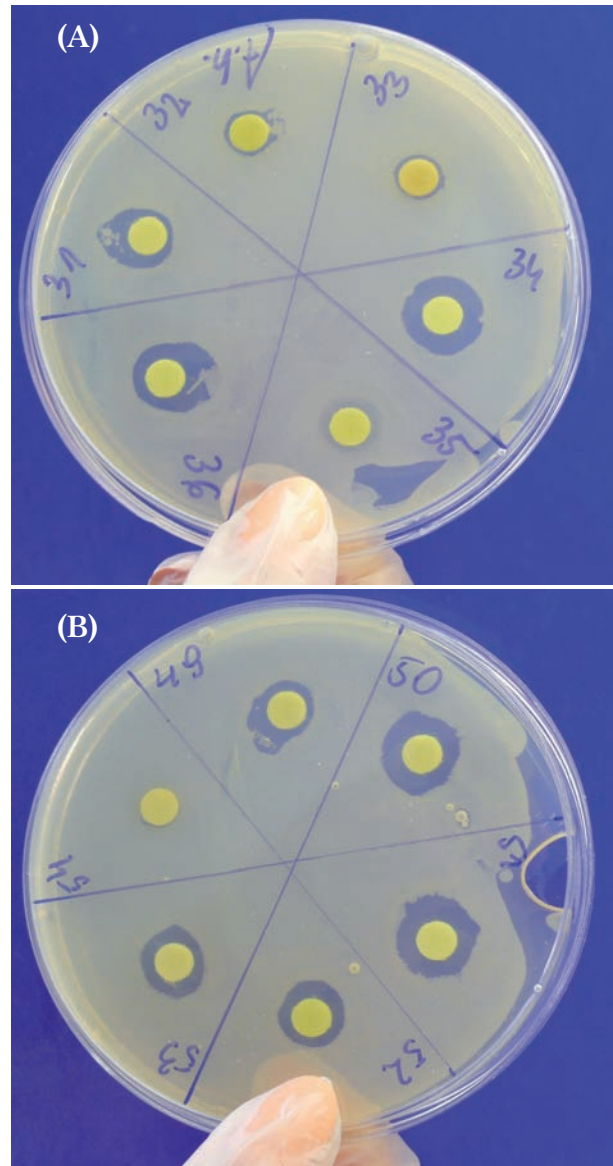


Figure 2. Antimicrobial activity of ethanolic extracts obtained from *F. natalensis* subsp. *leprieurii* (34) and *F. deltoidea* (36) (A), *F. palmeri* (50), *F. hispida* (51), and *F. natalensis* subsp. *natalensis* (52) against *A. hydrophila* (B) measured as inhibition zone diameter.

Ficus spp. indicates that these plants have been of increasing interest in recent years. For example, bergapten and oxypeucedanin hydrate were isolated from the chloroform extract of *F. pumila* (Juan et al. 1997). Bergapten was found to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi*, but it was inactive against *Trichophyton mentagrophytes*, *Mycobacterium pleie*, and *Candida*

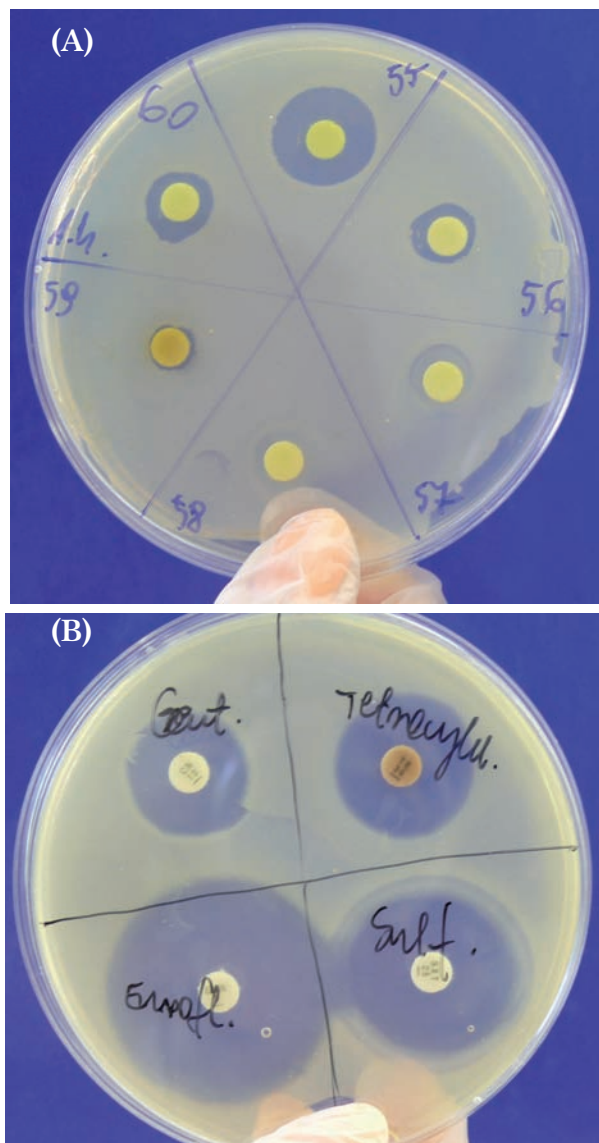


Figure 3. Antimicrobial activity of ethanolic extracts obtained from *F. pumila* (55) against *A. hydrophila* (A) and tested antibiotics measured as inhibition zone diameter.

albicans. Oxypeucedanin hydrate inhibited the growth of *S. typhi*, but was inactive against another five microorganisms (Juan et al. 1997).

Interestingly, ethanolic extracts obtained from *Ficus* species demonstrated different antibacterial activity against *A. hydrophila* inoculated with 200 µl and 400 µl of standardized inoculum (10^8 CFU ml⁻¹) of bacterium strain. Previous study using 200 µl of *A. hydrophila* inoculum revealed that *F. benghalensis*, *F. benjamina*, *F.*

binnendijkii, *F. cyathistipula*, *F. deltoidea*, *F. erecta*, *F. erecta* var. *sieboldii*, *F. hispida*, *F. luschanthiana*, *F. lyrata*, *F. macrophylla*, *F. mucoso*, *F. natalensis* subsp. *leprieurii*, *F. natalensis* subsp. *natalensis*, *F. palmeri*, *F. platypoda*, *F. pumila*, *F. rubiginosa*, *F. sur*, *F. sycomorus*, and *F. villosa* possessed good antibacterial activity (diameters of inhibition zones ranged from 10 to 14 mm), while *A. hydrophila* was resistant against ethanolic extracts from *F. aspera*, *F. benjamina* ‘Reginald’, *F. binnendijkii* ‘Amstel Gold’, *F. binnendijkii* ‘Amstel King’, *F. carica*, *F. craterostoma*, *F. drupacea*, *F. drupacea* ‘Black Velvet’, *F. elastica*, *F. elastica* ‘Variegata’, *F. religiosa*, *F. sagittata*, *F. septica*, and *F. vasta* (Tkachenko et al. 2016). On the other hand, in this study with 400 µl of *A. hydrophila* inoculum, bacterium demonstrated the highest susceptibility to *F. pumila* leaves extract (diameters of inhibition zones ranged from 16 to 18 mm), while intermediate susceptibility was demonstrated to ethanolic extracts obtained from *F. benghalensis*, *F. benjamina*, *F. benjamina* ‘Reginald’, *F. binnendijkii*, *F. carica*, *F. cyathistipula*, *F. deltoidea*, *F. drupacea*, *F. drupacea* ‘Black Velvet’, *F. elastica*, *F. erecta*, *F. erecta* var. *sieboldii*, *F. hispida*, *F. lyrata*, *F. macrophylla*, *F. natalensis* subsp. *leprieurii*, *F. natalensis* subsp. *natalensis*, *F. palmeri*, *F. religiosa*, *F. rubiginosa*, *F. septica*, and *F. sycomorus* leaves (Table 1).

In this study, among various species of *Ficus* with moderate activity against *A. hydrophila*, the highest antibacterial activity was noted in *F. benghalensis*, *F. benjamina*, *F. deltoidea*, *F. hispida*, and *F. lyrata*. The results demonstrate that *F. benghalensis* has intermediate *in vitro* activity against *A. hydrophila*. This data are consistent with the results of other authors. *F. benghalensis* bark exhibited significant antibacterial activity against *S. aureus*, *P. aeruginosa*, and *K. pneumoniae* (Gayathri et al. 2009). Aqueous and hexane aerial root extracts of *F. benghalensis* showed sustained activity against all bacterial strains and the highest activity was observed against *S. aureus* (Singh and Watal 2010). The antimicrobial activity of a methanolic extract was good and was more potent against *B. subtilis* (Jagtap et al. 2012). Verma et al. (2012, 2013) confirmed the immunostimulatory role of *F. benghalensis* (prop-roots) and *L. leucocephala* (pod

seed) in *Clarias gariepinus* when supplemented in artificial feed. These authors evaluated the antibacterial activity of methanolic extracts of *F. benghalensis* (prop-root) by measuring the inhibition zone against the pathogenic bacteria *E. coli* and *A. hydrophila*. Moreover, juvenile *C. gariepinus* were fed a 5% powder of *F. benghalensis* with respective feeds for 20 days prior to the experiment. The immunomodulatory response conferred by the feed supplement was studied by challenging the fish intraperitoneally with *A. hydrophila* weekly. One set of fish, which were not challenged with *A. hydrophila*, was used as a negative control, to analyze any detrimental effect of the feed supplement, while the positive control comprised challenged fish fed with non-supplemented feed. Another two groups of fish were fed supplemented feeds and challenged with *A. hydrophila*. The fish fed supplemented feed showed increased lysozyme activity and a higher phagocytic index indicating an increased non-specific immune response (Verma et al. 2013). Moreover, serum lysozyme, tissue superoxide dismutase, percentage phagocytosis, phagocytotic index, nitric oxide (NO), total serum protein, and immunoglobulin increased significantly in the treated fish compared to the control fish (Verma et al. 2012).

Similar antibacterial activity was noted for the ethanolic extract from *F. benjamina* (Fig. 1). *F. benjamina* also of high medicinal potential. The plant is also used as antimicrobial, antinociceptive, antipyretic, hypotensive, and anti-dysentery remedy (Imran et al. 2014). Sirisha et al. (2010) suggest that the leaves, bark, and fruits of *F. benjamina* contain various bioactive constituents like cinnamic acid, lactose, naringenin, quercetin, caffeic acid, and stigmasterol. The fruit extract of *F. benjamina* exhibits both anti-tumor activity and significant antibacterial activities (Parveen et al. 2009). The medicinal importance of this plant encouraged us to carry out the antimicrobial study of the ethanolic extract from the leaves of *F. benjamina* plants against *A. hydrophila*. Imran et al. (2014) reveal that the extracts and fractions of stem, root, and leaves of *F. benjamina* exhibited considerable antimicrobial activity against four bacterial (*P. aeruginosa*, *E. coli* ATCC 25922, *B. subtilis* JS 2004, *B. cereus*) and two fungal strains (*Aspergillus niger* ATCC 10595, *C.*

albicans ATCC 32612). These researchers clearly demonstrate that all the butanol fractions exhibited strong inhibitory activity. The methanolic extract and n-butanolic fraction of the stem showed substantial activity. Moderate values were recorded for n-hexane, chloroform, and ethyl acetate (Imran et al. 2014). Parveen et al. (2009) report on the isolation and characterization of a new triterpene, (9,11),(18,19)-disecoolean-12-en-28-oic acid (1) along with β -amyryn (2). Compound 1 exhibited significant antimicrobial activity against *Salmonella typhimurium* (MTCC-98), *C. albicans* (IAO-109), *S. aureus* (IAO-SA-22), and *E. coli* (K-12) and low activity against *A. niger* (lab isolate ICAR) and *A. brassicola* (Parveen et al. 2009).

The ethanolic extracts from *F. lyrata* and *F. hispida* leaves inhibited the growth of *A. hydrophila* (Fig. 1B, 2B). Considerable *F. lyrata* antimicrobial activity is also reported in other similar studies. The ethyl acetate extract of *F. lyrata* latex comprises compounds with antibacterial and anticandidal properties that can be used as antimicrobial agents in new drugs to treat infectious diseases (Bidarigh et al. 2011). The methanolic extract had no effect against bacteria except for *Proteus mirabilis*, while the ethyl acetate extract inhibited the multiplication of five bacteria species (*E. faecalis*, *Citrobacter freundii*, *P. aeruginosa*, *E. coli*, and *Proteus mirabilis*) (Bidarigh et al. 2011). *F. lyrata* is reported to have numerous bioactive compounds such as arabinose, β -amyryns, β -carotenes, glycosides, β -setosterols, and xanthotoxol (Jeong and Lachance 2001, Vaya and Mahmood 2006). To identify the active substance responsible for the antibacterial activity, Rizvi et al. (2010) screened isolated pure compounds (FL-1–FL8) from *F. lyrata* for their inhibitory effect against the growth of various bacterial strains. They observed that only two compounds, i.e., ursolic acid (FL-1) and acacetin-7-O-neohesperidoside (FL-2), exhibited antibacterial activity. The Minimal Inhibitory Concentrations (MICs) were significantly lower against bacterial strains sensitive to the crude extract but also against *S. typhi*, *S. paratyphi* A, *S. typhimurium*, *Vibrio cholerae*, *E. coli*, *K. pneumoniae* and to *E. coli* and *K. pneumoniae* that produced extended-spectrum β -lactamases (ESBL). Thus, the range of activity against

Gram negative bacteria was greatly enhanced on testing with pure compounds (Rizvi et al. 2010). Ursolic acid was more potent than acacetin-7-O-neohesperidoside. Ursolic acid is a triterpenoid saponin from the ursan group, whereas Acacetin-7-O-neohesperidoside is a flavonoid glycoside (Rizvi et al. 2010).

Experimental evidence presented by Rizvi et al. (2010) suggests that ursolic acid from *F. lyrata* has excellent antibacterial activity against several problematic bacteria like MRSA and the ESBL-producing bacteria *Pseudomonas*, *Salmonella*, *Shigella*, and *Vibrio cholerae* and other known pathogens that are drug resistant. Ursolic acid and Acacetin-7-O-neohesperidoside contribute significantly to the antimicrobial activity of the crude extract of *F. lyrata* (Rizvi et al. 2010). Ahmad and Beg (2001) have revealed that glycosides and saponins extracted from leaves using alcohol had biological effects, but they had no effects on *C. albicans*, *S. aureus*, or *E. coli*.

F. hispida was chosen for its abundance of alkaloids, carbohydrates, proteins and amino acids, sterols, phenols, flavonoids, gums and mucilage, glycosides, saponins, and terpenes (Ghosh et al. 2004). The broad antibacterial activities of this extract, apparently, is explained by the plant's secondary metabolites. Sirisha et al. (2010) and Salem et al. (2013) report that the therapeutic properties of *Ficus* species could be attributed to the presence of a wide range of phytochemical compounds. In general, *Ficus* species are rich sources of polyphenolic compounds. In particular, flavonoids and isoflavonoids are responsible for the extract's strong antioxidant activity that could be useful in preventing diseases involving oxidative stress (Sirisha et al. 2010). A wide variety of bioactive compounds from different phytochemical groups like alkaloids, carbohydrates, proteins and amino acids, sterols, phenols, flavonoids, gums and mucilage, glycosides, saponins, and terpenes are thought to be key to the antimicrobial activity of *F. hispida* (Ali and Chaudhary 2011). Two substantial phenanthroindolizidine alkaloids, 6-O-methyltylophorinidine and 2-demethoxytylophorine, and a novel biphenylhexahydroindolizine hispidine from stem and leaves of *F. hispida* were isolated by Venkatachalam and Mulchandani (1982). Recently, hispidin has been reported to have anticancer activity (Ali

and Chaudhary 2011). All the phenolic acids detected are known to have antimicrobial and antioxidant properties (Jaafar et al. 2012). The antimicrobial properties of *F. hispida* extract could stem from its constituents.

The antibacterial activity of this extract is possibly linked to the presence of flavonoid compounds. The antibacterial activity of flavonoid compounds isolated from plant species are well documented (Hendrich 2006, Ferrazzano et al. 2011, Farzaei et al. 2013). The high amount of epicatechin found in *F. deltoidea* could be responsible for the strong radical scavenging activities found in the extract. A positive correlation was observed between the flavonoid constituents present and the radical scavenging activities of aqueous extracts of *F. deltoidea* (Dzolin et al. 2015). Chemical analysis found four phenolic compounds (chlorogenic, p-coumaric, ferulic and syringic acids) in the roots, three (chlorogenic p-coumaric and ferulic acids) in the stems, and only one (caffeic acid) in the leaves of *F. benjamina* (Imran et al. 2014). A glucoside, bengalenside, was isolated from *F. benghalensis* and evaluated for hypoglycemic activity (Garg and Paliwal 2011). The phytochemical screening of *F. benghalensis* revealed the presence of saponins, tannins, and flavonoids in aqueous and methanolic extracts (Aswar et al. 2008). Levels of total phenolic, total flavonol, and total flavonoid compounds in aerial roots in 70 mg per g of extract, 3 mg per g quercetin equivalent, and 5 mg quercetin equivalent per g extract have also been reported (Sharma et al. 2009). Some natural compounds, i.e., glucoside, 20-tetatriacontene-2-one, 6-heptatriacontene-10-one, pentariacontan-5-one, β -sitosterol- α -D-glucose, and meso-inositol, have been isolated from the bark (Subramanian and Misra 1978).

Kumar and Pandey (2013) suggest that antibacterial flavonoids might have multiple cellular targets rather than one specific site of action. Moreover, they also suggest that one of their molecular actions is to form complexes with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. Thus, their mode of antimicrobial action could be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth.

Lipophilic flavonoids could also disrupt microbial membranes (Kumar and Pandey 2013).

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 their environment. Antibiotics are used widely in fish farms as bacterial infection prophylaxis and as growth promoters. Despite widespread use, the use of this drug class in fish is unregulated (Rigos and Troisi 2005). Therefore, plant extracts can be more effective for preventive and therapeutical aims in organic aquaculture. In the present study, the ethanolic extracts obtained from various species of *Ficus* leaves showed varying inhibitory activities against *A. hydrophila*. Consequently, the screening results of this study confirm the possible use of medicinal plants, *Ficus* in particular, as a source of antimicrobial agents.

Conclusions

The results obtained highlight the interesting antimicrobial potency of various *Ficus* species against *A. hydrophila*, and provide a scientific basis for the traditional use of these species. The results demonstrate that various species of *Ficus* had intermediate antibacterial in vitro activity against *A. hydrophila*. The highest antibacterial activity against *A. hydrophila* was noted in *F. pumila*, *F. benghalensis*, *F. benjamina*, *F. deltoidea*, *F. hispida*, and *F. lyrata*. *Ficus* spp. leaves possess great medicinal potential for bacterial and fungal infection therapy and could be used as natural antiseptics and antimicrobial agents in veterinary medicine. Further studies should be conducted to verify this activity against other pathogenic bacteria occurring in aquaculture and to confirm immune response involvement and their potential as virulence factor inhibitors. These products can be used in aquaculture as therapeutic and prophylactic agents with antimicrobial properties against fish pathogens. The results of the present study permit concluding that the plant extracts studied could be potential sources of new antimicrobial drugs. Finally, further investigation is necessary to identify the

bioactive compounds that can be subjected to detailed pharmacological studies and the development of clinical applications.

Author contributions. H.T. and L.B. performed the experiment, analyzed the data, and wrote the manuscript, E.T.-M. performed disk diffusion method, Z.O. analyzed the data.

References

- Ahmad L., Beg A.Z. 2001 – Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multidrug resistant human pathogens – *J. Ethnopharmacol.* 74: 113-123.
- Ahmad S., Rao H., Akhtar M., Ahmad I., Hayat M.M., Iqbal Z., Nisar-ur-Rahman 2011 – Phytochemical composition and pharmacological prospectus of *Ficus bengalensis* Linn. (Moraceae) - A review – *J. Med. Plants Res.* 5: 6393-6400.
- Ali M., Chaudhary N. 2011 – *Ficus hispida* Linn.: A review of its pharmacognostic and ethnomedicinal properties – *Pharmacogn. Rev.* 5: 96-102.
- Ardo L., Yin G., Xu P., Varadi L., Szigeti G., Jeney Z., Jeney G. 2008 – Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron enhance the non-specific immune response of Nile tilapia (*Oreochromis niloticus*) and resistance against *Aeromonas hydrophila* – *Aquaculture* 275: 26-33.
- Arunachalam K., Parimelazhagan T. 2013 – Anti-inflammatory, wound healing and *in-vivo* antioxidant properties of the leaves of *Ficus amplissima* Smith – *J. Ethnopharmacol.* 145: 139-145.
- Aswar M., Aswar U., Watkar B., Vyas M., Wagh A., Gujar K.N. 2008 – Anthelmintic activity of *Ficus benghalensis* – *Int. J. Green Pharma* 2: 170-172.
- Austin B., Austin D.A. 2007 – Diseases of Farmed and Wild Fish. 4th ed. – Springer-Praxis Publishing, Chichester, United Kingdom.
- Bauer A.W., Kirby W.M., Sherris J.C., Turck M. 1966 – Antibiotic susceptibility testing by a standardized single disk method – *Am. J. Clin. Pathol.* 45: 493-496.
- Bidarigh S., Khoshkholgh Pahlaviani M.R.M., Massiha A., Issazadeh Kh. 2011 – *In vitro* anti-*Candida* activity of *Ficus lyrata* L. ethyl acetate latex extract and nystatin on clinical isolates and standard strains of *Candida albicans* – International Conference on Biotechnology and Environment Management, IPCBEE, 18: 115-119.
- Brummitt R.K., Powell C.E. 1992 – Authors of Plant Names – Kew, Royal Botanic Gardens.

- Cipriano R.C. 2001 – *Aeromonas hydrophila* and motile aeromonad septicemias of fish – U.S. Department of the Interior, Fish and Wildlife Service, Washington, DC.
- Cipriano R.C., Austin B. 2011 – Furunculosis and Other Aeromonad Diseases – In: Fish Diseases and Disorders Vol. 3: Viral, Bacterial and Fungal Infections, 2nd Edition (Eds) P.T.K. Woo, D.W. Bruno, CAB International, Oxfordshire, United Kingdom: 424-483.
- Dangarembizi R., Erlwanger K.H., Moyo D., Chivandi E. 2012 – Phytochemistry, pharmacology and ethnomedicinal uses of *Ficus thonningii* (Blume Moraceae): a review – Afr. J. Tradit. Complement. Altern. Med. 10: 203-212.
- Davis E.B., Schmidt D. 1996 – Guide to information sources in the botanical sciences. 2 ed. – Englewood, Colo, Libraries Unlimited. p. 188.
- Dzolin S., Ahmad R., Zain M.M., Ismail M.I. 2015 – Flavonoid distribution in four varieties of *Ficus deltoidea* (Jack) – J. Med. Plant Herb. Ther. Res. 3: 1-9.
- Farsi E., Ahmad M., Hor S.Y., Ahamed M.B., Yam M.F., Asmawi M.Z. 2014 – Standardized extract of *Ficus deltoidea* stimulates insulin secretion and blocks hepatic glucose production by regulating the expression of glucose-metabolic genes in streptozotocin-induced diabetic rats – BMC Complement. Altern. Med. 14: 220.
- Farzaei M.H., Abbasabadi Z., Ardekani M.R., Rahimi R., Farzaei F. 2013 – Parsley: a review of ethnopharmacology, phytochemistry and biological activities – J. Tradit. Chin. Med. 33: 815-826.
- Ferrazzano G.F., Amato I., Ingenito A., Zarrelli A., Pinto G., Pollio A. 2011 – Plant polyphenols and their anti-cariogenic properties: a review – Molecules 16: 1486-1507.
- Friedman M., Henika P.R., Mandrell R.E. 2002 – Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica* – J. Food Prot. 65: 1545-1560.
- Garg V.K., Paliwal S.K. 2011 – Wound-healing activity of ethanolic and aqueous extracts of *Ficus benghalensis* – J. Adv. Pharm. Technol. Res. 2: 110-114.
- Gayathri M., Kannabiran K. 2009 – Antimicrobial activity of *Hemidesmus indicus*, *Ficus bengalensis* and *Pterocarpus marsupium* Roxb – Indian J. Pharm. Sci. 71: 578-581.
- Ghosh R., Sharatchandra K.H., Rita S., Thokchom I.S. 2004 – Hypoglycemic activity of *Ficus hispida* (bark) in normal and diabetic albino rats – Indian J. Pharmacol. 36: 222-225.
- Giri S.S., Sen S.S., Chi C., Kim H.J., Yun S., Park S.C., Sukumaran V. 2015 – Effect of guava leaves on the growth performance and cytokine gene expression of *Labeo rohita* and its susceptibility to *Aeromonas hydrophila* infection – Fish Shellfish Immunol. 46: 217-224.
- Gul-e-Rana, Karim S., Khurhsid R., Saeed-ul-Hassan S., Tariq I., Sultana M., Rashid A.J., Shah S.H., Murtaza G. 2013 – Hypoglycemic activity of *Ficus racemosa* bark in combination with oral hypoglycemic drug in diabetic human – Acta Pol. Pharm. 70: 1045-1049.
- Harikrishnan R., Balasundaram C. 2005 – Antimicrobial activity of medicinal herbs *in vitro* against fish pathogen, *Aeromonas hydrophila* – Fish Pathol. 40: 187-189.
- Hendrich A.B. 2006 – Flavonoid-membrane interactions: possible consequences for biological effects of some polyphenolic compounds – Acta Pharmacol. Sin. 27: 27-40.
- Ilyanie Y., Wong T.W., Choo C.Y. 2011 – Evaluation of hypoglycemic activity and toxicity profiles of the leaves of *Ficus deltoidea* in rodents – J. Complement. Integr. Med. 8.
- Imran M., Rasool N., Rizwan K., Zubair M., Riaz M., Zia-Ul-Haq M., Rana U.A., Nafady A., Jaafar H.Z. 2014 – Chemical composition and Biological studies of *Ficus benjamina* – Chem. Cent. J. 8: 12.
- Jagtap S.G., Shelar R.S., Munot N.M., Ghante M.R., Sawant S.D. 2012 – Antimicrobial activity of *Ficus glomerata* Linn. bark – Int. Res. J. Pharm. 3: 281-284.
- Jeong W.S., Lachance P.A. 2001 – Phytosterols and fatty acids in fig (*Ficus lyrata* var. *mission*) fruit and tree components – Food Chem. Toxicol. 66: 278-281.
- Jones S.D., Wipff J.K., Montgomery P.M. 1997 – Vascular Plants of Texas: A Comprehensive Checklist Including Synonymy, Bibliography, and Index – University of Texas Press.
- Juan E., Rideout J., Ragasa C. 1997 – Bioactive furanocoumarin derivatives from *Ficus pumila* (Moraceae) – Philipp. J. Sci. 126:143-153.
- Jaafar H.Z., Ibrahim M.H., Karimi E. 2012 – Phenolics and flavonoids compounds, phenylalanine ammonia lyase and antioxidant activity responses to elevated CO₂ in *Labisia pumila* (Myrsinaceae) – Molecules 17: 6331-6347.
- Kumar S., Pandey A.K. 2013 – Chemistry and biological activities of flavonoids: an overview – Sci. World J. 2013: 162750.
- OIE 2000 – Diagnostic Manual for aquatic animal diseases – Fish Diseases Commission, Paris, France.
- Okoth D.A., Chenia H.Y., Koorbanally N.A. 2013 – Antibacterial and antioxidant activities of flavonoids from *Lannea alata* (Engl.) Engl. (Anacardiaceae) – Phytochem. Lett. 6: 476-481.
- Parveen M., Ghalib R.M., Mehdi S.H., Rehman S.Z., Ali M. 2009 – A new triterpenoid from the leaves of *Ficus benjamina* (var. *comosa*) – Nat. Prod. Res. 23: 729-736.
- Rigos G., Troisi G.M. 2005 – Antibacterial agents in Mediterranean finfish farming: A synopsis of drug pharmacokinetics in important euryhaline fish species and possible environmental implications – Rev. Fish Biol. Fish. 15: 53-73.

- Rizvi W., Rizvi M., Kumar R., Kumar A., Shukla I., Parveen M. 2010 – Antibacterial activity of *Ficus lyrata* – An *in vitro* study – Int. J. Pharmacol. 8: 7.
- Sahu S., Das B.K., Pradhan J., Mohapatra B.C., Mishra B.K., Sarangi N. 2007 – Effect of *Magnifera indica* kernel as a feed additive on immunity and resistance to *Aeromonas hydrophila* in *Labeo rohita* fingerlings – Fish Shellfish Immunol. 23: 109-118.
- Salem M.Z.M., Salem A.Z.M., Camacho L.M., Ali H.M. 2013 – Antimicrobial activities and phytochemical composition of extracts of *Ficus* species: An over view – Afr. J. Microbiol. Res. 7: 4207-4219.
- Sharma Madhuri, Mandloi A.K., Pandey Govind, Sahni Y.P. 2012 – Antimicrobial activity of some medicinal plants against fish pathogens – Int. Res. J. Pharm. 3: 28-30.
- Singh R.K., Watal G. 2010 – Antimicrobial potential of *Ficus bengalensis* aerial roots – Int. J. Pharm. Biol. Sci. 1: 1-9.
- Sirisha N., Sreenivasulu M., Sangeeta K., Chetty C.M. 2010 – Antioxidant properties of *Ficus* species, a review – Int. J. Pharm. Tech. Res. 4: 2174-2182.
- Subramanian P.M., Misra G.S. 1978 – Chemical constituents of *Ficus bengalensis* (part II) – Pol. J. Pharmacol. Pharm. 30: 559-562.
- Sukumaran V., Park S.C., Giri S.S. 2016 – Role of dietary ginger *Zingiber officinale* in improving growth performances and immune functions of *Labeo rohita* fingerlings – Fish Shellfish Immunol. 57: 362-370.
- Tkachenko H., Buyun L., Terech-Majewska E., Osadowski Z., Sosnovskiy Y., Honcharenko V., Prokopiv A. 2016 – The antimicrobial activity of some ethanolic extracts obtained from *Ficus* spp. leaves against *Aeromonas hydrophila* – Trudy VNIRO 162: 172-183.
- Tukmechi A., Ownagh A., Mohebbat A. 2010 – *In vitro* antibacterial activities of ethanol extract of Iranian propolis (EPIP) against fish pathogenic bacteria (*Aeromonas hydrophila*, *Yersinia ruckeri* and *Streptococcus iniae* – Braz. J. Microbiol. 41: 1086-1092.
- Vaya J., Mahmood S. 2006 – Flavonoid content in leaf extracts of the fig (*Ficus carica* L.), carob (*Ceratonia siliqua* L.) and pistachio (*Pistacia lentiscus* L.) – Biofactors 28: 169-175.
- Venkatachalam S.R., Mulchandani N.B. 1982 – Isolation of phenanthroindolizidinealkaloids and a novel biphenylhexahydroindolizine alkaloid from *Ficus hispida* – Naturwissenschaften 69: 287-288.
- Verma V.K., Rani K.V., Sehgal N., Prakash O. 2012 – Immunostimulatory response induced by supplementation of *Ficus benghalensis* root powder, in the artificial feed the Indian freshwater murrel, *Channa punctatus* – Fish Shellfish Immunol. 33: 590-596.
- Verma V.K., Rani K.V., Sehgal N., Prakash O. 2013 – Immunostimulatory effect of artificial feed supplemented with indigenous plants on *Clarias gariepinus* against *Aeromonas hydrophila* – Fish Shellfish Immunol. 35: 1924-1931.
- Zepeda-Velázquez A.P., Vega-Sánchez V., Salgado-Miranda C., Soriano-Vargas E. 2015 – Histopathological findings in farmed rainbow trout (*Oncorhynchus mykiss*) naturally infected with 3 different *Aeromonas* species – Can. J. Vet. Res. 79: 250-254.