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PRELIMINARY *IN VITRO* SCREENING OF ANTIBACTERIAL ACTIVITY OF LEAF EXTRACT FROM *FICUS NATALENSIS* SUBSP. *NATALENSIS* HOCHST. (MORACEAE) AGAINST FISH PATHOGENS

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The aim of this study was to screen the antibacterial efficacy of ethanolic extract obtained from the leaves of *Ficus natalensis* subsp. *natalensis* Hochst. against fish pathogens, *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri* and to evaluate the possible use of this plant in preventing infections caused by these bacteria in aquaculture. The bacterial growth inhibition tests were carried out using agar well diffusion method with the use of crude ethanolic extract. Muller-Hinton agar plates were inoculated with 200 and 400 µL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium. In our study, the *A. hydrophila* strain (200 and 400 µl of standardized inoculum) revealed intermediate susceptibility to an ethanolic extract (inhibition zone diameters ranged from 10.0 to 11.8 mm). *C. freundii* (200 and 400 µl) was resistant to ethanolic extract (7.5 and 11.7 mm). The extract exhibited intermediate antibacterial activity against *P. fluorescens* causing a zone of inhibition, comprising at least 10.2–12.8 mm for 200 µL and 7.4–11.2 mm for 400 µL of bacterium strain. *Y. ruckeri* (200 and 400 µl) revealed intermediate susceptibility (8.0 and 16.5 mm). This may be because the herbs have more than one extractable active compound that provides antimicrobial activity. Further chemical analysis of the aforementioned plant extract should be performed to determinate chemical composition and identify the exact phytocompounds responsible for the antimicrobial activity.

Keywords: *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri*, antimicrobial activity, disc diffusion technique, ethanolic extract

Introduction

Currently, outbreaks of parasitic, bacterial, and fungal diseases act as major limiting factors for fish farming, meaning that producers have to make use of massive amounts of antibiotics,

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disinfectants, and pesticides in order to control mortality and avoid huge economic losses (Valladão et al., 2015). In this context, there is an urgent need for the development of alternative therapies against bacterial pathogens in aquaculture production. Consequently, several alternatives to the use of antibiotics have been applied successfully in aquaculture (Romero et al., 2012). The use of probiotics or beneficial bacteria, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment (Balcázar et al., 2006). Other sources of alternative treatment are essential oils and plant-derived extracts, which have been used *in vivo* as antibacterial agents to control bacterial infections (Cowan, 1999). There is documented evidence for the development of resistance of fish pathogens to antimicrobial agents (McPhearson et al., 1991; Founou et al., 2016; Gabriel et al., 2017). After the emergence of multi-drug resistant pathogens in aquaculture, the research for new remedy alternatives has led to the recognition of the potential of plant extracts for treating the infections associated to fish pathogens (McPhearson et al., 1991). These compounds may constitute alternative prophylactic and therapeutic agents in aquaculture because of their antibacterial properties (Turker and Yıldırım, 2015). Moreover, large numbers of plants have been proven to be rich sources of cheaper immune-enhancing and growth promoter substances, with a wide therapeutic and preventive spectrum of activity, potentially useful in solving the multiple health problems that characterize aquaculture (Dügenci et al., 2003; De Vico et al., 2018).

In our previous studies, therapeutic potential for the use of various plants of *Ficus* L. genus in the control of bacterial diseases was evaluated against fish pathogens in *in vitro* study with promising results (Tkachenko et al., 2016-2018). Antibacterial properties of plant extracts have been by far the most studied bioactivity with potential application in aquaculture systems (Reverter et al., 2014). Castro and co-workers (2008) have revealed by agar diffusion assay that 31 methanolic extracts of Brazilian plants presented antibacterial activity against the fish pathogenic bacteria, i.e. *Streptococcus agalactiae*, *Flavobacterium columnare*, and *A. hydrophila*. *F. columnare* being the most susceptible microorganism to most of the tested extracts. Similarly, Wei and Musa (2008) also studied the susceptibility by assay of minimum inhibitory concentration (MIC) of two Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*), four Gram-negative bacteria (*C. freundii*, *Escherichia coli*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*) and 18 isolates of Edwardsiella tarda to aqueous extract of garlic (500, 250, 125, 62.5 $mg.mL^{-1}$), and found that all garlic extracts were effective against the tested pathogenic bacteria.

The angiosperm family Moraceae Gaudich., or mulberry family, is a diverse group of nearly 1100 species, predominantly woody and with milky latex in all parts of their body, which are distributed throughout the tropics and subtropics and rarely extend to the temperate zone. They are represented by a variety of growth forms, such as terrestrial and hemi-epiphytic trees, shrubs, lianas, subshrubs, and herbs, with small unisexual flowers assembled into various, often peculiar inflorescences (Datwyler and Weiblen, 2004; Clement and Weiblen, 2009). The pantropical genus *Ficus*, with its approximately 750 species, is the largest within the family and one of the most speciose genera of flowering plants. Among all Moraceae, it is characterized by the presence of waxy glands on vegetative organs, heterostyly, and prolonged protogyny, that is the anthesis of staminate flowers in already mature fruits. These

features are functionally linked to the unique pollination mode in *Ficus* involving mutualistic relationships with agaonid wasps (order *Hymenoptera*). The closed urceolate inflorescences provide a shelter for the development of wasps, which, in turn, are the only pollinators of these plants ensuring their reproductive propagation (Cook and Rasplus, 2003; Berg and Corner, 2005). *Ficus* trees have a number of uses in various industries and fields of human activity. The reported biological activities of *Ficus* plants include antioxidant (Mohan et al., 2015), antiplasmodial (Muregi et al., 2003), anticancer (Mbosso et al., 2016a), antimicrobial (Awolola et al., 2017), antiulcer (Galati et al., 2001), antidiarrhoeal (Mandal and Kumar, 2002), anti-pyretic (Rao et al., 2002), and gastroprotective (Rao et al., 2008) properties. Virtually all parts of their body are utilized in ethnomedicine to cure disorders of digestive and respiratory systems, skin diseases, parasitic infections, etc. Some species have been cited to have analgesic, tonic, and ecbolic effects (Lansky and Paavilainen, 2011).

Ficus natalensis subsp. *natalensis* Hochst. is a monoecious evergreen tree up to 30 m tall or shrub, hemi-epiphytic or terrestrial, which naturally occurs in southern and eastern Africa. The leaves are 2.5–10.0 cm long and 1–5 cm across (sub)coriaceous and glabrous, elliptic to oblong or obovate, with plane margin and acuminate to the rounded apex. Figs are born in the leaf axils or just below the leaves, pedunculate, globose to ellipsoid to obovoid, 1.5–2.0 cm in diameter, glabrous, at maturity reddish, orange or yellowish to brown (Berg and Wiebes, 1992).

Figure 1 Leaves of *Ficus natalensis* subsp. *natalensis* Hochst.

Therefore, the aim of this study was to assess the antibacterial efficacy of ethanolic extract obtained from the leaves of *Ficus natalensis* subsp. *natalensis* against fish pathogens, *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri* and to evaluate the possible use of this plant in preventing infections caused by these bacteria in aquaculture.

The current investigation was conducted as a part of ongoing project between Institute of Biology and Environmental Protection (Pomeranian University in Slupsk, Poland), M.M. Gryshko National Botanic Gardens of National Academy of Sciences of Ukraine (Kyiv,

Ukraine), and Ivan Franko Lviv National University (Lviv, Ukraine) undertaken in the frame of cooperation program aimed at assessment of medicinal properties of tropical plants, cultivated *in vitro*.

Material and methodology

Collection of plant material and preparing plant extract

The leaves of *F. natalensis* subsp. *natalensis* were sampled at National Botanic Garden, National Academy of Science of Ukraine (Kyiv, Ukraine) and Botanic Garden of Ivan Franko Lviv National University (Lviv, Ukraine). The sampled leaves of *F. natalensis* subsp. *natalensis* were brought into the laboratory for antimicrobial studies. Freshly crushed leaves were washed, weighed, and homogenized in 96% ethanol (in proportion 1 : 10) at room temperature, and centrifuged at 3.000 g for 5 minutes. Supernatants were stored at -20 °C in bottles protected with the laminated paper until required.

Method of culturing pathological sample and identification method of the bacteria

Aeromonas hydrophila (strain E 2/7/15) and *Pseudomonas fluorescens* (strain E 1/7/15) isolated locally from internal organs of rainbow trout (*Oncorhynchus mykiss* Walbaum) with clinical features of furunculosis (kidneys were grey, the liver was pale and fragile, enlarged spleen with exudate in the body cavity), as well as *Citrobacter freundii* isolated locally from gill of eel (*Anguilla anguilla* L.) with clinical features of disease were used as test organisms. Samples of internal organs (kidneys, spleen, liver) weighing 2 g were taken and homogenized before preincubation in TSB broth (Trypticase Soy Broth, Oxoid®) for 24 hrs. After preincubation, bacterial culture was transferred to two different cultivation media: TSA (Trypticase Soy Agar, Oxoid®) and BHIA (Brain Heart Infusion Agar, Oxoid®) supplemented with 5% of sheep blood (OIE Fish Diseases Commission, 2000). After 48 hrs of incubation at 27 °C, characteristic pink colonies were selected for further examination.

The isolates of *Y. ruckeri* were collected from clinically healthy fish and fish with clinical symptoms of yersiniosis. Internal tissues (predominantly pronephros and gills), as well as 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.

Preliminary characterization of isolates

Bacterial species were identified with the use of the oxidase test and API E test kit (BioMérieux, France). The results of the test were interpreted in accordance with the manufacturer‘s protocol, after 24 hrs of incubation at 27 °C. Codes + + V-V--- + V + + + --- + -VV + in API E test were identified as *A. hydrophila*. The strain was obtained from Diagnostics Laboratory of Fish and Crayfish Diseases, Department of Veterinary Hygiene, Provincial Veterinary Inspectorate in Olsztyn (Poland).

For characterization of *Y. ruckeri* isolates, bacteria were Gram-stained and then morphologically evaluated. The 24h bacterial culture was wet-mounted and a microscopic smear on the slide was prepared. Following fixation over the flame, the slide was Gram-stained with a Gram color set (Merck) according to the manufacturer's instructions. The shape of the bacteria was determined by observing the microorganisms under a light microscope at 1000x with immersion oil (Kocwowa, 1981; Whitman and MacNair, 2004). Motility was examined on a wet mount. A drop of distilled water was put on a coverslip and bacteria were mounted on it with drops of distilled water put on the corners of a slip. The slip was then covered with a special microscopic slide with an indentation and the whole set was vigorously turned. The motility of the bacteria was evaluated under a light microscope at 400× (Kocwowa, 1981; Whitman and MacNair, 2004).

Oxidase test was performed according to the manufacturer's instruction (Merck). Biochemical properties of individual *Y. ruckeri* isolates were investigated with the API 20E system (BioMérieux, France). Tests were performed according to the manufacturer's instructions. The results, namely, the presence or a lack of reaction, were read based on the key featured in the operating procedure provided by the manufacturer of the assay. The results were analyzed with the Apiweb software (BioMérieux, France) to identify the investigated bacterium.

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

Strains tested were plated on TSA medium (Tryptone Soya Agar) and incubated for 24 hr at 25 °C. Subsequently, the microorganisms were suspended in sterile PBS and the turbidity adjusted equivalent to that of a 0.5 McFarland standard. The disc diffusion assay (Kirby-Bauer Method) was used to screen for antibacterial activity (Bauer et al., 1966). Muller-Hinton agar plates were inoculated with 200 and 400 μL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium and spread with sterile swabs.

Sterile filter paper discs impregnated by extract were applied over each of the culture plates, 15 min after bacteria suspension was placed. The antimicrobial susceptibility testing was done on Muller-Hinton agar by disc diffusion method (Kirby-Bauer disk diffusion susceptibility test protocol). A negative control disc impregnated by sterile ethanol was used in each experiment. The sensitivity of strain was also studied to the commercial preparation with extracts of garlic (in dilution 1 : 10, 1 : 100 and 1 : 1000). After culturing bacteria on Mueller-Hinton agar, the disks were placed on the same plates and incubated for 24 hrs at 25 °C. The diameters of the inhibition zones were measured in millimetres and compared with those of the control and standard susceptibility disks. Activity was evidenced by the presence of a zone of inhibition surrounding the well.

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 tested for normal distribution using the Kolmogorov-Smirnov test (p >0.05). In order to find significant differences (significance level, p <0.05) between groups, the Kruskal-Wallis test by ranks was applied to the data (Zar, 1999). All statistical analyses were performed using Statistica 8.0 software (StatSoft, Poland). The

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

Results and discussion

Data on antimicrobial activities of ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* against *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri* isolated from fish expressed as mean of diameters of inhibition zone are presented in Figs 2–6. The mean inhibition zone diameter induced by 96% ethanol was (8.4 ±0.6) mm. The ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* increased the zone of *Aeromonas hydrophila* growth inhibition to (11.5 ±0.9) mm (200 μL of standardized inoculum) and (11.3 ±1.2) mm (400 μL), respectively. The extract also increased the inhibition zone for *Citrobacter freundii* growth – (9.6 ±0.95) mm for 200 μL of standardized inoculum and (10.7 ±0.98) mm for 400 μL of standardized inoculum. Similarly, the growth of *Pseudomonas fluorescens* was inhibited by application of the ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis*. The inhibition zone was (11.1 ±1.5) mm for 200 μL of standardized inoculum and (9.8 ±1.1) mm for 400 μL of standardized inoculum. The highest degree of growth inhibition zone (14.4 ±1.4) mm was observed for 200 μL of *Yersinia ruckeri* isolated (increased by 71%, p <0.05, compared to control sample), while 400 μL of standardized inoculum was resistant to an ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* (Figure 2).

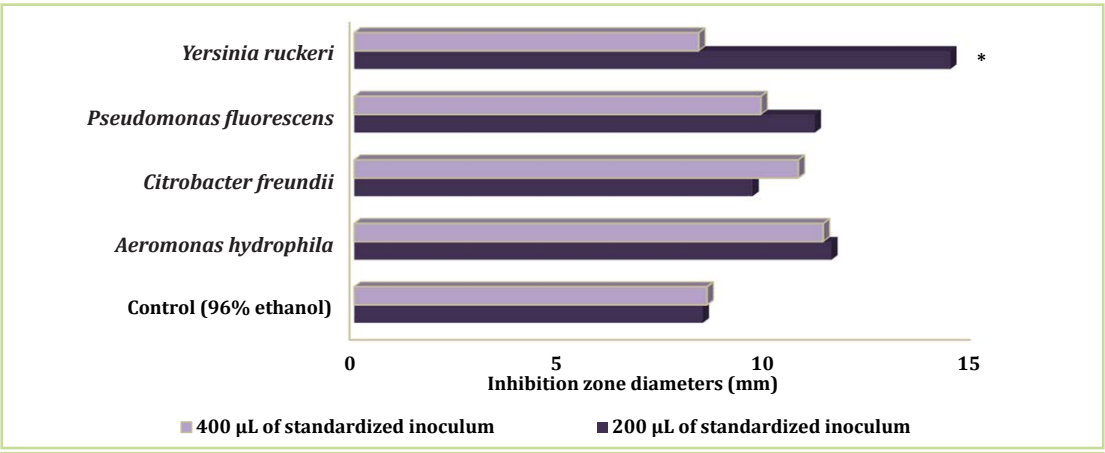

Figure 2 The mean inhibition zone diameters induced by ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* against *Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri*. Muller-Hinton agar plates were inoculated with 200 and 400 μL of standardized inoculum (10^8 CFU.mL^{-1}) of bacteria
* changes were statistically significant (p <0.05) compared to control sample (96% ethanol)

In our study, the *A. hydrophila* strain (200 and 400 μl of standardized inoculum) revealed intermediate susceptibility to an ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* (inhibition zone diameters ranged from 10.0 to 11.8 mm) (Figures 2, 3).

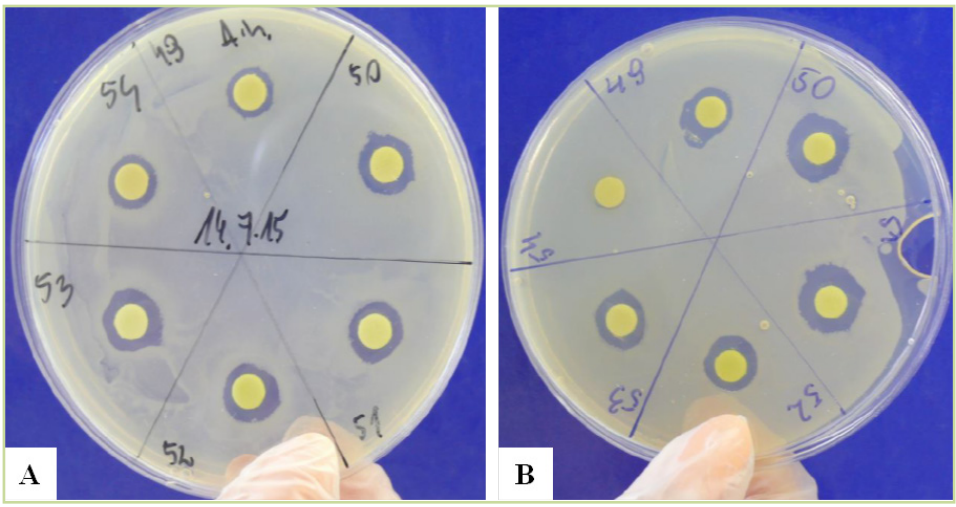

Figure 3 Antimicrobial activity of ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* (52) against *Aeromonas hydrophila*. Muller-Hinton agar plates were inoculated with 200 (A) and 400 µL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium (B)

Our results also demonstrated that the *C. freundii* (200 and 400 µl of standardized inoculum) was resistant to an ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* (inhibition zone diameters ranged between 7.5 and 11.7 mm) (Figure 4).

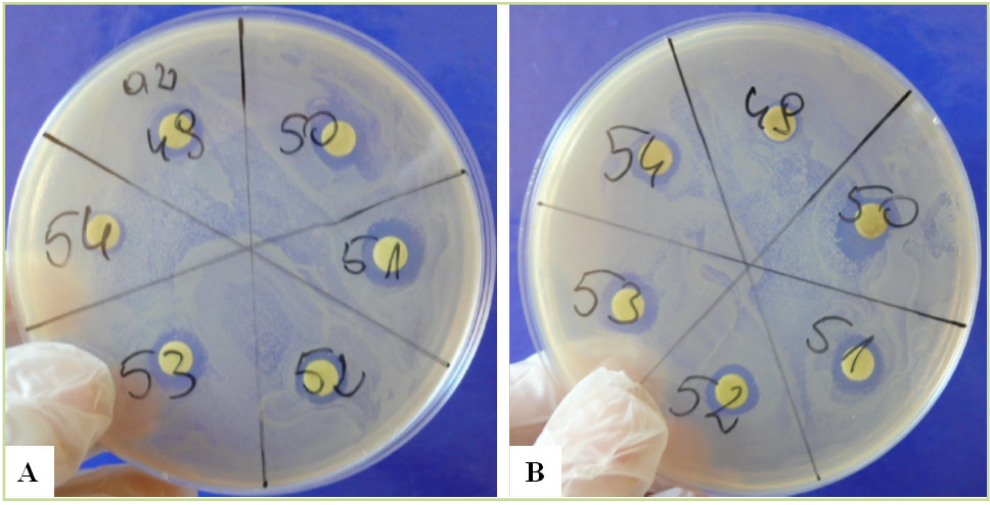

Figure 4 Antimicrobial activity of ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* (52) against *Citrobacter freundii*. Muller-Hinton agar plates were inoculated with 200 (A) and 400 µL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium (B)

The ethanolic extract derived from *F. natalensis* subsp. *natalensis* leaves exhibited intermediate antibacterial activity against *Pseudomonas fluorescens* causing a zone of

inhibition, comprising at least 10.2–12.8 mm for 200 μL and 7.4–11.2 mm for 400 μL of standardized inoculum (10^8 CFU.mL^{-1}) of bacterium strain (Figures 2, 5).

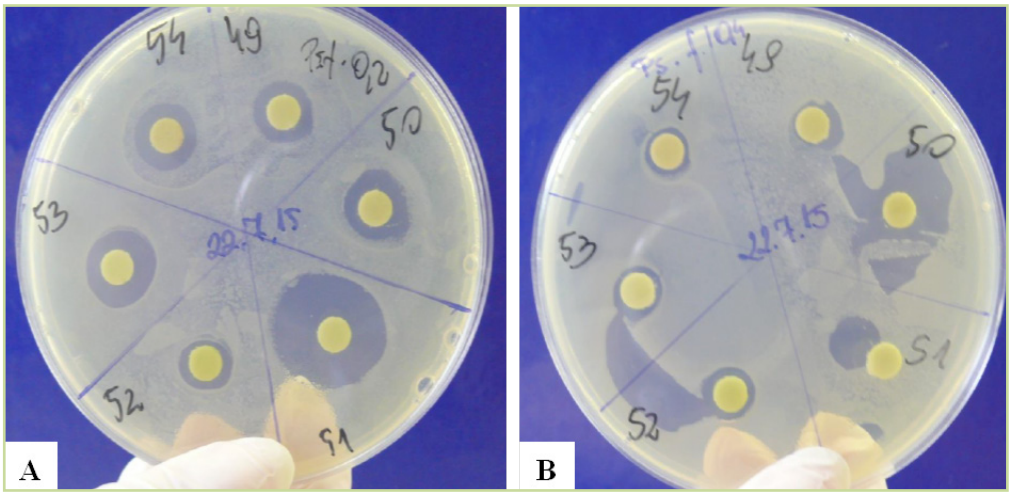

Figure 5 Antimicrobial activity of ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* (52) against *Pseudomonas fluorescens*. Muller-Hinton agar plates were inoculated with 200 (A) and 400 μL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium (B)

Y. ruckeri (200 and 400 μl of standardized inoculum) revealed intermediate susceptibility to an ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* (inhibition zone diameters were ranged between 8.0 and 16.5 mm) (Figure 6). Moreover, statistically significant increase (by 71%, p <0.05) of inhibition zone diameters induced by ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* compared to control (96% ethanol) was observed.

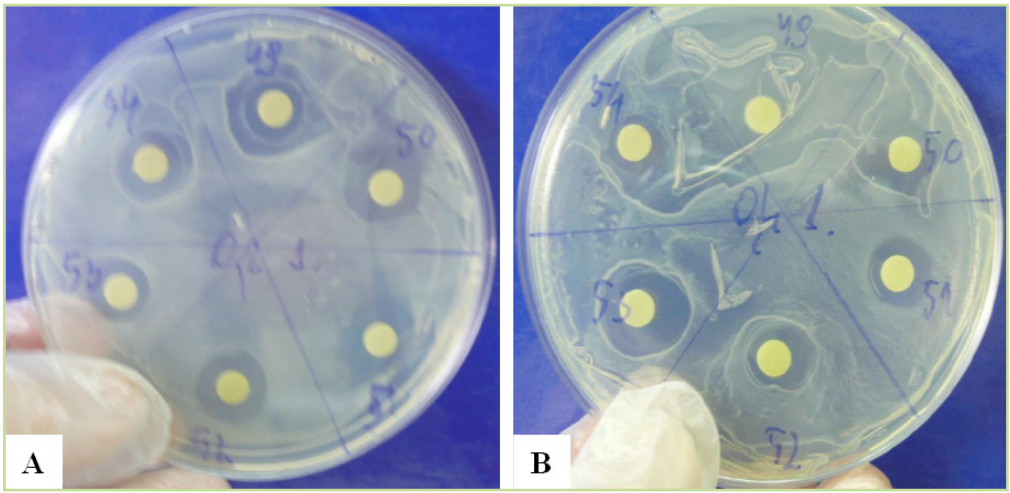

Figure 6 Antimicrobial activity of ethanolic extracts obtained from leaves of *F. natalensis* subsp. *natalensis* (52) against *Yersinia ruckeri*. Muller-Hinton agar plates were inoculated with 200 (A) and 400 μL of standardized inoculum (10^8 CFU.mL^{-1}) of the bacterium (B)

This investigation is in line with our previous works which have revealed a great potential of *Ficus* species as plants with potent antimicrobial properties. In our previous study, the *in vitro* antimicrobial activity of the ethanolic leaf extracts of various *Ficus* species against *Citrobacter freundii* was evaluated. The results proved that the extracts derived from *F. drupacea*, *F. septica*, *F. deltoidea* as well as *F. hispida*, *F. mucuso*, *F. pumila*, *F. craterostoma* leaves exhibit a favorable antibacterial activity against *C. freundii* (200 μL of standardized inoculum) (Tkachenko et al. 2016b). Our results also proved that the ethanolic extracts obtained from *F. pumila*, *F. binnendijkii* 'Amstel Gold', *F. carica*, *F. erecta*, *F. hispida*, *F. mucuso*, *F. palmeri*, *F. religiosa* lan eaves possess considerably sufficient antibacterial potential against *C. freundii* (Tkachenko et al., 2017c). Among various species of *Ficus* plants screened ethanolic extracts of the leaves of ten *Ficus* species: *F. hispida*, *F. binnendijkii*, *F. pumila*, *F. rubiginosa*, *F. erecta*, *F. erecta* var. *sieboldii*, *F. sur*, *F. benjamina*, *F. craterostoma*, *F. lyrata*, *F. palmeri* (the species are listed in the order of effectiveness against pathogen tested) were the most effective against *P. fluorescens* (200 μL of standardized inoculum) (Tkachenko et al., 2016a). Moreover, previous investigation has shown that the most effective against *P. fluorescens* (400 μL of standardized inoculum) were the ethanolic extracts obtained from leaves of ten *Ficus* species: *F. craterostoma*, *F. cyathistipula*, *F. drupacea* 'Black Velvet', *F. hispida*, *F. macrophylla*, *F. mucuso*, *F. pumila*, *F. villosa* (Tkachenko et al., 2016e). In our study, most ethanolic extracts obtained from *Ficus* spp. proved effective against the bacterial strain of Gram-negative *A. hydrophila* tested, with 10–12 mm zones of inhibition being observed. Interestingly, *A. hydrophila* demonstrated the highest susceptibility to *F. pumila* leaf extract. The highest antibacterial activity against *A. hydrophila* (200 μL of standardized inoculum) was displayed by *F. benghalensis*, *F. benjamina*, *F. deltoidea*, *F. hispida*, *F. lyrata* leaf extracts (Tkachenko et al., 2016c,d,f). Among various species of *Ficus* genus exhibiting moderate activity against *A. hydrophila* (400 μL of standardized inoculum), the highest antibacterial activity was displayed by *F. benghalensis*, *F. benjamina*, *F. deltoidea*, *F. hispida*, *F. lyrata* leaf extracts (Tkachenko et al., 2016c,d,f).

Our results also demonstrated that the *C. freundii* revealed intermediate susceptibility to *F. hispida* (inhibition zone diameters ranged between 11 and 15 mm). *A. hydrophila* revealed intermediate susceptibility concerning to ethanolic extract obtained from leaves of *F. hispida* (inhibition zone diameters were ranged from 8 to 12 mm). The most effective at least causing a zone of inhibition within 14–16 mm was an ethanolic extract from *F. hispida* leaves against *P. fluorescens* both in 200 μL of standardized inoculum of the bacterium (inhibition zone ranged from 15 to 16 mm in diameter) and 400 μL (14–15 mm) (Tkachenko et al., 2017d). Our results also indicated that extract obtained from *F. mucuso* offer a promising alternative to the use of antibiotics in controlling of infection caused by *A. hydrophila*, *C. freundii*, *P. fluorescens*, *Y. ruckeri*. In our study, ethanolic extracts obtained from *F. mucuso* proved effective against bacteria tested, with 10–15 mm zones of inhibition being observed. *F. mucuso* demonstrated the highest antibacterial activity against *C. freundii* and *P. fluorescens*. Among various bacteria tested, the highest susceptibility for 400 μL of standardized inoculum of *C. freundii* and *P. fluorescens* was noted (Tkachenko et al., 2017e). Moreover, the *A. hydrophila* (200 and 400 μl of standardized inoculum) revealed intermediate susceptibility to an ethanolic extract obtained from leaves of *F. benghalensis* (inhibition zone diameters ranged from 8 to 12 mm). The *C. freundii* strain (200 and 400 μl of standardized inoculum) displayed mild susceptibility to an ethanolic

extract obtained from leaves of *F. benghalensis* (inhibition zone ranged between 8 and 10 mm in diameter). The ethanolic extract derived from *F. benghalensis* leaves exhibited the highest antibacterial activity against *Pseudomonas fluorescens* causing a zone of inhibition, comprising at least 8–14 mm, both in 200 µL and 400 µL of standardized inoculum (10^8 CFU.mL^{-1}) of bacterium strain. *Y. ruckeri* isolate (200 and 400 µl of standardized inoculum) revealed good susceptibility to an ethanolic extract obtained from leaves of *F. benghalensis* (inhibition zone diameters ranged between 8 and 15 mm) (Tkachenko et al., 2017b). The antimicrobial activity profile of ethanolic extract obtained from leaves of *F. pumila* against the tested pathogen strains indicated that *Y. ruckeri* was the most susceptible bacterium (200 and 400 µl of standardized inoculum) among all the bacterial test strains. Similarly, *P. fluorescens* was found to be a sensitive strain (13–14 mm for 200 µL and 9–10 mm for 400 µL of standardized inoculum of bacterium strain) although *A. hydrophila* and *C. freundii* was found to be least susceptible to an ethanolic extract obtained from leaves of *F. pumila*. Of all the bacterial strains included in the test, *Y. ruckeri* (200 and 400 µl of standardized inoculum dilution) and *P. fluorescens* (200 µl) were found to be the most susceptible and *C. freundii*, which is an isolate from gills of eel, was found to be the least inhibited bacterium (Tkachenko et al., 2018).

Ajaib et al. (2016) also have concluded that methanolic extract of *F. natalensis* bark was potentially a promising candidate to be used as a natural source of antifungal medicine. The antimicrobial potential assessment was carried out by using four bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa* (Gram-negative), *Staphylococcus aureus* and *Bacillus subtilis* (Gram-positive) and two fungal strains *Aspergillus niger* and *Aspergillus oryzae*. The maximum antibacterial activity against *S. aureus* was produced by petroleum ether extract of leaves and bark with a zone of inhibition of 50 ±0.51 mm and 55.7 ±1.15 mm, respectively. The chloroform leaves extract also revealed an inhibition zone of 50 ±2 mm against *S. aureus*. Similarly, the petroleum ether extract of leaves showed significant activity with an inhibition zone of 40 ±0.4 mm against *B. subtilis*. The petroleum ether extract of leaves showed an inhibition zone of 44.7 ±0.57 mm against *E. coli*. The petroleum ether extract of bark and distilled water extract of leaves also exhibited good inhibition of 30 ±0.57 mm and 30 ±0.26 mm respectively against *E. coli*. The results of antibacterial activity evaluation against *P. aeruginosa* were reported by the petroleum ether extract of bark (47 ±0.4 mm). Chloroform and methanol extracts of bark and petroleum ether and methanolic extracts of leaves gave promising results. The MIC value of the methanol extracts of bark and leaves at concentration 1.25 mg.mL^{-1} and 5 mg.mL^{-1} against *E. coli* showed less effective, whereas methanolic extracts of bark against *P. aeruginosa* inhibited the growth up to concentration of 0.625 mg.mL^{-1} but the leaves extract of methanol against *P. aeruginosa* lost its activity beyond at concentration of 5 mg.mL^{-1}. The methanolic extracts of bark and leaves reduced the growth of *S. aureus* up to 0.625 mg.mL^{-1} and *B. subtilis* was grown only below the concentration of 0.625 mg.mL^{-1}. Assessment of antifungal activity revealed that *A. niger* was inhibited by all the extracts with variable potency. The methanol extract of bark was found to be most potent against *A. niger* with a zone of inhibition 43.7 ±1.527 mm. The petroleum ether extract of leaves was least effective against fungal strain (9 ±0 mm). The petroleum ether extract of bark showed a considerably high activity with a zone of inhibition 37 ±0.577 mm. All extracts showed a moderate level of inhibition against *A. oryzae* between 23 ±2.645 mm to 34.8 ±1.607 mm. The estimation of MIC also supported

the activity of methanol extract of bark. The methanol extract of bark showed inhibition of *A. niger* and *A. oryzae* up to the concentration of 0.625 mg.mL^{-1}. The methanol extract of leaves showed low inhibition towards both fungal strains (10 mg.mL^{-1} against *A. niger* and 5 mg.mL^{-1} against *A. oryzae*). *A. niger* was more susceptible to the extracts of the plant, whereas *A. oryzae*, was somewhat resistant to the tested extracts (Ajaib et al., 2016).

The crude stem bark and fruit extracts of *F. natalensis* subsp. *natalensis* were tested for their antibacterial activity against five Gram-negative and seven Gram-positive strains and for their potential anti-biofilm activity was evaluated by Awolola et al. (2017). As the result, the dichloromethane-soluble fruit extract was active against sensitive and resistant *Staphylococcus aureus* strains, *Enterococcus faecalis*, and *Staphylococcus xylosus*. In the anti-biofilm assay, exposure to ethyl acetate, methanol and aqueous methanol leaf, stem bark and fruit extracts decreased adhesion with a biofilm reduction of ≥100% for all three tested organisms: *E. coli*, *P. aeruginosa*, and *S. aureus*. The methanol leaf extract demonstrated the most potent anti-adhesion potential against *E. coli* (218% biofilm reduction) (Awolola et al., 2017).

Consequently, the antimicrobial property of *F. benghalensis* leaf extract may be manifested due to its constituents. As was shown, the phytochemical screening of the leaf, stem bark and fruit extracts of *F. natalensis* subsp. *natalensis* (Awolola et al., 2017) detected the presence of four triterpenoids, ergosta-4,6,8(14),22-tetraene-3-one (1), stigma-4-ene-3-one (2), 3β-hydroxy-21β-H-hop-22(29)-ene (3), sitosterol and a quinone, tectoquinone (4). These compounds (2, 3 and 4) demonstrated broad-spectrum antibiotic effects against eight of the twelve bacterial strains tested in a study by Awolola et al. (2017). The isolated compounds exhibited strain-specific anti-adhesion potential, with biofilm reduction against *P. aeruginosa*, but not *E. coli* or *S. aureus* (Awolola et al., 2017). The finding that quinone in terpenoids is an essential substructure for anti-Gram-positive-bacteria activity has been reported previously (Saruul et al., 2015). Menaquinone is a component of electron transport chains in a majority of anaerobic bacteria and Gram-positive bacteria. Due to its exclusivity in bacteria, menaquinone is thought to be a potential target for the development of therapeutically effective antibacterial agents without side effects (Paudel et al., 2016).

Conclusions

An *in vitro* test for antibacterial activity revealed that 96% ethanolic leaf extract of *F. natalensis* subsp. *natalensis* used in this study was able to inhibit the growth of fish pathogens (*Aeromonas hydrophila*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *Yersinia ruckeri*). In our study, the *A. hydrophila* strain (200 and 400 µl of standardized inoculum) displayed intermediate susceptibility to an ethanolic extract obtained from leaves of *F. natalensis* subsp. *natalensis* (inhibition zone diameters ranged from 10 to 11.8 mm). *C. freundii* (200 and 400 µl of standardized inoculum) was resistant to ethanolic extract (inhibition zone diameters ranged between 7.5 and 11.7 mm). The ethanolic extract derived from *F. natalensis* subsp. *natalensis* leaves exhibited intermediate antibacterial activity against *Pseudomonas fluorescens* causing a zone of inhibition, comprising at least 10.2–12.8 mm for 200 µL and 7.4–11.2 mm for 400 µL of standardized inoculum (10^8 CFU.mL^{-1}) of bacterium strain. *Y. ruckeri* (200 and 400 µl of standardized inoculum) revealed intermediate susceptibility to an ethanolic extract obtained

from leaves of *F. natalensis* subsp. *natalensis* (inhibition zone diameters were ranged between 8.0 and 16.5 mm). This may be because the herbs have more than one extractable active compound that provides antimicrobial activity. Overall, our findings provide support that further chemical analysis of the aforementioned plant extracts should be performed to determinate their chemical composition and identify the exact phytocompounds responsible for the antimicrobial activity. In addition, they should be subjected to pharmacological evaluations with the aim of assessing theirs *in vivo* efficacy, toxicity, potential adverse effects, interactions, and contraindications.

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