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IN VITRO EVALUATION OF OXIDATIVE STRESS BIOMARKERS IN THE MUSCLE TISSUE OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM) EXPOSED TO LEAF EXTRACT OF *FICUS BENJAMINA* L. AND ITS CULTIVARS

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The aim of this study was to evaluate the *in vitro* effect of extracts obtained from leaves of *Ficus benjamina* L. and its cultivars on the oxidative stress biomarkers (carbonyl content of the oxidatively modified proteins, total antioxidant capacity) in the muscle tissue of the rainbow trout. The leaves of *F. benjamina* and its cultivars, i.e. *F. benjamina* 'Safari', 'Baroque', 'Amstel Gold', 'Reginald' were sampled for our study. Our results showed that extracts obtained from leaves of *F. benjamina* 'Safari' and *F. benjamina* 'Reginald' decreased non-significantly the lipid peroxidation biomarker in the muscle tissue. Extracts obtained from leaves of *F. benjamina* and its cultivars decreased the ketonic derivatives of oxidatively modified proteins in the muscle tissue. Our results showed that extracts obtained from leaves of *F. benjamina* and its cultivars increased efficiently the total antioxidant capacity in muscle tissue by 76.9% (*F. benjamina*), 66.9% (*F. benjamina* 'Safari'), 70.5% (*F. benjamina* 'Baroque'), 49.4% (*F. benjamina* 'Amstel Gold'), and 42.8% (*F. benjamina* 'Reginald') (p <0.05). The results of this study provide a new perspective on the use of various *Ficus* species as a medicinal plant to improve the antioxidant response of rainbow trout. Further studies including the use of other medicinal plants as food additives in aquaculture, the assessment of their antioxidant effects on various tissues of salmonids are in progress.

Keywords: *Ficus benjamina* L., rainbow trout (*Oncorhynchus mykiss* Walbaum), muscle tissue, lipid peroxidation, oxidatively modified proteins, total antioxidant capacity

Introduction

Herbs are currently used in commercial aquaculture as growth-promoting substances, antimicrobial agents, nutrients as well as many other applications. Their potential to prevent

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and control fish diseases is also being studied (Galina et al., 2009). The use of immunostimulants, as dietary supplements, can improve the innate defence of animals providing resistance to pathogens during periods of high stress, such as grading, reproduction, sea transfer and vaccination (Bricknell and Dalmo, 2005). During the functioning of the immune system, such as in phagocytosis, reactive oxygen and nitrogen species are generated. If they are left unchecked they can affect the components of the immune system by inducing oxidative damage. Natural compounds from medicinal plants having antioxidant and immunomodulatory activities have potential as therapeutic agents in this regard (Devasagayam and Sainis, 2002).

Many studies have proved that plant-derived additives enhanced the growth of fishes and protected from diseases. The non-specific immune system of fish is considered to be the first line of defence against invading pathogens (Ahilan et al., 2010).

In this study, attention was focused on *Ficus* L., a genus with diverse ethnobotanical uses in its geographical distribution range. The genus has occupied an important place among plant genera applied for treatment of a broad spectrum of diseases and disorders. Along with being an object of extreme interest for researchers during the last two centuries, *Ficus* has a long history of use by humans as a food source, in medicine, planting, and other industries and fields of human activity, partly owing to its great diversity and wide distribution range. Among popular ethnomedicinal uses of *Ficus* are treatments of skin damages, disorders of the digestive system and related organs, and parasitic infections. Besides these, the range of healing targets for particular *Ficus* species compiled from local medicines can be competitive with that of broad-spectrum traditional remedies (Lansky and Paavilainen, 2011).

Ficus benjamina L. (Moraceae Gaudich.) is a multipurpose tree found in a large area, including India, southern China, Southeast Asia, Malaysia, the Philippines, northern Australia, and the islands of the South Pacific. It grows as a large evergreen shrub, up to 8 m tall, with nearly 10 m wide-spreading crown and drooping shoots with young slender twigs (Imran et al., 2014). The plant is well known due to its medicinal potential. Its latex and some fruit extracts are used by indigenous communities to treat skin disorders, inflammation, piles, vomiting, leprosy, malaria, nose-diseases, and cancer besides the use as a general tonic. The plant is also used as an antimicrobial, antinociceptive, antipyretic, hypotensive and anti-dysentery remedy. The leaves and twigs are used as an insect repellant (Imran et al., 2014). The leaves, bark, and fruits of *F. benjamina* contain various bioactive constituents like cinnamic acid, lactose, naringenin, quercetin, caffeic acid and stigmasterol (Sirisha et al., 2010). The ability of plant extracts to inhibit the activity of bacteria having potential interest as fish pathogens have been well documented. Nevertheless, although antimicrobial activities of extracts obtained from the leaves of various species of *Ficus* genus were investigated (Solomon-Wisdom et al., 2011; Olusesan et al., 2010; Namita and Mukesh, 2012; Tkachenko et al., 2016–2018), studies regarding their antioxidant properties *in vitro* model with the muscle tissue of the rainbow trout (*Oncorhynchus mykiss* Walbaum) have not been undertaken. To estimate oxidative stress using animal models, many markers of oxidative stress are used. One of the oldest but still widely used assays for the determination of oxidative stress in serum is the TBARS (thiobarbituric acid reactive substances) assay (Dasgupta, Klein, 2014). TBARS are a common way to measure lipid peroxidation products in cells, tissues, and body fluids. TBARS is probably

the oldest and one of the most widely used assays for measuring lipid peroxidation end product malondialdehyde, a reactive aldehyde produced by lipid peroxidation of polyunsaturated fatty acids (Marrocco et al., 2017).

Therefore, the purpose of this study was to evaluate the *in vitro* effect of extracts obtained from leaves of *Ficus benjamina* and its cultivars on the oxidative stress biomarkers (carbonyl content of the oxidatively modified proteins, total antioxidant capacity) in the muscle tissue of the rainbow trout.

Our current scientific project undertaken in the frame of cooperation programme 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) directed to assessment of medicinal properties of tropical plants.

Figure 1 The growth habit of *Ficus benjamina* L. (A) and twigs with syconia (B)

Material and methodology

Collection of plant material

The leaves of plants, cultivated under glasshouse conditions, were sampled at M.M. Gryshko National Botanic Garden (NBG), National Academy of Science of Ukraine. Specifically, the leaves of *F. benjamina* and its cultivars, i.e. *F. benjamina* 'Safari', 'Baroque', 'Amstel Gold', 'Reginald' were sampled for our study.

Preparation of plant extracts

Freshly collected leaves were washed, weighted, crushed, and homogenized in 0.1 M phosphate buffer (pH 7.4) (in proportion 1 : 19, w/w) at room temperature. The extracts were then filtered and investigated. The extract was stored at -20 °C until use.

Experimental fish

Clinically healthy rainbow trout (*Oncorhynchus mykiss* Walbaum) with a mean body mass of 80–120 g were used in the experiments. The study was carried out in a Department of Salmonid Research, Inland Fisheries Institute (Rutki, Poland). The experiments were performed in water at 14.5 ±0.5 °C and pH 7.2–7.4. The dissolved oxygen level was about 9 ppm with additional oxygen supply, with a water flow of 25 $L.min^{-1}$, and a photoperiod of 12 h per day. The same experimental conditions were used during the whole research. The water parameters were maintained under constant surveillance. The fish were held in square tanks (150 fish per tank) and fed commercial pelleted diet.

Muscle tissue samples

The trout muscle tissue samples were homogenized in ice-cold buffer (100 mM Tris-HCl, pH 7.2). The minced muscle tissue was rinsed clear of blood with cold isolation buffer and homogenized in a homogenizer H500 with a motor-driven pestle on ice. Homogenates were centrifuged at 3,000 g for 15 min at 4 °C. After centrifugation, the supernatant was collected and frozen at -20 °C until analyzed. Protein contents were determined with the method described by Bradford (1976) with bovine serum albumin as a standard. Absorbance was recorded at 595 nm. All enzymatic assays were carried out at 22 ±0.5 °C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) (n = 8). The enzymatic reactions were started by adding the tissue supernatant.

Experimental design

The supernatant of the muscle tissue was used to incubate with extracts obtained from leaves of *F. benjamina* and its cultivars (in a ratio 19 : 1) at room temperature. The control group (trout muscle tissue) was incubated with 100 mM Tris-HCl buffer (pH 7.2) (in a ratio 19 : 1). The incubation time was 2 hours. Oxidative stress biomarkers were studied in the incubated homogenate (control group and in samples with extracts obtained from leaves of *F. benjamina* and its cultivars).

The 2-thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation was evaluated by the production of 2-thiobarbituric acid-reactive substances (TBARS). An aliquot of the homogenate was used to determine the lipid peroxidation status of the sample by measuring the concentration of 2-thiobarbituric-acid-reacting substances (TBARS), according to the method of Kamyshnikov (2004). Reaction mixture contained sample homogenate (2.1 mL, 10% w/v) in tris-HCl buffer (100 mM, pH 7.2), 2-thiobarbituric acid (TBA; 0.8%, 1.0 mL), and trichloracetic acid (TCA; 20%, 1.0 mL). The total volume was kept in a water bath at 100 °C for 10 min. After cooling, the mixture was centrifuged at 3,000 g for 10 min. The absorbance of the supernatant was measured at 540 nm. TBARS values were reported as nmoles malonic dialdehyde (MDA) per mg protein.

Carbonyl groups of the oxidatively modified proteins assay

Carbonyl groups were measured as an indication of oxidative damage to proteins according to the method of Levine and co-workers (1990) in the modification of Dubinina and

co-workers (1998). Samples were incubated at room temperature for 1 h with 10 mM 2,4-dinitrophenylhydrazine (DNTP) in 2M HCl. Blanks were run without DNTP. Afterwards, proteins were precipitated with 20% TCA and centrifuged for 20 min at 3,000 g. The protein pellet was washed three times with ethanol: ethyl acetate (1 : 1) and incubated at 37 °C until complete resuspension. The carbonyl content was measured spectrophotometrically at 370 nm (aldehydic derivatives, OMP_{370}) and at 430 nm (ketonic derivatives, OMP_{430}) (molar extinction coefficient 22,000 $M^{-1} \cdot cm^{-1}$) and expressed as nmol per mg protein.

Total antioxidant capacity (TAC) assay

The TAC level in the sample was estimated by measuring the 2-thiobarbituric acid reactive substances (TBARS) level after Tween 80 oxidation. This level was determined spectrophotometrically at 532 nm (Galaktionova et al., 1998). Sample inhibits the Fe^{2+}/ascorbate-induced oxidation of Tween 80, resulting in a decrease in the TBARS level. Briefly, 0.1 mL of sample was added to 2 mL of 1% Tween 80 reagent, 0.2 mL of 1 mM $FeSO_4$, and 0.2 mL of 10 mM ascorbic acid. In the blank assay, 0.1 mL of distilled water was used instead of the sample. The mixture was incubated for 48 hrs at 37 °C. After cooling, 1 mL of 20% trichloroacetic acid was added. The mixture was centrifuged at 3000 g for 10 min. After centrifugation, 1 mL of supernatant and 2 mL of 0.25% 2-thiobarbituric acid were mixed. The mixture was heated in a water bath at 95 °C for 15 min. The absorbance of the obtained solution was measured at 532 nm. The absorbance of the blank was defined as 100%. The level of TAC in the sample (%) was calculated with respect to the absorbance of the blank sample.

Statistical analysis

The mean ± S.E.M. values were calculated for each group to determine the significance of the intergroup difference. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test (p >0.05). The significance of differences between the total antioxidant capacity level (significance level, p <0.05) was examined using the Mann-Whitney U test (Zar, 1999). All statistical calculation was performed on separate data from each individual with Statistica 8.0 software (StatSoft, Krakow, Poland).

Results and discussion

In a present study, we have studied the influence of extracts derived from leaves of *F. benjamina* and its cultivars, grown under glasshouse conditions, on the lipid peroxidation measured by the quantity of TBARS level in the muscle tissue of rainbow trout after incubation with extracts *in vitro*. As presented in Figure 2, our results showed that extracts obtained from leaves of *F. benjamina* 'Safari' and *F. benjamina* 'Reginald' decreased non-significantly the TBARS level in muscle tissue by 21.4% and by 9% (p >0.05), respectively. On the other hand, extracts obtained from leaves of *F. benjamina* 'Baroque' and 'Amstel Gold' increased non-significantly the TBARS level in muscle tissue by 11.6% and by 10.4% (p >0.05), respectively (Figure 2).

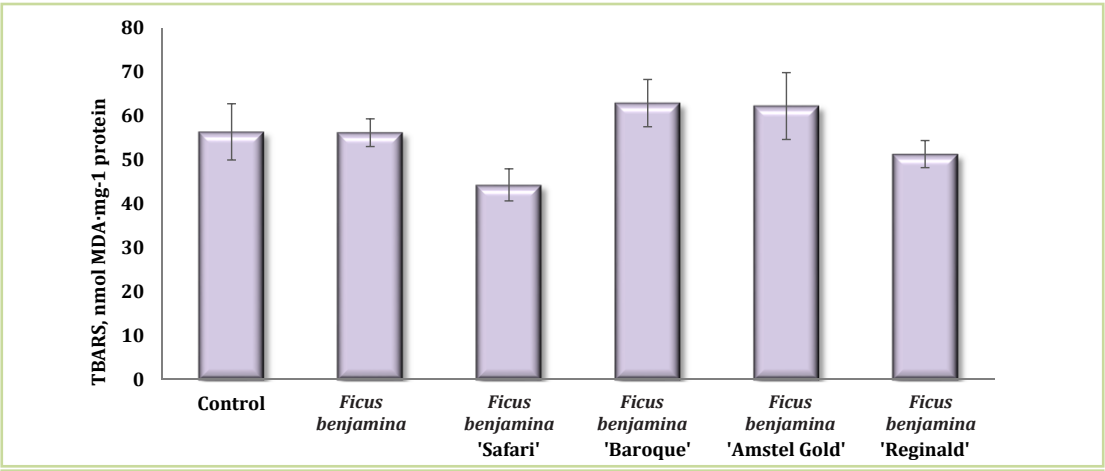

Figure 2 Lipid peroxidation measured by the quantity of TBARS level (nmol MDA·mg^{-1} protein) in the muscle tissue of rainbow trout after incubation with buffer extracts obtained from leaves of *Ficus benjamina* and its cultivars (M ±m, *n* = 8)

Our results also showed that extracts obtained from leaves of *F. benjamina* and its cultivars decreased the ketonic derivatives of oxidatively modified proteins in the muscle tissue by 5.1% (*F. benjamina*), 5.6% (*F. benjamina* 'Safari'), 13.1% (*F. benjamina* 'Baroque'), 5.9% (*F. benjamina* 'Amstel Gold'), and 1.8% (*F. benjamina* 'Reginald'). This decrease was non-statistically significant (p >0.05). Aldehydic derivatives content was ranged on the control group level (Figure 3).

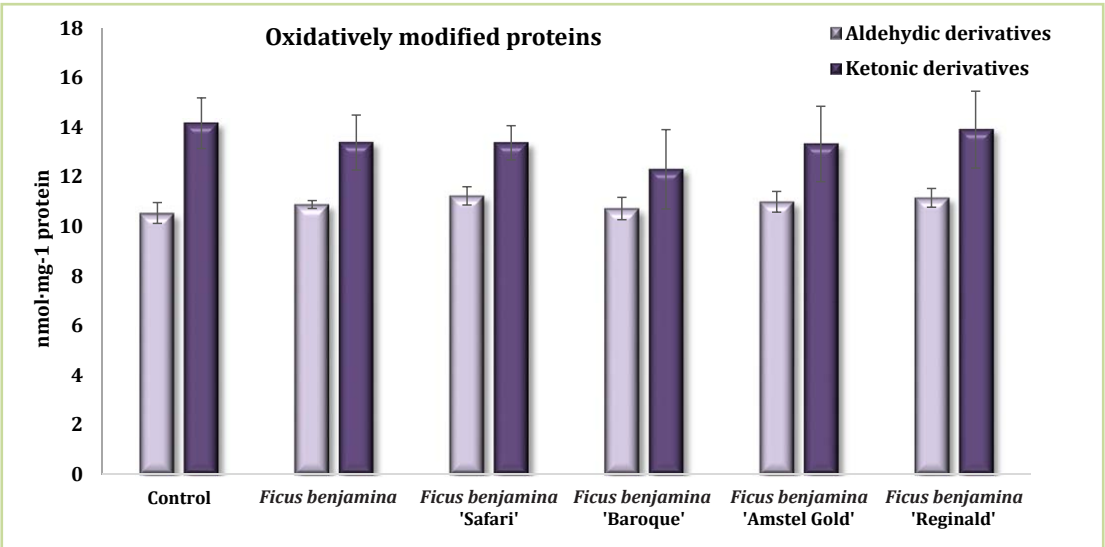

Figure 3 Level of the aldehydic and ketonic derivatives (nmol·mg^{-1} protein) in the muscle tissue of rainbow trout after incubation with buffer extracts obtained from leaves of *Ficus benjamina* and its cultivars (*M* ±m, n = 8)

In a present study, we also have investigated the influence of extracts derived from leaves of *F. benjamina* and its cultivars on the total antioxidant capacity in the muscle tissue of rainbow trout after incubation with extracts *in vitro*. Our results showed that extracts obtained from leaves of *F. benjamina* and its cultivars increased efficiently the TAC level in muscle tissue by 76.9% (*F. benjamina*), 66.9% (*F. benjamina* 'Safari'), 70.5% (*F. benjamina* 'Baroque'), 49.4% (*F. benjamina* 'Amstel Gold'), and 42.8% (*F. benjamina* 'Reginald') (p <0.05) (Figure 4).

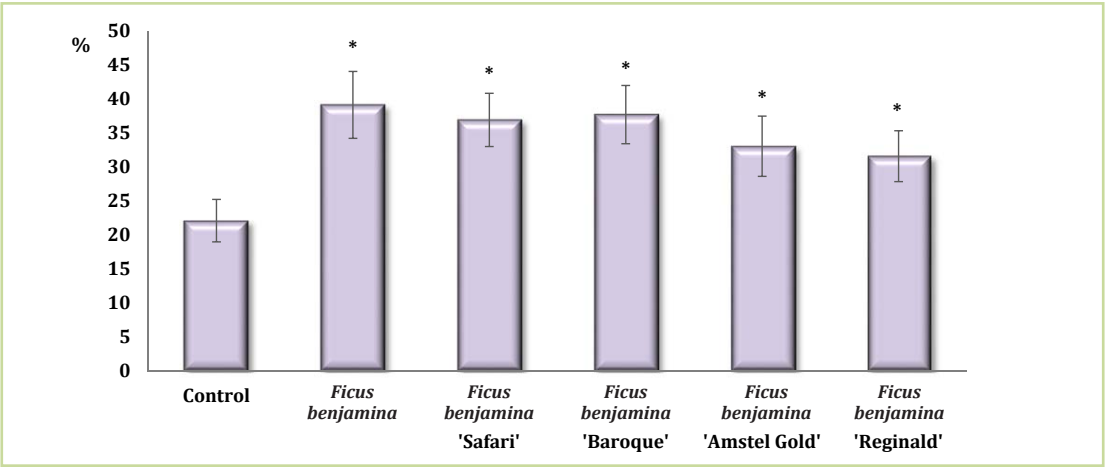

Figure 4 The total antioxidant capacity in the muscle tissue of rainbow trout after incubation with buffer extracts obtained from leaves of *Ficus benjamina* and its cultivars (M ±m, n = 8).* – the changes are statistically significant (p <0.05) compared to the control group

Therefore, the results suggested that the extracts screened could be a potential source of natural antioxidants. Supplementation of extracts obtained from leaves of *F. benjamina* and its cultivars caused to increase of antioxidant responses in muscle tissue of trout. It would be reasonable to suggest that these antioxidant effects are determined by their by-products, i.e. flavonoids. Indeed, the results of Imran et al. (2014) indicated that *F. benjamina* is a good source of antioxidants with high reducing power. *F. benjamina* disclosed substantial bioactivity, and this plant can be regarded as a potential source of antioxidant agents. The root and leaves showed good antioxidant activity, whereas stem extract and fractions revealed good antimicrobial activity. *F. benjamina* disclosed substantial bioactivity, being root extract and fractions the most active. This plant can be regarded as a potential source of antioxidant and antimicrobial agents. 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 fish pathogens was evaluated (Tkachenko et al., 2016–2017).

Imran et al. (2014) in their study revealed that the methanol extract and n-butanol fraction showed greater percent inhibition of linoleic acid system, compared to other fractions. The percent inhibition in the linoleic acid system for stem was in the range of 16.94–78.16, in root 20.57–85.87 and leaves 26.82–69.81%. The maximum percent inhibition was determined

by methanol extract (85.87) and butanol fraction (81.48) of the root. The results of Imran et al. (2014) experiments revealed that the antioxidant potential of plant increased linearly with the increase in concentration. The methanol extract as well as fractions of root exhibited a linear rise in absorbance value for various concentrations 0.56 nm: 2.5 ($mg.mL^{-1}$), 0.87 nm: 5 ($mg.mL^{-1}$), 1.03 nm: 7.5 ($mg.mL^{-1}$) and 1.49 nm: 10 ($mg.mL^{-1}$). The presence of phenolic compounds might be the reason for reducing power. The results of this assay indicated that the plant is a good source of antioxidants with high reducing power. Methanol extracts of stem, root, and leaves exhibited IC_{50} values of 50.1, 58.81 and 49.86 $\mu g.mL^{-1}$, respectively. The maximum value of IC_{50} was demonstrated by root's fraction of n-hexane (580.75 $\mu g.mL^{-1}$), indicating that this fraction showed minimum free radical scavenging activity. Unlike the n-hexane fraction, chloroform and ethyl acetate fractions exhibited lower values of IC_{50}. The methanol extract and *n*-butanolic fractions showed maximum free radical scavenging activity. The *n*-hexane fractions of root revealed the maximum value of IC_{50} (580.75 $\mu g.mL^{-1}$). Methanol and n-butanol fractions exhibited the lowest IC_{50} values, showing a maximum value (158.34 $\mu g.mL^{-1}$) for root (Imran et al., 2014).

Literature reports indicate that the power of bioactive compounds is associated with the antioxidant activity of *F. benjamina* (Mousa et al., 1994; Parveen et al., 2009; Ogunwande et al., 2012; Yarmolinsky et al., 2012; Imran et al., 2014). Essential oils obtained by hydrodistillation of leaves of *F. benjamina* were analyzed for their constituents by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) by Ogunwande et al. (2012). The leaf oil of *F. benjamina*, collected during the day, contained high contents of alpha-pinene (13.9%), abietadiene (9.7%), cis-alpha-bisabolene (8.2%) and germacrene-D-4-ol (8.4%), while the night sample was dominated by germacrene-D-4-ol (31.5%), 1,10-di-epi-cubenol (8.8%) and hexahydro farnesyl acetone (8.3%). This could be a possible indication of differences in emissions of volatiles by *F. benjamina* during the day and night (Ogunwande et al., 2012). Imran et al. (2014) showed that the HPLC analysis for the presence of phenolic acids permitted the identification of 5 phenolic acids, three in the stem, four in root and one in leaves. *F. benjamina* fruit extracts also showed antitumor and antibacterial activity (Mousa et al., 1994), while aqueous and alcoholic leaf extracts had significant antinociceptive activity (reducing sensitivity to painful stimuli) in analgesia test (Parveen et al., 2009). The fruit extracts of *F. sycomorus*, *F. benjamina*, *F. benghalensis*, and *F. religiosa* were screened for bioactivity. *F. bengalensis* and *F. religiosa* demonstrated activity in the brine shrimp test (*Artemia salina*) which indicates toxicity, whereas *F. sycomorus* and *F. benjamina* showed no activity. All the fruit extracts exhibited antitumor activity in the potato disc bioassay. None of the tested extracts showed any marked inhibition of the uptake of calcium into rat pituitary cells GH4C1. The extracts of the four tested *Ficus* species had significant antibacterial activity, but no antifungal activity (Mousa et al., 1994). The aqueous and alcoholic extracts of leaves of F. bejamina showed significant antinociceptive activity in an analgesiometer test (Parveen et al., 2009). The presence of polyphenols in *F. benjamina* glandular epithelium has been reported (Pennisi et al., 1999). A new triterpene, named serrate-3-one, along with phytoconstituents pentacontanyl decanoate, friedelin and beta-sitosterol have been detected in *F. benjamina* (var. *comosa*) benzenoid extracts (Parveen et al., 2009). All flavone glycosides quercetin 3-O-rutinoside (1), kaempferol 3-O-rutinoside (2) and kaempferol 3-O-robinobioside (3)

from *F. benjamina* showed high antiviral activity against Herpes Simplex Virus 1 and 2 (HSV-1 and HSV-2) without any significant activity against Varicella Zoster Virus (VZV) in the study of Yarmolinsky et al. (2012). Kaempferol 3-O-robinobioside showed the highest antiherpetic activity, similar to that of acyclovir (ACV). The highest antiviral activity of all these flavone glycosides was obtained when the infected cells were treated during and after infection (Yarmolinsky et al., 2012).

Flavonoids and phenolic acids may be responsible for antioxidant activities of *Ficus benjamina* and its cultivars in the muscle tissue of rainbow trout after *in vitro* incubation. Indeed, flavonoids act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and light screening. Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce the free radical formation and to scavenge free radicals (Pietta, 2000). Mechanisms of antioxidant action can include (1) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in a free radical generation; (2) scavenging ROS; and (3) upregulation or protection of antioxidant defences (Kumar and Pandey, 2013). Flavonoids inhibit the enzymes involved in ROS generation, that is, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, and so forth (Brown et al., 1998).

Antioxidant activity usually means the ability of a compound to delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and reducing oxidative stress (Bhanwase and Alagawadi, 2016). Therefore, the antioxidant containing in *F. benjamina* leaf extracts may offer resistance to rainbow trout against the oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation and by many other mechanisms and thus prevents diseases by elevating the specific immune response.

Conclusions

Present study ascertained the antioxidant potency of the extracts obtained from leaves of *F. benjamina* and its cultivars as a potential source of natural antioxidants. *Ficus benjamina* disclosed substantial bioactivity, and this plant can be regarded as a potential source of antioxidant agents. Thus, the results of this study provide a new perspective on the use of various *Ficus* species as a medicinal plant to improve the antioxidant response of rainbow trout. Further studies including the use of other medicinal plants as food additives in aquaculture, the assessment of their antioxidant effects on various tissues of salmonids are in progress.

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