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DETERMINATION OF THE ANTIBACTERIAL EFFECTS OF CAFFEIC ACID ON FISH PATHOGENS

Fatih TAPAN*, Sebahattin ERGÜN, Sevdan YILMAZ

Çanakkale Onsekiz Mart University, Faculty of Marine Sciences and Technology, Aquaculture Department, 17020-Çanakkale, Türkiye

Fatih TAPAN: fatihtapan99@gmail.com, https://orcid.org/0000-0002-4861-2546
Sebahattin ERGÜN: sergun@comu.edu.tr, https://orcid.org/0000-0003-3685-0691
Sevdan YILMAZ: sevdanyilmaz@comu.edu.tr, https://orcid.org/0000-0002-4809-5809

**Corresponding author: Fatih TAPAN, fatihtapan99@gmail.com, +90-538-6036039*

Abstract

The use of phenolic (organic) acids as an alternative to antibiotic agents in aquaculture prevents the multiplication of pathogenic bacteria and demonstrates the ability of fish to cure animals by promoting the growth of beneficial bacteria in the intestinal flora. It is thought that the use of organic acids as an additive and antibacterial agent in aquaculture will increase in the future. In this study, the antibacterial effect of the caffeic acid, one of the organic acids, on *Yersinia ruckeri* E42, *Listonella anguillarum* SY-L24, *Streptococcus iniae* ATCC 2917, *Edwardsiella tarda* SY-ED14, *Citrobacter* sp. SY-C10 bacteria was investigated by using the Disc Diffusion (DISC) and Minimum Inhibitory Concentration (MIC) methods. Our results showed that caffeic acid had antimicrobial activity against all five bacteria, but it was the most effective on *E. tarda* SY-ED14, among the investigated fish bacteria pathogens. According to the results of this research, it is suggested that caffeic acid can be used as an antimicrobial agent against fish bacterial pathogens in aquaculture facilities. However, further studies are necessary to confirm caffeic acid's therapeutic efficacy *in vivo* against These studied pathogenic bacteria.

Keywords: Caffeic Acid, Bacterial Fish Pathogen, Antibacterial Activity, Minimum Inhibitory Concentration (MIC), Disk Diffusion (DISC)

Introduction

World aquaculture suffers a rapid increase in fish diseases recently. It is also known that many different factors such as feed additives and stock density may accelerate the increase in fish diseases. Consequently, epidemic diseases are seen more and more. For the treatment of diseases, synthetic materials such as antibiotics have been used for a long time causing the

accumulation of antibiotics in the body and deterioration of the water quality. As the use of antibiotics increases, concerns arise for reaching safe food products. Therefore, prohibitions and/or restrictions are imposed on the use of synthetic materials.

Harmful bacteria in aquaculture can cause deaths and serious economic losses in aquaculture facilities. For this reason, various natural feed additives have been studied and their effects on fish have been investigated in order to keep a strong immune system.

Organic acids prevent spoilage caused by microorganisms such as yeast, mold and bacteria which are widely used as a preservative in feeds (Ricke, 2003).
It is known that antimicrobial compounds in medicinal plants are used as a defense mechanism against microbial pathogens. The medicinal plant family that has contributed to the largest number of antimicrobial drugs in the pharmacology industry is the secondary metabolites such as alkaloids, phenolics and other compounds. Safer, biodegradable, plant-derived compounds offer a promising solution to the problem of resistant microbes (Perumal & Gopalakrishnakone, 2010).

Caffeic acid (Figure 1) is an organic compound that is classified as a hydroxycinnamic acid. It is known as 3,4-hydroxy cinnamic acid and one of the phenolic acids found in some plants, fruits and vegetables. The most known source of caffeic acid is coffee and is a separate substance from caffeine (Higdon & Frei, 2006). It lowers uric acid, serum creatinine and urea in the blood and causes an increase in total albumin and protein values. Short-chain phenolic acids, their salts or mixtures, which are derivatives of phenolic compounds, are widely used and are reported to be seen as an alternative to antibiotics (Yılmaz & Hunt, 2017).

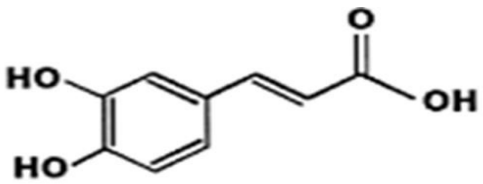

Figure 1. Caffeic acid (Akyol et al., 2011)

The antimicrobial and bacteriostatic effect of phenolic acids, including caffeic acid, is due to their ability to pass through the semipermeable membrane of bacteria and decompose in the cytoplasm to neutralize pH (Cherrington et al., 1991; Booth & Stratford, 2003). Accumulation of excess protons in the cell lowers the cytoplasm pH, thus inhibiting the pyruvate decarboxylase enzyme, which is used as metabolic energy, and inhibition of certain cell enzymes, thereby preventing bacterial cell metabolism. If the cytoplasmic pH falls below the physiological optimal range, cell death occurs (Smigic et al., 2009).

The antimicrobial effect of feeding fish with feed containing organic acids and salts in their feces and intestines was first reported by Ng et al (2009). The antimicrobial effect of organic acids and their salts on harmful microorganisms on the intestine, *Oreochromis sp.* (Tilapia) (Koh et al., 2016), *Paralichthys olivaceus* (flounder) (Park et al., 2011), *L. vannamei* (pacific white shrimp) (Da Silva et al. 2016) also stated that. It has been determined that changes in intestinal flora and composition have significant effects on animal growth, nutrient use, immune response, and resistance to pathogens (Chuchird et al., 2015). Yilmaz (2019) reported that in Nile tilapia fed caffeic acid supplemented feed for sixty days, it may be sufficient to improve fish immune parameters, antioxidant status and survival rate against *Aeromonas veronii*, similar

to antibiotic treatment. The findings of this research are a very interesting result showing that caffeic acid may also be effective against fish pathogens. For this purpose, the antibacterial effects of caffeic acid on the common fish pathogens *Yersinia ruckeri* E42, *Listonella anguillarum* SY-L24, *Streptococcus iniae* ATCC 2917, *Edwardsiella tarda* SY-ED14, *Citrobacter sp*. SY-C10 were investigated in vitro.

Material and Method

Pathogen Bacteria Material and Caffeic Acid

Pathogenic bacteria were obtained from the disease laboratory of Çanakkale Onsekiz Mart University, Faculty of Marine Sciences and Technology, Department of Aquaculture. Pathogenic bacteria *Y. ruckeri* E42, *L. Anguillarum* SY-L24, *S. iniae* ATCC 2917, *E. tarda* SY-ED14, *Citrobacter sp.,* SY-C10, which are commonly encountered in fish, were used (Table 1).

Table 1. Fish pathogenic bacteria used in in vitro experiments

Bacteria	Description	Culture Media	(°C)
Citrobacter sp., SY-C10	Pathogen	MH,TS	28
E. tarda SY-ED14	Pathogen	MH,TS	28
L. anguillarum SY-L24	Pathogen	MH,TS*	24
S. iniae ATCC 2917	Pathogen	BH	37
Y. ruckeri E42	Pathogen	MH,TS	22

*% plus 1.5 NaCI, MH-Mueller Hinton broth, TS: Triptic soy media, BH: Brain Heart media

Disc Diffusion Method

Isolates were tested according to Bauer disc diffusion method (Bauer et al., 1966). Bacteria colonies were grown in suitable temperature and liquid media and their density was adjusted to 0.5 McFarland. Afterwards, the density was adjusted on the solid medium suitable for the bacterial species (0.5 McFarlant). Bacteria grown in liquid media were transferred with sterile cotton swab. After transfer, sterile discs were placed on solid medium for disc diffusion test and 10 µL (2 mg/disc) of caffeic acid (Carl Roth GmbH + Co. KG) were applied and placed in the incubator for incubation. It was determined whether the petri dishes had an antibacterial effect by measuring the inhibition zone diameters formed after 24 hours of incubation. The study was carried out as two parallel sets, and zones of inhibition were detected by eye vision and the diameter was measured. The scale of the measurement was as follows (disc diameter included): 24 mm or greater — good antibacterial activity; 12–13 mm — moderate antibacterial activity; 10–11 mm — weak antibacterial activity; 8–9 — very weak antibacterial activity (Ali et al., 2001).

Minimum Inhibition Concentration (MIC)

Minimum inhibitor concentration analysis was conducted according to methods specified by the Clinical and Laboratory Standards Institute (CLSI, 2006). After the organic acid stock solution (5000 µg/mL) was prepared in the appropriate growth medium, two-fold dilutions (2500-0.15 µg/mL) were made in 96 plates in which 100 µL of the medium was added and 100 µL of the stock solution was added. Subsequently, 100 µL of bacterial suspension (10^5

CFU/mL) was added to the plates. To the control plates:
1- Only 2500 µL of bacterial media
2- 2500 µL of bacteria-free medium containing each concentration of ethanol and caffeic acid
3- Only 2500 µL of bacteria-free medium was added.

Subsequently, the plates were placed in the incubator for 24 hours at optimum growth temperatures and nutrient media for bacterial growth. MIC value was determined according to the concentration inhibiting growth. The MIC value was evaluated as the lowest organic acid concentration without growth in microorganisms. MIC values of caffeic acid against five bacteria used in this study were determined using the microplate microdilution method. Bacteria inoculated into Müller Hinton Broth (MHB) were incubated for 12 hours and then their density was adjusted to McFarland 0.5 (10^8 CFU/mL) using sterile PBS. Working stocks of 5000 µg/mL of caffeic acid were prepared using ethanol. Mixing the same amount of ethanol (1:1) as from working stock and halving each time (2500; 1250; 625; 312.50; 156.25; 78.12; 39.06; 19.53; 9.76; 4.88; 2.44; 1.22; 0.61; 0.30; 0.15 µg/mL) 15 different concentrations were generated 100 µL of the prepared concentrations and 100 µL of the prepared bacterial culture were added to each well of the 96-well microplates. Equal volumes of ethanol and MHB were mixed and used as control. After the microplates were incubated for 24 hours at the temperatures indicated in Table 1, the microbial growth in the samples was observed visually.

Results

Disc diffusion test results of caffeic acid against pathogens were measured as 8 mm for *Y. Ruckeri* E42, 10 mm for *L. Anguillarum* SY-L24, 8 mm for *S. İniae* ATCC 2917, 10 mm for *E. Tarda* SY-ED14, 0 mm for *Citrobacter sp*., SY-C10. Inhibition zone values in the discs are given in Figure 2.

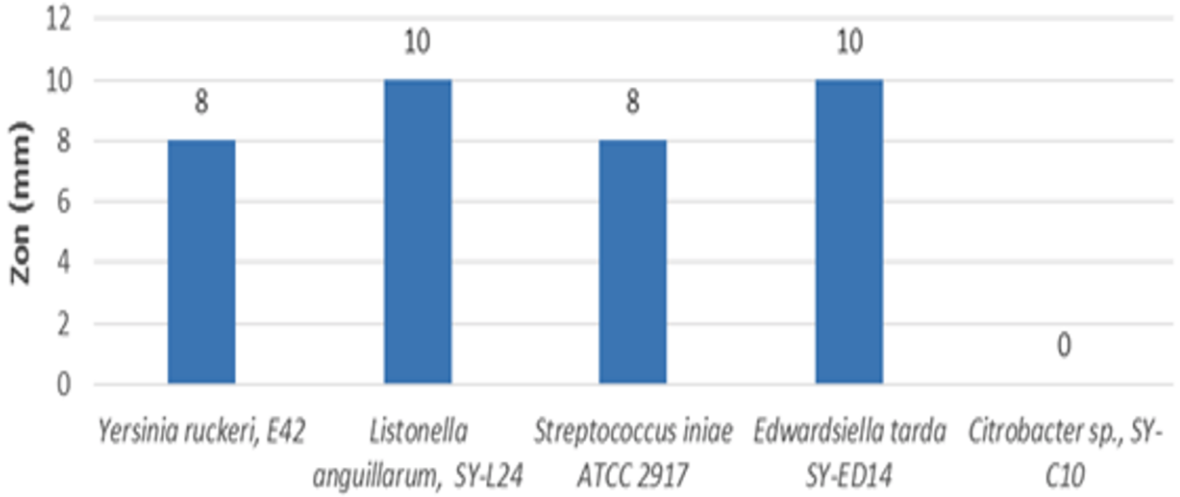

Figure 2. Inhibition zone diameter (mm)

The minimum inhibition concentration (MIC) test results of caffeic acid against pathogens were found as 2500 µg/mL for *Y. ruckeri* E42, 1250 µg/mL for *L. anguillarum* SY-L24, 2500 µg/mL for *S. iniae* ATCC 2917, 312 µg/mL for *E. Tarda* SY-ED14, 2500 µg/mL for 5 µg/mL *Citrobacter* sp., SY-C10. The results show that among five bacterial strains caffeic acid exhibited potent inhibitory effect (MIC: 312.50 µg/mL) on the fish pathogen *E. tarda* SY-ED14 (Table 2).

Table 2. MIC values of caffeic acid against fish pathogens (µg/mL)

Concentration (µg/ml)	Pathogenic Bacteria				
	Y. ruckeri E42	*L. anguillarum* SY-L24	*S. iniae* ATCC 2917	*Citrobacter* sp. SY-C10	*E. tarda* SY-ED14
2500	-	-	-	-	-
1250	+	-	+	+	-
625	+	+	+	+	-
312.50	+	+	+	+	-
156.25	+	+	+	+	+
78.12	+	+	+	+	+
39.06	+	+	+	+	+
19.53	+	+	+	+	+
9.76	+	+	+	+	+
4.88	+	+	+	+	+
2.44	+	+	+	+	+
1.22	+	+	+	+	+
0.61	+	+	+	+	+
0.30	+	+	+	+	+
0.15	+	+	+	+	+
Control	+	+	+	+	+

Discussion

The study findings showed that caffeic acid had antimicrobial activity against all five bacteria, but it was most effective on *Edwardsiella tarda* SY-ED14. Similarly, previous studies reported that organic acids as chlorogenic acid, 4,5-O-dicaffeoylquinic acid, 3,5-O-dicaffeoylquinic acid, gallic acid (Zhu et al., 2004), p-anisic acid, cinnamic acid (Prasad et al. et al., 2014), cinnamic acid (Yilmaz et al., 2018), 2-(3-m-toluyl-1,2,4-oxadiazol-5-yl)-3,4-(methylenedioxy)-cinnamyl and 2-(3-pyrimidyl-1,2,4-oxadiazol-5-yl)-3,4-(methylenedioxy)-cinnamyl (de Freitas Filho et al., 2022) have antimicrobial activity against pathogens. However, only one report has been published on the *in vitro* antimicrobial effects of caffeic acid on fish pathogens. Kırıcı et al., (2016) reported that caffeic acid concentration of 0.5 and 1% or greater significantly decreased the number of *Yersinia ruckeri* colonies per plate compared with the control.

The zone diameter evaluated as 12 mm and above, which is generally accepted moderate or high antimicrobial activity in the literature (Rota et al., 2008; Yılmaz et al., 2018). Assuming that a zone diameter of 8 mm and above has a weak effect, it can be concluded that caffeic acid showed weak effect in disc diffusion tests against pathogens in this study. However, the results of MIC test showed that among five bacterial strains caffeic acid exhibited potent inhibitory effect with an MIC value 312.50 µg/mL on the fish pathogen *E. tarda* SY-ED14. Moreover, promising results were also found when caffeic acid was added to fish feeds. For instance, Yılmaz (2019) found that feeding Nile tilapia with a diet containing 5 g kg^{-1} caffeic acid over a period of 60 days might be adequate to improve fish immune parameters, antioxidant status,

as well as survival rate against *A. veronii*, similar to antibiotic treatment.

When the above studies were evaluated, it has been found that caffeic acid and/or its derivatives have different antimicrobial effects on different pathogenic bacteria. Unfortunately, differences in the evaluation criteria of disc diffusion zone diameters in studies make it difficult to compare our findings with those of other studies. Thus, when the accepted effective zone diameter was 12 mm and above caffeic acid did not have a strong effect on the disc diffusion test in our study. Therefore, more detailed studies on the effects of caffeic acid would be suggested.

Conclusions

According to the results of in vitro research, caffeic acid has a higher antibacterial effect on the *E. tarda* SY-ED14 than tested pathogens. In our study, the antibacterial effect of caffeic acid against few pathogenic fish bacteria were investigated in vivo. Further investigations are necessary to study the *in vivo* effect of the drug and validate the benefits of using dietary caffeic acid in preventing bacterial infection in aquaculture.

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Ethical approval

The experiment was performed under the approval of the Committee on Animal Ethics, Çanakkale Onsekiz Mart University, Türkiye, as a partial fulfilment of the M.Sc. Thesis of first author.

Informed consent

Not available.

Data availability statement

The authors declare that data are available from authors upon reasonable request.

Conflicts of interest

There is no conflict of interests for publishing of this study.

Funding organizations

No funding available for this study.

Contribution of authors

All authors in this study have equally contributed in terms of conceptualization, data curation, formal analysis, writing original draft, funding acquisition, investigation, methodology, resources, validation, and visualization, and finalizing paper.

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