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SHORT COMMUNICATION

Antibacterial Synergy between Oxytetracycline and Selected Polyphenols against Bacterial Fish Pathogens

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ABSTRACT

Objective: Oxytetracycline is an approved antibiotic and most frequently used drug in commercial aquaculture all over the world for treating bacterial diseases in fish. The intensive use of oxytetracycline has resulted development of bacterial resistance to oxytetracycline. It is reported that phytochemicals like quercetin, gallic acid, cinnamic acid and *p*-anisic acid have antibacterial properties. An attempt was made to observe the type of interaction upon incorporation of phytochemicals in combination with conventional antimicrobials like oxytetracycline with a view to decrease the antimicrobial load in aquaculture for minimizing resistance in bacterial pathogens in case combination of phytochemicals and oxytetracycline do possess synergy or additive effect. Combinations of oxytetracycline with polyphenolic compounds (quercetin, gallic acid, *p*-anisic acid, and cinnamic acid) against common gram negative bacterial pathogens of fish, such as *Aeromonas hydrophila, Aeromonas salmonicida,* and *Edwardsiella tarda* were tested. **Methods:** Antibacterial activity of oxytetracycline, quercetin, gallic acid, *p*-anisic acid and cinnamic acid was determined *in vitro* against selected bacterial pathogens individually followed by combination of oxytetracycline with polyphenols using serial microplate dilution method measuring minimum inhibitory concentrations (MIC). Fractional inhibitory concentration (FIC) indices were calculated. **Results:** Oxytetracycline and other polyphenolic compounds exhibited antibacterial action against the selected fish pathogens with mean MIC ranging from 0.5 to 2.5 mg ml^{-1}. Based on FIC indices, synergistic interaction was observed for combination of oxytetracycline with quercetin or cinnamic acid against *Aeromonas salmonicida,* and with gallic acid against *Edwardsiella tarda.* However, combinations of oxytetracycline with gallic acid, *p*-anisic acid and cinnamic acid against *Aeromonas hydrophila*; Combinations of oxytetracycline with gallic and *p*-anisic acid against *Aeromonas salmonicida* and the combinations of oxytetracycline with *p*-anisic acid and cinnamic acid against *Edwardsiella tarda* revealed additive antimicrobial interaction **Conclusion:** Positive antibacterial interaction was evident between oxytetracycline and selected polyphenols *in vitro* against *Aeromonas hydrophila, Aeromonas salmonicida,* and *Edwardsiella tarda*.

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INTRODUCTION

Fish are susceptible to several bacterial infections especially when they are reared in high density conditions. In commercial fish farming, disease outbreaks are imminent in high density conditions that may further elevate mortality rates and decrease the productivity, causing high economic losses to the fish farmers.

Aeromonas hydrophila, Aeromonas salmonicida, and *Edwardsiella tarda* are common gram negative bacterial pathogens of fish. *Aeromonas hydrophila* induces skin infections, septicaemia, and gastroenteritis in fish and humans (Yu *et al*., 2007), *Aeromonas salmonicida* causes furunculosis, and *Edwardsiella tarda* causes edwardsiella septicaemia in fish. Indiscriminate uses of various antibiotics to control these infections result in emergence

of resistance. Therefore, to control bacterial diseases, alternative therapies were approached (Castro *et al.*, 2008) in order to prevent the emergence of resistance. Phytotherapy to treat the infections and mitigate many of the side effects that are associated with commercial antimicrobials is gaining importance. In addition, alternative medicines are relatively cheaper than the commercial chemotherapeutic agents (Punitha *et al.*, 2008).

Many reports indicated the antibacterial actions of polyphenols (Karou *et al.*, 2005). Polyphenols are a group of highly hydroxylated phenolic compounds present in the extractive fraction of several plant materials. Oxytetracycline (OTC) is one of the common antimicrobial agents used in the aquaculture and the emergence of resistance to OTC has been reported (Furushita *et al*., 2003).

Keeping in view, an attempt was made in the present study to screen the combination of OTC with selected polyphenols against common bacterial pathogens of fish.

MATERIALS AND METHODS

Oxytetracycline (OTC) and polyphenolics, namely gallic acid, *p*-anisic acid, and cinnamic acid were obtained from Himedia, Mumbai, India. *p*-iodonitrotetrazolium violet (INT) and quercetin were from SRL, Mumbai, India. Dimethyl sulfoxide (DMSO) was obtained from Merck, Mumbai, India.

Bacterial cultures

The bacteria used in the present study, *Aeromonas hydrophila* MTCC: 646 (AH); *Aeromonas salmonicida* MTCC: 1522 (AS) and *Edwardsiella tarda* MTCC: 2400 (ET) were obtained from Mother Type Culture Collection, Chandigarh, India. The bacterial strains were cultured onto nutrient agar and incubated at 37°C for 16-18 h. Isolated colonies were selected and inoculated into Muller-Hinton broth prior to use in the microdilution assay (Tukmechi *et al*., 2010).

Determination of minimum inhibitory concentration (MIC)

The MIC of individual polyphenolic acids and combinations of quercetin with other polyphenolic acids was determined using serial microplate dilution method of Eloff, (1998) with few modifications. Pure compounds were dissolved in DMSO to give a stock concentration of 5 mg ml^{-1}, while, OTC was dissolved in DMSO to give a stock concentration of 1 mg. ml^{-1}. Two fold serial dilutions of test compounds (100 µl) in sterile normal saline was prepared in 96-well microtitre plate. Fifty µl overnight fresh bacterial cultures of *Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Edwardsiella tarda* were adjusted to one McFarland unit and added to each well. The plates were incubated overnight at 37 ^{0}C and bacterial growth was detected by adding 20 µl of *p*-iodonitrotetrazolium violet (INT) to each well. After incubation at 37 ^{0}C for 30 min, INT is reduced to a red formazan by biologically active organisms. Bacterial growth was shown to be inhibited when the solution in the well remained clear. This concentration was taken as the minimum inhibitory concentration (MIC). Solvent controls were included in each experiment.

Determination of interaction between polyphenolic compounds

Interaction between compounds was determined by calculating the fractional inhibitory concentration (FIC).

FIC of compound A (FIC_A) = MIC of compound A in combination / MIC of compound A alone

FIC of compound B (FIC_B) = MIC of compound B in combination / MIC of compound B alone

The sum of FIC indices (FICs) = $FIC_A + FIC_B$

Synergism has been defined as an FIC index of 0.5 or less, addition as an FIC index of more than 0.5 and less than 4, and the antagonism as an FIC index of more than 4 (Odds, 2003; Braga *et al.*, 2005).

RESULTS

The mean MIC (n=6) values for individual agents, quercetin, gallic acid, *p*-anisic acid, cinnamic acid, and OTC against the selected bacterial pathogens were presented in Table 1. The MICs of tested compounds were in the range of 0.5 to 2.5 mg. ml^{-1}. Among polyphenolics tested, gallic acid and quercetin showed the lowest (0.96 $mg.ml^{-1}$) and the highest (1.67 $mg.ml^{-1}$) MIC, against *Aeromonas hydrophila*, respectively and MIC of OTC was 0.5 mg ml^{-1}. MICs of cinnamic acid, gallic acid, and *p*-anisic acid against *Aeromonas salmonicida* were in the range of 0.83-1.25 $mg.ml^{-1}$ and that of OTC was 0.75 mg ml^{-1}. Cinnamic acid and *p*-anisic acid showed MIC of 1.04 mg ml^{-1}, whereas, OTC and quercetin showed 1 $mg.ml^{-1}$ and 2.5 $mg.ml^{-1}$ against *Edwardsiella tarda*, respectively.

Table 1: Individual minimum inhibitory concentration (MIC) of polyphenolic compounds (mg. ml^{-1})

Compound	Organism		
	AH	AS	ET
Oxytetracycline	0.5±0	0.75±0.35	1.0±0
Quercetin	1.67±0.72	1.04±0.32	2.50±2.1
Gallic Acid	0.96±0.53	1.25±0	1.25± 0
Anisic Acid	1.14±0.31	1.25±0	1.04±0.36
Cinnamic Acid	1.14±0.31	0.83±0.32	1.04±0.36

Values are expressed as Mean±SD of 6 observations; AH = *Aeromonas hydrophila*, AS = *Aeromonas salmonicida*, ET = *Edwardsiella tarda*

Antibacterial activity of combinations

The MIC values of OTC in combination with other polyphenols were presented in Table 2. The combination of OTC with quercetin or cinnamic acid against *Aeromonas salmonicida* showed synergistic effect with FIC indices of 0.095 and 0.45, respectively. The combination of OTC with gallic acid against *Edwardsiella tarda* was synergistic with an FIC of 0.31. The remaining combinations of OTC with polyphenols were found to be additive against tested pathogens with FIC indices in the range of 0.65 to 1.24.

DISCUSSION

The emergence and transfer of bacterial resistance to existing antimicrobials is creating a constant need to develop new antimicrobial agents. Since long, plant derived products have shown the potential to inhibit bacterial growth (Proestos *et al.*, 2005) and there are certain reports on the utility of phytochemicals for

Table 2: Minimum inhibitory concentration (MIC; mg. ml^{-1}) and FIC indices of oxytetracycline and polyphenolic compounds in combinations

Combination	AH	FIC	AS	FIC	ET	FIC
O+Q	0.25+1.25	1.249	0.0156+0.078	0.095**	0.5+1.25	1.0
O+G	0.125+0.625	0.898*	0.125+0.625	0.66*	0.06+0.312	0.31**
O+A	0.125+0.625	0.795*	0.125+0.625	0.66*	0.06+0.625	0.659*
O+C	0.125+0.625	0.795*	0.0625+0.312	0.458**	0.06+0.625	0.659*

Values are expressed as means of 6 observations; No deviations were observed (in this study MIC was determined by serial microplate dilution method and when a given combination was tested we obtained MIC at the same well for all the six replicates, so there is no standard deviation and its value is zero. Zero in the table was not represented deliberately to avoid confusion); * = FIC >0.5 & < 1.0 (indicating additive interaction), ** = FIC < 0.5 (indicating synergistic interaction); AH = *Aeromonas hydrophila*, AS = *Aeromonas salmonicida*, ET = *Edwardsiella tarda;* O+Q = OTC in combination with quercetin, O+G = OTC in combination with gallic acid, O+A = OTC in combination with anisic acid, O+C = OTC in combination with cinnamic acid

antimicrobial actions (Saravanakumar *et al.,* 2009; Ozdemir, 2009; Sudjana *et al.,* 2009). The emergence and transfer of bacterial resistance to existing antimicrobials is creating a constant need to develop new antimicrobial agents. Since long, plant derived products have shown the potential to inhibit bacterial growth (Proestos *et al.,* 2005). In this study, synergistic and additive interactions between OTC and selected polyphenolic compounds against common bacterial pathogens of fish were reported.

The combination of OTC with quercetin or cinnamic acid against *Aeromonas salmonicida* reduced the MIC of OTC by 48 and 12 times, respectively, indicating an obvious synergistic interaction. The reduction in the MIC of OTC was 16.6 times when used in combination with gallic acid against *Edwardsiella tarda*. Synergistic interaction between tetracyclines and gallic acid against *Pseudomonas aeruginosa* was reported by Jayaraman *et al.,* (2010), whereas the remaining test combinations proved to be additive as their FICs are in the range of 0.65 to 1.24.

Drug efflux pumps play a key role in drug resistance apart from other physiological functions in bacteria (Li & Nikaido, 2009). Efflux of drug is the major contributor of drug resistance in *Aeromonas* (Balotescu *et al.,* 2003). The presence of putative drug efflux systems including RND transporters, such asAheB in *A. hydrophila* and RND exporters (AcrAB, MexF, and MexW), MFS pumps, MATE, SMR, ABC transporters and *tetA(E)* gene encoding tetracycline efflux protein in *A. salmonicida*, explains the mechanism of multidrug resistance in *Aeromonas* (Seshadri *et al.,* 2006; Marshall *et al.,* 1986). Antimicrobial action of quercetin can be attributed partly to the inhibition of DNA gyrase (Tim Cushnie & Lamb, 2005), which is important for supercoiling of DNA. Quercetin is capable of inhibiting several families of efflux proteins that play an important role in multidrug resistance of bacteria. In addition, binding of quercetin to porins changes the tridimensional conformation, there by exposing the hydrophilic character of the pore (Alvarez *et al.,* 2006) facilitating the transport of drugs into the bacteria**.** Further, Polyphenolic compounds, such as gallic acid, cinnamic acid *etc*. produce irreversible changes in membrane properties through hydrophobicity changes and occurrence of local rupture or pore formation in the cell membranes with consequent leakage of essential intracellular constituents (Borges *et al.,* 2013). The interaction of phenolic acids with membrane of the bacterial cell might be the reason for enhancing the antimicrobial activity of the antibiotics (Shanmugam & Doble, 2010). The synergistic interaction between OTC and quercetin or cinnamic acid against *A. salmonicida* might be attributed to the combination of above mechanisms.

In conclusion, the results suggest positive interaction between OTC and selected polyphenolic compounds. Incorporation of phytochemicals in combination with conventional remedies decreases the problem of antimicrobial resistance effectively and also reduces the antibiotic load in fish ponds and food chain.

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