Antibacterial synergy between quercetin and polyphenolic acids against bacterial pathogens of fish

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Objective: To evaluate combinations of quercetin with gallic acid, p-anisic acid and cinnamic acid in vitro for synergistic activity against common Gram–negative bacterial pathogens of fish viz., Aeromonas hydrophila, Aeromonas salmonicida and Edwardsiella tarda.

Methods: Antibacterial activity of quercetin, gallic acid, p-anisic acid and cinnamic acid was determined against selected bacterial pathogens individually, followed by combination of quercetin with polyphenolic acids using serial microplate dilution method measuring minimum inhibitory concentrations. Fractional inhibitory concentration indices were calculated.

Results: Quercetin and other polyphenolic compounds exhibited antibacterial action against the selected fish pathogens with mean minimum inhibitory concentrations ranging from 0.83 to 2.5 mg/mL. It was observed that fractional inhibitory concentration indices for combination of quercetin with gallic acid, p-anisic acid or cinnamic acid against Aeromonas salmonicida were less than 0.5, indicating synergistic interaction. However, the above combinations produced additive antimicrobial activity against Aeromonas hydrophila and Edwardsiella tarda.

Conclusions: Positive antibacterial interaction was evident between quercetin and selected polyphenolic acids in vitro.

KEYWORDS
Bacterial pathogens of fish, Fractional inhibitory concentration index, Polyphenolic acids, Quercetin

1. Introduction

Fish are known to be susceptible to several bacterial infections when they were reared in high density conditions like commercial fish farming. Disease outbreaks are imminent in high density conditions, and may further elevate mortality rates in fish and decrease the productivity, causing high economic losses to the fish farmers.
Aeromonas hydrophila (A. hydrophila), Aeromonas salmonicida (A. salmonicida) and Edwardsiella tarda (E. tarda) are some of the common Gram-negative bacterial pathogens of fish. A. hydrophila is responsible for cases of skin infections, septicemia and gastroenteritis in fish and humans. A. salmonicida causes furunculosis and E. tarda causes Edwardsiella septicemia in fish. Wide use of various antibiotics indiscriminately to control these bacterial infections resulted in emergence of resistance among the bacteria. This situation forced the aquaculture farmers to use the antibiotics beyond prescribed limit, leading to accumulation of antibiotic residues in the edible portion of fish that ultimately enter the human body via food chain that result in further complication in therapeutics in human practice. Therefore, alternative therapies were approached to control bacterial diseases in order to prevent the emergence of resistance and to avoid public health problems. Polyphenols act synergistically with antimicrobials against resistant organisms and mitigate many of the side effects that are associated with synthetic antimicrobials.

Polyphenols are a group of highly hydroxylated phenolic compounds present in the extractive fraction of several plant materials. Polyphenols in plants include flavanols, flavonoids, flavonones, flavones, anthocyanins, proanthocyanidins (tannins), hydroxystilbenes, aurones etc. Many reports indicating the antibacterial actions of polyphenols are available. In spite of the fact that plant-derived antibacterials are less potent, plants still fight with infections successfully. Hence, it becomes apparent that plants adopt a different paradigm synergy to combat infections. Quercetin is a flavonoid present in grapes, onions and apples etc. and many reports stating its antibacterial activity are available.

Keeping the above facts in view, an attempt was made in the present investigation to screen the antibacterial activity of quercetin and other selected polyphenolic compounds individually and the combination of quercetin with gallic acid, anisic acid and cinnamic acid against common bacterial pathogens of fish.

2. Material and methods

Enrofloxacin and polyphenolic acids, namely, gallic acid, p-anisic acid and cinnamic acid used in this study were obtained from Himedia, Mumbai, India. P-iodonitrotetrazolium violet (INT) and quercetin were from SRL, Mumbai, India. Dimethyl sulfoxide was obtained from Merck, Mumbai, India.

2.1. Bacterial cultures

The bacteria used in the present study included A. hydrophila MTCC 646; A. salmonicida MTCC 1522 and E. tarda MTCC 2400 obtained from Mother Type Culture Collection, Chandigarh, India. These bacterial strains were maintained in the Department of Veterinary Public Health, NTR College of Veterinary Science, Gannavaram, Andhra Pradesh (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.

2.2. 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 with few modifications. Pure compounds were dissolved in dimethyl sulfoxide to give a stock concentration of 5 mg/mL, while the antibiotic enrofloxacin used as a positive control was dissolved in ultrapure water to give a stock concentration of 250 µg/mL. Two fold serial dilution of test polyphenolic compounds (100 µL) in sterile normal saline was prepared in 96-well microtitre plate and 50 µL overnight fresh bacterial cultures of A. hydrophila, A. salmonicida and E. tarda adjusted to one McFarland unit were added to each well. The plates were incubated overnight at 37 °C and bacterial growth was detected by adding 20 µL of INT to each well. After incubation at 37 °C for 30 min, INT is reduced to a red formazan by biologically active organisms, in this case the dividing bacteria. Bacterial growth was shown to be inhibited when the solution in the well remained clear. This concentration was taken as MIC. Solvent controls and standard antibiotic enrofloxacin were included in each experiment.

2.3. Determination of interaction between the polyphenolic compounds

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

FIC of compound A (FICA) = \[
\frac{MIC of compound A in combination}{MIC of compound A alone}
\]

FIC of compound B (FICB) = \[
\frac{MIC of compound B in combination}{MIC of compound B alone}
\]

The sum of FIC indices (FICs) = FICA + FICB

Synergism has been defined as an FIC index of 0.5 or less, addition as a FIC index of more than 0.5 and less than 4, and the antagonism as FIC index of more than 4.

3. Results

The mean MIC (n=6) values for individual agents, quercetin, gallic acid, anisic acid, cinnamic acid and enrofloxacin (standard antibiotic) against the selected bacterial pathogens are presented in Table 1. The results showed that the polyphenolic compounds had antibacterial activity against the test organisms. Their MICs were in the range of 0.83 to 2.5 mg/mL, which were higher than that of enrofloxacin that is used for therapeutic purposes in fish farming. Among the polyphenolics tested, gallic acid and quercetin showed the lowest (0.96 mg/mL) and the highest (1.67 mg/mL) MIC against A. hydrophila, respectively. MICs of cinnamonic acid, gallic acid and anisic acid against A. salmonicida were in the range of 0.83–1.25 mg/mL. Cinnamic acid and anisic acid showed MIC of 1.04 mg/mL.
whereas quercetin showed 2.5 mg/mL against *E. tarda*.

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Organism</th>
<th>AH</th>
<th>AS</th>
<th>ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>1.67</td>
<td>1.04</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.96</td>
<td>1.25</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>Anisic acid</td>
<td>1.14</td>
<td>1.25</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>1.14</td>
<td>0.83</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>1.95</td>
<td>1.95</td>
<td>3.90</td>
<td></td>
</tr>
</tbody>
</table>

Mean values are expressed with n=6. AH=A. hydrophila, AS = A. salmonicida, ET = E. tarda.

The MIC values of quercetin in combination with other polyphenols are presented in Table 2. The combination of quercetin with gallic acid, anisic acid or cinnamic acid against *A. salmonicida* showed synergistic effect with FIC indices of 0.32, 0.32 and 0.28, respectively. These combinations were found to be additive against *A. hydrophila* and *E. tarda* with FIC indices of 0.85, 0.92 and 0.92, and 0.75, 0.85 and 0.85, respectively.

**Table 2**

<table>
<thead>
<tr>
<th>Combination</th>
<th>AH</th>
<th>AS</th>
<th>ET</th>
<th>FIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q+G</td>
<td>0.5250+0.5250</td>
<td>0.85</td>
<td>0.18216+0.18216</td>
<td>0.32</td>
</tr>
<tr>
<td>Q+A</td>
<td>0.6250+0.6250</td>
<td>0.92</td>
<td>0.18216+0.18216</td>
<td>0.32</td>
</tr>
<tr>
<td>Q+C</td>
<td>0.6250+0.6250</td>
<td>0.92</td>
<td>0.13000+0.13000</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Mean values are expressed with n=6. AH=A. hydrophila, AS=A. salmonicida, ET=E. tarda. Q+G=quercetin in combination with gallic acid, Q+A=quercetin in combination with anisic acid, Q+C=quercetin in combination with cinnamic acid. *: 0.5 < FIC < 1.0 (indicating additive interaction), **: FIC<0.5 (indicating synergistic interaction).

### 4. Discussion

The emergence and transfer of bacterial resistance to existing antibiotics creates constant need to develop new antimicrobial agents. Thus, many studies were focused on antimicrobial properties of plant-derived active principles[9,10]. Since long, bioactive compounds from natural sources have shown the potential to inhibit bacterial growth[11]. Here, we report on the synergistic and additive interactions between quercetin and selected polyphenolic acids against common bacterial pathogens of fish.

Polyphenolic acids revealed a weak antibacterial action when used alone with MICs up to 2.5 mg/mL, but their combinations reduced MICs by 8 times. Against *A. salmonicida*, the MIC of quercetin was reduced by 5.7, 5.7 and 8 times, respectively in combination with gallic acid, anisic acid and cinnamic acid, while it was reduced by 3.2, 2.67 and 2.67 times when used in combination with gallic acid, anisic and cinnamic acids, respectively against *A. hydrophila*. The reduction in MIC of quercetin was 4 times when used in combination with other polyphenolic acids against *E. tarda*. The above combinations of quercetin with polyphenolic acids proved to be synergistic against *A. salmonicida* with FIC indices less than 0.5, indicating a synergistic interaction. There was an additive interaction when the combinations were used against *A. hydrophila* and *E. tarda* with FIC indices between 0.5 and 1.

Binding of one flavonoid facilitates an easier passage of another flavonoid by diffusion across structural membrane proteins of bacteria. Such binding of quercetin to porins changes the tridimensional conformation, thereby exposing the hydrophilic character of the pore[12]. In addition, some polyphenolic acids like 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[13]. The interaction of phenolic acids with membrane of the bacterial cell might be the reason for enhancing the antimicrobial activity of the antibiotics[14]. Antimicrobial action of quercetin can be attributed partly to the inhibition of DNA gyrase[15], which is important for supercoiling of DNA. The positive interaction between quercetin and polyphenolic acids against the test microbes might be attributed to the combination of above mechanisms. Synergistic interaction between epicatechin and quercetin against *Stenotrophomonas maltophilia*, a nosocomial pathogen, was reported[16]. Synergistic interaction of test compounds against *A. salmonicida* suggests that the organism is highly susceptible to the combination of quercetin with other polyphenolic acids, while the combinations of test chemicals proved to be additive against the other two organisms in test, for which the microbiological and drug factors need to be explored further.

In conclusion, the results suggest positive interaction between quercetin and selected polyphenolic acids and hence these combinations may be helpful in the treatment of susceptible bacterial infections in fish, reducing the synthetic antibiotic load in aquaculture.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Comments

#### Background

*A. hydrophila*, *A. salmonicida* and *E. tarda* are the most common Gram-positive pathogens of fish that cause skin and gastrointestinal infections. Indiscriminate use of antibacterials to control such infections is the leading cause of resistant pathogens and development of adverse effects in humans and livestock due to entry of such antibiotics into...
biological system via food chain. Hence, there is a need for introducing more safer and cost effective chemicals. Such alternatives can be obtained in the form of polyphenolic compounds in plants. Hence this study attempted to use quercetin and other polyphenolic acids individually and in combinations against the above pathogens in vitro.

Research frontiers

This paper deals mainly with the antibacterial action of selected polyphenolic compounds with known antioxidant properties. It is a very useful work in the field of alternative medicine in the area of chemotherapy in aquaculture.

Related reports

Polyphenols act synergistically with antimicrobials against resistant organisms and mitigate many of the side effects that are associated with synthetic antimicrobials. Many reports indicating the antibacterial actions of polyphenols are available.

Innovations & breakthroughs

This is a novel work and literature is scanty in this type of work. The results of this study revealed that quercetin and other polyphenolic compounds exhibited antibacterial action against the selected fish pathogens with mean MIC ranging from 0.83 to 2.5 mg/mL. It was observed that FIC indices for combination of quercetin with gallic acid, p-anisic acid or cinnamic acid against A. salmonicida were less than 0.5, indicating synergistic interaction. However, the above combinations produced additive antimicrobial activity against A. hydrophila and E. tarda.

Applications

The results suggest positive interaction between quercetin and selected polyphenolic acids and hence these combinations may be helpful in the treatment of susceptible bacterial infections in fish, reducing the synthetic antibiotic load in aquaculture and hence reducing selection pressure. These tested compounds can better be utilized as alternatives to the available antibacterials, which can reduce the incidence of adverse drug reactions, which occur due to entry of the antibiotics via food chain into human beings.

Peer review

The work presented in this article is novel and is very relevant to the current clinical scenario of aquaculture. The authors have designed the experiment using appropriate in vitro tests. The research methodology, presentation of results and discussion were meticulous. The findings revealed good efficacy (synergism/addition) of the test combinations of polyphenolic compounds against the fish pathogens in test. These findings can be applied in the field of aquaculture to improve the safety and reduce the adverse effects of existing antibiotics for better productivity and economics. This work is suitable for publication.

References