Toxicology International, *32(1) : 171-179; 2025. DOI: 10.18311/ti/2025/v32i1/47581*

Online ISSN : 0976-5131; Print ISSN : 0971-6580

Toxicology International

Contents available at: www.informaticsjournals.com/index.php/toxi



In Vitro Validation of Quercetin's Antibacterial Potency Against Hemorrhagic Septicemia in *Oreochromis niloticus*

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Abstract

Hemorrhagic septicemia caused by *Aeromonas hydrophila* is a major bacterial infection threatening sustainable aquaculture, particularly in species like Nile Tilapia *(Oreochromis niloticus).* Building on previous computational studies that identified quercetin as a potential antibacterial agent against *Aeromonas hydrophila*, this study validates quercetin's efficacy on fish cell lines *by examining* a multifaceted approach, conducted FTIR to confirm the presence of quercetin and its functional group, evaluated the impact on DNA gyrase supercoiling inhibition and cytotoxicity assessment of quercetin on a fish cell line using the MTT assay. Our findings show that 80 µg/mL quercetin is safe for OnIL (*Oreochromis niloticus* liver cell line). Whereas 50, 60, 70, and 80 µg/mL, reveal potent inhibitory effects against *Aeromonas hydrophila* cultured with OnIL cells. This study establishes the effectiveness of quercetin against *Aeromonas hydrophila* in OnIL cells. This indicates that quercetin could be a sustainable approach to managing bacterial hemorrhagic septicemia in aquaculture.

Major Findings: 1) The study investigates the use of quercetin, a natural polyphenol, as a potential antibacterial agent against *Aeromonas hydrophila*, the pathogen responsible for hemorrhagic septicemia. 2) Quercetin concentrations of 50, 60, 70, and 80 µg/mL showed strong inhibitory effects *against A. hydrophila* when cultured with OnIL cells while Quercetin at a concentration of 80 µg/mL was found to be safe for the *Oreochromis niloticus* liver cell line (OnIL). 3) The study confirmed quercetin's antimicrobial activity through its ability to inhibit DNA gyrase supercoiling in bacterial cells.

Keywords: DNA Gyrase Supercoiling Inhibition, FTIR, Hemorrhagic Septicemia, MTT Assay, *Oreochromis niloticus,* Quercetin

1. Introduction

Quercetin is a natural polyphenol that is present in many fruits and vegetables. The basic structure of quercetin has two aromatic rings (A and B) connected by an oxygen heterocycle (ring C) comprising a double bond, one keto-carbonyl, and five hydroxyl groups[1]. Its phenolic hydroxyl group and double bonds show antioxidant and anti-inflammatory properties that help treatment of cardiovascular diseases, inflammation tumours and cancer while hydroxyl groups and the keto groups form the metal complexes, has been reported as potential treatment options against a wide range of gram-positive and gram-negative bacterial infections, attained

Article Received on: 04.12.2024 Revised on: 03.02.2025 Accepted on: 06.02.2025

momentum, especially in antibiotic-resistant strains due to its mechanistic approach of chelation with metal ions that depend on the formation of hydrogen bonds between the hydroxyl groups (OH) and cooperative bonds form between oxygen atoms in the carbonyl groups of quercetin and metal ions, providing greater stability to the complexes[2-4]. Primary Quercetin disrupts cell membrane integrity leading to an outflow of cellular components. Furthermore, it catalyzes DNA gyrase supercoil activity, inhibits nucleic acid metabolism, blocks biofilm formation, and prevents virulence factor expression[5].

Aquaculture industries are affected predominantly by hemorrhagic septicemia, which is largely caused by bacteria *Aeromonas hydrophila* is a significant concern, raising a substantial risk to fish health and economic viability[6]. Although regular antibiotic treatments are effective, concerns regarding antibiotic resistance and their impact on the environment are raised[7]. In prior research, molecular docking and MD (molecular dynamics) simulations identified quercetin, as a promising antibacterial agent, exhibiting a strong binding affinity to DNA gyrase in *Aeromonas hydrophila*[8]. This computational insight established a theoretical basis, paving the way for the current study to explore quercetin's practical efficacy on fish cell lines in treating hemorrhagic septicemia in Nile Tilapia[9-11,13,14]. We are using a thorough approach to investigate quercetin's effects on the pathogenic bacteria and the host fish cells to better understand its possible uses in the management of fish farms.

We use FTIR (Fourier-transform infrared spectroscopy) measurements to verify that our sample contains quercetin[15,16]. FTIR is a novel method that uses different wavelengths or frequencies to determine the infrared light's intensity. It helps us to determine the range of absorption from liquids, solids, or gases. In this study, FTIR analysis is used to pinpoint the locations of functional groups within the molecular structure of quercetin. This confirms the purity and structural correctness of quercetin, thereby sustaining the validity of our results by connecting the biological effect to the chemical composition of quercetin[17].

The study explores the inhibition of DNA gyrase supercoiling, a crucial process for transcription and bacterial DNA replication[18]. DNA (deoxyribonucleic acid) is given negative supercoils by the essential enzyme DNA gyrase, which facilitates the unwinding of DNA necessary for transcription and replication[19]. By inhibiting this enzyme, quercetin inhibits bacterial growth and provides important information about the mechanism behind its antibacterial action[20].

The study involves a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay to determine the cytotoxic and inhibitory effect of quercetin on cell lines (OnIL). Toxic compounds often first damage the liver cells, which metabolize internal and external toxins[21,22]. We can investigate metabolic processes and identify significant toxicity markers to fix this. Furthermore, the liver cells' regeneration potential and ease of culture make them suitable for long-term and comparative studies[23,24]. We could observe quercetin's effects on bacterial cells and changes in cell morphology through microscopy[25].

This study builds upon our previous computational findings[8] by assessing the cytotoxicity, cellular interactions, and antibacterial effects of quercetin in vitro using an OnIL. The objectives are to validate quercetin's inhibitory effects on *Aeromonas hydrophila* in a biological context and to determine safe concentration ranges for application in aquaculture. This leads to ways that can help us find effective and sustainable replacements for traditional antibiotics while tackling both health issues and environmental worries.

2. Materials and Methods

2.1 Quercetin Preparation and FTIR Analysis

Quercetin, with more than 95% purity, was obtained from Sigma Aldrich. A stock solution was prepared using DMSO (dimethylsulfoxide) and stored at -20°C until needed[17]. All samples were freeze-dried first. Then, they were analyzed with a Fourier-transform infrared spectrometer, akin to Elmer Spectrum-Two from the USA, to be specific. It had an ATR (attenuated total reflectance) accessory. The measurement range spanned from 500 cm^{-1} to 4000 cm^{-1}, with 32 scans per sample[26,27].

2.2 DNA Gyrase Supercoiling Inhibition

The effect of quercetin on bacterial DNA gyrase activity was assessed using a supercoiling assay. Plasmid DNA was exposed to various concentrations of quercetin, and changes in DNA supercoiling were observed through gel electrophoresis. DNA gyrase and pBR322 (plasmid Bolivar Rodriguez 322) DNA were sourced from Sigma

Aldrich. The supercoiling reaction mixtures (30 µl), which included 3.4 nM gyrase, 1 mM ATP, and 5 nM (0.5 µg) relaxed pBR322 DNA, were incubated at 37°C for 30 to 60 minutes. The reactions were stopped by adding 0.5 volume of STEB (20% [wt/vol] sucrose, 0.05 M Tris-HCl [pH 7.5], 0.05 M EDTA, 50 µg/ml bromophenol blue) and 2 volumes of chloroform-isoamyl alcohol (24:1)[28]. The samples were treated with quercetin at 5, 10, 15, 20, and 25 µM concentrations, dissolved in 1% DMSO, and then subjected to electrophoretic analysis on 1% agarose gels. The gels were run in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) at a maximum voltage of 90V for 2 to 3 h and visualized using a transilluminator[29].

2.3 Culturing *Aeromonas hydrophila*

Aeromonas hydrophila was obtained from MTCC Chandigarh and cultured on nutrient agar at 37°C for 48 h. The bacteria were then inoculated into nutrient broth supplemented with 1% (w/v) sodium chloride (NaCl, Sigma-Aldrich) and cultured under continuous shaking (100 rpm) for 24 h to achieve exponential growth. The bacteria were subsequently harvested, resuspended in sterile PBS (phosphate-buffered saline), and adjusted to a concentration of 10^8 CFU (colony-forming units) per mL[30-32].

2.4 Preparation of OnIL Cell Line

The OnIL was sourced from the National Repository of Fish, NBGRF, Lucknow. Cells were cultivated in Leibovitz's L-15 Medium (1X) in 25 cm^2 cell culture flasks supplemented with 10% fetal calf serum and 1% Penicillin- Neomycin-Streptomycin solution (5,000 units penicillin-G,5 mg streptomycin and 10 mg neomycin per ml, serum (Sigma-Aldrich, Cat. No: P4083)[33]. The cells were maintained at 22°C. A suspension of OnIL cells containing 1×10^5 cells per millilitre was prepared for the cytotoxicity test, allowing cells to adhere and stabilize for 24 h under standard culture conditions at 28°C[34].

2.5 Bacterial Inoculation and Cell Infection

Aeromonas hydrophila inoculation was performed using the absorption method after adding the cell production medium. Following the incubation period, L-15 medium supplemented with bovine serum was added, and the flask was placed in a 15°C incubator. Cytopathic Effect (CPE) formation was observed after approximately 48 h. The infected cells were then frozen at -80°C, subjected to three freeze-thaw cycles, and centrifuged at 4°C and 3000 rpm. The resulting bacterial supernatants were aliquoted into vials and stored at -80°C for future use[35].

2.6 Assessing the Safe and Inhibitive Dosages of Quercetin

A 96-well plate had samples of 20 µL of quercetin at 50, 60, 70, and 80 mM added to the wells. PBS solution was added to some wells as a positive control. Aliquots of 20 µL of the previously infected cell lines were added to the wells and incubated for 48 h at 28°C. Then, 25 µL of 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) was added to each well, and the plates were incubated for an additional 3 h at 28°C to allow for formazan formation. The plates were centrifuged (2000 g for 10 min)[36], and the precipitates were dissolved in 200 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) and then moved to a 96-well plate. The absorbance of the dissolved formazan was measured at 570 nm[37].

Non-infected OnIL cells were treated with quercetin at doses of 80, 90, 100, 150, and 200 µg/mL to establish a safe quercetin concentration. The difference in absorbance between the bacteria that survived and the bacteria from the positive controls (100%) was used to compute the percentage of non-viable bacteria, which was used to express the bactericidal action[38].

2.7 Statistical Analysis

Every experiment had suitable positive and negative controls. Data were analyzed using IBM SPSS 16.0 software, and results were presented as mean ± standard deviation[39]. Statistical significance was evaluated using the student's t-test, with significance levels indicated by * for $p<0.05$ and ** for $p<0.01$[40].

3. Results

3.1 FTIR Analysis

The structural organization of quercetin was analyzed using FTIR spectroscopy. The FTIR spectrum of quercetin is presented in Figure 1, revealing its characteristic bands.

The OH group stretching appeared at 3397 and 3315 cm^{-1}, indicating the presence of hydroxyl groups in the structure. The broadness and intensity of this peak suggest strong hydrogen bonding interactions. C-O and C-O-C stretching bands were identified at 1031, 1078, 1217, and 1295 cm^{-1}, corresponding to the C-O stretching in the aryl

ether ring, the C-O stretching in phenol, and the C-CO-C stretch and bending in ketone, respectively. Multiple peaks in the 1000-1300 cm^{-1} region indicate the complexity and substitution pattern of the aromatic rings. The C=O aryl ketonic stretch was observed at 1673 cm^{-1}, and C=C aromatic ring stretches were seen at 1726 and 1673 cm^{-1}. A significant peak around 1660-1750 cm^{-1} indicates the presence of a conjugated carbonyl group, a key feature of quercetin's structure. In-plane bending bands of C-H in aromatic hydrocarbons were found at 1366, 1419, and 1457 cm^{-1}, and out-of-plane bending bands were detected at 979, 908, 815, 713, 657, and 600 cm^{-1} [41].

3.2 DNA Gyrase Supercoiling Inhibition

Quercetin decreases DNA gyrase activity, as demonstrated by the DNA gyrase supercoiling inhibition experiment. As illustrated in Figure 2, different concentrations of quercetin (5, 10, 15, 20, and 25 µM) progressively inhibited supercoiling activity. Lane C displays the control with only relaxed DNA, representing the plasmid DNA in its initial state with no supercoiling[41]. Lanes 1 to 5 show the effects of quercetin at different concentrations, with inhibition of supercoiling apparent at each level. (Lane 1 to 5), substantial inhibition was observed, with the presence of relaxed DNA and minimal supercoiled DNA, indicating effective inhibition of DNA gyrase activity. Lanes 6 to 8 serve as positive controls, showing DNA gyrase activity in the absence of quercetin. These lanes confirm the enzyme's activity in the absence of the inhibitor as exhibited in the supercoiled DNA forms, Open circular DNA (OC) or nicked DNA was also seen across all lanes to reference plasmid DNA without supercoiling.

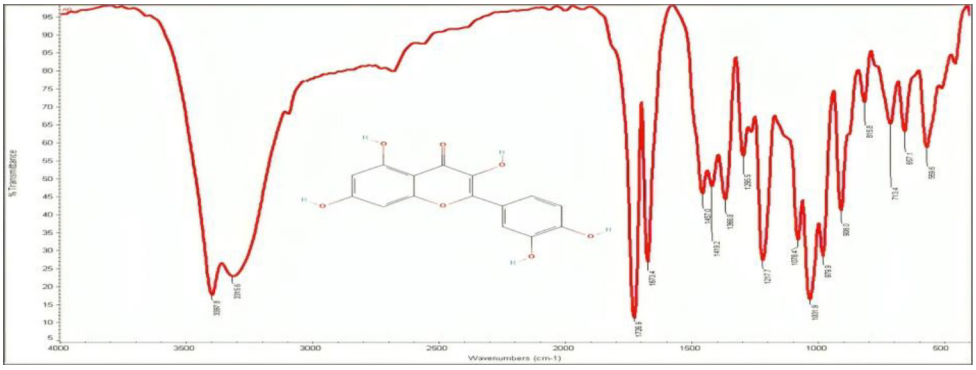


Figure 1. Illustrates the FTIR spectra of the Quercetin (characteristic peaks of stretching and bending of the hydroxyl groups and the stretching of the C=O and C=C bonds in the regions of 1100–1600 cm^{-1} and 3200–3400 cm^{-1}).

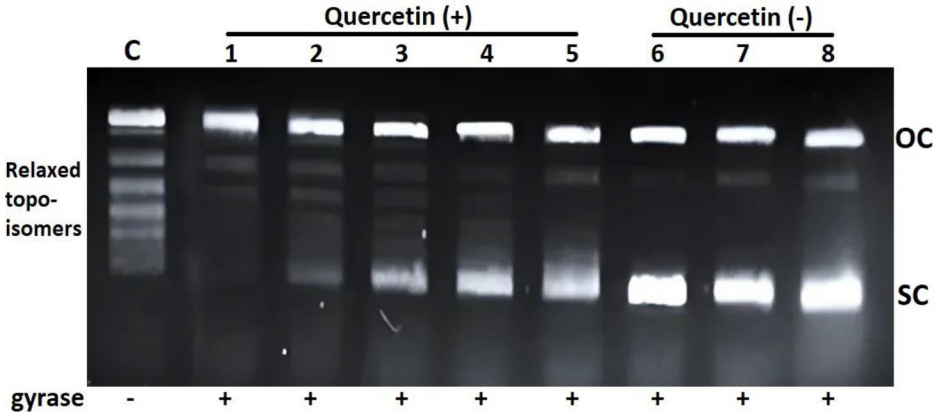


Figure 2. The Analysis of quercetin effect on DNA gyrase activity. The control sample (lane C) contains only relaxed DNA, with no supercoiling present. Lanes 1 to 5 show the DNA gyrase inhibition by quercetin at concentrations of 5, 10, 15, 20, and 25 µM, respectively. These lanes show a reduction in supercoiled DNA and an increase in relaxed DNA, indicating effective inhibition. Lanes 6 to 8 show the enzyme's ability to introduce supercoils into DNA in the absence of quercetin. Open circular DNA (OC), which is nicked or relaxed, is noted across all lanes.

3.3 Assessing the Safe and Inhibitive dosages of Quercetin

To identify the safe working concentration of quercetin, OnIL cells were exposed to quercetin at concentrations of 80, 100, 125, 150, 200, and 250 µg/mL. Notable changes in cell morphology were observed at concentrations above 80 µg/mL. In contrast, OnIL cells treated with 80 µg/mL quercetin exhibited normal morphology with no adverse effects. This concentration was confirmed to have no impact on cell viability. Multiple repetition of experiments was used to verify that 80 µg/mL quercetin is a safe concentration[42]. Figure 3 shows that OnIL cells treated with this concentration showed normal cell viability, similar to control cells, with no signs of damage or stress, indicating that 80 µg/mL does not cause cytotoxic effects.

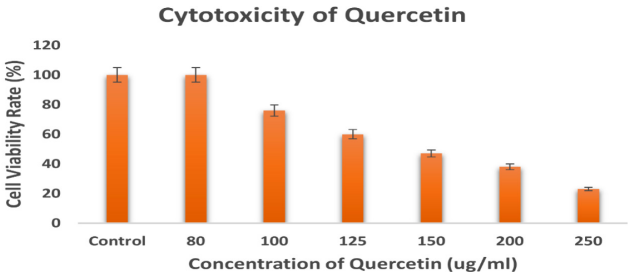


Figure 3. Safe working Concentration of quercetin for the cytotoxic effect of quercetin on OnIL (A) Cell activity analysis indicated that a concentration of 80 µg/mL of quercetin did not impact cell viability. Above 80 µg/mL showed the cytotoxic effect. The data are presented as mean ± SD (n = 3), with ** denoting p≤0.01 and Ns indicating p>0.05.

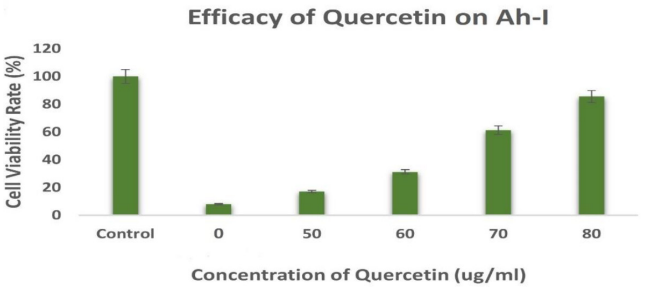


Figure 4. Cell viability tests (Antimicrobial effects of Quercetin) on *A hydrophilic-infected OnIL cells* (Ah-I) were evaluated at different concentrations. CPEs in cell morphology occurred at quercetin concentrations above 50 µg/mL, with more pronounced alterations observed at 60, 70, and 80 µg/mL. Control represents Non-infected OnIL cells. The data are presented as mean ± SD (n = 3), with ** indicating p≤0.01 and Ns denoting p>0.05.

To assess the effect of quercetin on Ah-I cells (OnIL cells infected with *Aeromonas hydrophila*), cells were treated with quercetin at concentrations of 50, 60, 70, and 80 µg/mL, and compared to a control group of uninfected OnIL cells, revealed minimal Cytopathic Effects (CPEs) in Ah-I cells treated with quercetin, with observable morphological changes at concentrations above 50 µg/mL (Figure 4). Infected cells showed typical CPEs, such as cell rounding, detachment, and lysis, with significant changes in morphology at higher concentrations of 60, 70, and 80 µg/mL. Observations were made at a scale of 100 µm. Our previous study highlighted quercetin's favourable binding affinity to *Aeromonas hydrophila* DNA gyrase, with a docking score of -7.812 [8]. In this study, quercetin demonstrated no cytotoxicity at concentrations up to 80 µg/mL in OnIL cells and showed effective inhibition of *Aeromonas hydrophila* at concentrations between 50-80 µg/mL. These findings validate the predicted efficacy of quercetin and suggest its practical applicability as an antibacterial agent.

4. Discussion

Many studies described that flavonoids possessed antibacterial activity against a broad spectrum of bacteria, including quercetin, due to the presence of its phenolic hydroxyl group and double bonds attributed to disrupting bacterial cell membranes by altering their permeability, leading to leakage of cellular components. The FTIR spectrum of quercetin reveals several key peaks that correspond to its molecular structure. The broad O-H stretching band indicates the presence of hydroxyl groups involved in hydrogen bonding that possess the ability to chelate metal, contributing to antibacterial activity by depriving the bacteria of these essential elements like iron thereby disrupting their cellular processes and hindering their growth as certain metal ions, such as iron, manganese, and zinc, are necessary for bacteria to perform essential biological activities like DNA replication and enzyme activity[2]. A prominent C=O stretching peak signifies the presence of a conjugated carbonyl group, a critical feature of quercetin. Double bond makes it easier to interact with the lipid bilayer found in bacterial cell membranes due to its contribution to planarity and lipophilicity. The C=C stretching vibrations confirm the presence of aromatic rings, allowing it to effectively interact with bacterial membranes and enhancing the molecule's ability to leak cellular components contributing to its antimicrobial

properties. The quercetin molecule's polar regions, which are formed by the C-O and C-O-C stretching peaks provide details about the substitution patterns of these rings. These stretches enable quercetin to interact with the polar components of the bacterial cell membrane, which are mainly made up of phospholipids, and create hydrogen bonds with the membrane, which compromises its stability[3].

Wang S. *et al*., 2018 revealed that the cell wall was damaged and showed abnormalities including separation of the cytoplasmic membrane from the cell wall of *S. aureus* and *E. coli* treated with quercetin at 50× MIC, and the bacteriostatic effect of quercetin on gram-positive bacteria was stronger than that on gram-negative bacteria, possibly because of the differences in the structure and composition of cell wall[43].

Looking at other studies, like those from *Ol'ha O. Brovarets* and *M. Catauro,* we can see that peak positions and intensities are coordinated with known values for quercetin[44]. Deviations in these statistics (Figure 1) can happen due to differences in sample preparation, purity, or even how measurements are taken.

These FTIR findings fit nicely with the known structure of quercetin. They confirm that hydroxyl, carbonyl, aromatic, and ether groups are all present. So, this analysis helps confirm the purity of quercetin and improves our understanding of its antibacterial properties and interaction with bacterial cell membranes.

The research illustrates quercetin's effects on DNA gyrase activity focused on inhibiting the enzyme based on concentration levels. Different concentrations of quercetin (5, 10, 15, 20, and 25 µM) reduced the enzyme's ability to introduce supercoiling in DNA. Gel electrophoresis results show a decrease in supercoiled DNA and an increase in DNA relaxation in the presence of quercetin. Quercetin may bind to enzymes at the ATP (adenosine triphosphate) binding site or the DNA binding site, inhibiting their ability to supercoil. Control samples and Lane 6 to 8 (absence of quercetin) confirm that DNA gyrase is in an active state and capable of introducing supercoiling.

The way quercetin inhibits DNA gyrase is similar to other natural inhibitors like coumarins and flavonoids. This suggests it might be a good antibacterial option targeting this enzyme. It could have a big effect on bacterial replication and transcription, which makes quercetin promising for fighting bacterial infections—especially with antibiotic resistance growing! More work is needed to figure out exactly how quercetin binds using methods like NMR (nuclear magnetic resonance) and crystallography and to check how effective it is in live organisms.

Studies on cell viability revealed that quercetin at 80 µg/mL doesn't harm OnIL cell viability with normal cell morphology. Cell viability assessment of cells treated with this concentration reveals no signs of cytotoxicity, indicating that 80 µg/mL is a safe concentration[45].

For Ah-I cells (OnIL cells infected with *Aeromonas hydrophila*), significant morphological changes were observed at quercetin concentrations above 50 µg/mL, indicating an antimicrobial effect. These changes were more pronounced at 60, 70, and 80 µg/mL concentrations. While quercetin is not toxic to OnIL cells at up to 80 µg/mL, higher doses exhibit cytotoxicity[12,37]. The increase in cell viability of Ah-I cells treated with quercetin at 60, 70, and 80 µg/mL ($p \leq 0.01$) confirms its antimicrobial efficacy, though higher concentrations can harm host cells. Overall, these studies support quercetin's role as a potential antimicrobial agent, while also highlighting the importance of concentration in balancing efficacy and cytotoxicity. Optimized MTT incubation time and growth conditions were carried out to prevent saturate signals or weak absorbance signals if the incubation time is too long or too short respectively.

Zhao *et al.*[14] stated that quercetin showed synergistic results against *Aeromonas hydrophila* combinations with florfenicol increasing the sensitivity from 2.5 µg/mL to 0.078 µg/mL. Resulting in less dose of antibiotics that lower bacterial load in the liver, spleen, and kidney tissues of infected fish enhancing fish health, and minimizing environmental pollution. Although florfenicol is an effective antibiotic its overuse can lead to antibiotic resistance and environmental risks. Quercetin, as a natural alternative, offers antimicrobial benefits while enhancing fish immunity, its addition to feed controls microbial loads, supports gut health, optimizes nutrient absorption and metabolism, reduces oxidative stress and promotes sustainability in aquaculture[14].

The successful inhibition of *Aeromonas hydrophila* in fish cell lines aligns with computational predictions from prior research[8], supporting quercetin's binding affinity as indicative of its practical antibacterial efficacy. Moreover, the absence of cytotoxic effects at effective concentrations suggests that quercetin is a safe candidate for managing bacterial infections in aquaculture. These findings bridge theoretical and experimental approaches, highlighting the potential of phytochemicals like quercetin as sustainable alternatives to antibiotics in aquaculture.

5. Conclusion

This study confirms the antibacterial efficacy of quercetin, previously identified through in silico screening, against *Aeromonas hydrophila* in Nile tilapia cell lines. FTIR spectroscopy confirms the presence of crucial functional groups in quercetin, aiding in its structural correlation with biological response and understanding of broad-spectrum antimicrobial properties. Quercetin was shown to be safe for OnIL cells at concentrations up to 80 µg/mL and effectively inhibited bacterial growth at concentrations as low as 50 µg/mL, offering a promising alternative to traditional antibiotics for managing bacterial infections like hemorrhagic septicemia. Careful dosing is necessary due to its cytotoxicity at higher concentrations. Overall, the antibacterial mechanism of quercetin shows potential as a sustainable alternative for managing hemorrhagic septicemia in aquaculture, and further research should explore its detailed mode of action and therapeutic potential.

6. Acknowledgements

We would like to express our gratitude to Amity Institute of Biotechnology, Amity University, Lucknow Campus. We would also like to thank everyone who helped us, directly or indirectly.

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