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Design, Synthesis, and Biological Evaluation of a Quercetin-4-(Methylthio)phenylboronic Acid Complex with Antibacterial, Antioxidant, and Anti-Inflammatory Activities

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ABSTRACT

Quercetin is a natural occurring flavonol that has been proven to have antioxidant, antimicrobial and anti-inflammatory properties, but it is not applicable in the pharmaceutical industry because of its poor physicochemical properties. The present study was aimed to find out whether the complexation with 4-(Methylthio) phenylboronic acid (4MTPBA) could be helpful in enhancing the biological performance of quercetin. A boronate complex of quercetin with 4MTPBA was prepared by condensation at 3'4'-catechol site and further characterized by FTIR and LC-MS. Successful complex formation was achieved by the presence of characteristic B-O and B-O-C vibrations and a deprotonated molecular ion at m/z 433.06. They assessed their antibacterial activity against *Salmonella typhi*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* by measuring zones of inhibition, MIC and MBC. The 4MTPBA-Q complex was more active than the individual quercetin and 4MTPBA against all tested bacteria with the greatest activity noted against *S. aureus* (24.9 mm inhibition zone, MIC 1.5625 g/100 µL, and MBC 3.125 g/100 µL). Antioxidant testing also showed that the complex had a higher percentage of DPPH and ABTS radicals scavenging activity of 88.9 ± 1.6% and 91.5 ± 1.7, respectively and a higher FRAP value of 356.9 ± 4.5 µmol Fe²⁺/g compared with the others. The complex showed the highest inhibitory activity towards the secretion of TNF-α, IL-6, IL-1β, PGE₂, nitrite and the expression of COX-2 in the in vitro inflammatory model, compared to the control. These results show that boronate complexation enhanced the antibacterial, antioxidant and anti-inflammatory properties of quercetin. The 4MTPBA-Q complex will have a therapeutic potential, which is supported by further in vivo and pharmacological studies.

Introduction

Quercetin is a flavonol compound found in the plant kingdom which is commonly consumed in fruits, vegetables and medicinal plants. Its biological activity is related to the polyphenolic scaffold, responsible for the antioxidant action, regulation of inflammatory mediators and antimicrobial action [1]. However, the use of quercetin in therapy is hampered by its poor physicochemical characteristics, low water solubility, poor stability and variable bioavailability. The structural modification of the parent molecule could provide a method of enhancing the functional properties without removing the pharmacologically relevant core of the molecule, the flavonoid. Boronic acids have been shown to be good candidates for this modification, as they are able to easily form cyclic boronate esters with vicinal diols[2]. Quercetin contains the adjacent hydroxyl groups at the 3' and 4' positions of the B-ring that are suitable for the formation of boronate ester[3]. In this study, 4-(Methylthio) phenylboronic acid (4MTPBA) was used to react with quercetin to form the corresponding quercetin 3',4'-O, O-[4-(Methylthio)phenyl] boronate complex. In this modification one phenylboronate group is added and the main structural elements of the flavonoid molecule are retained. The reasons for creating such a derivative are also associated with the biological association between oxidative stress, inflammation and microbial infection. Overproduction of ROS can amplify inflammatory reactions and bacterial infection can trigger additional ROS and cytokine production and oxidative damage. Chemicals that can exert effects on these interdependent processes could thus be more broadly pharmacologically relevant than compounds that exert effects on a single biological pathway. In the present investigation, the quercetin-4MTPBA complex was attempted to be synthesized and characterized and its biological properties were compared with that of quercetin and 4MTPBA alone [4]. FTIR and LC-MS analyses were used to confirm the structure. The zone of inhibition, minimum inhibitory concentration and minimum bactericidal concentration assays were used to determine the antibacterial potential. DPPH, ABTS and FRAP were used to measure antioxidant activity while TNF- α , IL-6, IL-1 β , PGE₂ and nitric oxide, as well as the expression of COX-2 were measured to assess anti-inflammatory activity. This experimental design allows an evaluation of the effects of boronate complex formation on the multifunctional biological profile of quercetin.

Methodology

Synthesis of Quercetin 3',4'-O, O-[4-(Methylthio)phenyl] boronate

Quercetin 3',4'-O, O-[4-(Methylthio) phenyl] boronate was obtained by condensation of quercetin with 4-(Methylthio)phenylboronic acid at catechol functionality of the flavonoid. Quercetin was added (1.0 mmol, 302.2 mg) into a dry round bottom flask and dispersed in anhydrous acetonitrile (20-30 mL). Then 4-(Methylthio) phenylboronic acid (1.0-1.1 mmol, 168.0-184.8 mg) was added to the reaction mixture. The contents were stirred under inert atmosphere to minimize encounter with atmosphere moisture. Then this mixture was refluxed for 24 h in anhydrous acetonitrile with stirring. In such conditions, the boronic acid is selectively reacting with the neighbouring 3'- and 4'-hydroxyl groups of quercetin to form a cyclic boronate ester with the loss of two molecules of water. The boronate esterification is an equilibrium reaction and the process is sensitive to water, therefore the reaction was carried out under anhydrous conditions which would aid the formation of the product and limit the hydrolysis of the boronate ester.

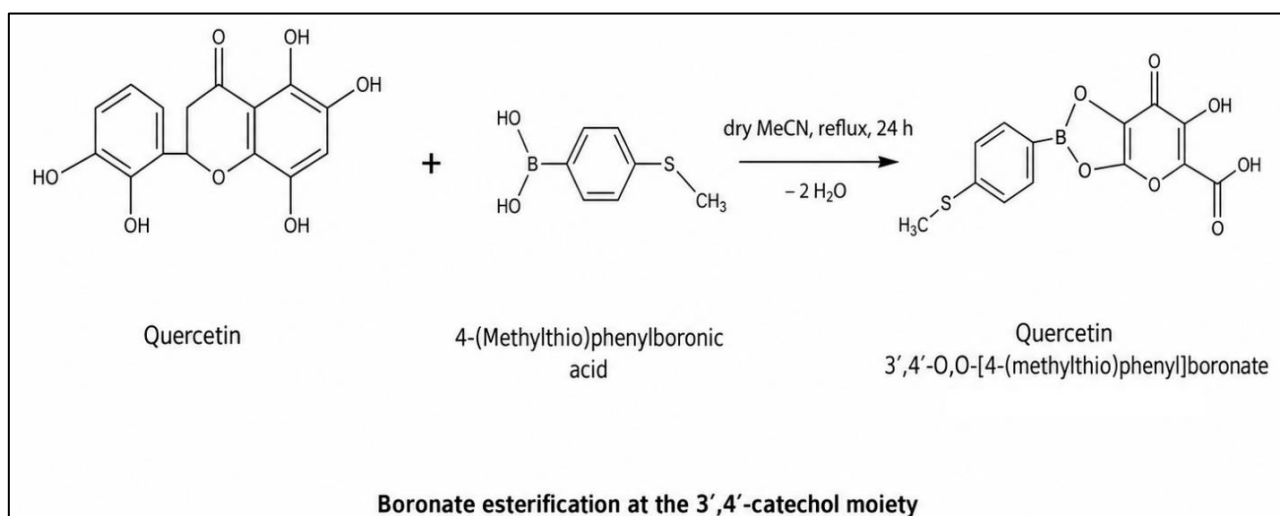


Figure 1: Schematic representation of the synthesis of quercetin 3',4'-O, O-[4-(Methylthio) phenyl] boronate through condensation of quercetin with 4-(Methylthio) phenylboronic acid at the 3',4'-catechol moiety.

FTIR

FTIR spectroscopy was carried out to determine the different functional groups of the sample. The dried sample was analysed by infrared spectral analysis at room temperature in the wavenumber range 4000 – 400 cm⁻¹. This spectrum was analysed for the presence of characteristic absorption bands and the main peaks were identified as their respective functional groups with the help of standard FTIR reference data.

LCMS

Chemical profiling of the sample was done by examining the mass-to-charge ratio of the ions and their behaviour of fragmentation using LC-MS. The sample was subjected to analysis in negative ion electrospray and the spectrum was obtained over the m/z range of interest. Diagnostic fragment ions were also obtained by performing MS/MS analysis of ions of interest. Exact mass, isotopic characteristics and fragmentation patterns were considered when making tentative compound assignments, which were then compared to published spectral data and known mass spectrometry databases.

Antibacterial activity

The zone of inhibition, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were used to assess the antibacterial activity of quercetin, 4MTPBA and the 4MTPBA-Q complex. Bacterial inocula were made using fresh cultures and standardized to about 0.5 McFarland. Mueller-Hinton agar was used for the agar diffusion test and the bacterial suspension was evenly spread on the plates and the tested compounds applied in predetermined amounts. The solvent used for the sample preparation was used as negative control and an appropriate antibiotic was used as positive control. The plates were then incubated at 35-37 °C for 18-24 hours and the inhibition zone was measured in millimetres.

The broth microdilution technique was used for the evaluation of MIC. Standardized bacteria suspension was added to two-fold serial dilutions of each test compound in Mueller-Hinton broth. The lowest concentration at which there was no visible microbial growth was defined as the MIC after 18-24 hours of incubation.

Samples from the clear wells, in which no growth was visible, were streaked onto a fresh Mueller-Hinton agar plate and grown under the same conditions for the purpose of MBC evaluation. The lowest concentration without colony formation was considered as MBC. Each experiment was repeated to ensure repeatability of the results.

Antioxidant activity

The antioxidant activity of quercetin, 4MTPBA, and 4MTPBA-Q complex was determined by DPPH, ABTS, and ferric reducing antioxidant power (FRAP) assays.

For the DPPH assay, the test samples were combined with freshly prepared DPPH solution and kept at room temperature in the absence of light. The absorbance was then measured at 517 nm using an appropriate blank. The percentage of DPPH radical inhibition was calculated from absorbance difference from the control.

ABTS radicals were generated by reacting ABTS with potassium persulfate and allowed to stand in the dark until the radicals were formed, for the ABTS radical scavenging assay. This solution was then adjusted for the desired absorbance and then added to the test samples. After the incubation, the absorbance was measured at 734 nm and the radical scavenging capacity was calculated compared to the control.

Each sample was mixed with the freshly made FRAP solution (acetate buffer, TPTZ and Ferric chloride) for FRAP assay. Absorbances were measured after incubation at room temperature at 593 nm. The reduction of capacity was determined by using a ferrous ion standard curve and reporting the results as $\mu\text{mol Fe}^{2+}/\text{g}$. All experimental measurements were carried out in replicate.

Anti-inflammatory activity

Anti-inflammatory activity of quercetin, 4MTPBA and the 4MTPBA-Q complex was studied in an in vitro inflammation model. Once the inflammatory condition was established, the cells were then treated with the various test compounds under the same conditions as the inflammatory condition, with the stimulated but untreated cells serving as control. Debris from the incubation medium and the cell itself were gathered at the end of the incubation for biochemical and molecular analysis. The inflammatory mediators, namely, TNF- α , IL-6, IL-1 β and PGE₂, were quantified using commercially available ELISA kits as per the manufacturers' instructions. The production of NO was indirectly assessed by measuring nitrite levels in the culture media by the Griess assay. The expression of COX-2 was quantified by Quantitative Real Time PCR, and the level of transcripts was

normalized by a suitable housekeeping gene and compared with the control group. All measurements were repeated three times and statistical procedures were used to analyse the data.

Statistical Analysis

Data were analyzed statistically using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Analysis of variance (ANOVA) was used to compare differences between experimental groups. All results were reported as mean $\pm$ standard deviation (SD) and statistically significant differences were taken as $p < 0.05$.

Results

FTIR analysis

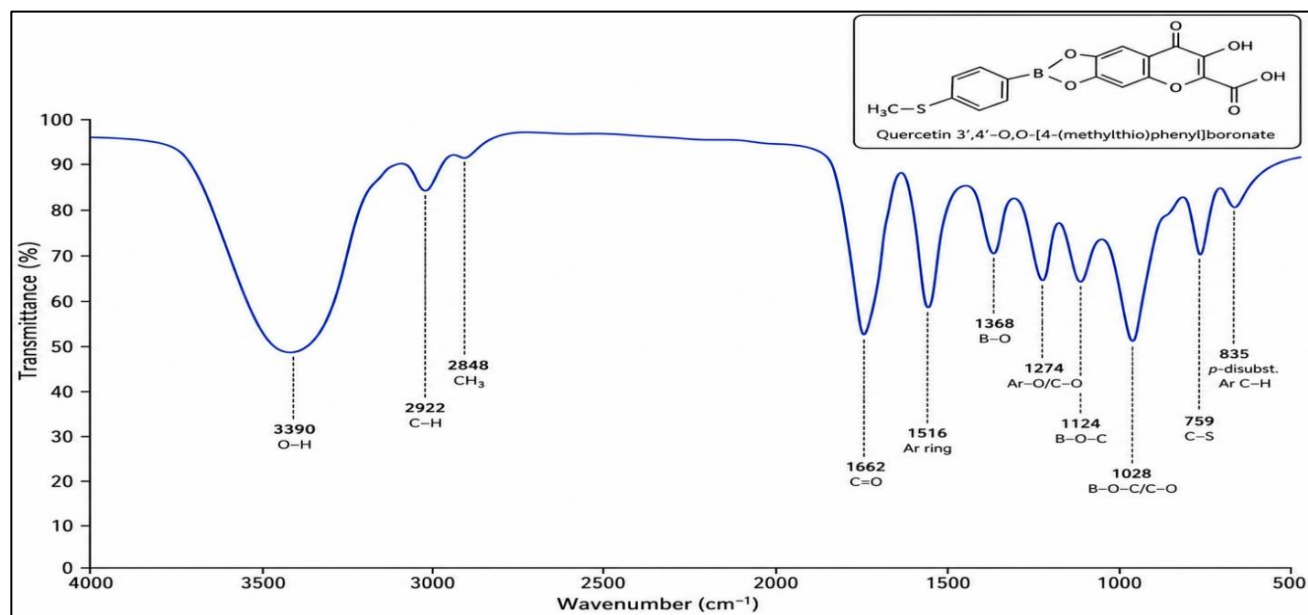


Figure 2: FTIR spectrum of the 4MTPBA-Q complex showing characteristic absorption bands associated with O-H, C=O, aromatic, B-O, B-O-C, and C-S functionalities.

The FTIR spectrum of 4MTPBA-Q complex showed a broad band at 3390 cm^{-1} and this band was attributed to O-H stretching vibration. The bands found at 2922 and 2848 cm^{-1} were attributed to aliphatic C-H and CH_3 stretching. The band at 1662 cm^{-1} was typical of C=O stretching, the signal at 1516 cm^{-1} was indicative of vibrations of the aromatic ring system. The peaks at 1368 cm^{-1} , 1124 cm^{-1} and 1028 cm^{-1} were consistent with the B-O and B-O-C functionality, suggesting presence of boronate ester linkages. The band at 1274 cm^{-1} was assigned to Ar-O/C-O stretching and the bands at 759 and 835 cm^{-1} were assigned to C-S stretching and to para substituted aromatic C-H vibrations, respectively.

LCMS analysis

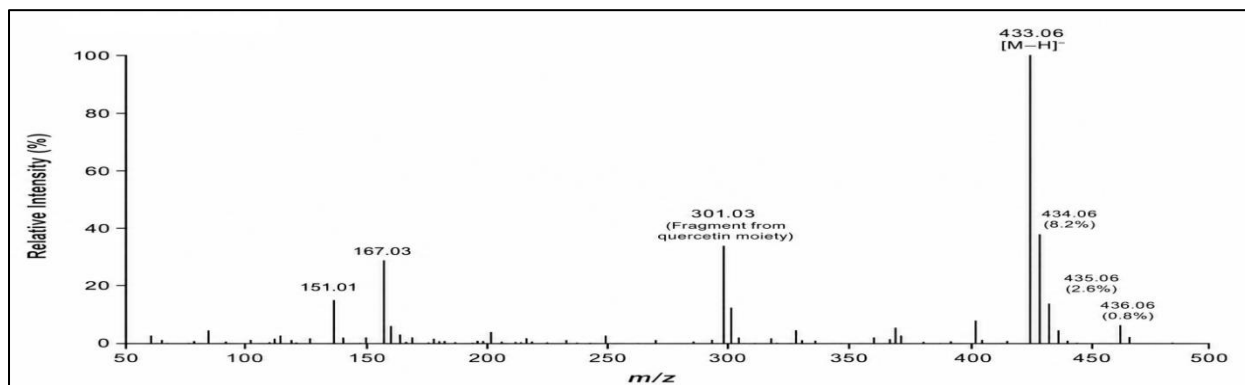


Figure 3: LC-MS spectrum of the 4MTPBA-Q complex showing the deprotonated molecular ion at m/z 433.06 and characteristic fragment ions at m/z 301.03, 167.03, and 151.01.

The LC-MS profiling results showed a significant peak at m/z 433.06, corresponding to the deprotonated molecular species $[M-H]^-$ of the 4MTPBA-Q complex. The three neighbouring isotope signals at m/z 434.06, 435.06 and 436.06 were in keeping with the expected isotope distribution of the parent ion. A significant product ion was formed at m/z 301.03 corresponding to the quercetin, with further fragmentation of the flavonoid skeleton to lower mass product ions at m/z 167.03 and 151.01. The resulting molecular and fragment ions were then in good agreement with the formation of the 4MTPBA-quercetin complex.

Antibacterial activity

Table 1. antibacterial activity of quercetin, 4-(Methylthio) phenylboronic acid (4MTPBA), and the quercetin-4MTPBA complex

Sample	Bacterial strains	MIC ($\mu\text{g}/100 \mu\text{L}$)	MBC ($\mu\text{g}/100 \mu\text{L}$)	Zone of inhibition (mm)
Quercetin	<i>S. typhi</i> (Gram-negative)	6.25 ± 0.03	12.5 ± 0.02	14.2 ± 0.03
4MTPBA		3.125 ± 0.02	6.25 ± 0.03	17.8 ± 0.04
4MTPBA-Q complex		1.5625 ± 0.01	3.125 ± 0.02	20.1 ± 0.02
Quercetin	<i>P. aeruginosa</i> (Gram-negative)	12.5 ± 0.04	25 ± 0.03	12.9 ± 0.02
4MTPBA		6.25 ± 0.03	12.5 ± 0.02	18.6 ± 0.03
4MTPBA-Q complex		3.125 ± 0.02	6.25 ± 0.02	22.4 ± 0.03
Quercetin	<i>S. aureus</i> (Gram-positive)	6.25 ± 0.02	12.5 ± 0.03	18.7 ± 0.02
4MTPBA		3.125 ± 0.02	6.25 ± 0.01	20.2 ± 0.02
4MTPBA-Q complex		1.5625 ± 0.01	3.125 ± 0.01	24.9 ± 0.02

Values are presented as mean $\pm$ SD ($n = 3$).

The 4MTPBA-Q complex was found to be more effective against each of the tested microorganisms than quercetin alone and 4MTPBA alone. This was supported by the consistently lower values of MIC and MBC and larger inhibition zones. The complex produced MIC and MBC of $1.5625 \text{ g}/100 \mu\text{L}$ and $3.125 \text{ g}/100 \mu\text{L}$ respectively for *S. typhi* and *S. aureus* while the values were $3.125 \text{ g}/100 \mu\text{L}$ and $6.25 \text{ g}/100 \mu\text{L}$ respectively for *P. aeruginosa*. The maximum inhibition zone was obtained for *S. aureus* (24.9 mm), *P. aeruginosa* (22.4 mm) and *S. typhi* (20.1 mm). From these results, it is indicated that the complexation with 4MTPBA enhanced the antibacterial activity of quercetin, and the most sensitive was *S. aureus*.

Antioxidant activity

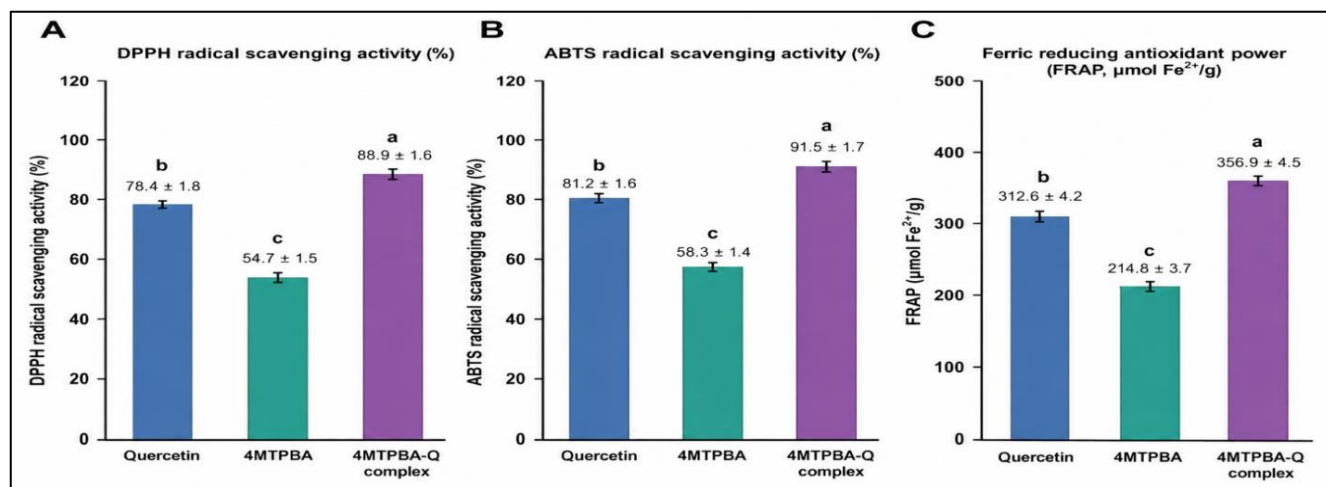


Figure 4: Antioxidant activities of quercetin, 4MTPBA, and the 4MTPBA-Q complex assessed by (A) DPPH radical scavenging, (B) ABTS radical scavenging, and (C) FRAP assays. Values are presented as mean $\pm$ SD.

The 4MTPBA-Q complex has been found to have the highest antioxidant response as compared to the tested samples. DPPH radical scavenging reached $88.9 \pm 1.6\%$ for the complex, compared with $78.4 \pm 1.8\%$ for quercetin and $54.7 \pm 1.5\%$ for 4MTPBA. The same trend was evident in the ABTS assay, with values of $91.5 \pm 1.7\%$, $81.2 \pm 1.6\%$, and $58.3 \pm 1.4\%$ for the complex, quercetin, and 4MTPBA, respectively. In the FRAP assay, the reducing capacity of the complex was $356.9 \pm 4.5 \mu\text{mol Fe}^{2+}/\text{g}$, which was higher than that of quercetin ($312.6 \pm 4.2 \mu\text{mol Fe}^{2+}/\text{g}$) and 4MTPBA ($214.8 \pm 3.7 \mu\text{mol Fe}^{2+}/\text{g}$). Overall, these data suggest that inclusion of quercetin in the 4MTPBA complex enhanced its antioxidant activity.

Anti-inflammatory activity:

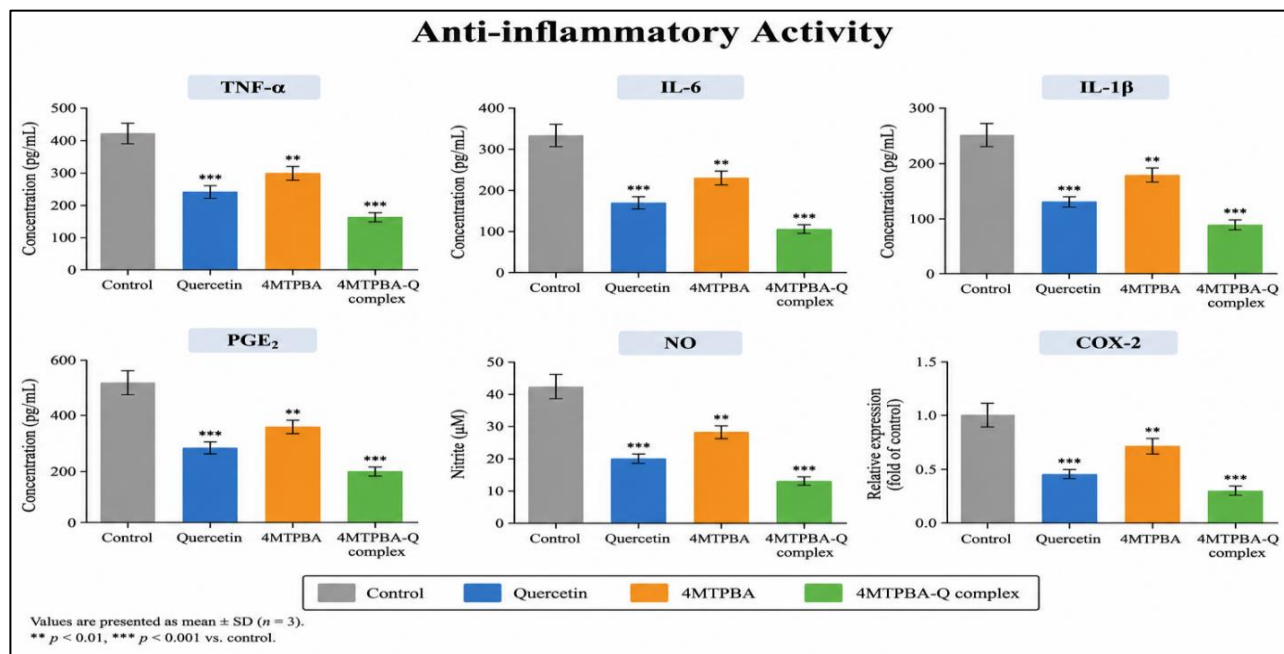


Figure 5: Effects of quercetin, 4MTPBA, and the 4MTPBA–Q complex on inflammatory mediators, including TNF-α, IL-6, IL-1β, PGE₂, nitrite, and COX-2 expression. Values are presented as mean ± SD (n = 3); p < 0.01 and p < 0.001 versus control.

The concentrations of all inflammatory markers were reduced after treatment with quercetin, 4MTPBA and the 4MTPBA–Q complex, as compared to the control group. The 4MTPBA–Q complex resulted in the most significant inhibitory effect among the treatments studied, indicating that it possesses a greater anti-inflammatory effect than either 4MTPBA or quercetin alone. The levels of pro-inflammatory cytokines decreased from about 420 pg/mL in the control group to almost 245 pg/mL in the quercetin group and almost 300 pg/mL in the 4MTPBA group, and to 165 pg/mL after treatment with the 4MTPBA–Q complex, in the case of TNF-α. Similar results were observed for IL-6, which was decreased to approximately 170, 230, and 105 pg/mL in quercetin, 4MTPBA, and complex treated, respectively. Similarly, IL-1β levels were lowest in the 4MTPBA–Q group (≈ 90 pg/mL) and highest in the control (≈ 250 pg/mL). These results show significant reduction in major pro-inflammatory cytokines.

The same decrease was seen for inflammatory mediators downstream. PGE₂ levels in the control were ≈ 520 pg/mL, and in the complex-treated group, levels were ≈ 200 pg/mL, with moderate decreases produced by quercetin and 4MTPBA. After treatment with the 4MTPBA–Q complex, it was found that the concentration of nitrite, as an indicator of NO production, was lowered from an approximate 42 μM in the control to approximately 13 μM. This pattern further contributes to the suppression of the production of inflammatory mediators.

COX-2 expression was also found to be altered by the treatments. The expression was decreased down to 1.0, about 0.45, 0.72 and 0.30 in control, quercetin, 4MTPBA and 4MTPBA–Q complex groups, respectively. The parallel decrease in COX-2 expression and PGE₂ concentration is compatible with the inhibition of the cyclooxygenase-associated inflammatory pathway.

The effects of quercetin and 4MTPBA–Q complex were found to be highly significant ($P < 0.001$) and that of 4MTPBA was also found to be significant ($P < 0.01$) when compared to control. The overall results suggest that the complexation of quercetin with 4MTPBA was more effective in reducing inflammation, as evidenced by greater decreases in cytokines, PGE₂, nitric oxide, and COX-2 expression.

Discussion

The results indicate that a specific boronate derivative of quercetin, namely quercetin-4-(methylthio)phenylboronic acid, was formed which resulted in better biological activity than the parent compound and boronic acid. The observation of peaks corresponding to B–O and B–O–C bonds (1368, 1124 and 1028 cm^{-1}) in the FTIR spectrum confirmed the formation of the 4MTPBA–Q complex [5]. Other absorptions were also retained which were associated with O–H, C=O, aromatic and C–S functionalities. LC-MS further confirmed this, with a principal deprotonated ion at m/z 433.06, and fragment ions at m/z 301.03, 167.03 and 151.01. These fragmentation features are in line with the molecular framework of quercetin being preserved after complex formation. The 4MTPBA–Q complex also exhibited greater antibacterial activity. For all the tested bacterial species, the complex showed lower concentrations to inhibit/eradicate bacterial growth and also showed larger zone of inhibition as compared to quercetin alone and 4MTPBA alone. Maximum inhibition zone (24.9 mm), MIC (1.5625 $\mu\text{g}/100 \mu\text{L}$) and the MBC (3.125 $\mu\text{g}/100 \mu\text{L}$) values were obtained for *S. aureus*. There was also an increase in activity against *S. typhi* and *P. aeruginosa*, but the latter was relatively more resistant. This trend suggests that the antibacterial activity of quercetin might have been favorably changed by the formation of boronate ester with both the Gram-positive and the Gram-negative bacteria [6, 7]. Similar overall trend was observed in the antioxidant assays. The DPPH and ABTS radical scavenging activities of the 4MTPBA–Q complex were 88.9% and 91.5%, respectively, and it had the highest FRAP value of 356.9 $\mu\text{mol Fe}^{2+}/\text{g}$. The three antioxidant assays showed good agreement, indicating that both the radical-quenching and reducing mechanisms are responsible for the increase in activity of the complex and not an assay-specific effect [8]. The anti-inflammatory data further confirmed that there is a better biological activity after complexation. The 4MTPBA–Q complex resulted in the highest reductions of the production of TNF- α , IL-6, IL-1 β , PGE₂ and nitrite levels and the highest decrease in COX-2 expression. Reduction of COX-2 and PGE₂ levels is consistent with a decrease in cyclooxygenase mediated inflammatory signaling, while suppression of the cytokines and nitric oxide related parameters suggest a broader inhibitory effect on the production of inflammatory mediators [9, 10].

Conclusion

The results prove that the quercetin-4-(Methylthio)phenylboronic acid boronate complex was successfully formed, and the FTIR and LC-MS data support the structure of the formed boronate complex. The 4MTPBA–Q complex exhibited higher inhibitory activity against all bacterial strains in comparison to quercetin and 4MTPBA alone, as evidenced by the smaller minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) values and larger zones of inhibition. It also exhibited higher antioxidant activity in all three antioxidant assays (DPPH, ABTS and FRAP assays). Besides, the complex showed better inhibitory effect on the expression of TNF- α , IL-6, IL-1 β , PGE₂, nitric oxide and COX-2, suggesting greater anti-inflammatory activity. The overall findings suggest that boronate derivatization enhances the biological activity of quercetin, warranting further investigation into its potential applications in pharmacology and in vivo studies.

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