Evaluation of Anti-Quorum Sensing Effect and Biological Activity studies of Novel Synthesized Boron Compounds As An Alternative Portential Against Microbial Resistance

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Abstract

Boronic acid compounds and natural flavonoid compound quercetin were handled to synthesize novel two ligands encoded as B1(2,2'-(1,4-phenylenebis (benzo [1,3,2] dioxaborole-2,5-diyl)) bis (3,5,7-trihydroxy-4H- chromen-4-one) and B2 (3.3.6. 3,5,7-trihydroxy-2-(2-(6-methoxypyridin-3-yl)benzo[d][1,3,2]dioxaborol-5-yl)-4H-chromene-4). Antioxidant activities of synthesized compounds were examined *in vitro*. Antioxidant features of B1 and B2 were investigated by 2,2-diphenyl picryl hydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and CUPRIC reducing antioxidant capacity (CUPRAC) methods. Anticholinesterase effects of ligands were determined by acetylcholinesterase and butyrylcholinesterase methods, cytotoxic effects of ligand (B2) were applied to healthy breast and colon cancer cell lines by MTT method, as well as urease and tyrosinase enzyme activities were determined. Moreover, antibacterial properties of the compounds were analyzed by detecting their anti-QS potentials on Chromobacterium violaceum biosensor system. Both compounds were found to have significant antioxidant effects compared to controls. It was determined that the compound B1 at 1-10 µg / mL was more active than the standards (α-TOC and BHT). It was determined from some enzyme activity studies of B1 and B2 compounds that acetylchoinesterase and butyrylcholinesterase enzyme inhibitions were higher than the standard used. In addition, in vitro antiquorum sensing and antibiofilm tests were performed. As expected, boron derivatives exhibited good activity against the biofilm of Escherichia coli (E. coli) and P. aeruginosa (P. aeruginosa). Biofilm analysis proved the inhibitory effect of boron derivatives by disrupting biofilm formation, reducing the thickness of biofilms and the number of viable bacteria. These results demonstrate the potential applicability of boron derivatives in the treatment of biofilm-associated infections and provide a practical strategy for the design of new boron-based antimicrobial materials. It was determined that compound B2 did not show any toxic effect on living cell lines and breast and colon cancer cell lines.

Introduction

Boron (B) is a trace element with atomic number 5 found in soil, water and air (Bingham 1983; Loomis and Durst 1992). B is considered as an element having semiconductivity between metal and nonmetal, havin more than 230 mineral derivatives (Borokhov and Schubert 2007; Renaud et al. 2007). Studies to determine its toxicity have shown that it beneficially affects the effectiveness of biological functions on humans, plants and animals. Boron compounds provide this activity by binding to the cis-hydroxyl groups in the cell membrane (Flores-Parra and Contreras 2000; Benderdour et al. 2000). Late researches suggest that trace mineral B has vital roles in human metabolism and health (Benderdour et al. 1998); mainly it reduces levels of inflammatory biomarkers (Nikkhah et al. 2015), greatly improves wound healing; is essential for the growth and maintenance of bone; beneficially impacts the body’s use of estrogen, testosterone, and vitamin D (Pizzorno 2015); boosts magnesium absorption (Penland 1994, 1996); improves the cognitive performance, and short-term memory for elders; reduces the levels of tumor necrosis factor α (TNF-α) (Armstrong and Spears 2003). Moreover, derivatives of B has demonstrated preventive and therapeutic effects in a number of cancers, such as prostate, cervical, and lung cancers,

and multiple and non-Hodgkin's lymphoma (Das et al. 2013); raises levels of antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (Türkez et al. 2007; Bhasker et al. 2016). Its effectiveness on infectious agents affecting agriculture and human health has not been fully elucidated. It is suggested that the antimicrobial activity of boron compounds is achieved by disrupting the activities of "serine-protease", "β-lactamase" and "amino-acyl tRNA synthetase" enzymes in microorganisms (Baker et al. 2009; Adriztina et al. 2018; Jakubczak et al. 2021). In recent years, there has been an increase in the frequency of infections caused by Enterobacteriaceae isolates producing extended-spectrum beta-lactamase (ESBL) (Machado et al. 2007). Therefore, infections caused by ESBL positive isolates, increased treatment failure and higher mortality rates come to the fore (Menashe et al. 2001). There is a need for agents that can replace or increase the effectiveness of synthetic drugs used in clinical practice (Lavigne et al. 2004). Since the isolates of Enterobacteriaceae, for which resistance problems arise, are also resistant to many other antibiotics and are considered virulent pathogens, serious measures must be taken to prevent the spread of these microorganisms.

Antimicrobial resistance is increasing dangerously due to the increasing use of various antibiotics in the community, the increase in the number of patients with impaired immune systems, the increase in intensive care units, and the use of antibiotics in the food industry. The main cause of antibiotic resistance is the communication of bacteria with each other (Dadgostar 2019). This situation is in parallel with the ability of microorganisms to form biofilms (Cepas et al. 2019). Known as a bacterial communication mechanism, quorum sensing was first discovered in the early 90s by bioluminescence created by a marine bacterium (Schauder and Bassler 2001; Miller and Bassler 2002; Camilli and Bassler 2006). The discovery that pathogenic bacteria regulate the production of pathogenicity and virulence factors by the QS mechanism has made this mechanism an attractive target for the treatment of infectious diseases (Bassler and Losick 2006). Targeting the pathogenesis instead of killing bacteria, blocking the QS mechanism using bacterial communication inhibitors is seen as a great source of hope for fighting bacterial resistance problems and bacterial infections. In addition, natural compounds such as boron compounds that block this mechanism suggest that they could potentially be used in combination with conventional antibiotics to increase the effectiveness of disease control and extend the life of existing antimicrobials (De Kievit and Iglewski 2000). Future studies will open the possibility of using QS inhibitors to treat bacterial infections in animals and humans while reducing the use of antibiotics that cause resistance.

Those recent findings reveal that there is an enormous deal of health benefits and also unexplored potential in medicinal aspects of B and Boron-including compounds. Therefore, in this study, newly synthesized Boron and natural flavonoid quercetin containing ligands (B1 and B2) were investigated for their antioxidant capacity via DPPH, CUPRAC, ABTS. The anticholinesterase effects of ligands are determined by acetylcholinesterase and butyrylcholinesterase methods. Cytotoxic studies were applied to healthy, breast and colon cancer cell lines and determined by MTT method. In addition, urease and tyrosinase enzyme activities were also determined. Various studies have been conducted on the antibacterial activities of some boron compounds, but the effect of these compounds on the bacterial communication system has not been demonstrated. Therefore, in this study, the anti-quorum sensing

activity of quercetin-based B1 and B2 ligands of newly synthesized Boron compounds was investigated. In this context, although there are studies on antioxidant and antibacterial effects of B-including compounds, this will be the first study investigating the activities of plant originated flavonoids and B including synthesized compounds.

Material And Methods

In this study, two types of new boron-phenyl including compounds were synthesized with the inclusion of quercetin, a flavonoid compound naturally found in plants. The structures of the synthesized compounds were illuminated by various spectroscopic methods such as Fourier Transform Infrared (FTIR) spectroscopy, Ultra Violet Visible (UV Vis) spectroscopy, Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) and Liquid Chromatography-Mass Spectroscopy (LC-MS). All the reagents and chemicals were supplied by Sigma-Aldrich (Steinheim, Germany) and Fluka (St. Gallen, Switzerland). ^{1}H and ^{13}C NMR spectrums were recorded on an Agilent 600 MHz spectrometer. FTIR spectra were recorded on an attenuated total reflectance (ATR) apparatus on a Perkin Elmer Spectrum 100 Fourier transform spectrophotometer. LC-MS analyses were performed by a Shimadzu LC/MS 8040 instrument (Kyoto, Japan). Melting points were measured by a Barnstead Electrothermal 9100 (San Francisco, USA).

1.1. Methods

Synthesis of B1 (2,2'-(1,4-phenylenebis (benzo [1,3,2] dioxaborole-2,5-diyl)) bis (3,5,7-trihydroxy-4H-chromen-4-one).

Quercetin (2 mmol, 0.604 g) was dissolved in 25 mL THF and refluxed at 120 ^{o}C. After about 10 minutes, 1 mmol 1,4-phenyl diboronic acid (0.165 g) of solution in 15 mL THF was added to this mixture and reflux was continued for 24 hours. The solid product (B1) was precipitated after removal of the solvent and water, which was washed with ethanol and water and then dried in an oven (Figure 1.).

Melting point: >350 ^{o}C. ^{1}H NMR (ppm, EtOH-d_1): δ = 9.30, 10.74, 12.47 (Ar-OH), δ = 6.17-7.73 (Ar-H). ^{13}C NMR (ppm, EtOH- d_1): δ = 94, 97, 104, 155, 115-146 (Ar-C), δ = 175 (C=O), δ = 136, 161, 164 (C-OH), δ = 132 (Ar-C-B). FT-IR (cm^{-1}) : 1355-1316 υ(BO), 1043, 1008 υ(B-C), 879υ(B-Ph), 1160 υ(Ar-O), 2959 υ(Aliph. C-H), 1649 υ(C=O), 1597 υ(C=C). UV-Vis (nm) : λ_1 = 256, λ_2 = 302, λ_3 = 371, λ_4 = 390. m/z: 697 [B1-H^+] $C_{36}H_{20}O_{14}B_2$ (MW: 698.16 g/mol).

Synthesis of B2 (3,5,7-trihydroxy-2-(2-(6-methoxynaphthalen-2-yl)benzo[d][1,3,2]dioxaborol-5-yl)-4H-chromene-4-one).

Quercetin (1 mmol, 0.302 g) was dissolved in 25 mL THF and refluxed at 120 ^{o}C. After about 10 minutes, 1 mmol (0.153 g) of 6-methoxy-3-pyridinylboronic acid solution in 15 mL THF was added to this mixture and reflux was continued for 24 hours. The solid product (B2) was precipitated after removal of the solvent and water, which was washed with ethanol and water and then dried in an oven (Figure 2.).

Melting point: 180 ^{o}C. ^{13}C NMR (ppm, EtOH-d1): δ = 94, 99, 102, 154, 110-148 (Ar-C), δ = 177 (C=O), δ = 134, 160, 163 (C-OH), δ = 122 (Ar-C-B), δ = 25(O-CH_3). FT-IR (cm^{-1}) : 1368-1316 υ(BO), 1021 υ(B-C), 811 υ(B-Ph), 1162 υ(Ar-O), 2953 υ(aliph. C-H), 1639 υ(C=O), 1590 υ(C=C). UV-Vis (nm) : λ_1 = 216, λ_2 = 223, λ_3 = 228, λ_4 = 257, λ_5 = 303, λ_6 = 368, λ_7 = 374, λ8=387. m/z: 418 [B2-H^+] $C_{21}H_{14}O_8NB$ (MW: 419.09 g/mol). ^{1}H NMR could not be obtained due to the low solubility of the B2 ligand.

1.2. Antioxidant activity

Antioxidant activities of novel boron compounds synthesized were determined by three methods, DPPH (Diphenylpicrylhydrazyl), ABTS (2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and CUPRAC (Cupric Reducing Antioxidant Capacity). DPPH is commonly used as reference for the evaluation of molecules, which are able to act as radical scavengers in the antioxidant activity experiments. 10 mg of each compound was dissolved in 10 mL of absolute ethanol to prepare the stock solutions. 2, 5, 10 and 20 µL of stock solutions were diluted into 40 µL with the absolute ethanol. Then 160 µL of DPPH solutions were added into each well in the micro plate. After incubation under dark condition at room temperature, the absorbances were measured at 517 nm. Free radical scavenging activities (% Inhibition) were calculated according to equation 1:

% Inhibition = (Acontrol – Asample) / Acontrol x 100

where A is the absorbance.

Each of the samples was applied three times to verify the results. α-Toc and BHT were also used as standard compounds. ABTS is an assay depending on the radical cation decolorization. In this assay, the addition of antioxidants into the solution including radical cation form of the ABTS converted this compound back into the neutral form in a variety of time-sclae. The different antioxidant efficiencies of boronic compounds were responsible for this issue. The degree of decolorization as percentage inhibition of ABTS•+ radical cation is obtained as a function of time and concentration, and calculated relative to the activity of α-Toc and BHT. The preparation of stock solutions was carried out by dissolving 10 mg of each compound in 10 mL absolute ethanol. 2, 5, 10 and 20 µL of stock solutions were diluted into 40 µL with the absolute ethanol. Then 160 µL of ABTS solutions were added into each well in the micro plate. After keeping them in tightened place for 6 min in the absence of sunlight at room temperature, the absorbances were measured at 734 nm. ABTS radical cation decolorization activities as % Inhibition were determined by using the equation 2:

% Inhibition = (Acontrol – Asample) / Acontrol x 100

where A is the absorbance.

CUPRAC method comprises the reduction of Cu(II)-neocuproine into its colored form Cu(I)-Neocuproine chelate in the presence of antioxidant compounds. The absorbance at 450 nm was measured when the complex was obtained. Cu(II), neocuproine and NH_4OAc were added into the prepared solutions to adjust

the as concentrations of 10, 25, 50, 100 µg/mL. The absorbance values were compared with the standard molecules α-Toc and BHT. Each of samples was applied three times to verify the results.

1.3. Anticholinesterase Activity Determination Method

Ellman method, which is a colorimetric method in which thiocholine reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as a result of the decomposition of Acetylcholine into thiocholine by AChE, yields the yellow 5-thio-2-nitrobenzoate anion, was carried out in 96-well microplates(Ellman et al. 1961).

1.4. AChE Activity Test

In this test, acetylcholinesterase is used as the enzyme and acetylthiocholine iodide is used as the substrate. The concentration of the 5-thio-2-nitrobenzoate anion is measured at 412 nm with a microplate reader.

130 µL of phosphate buffer (pH = 8), 10 µL of 4000 ppm solutions of boron compounds prepared using ethanol, and 20 µL of enzyme solution were added to the wells of the plate. This solution was incubated at 25ºC for 10 minutes. After 10 minutes, 20 µL of DTNB reagent and substrate (acetylthiocholine iodide) were added. Galantamine was used as standard. The plate was placed in the ELISA device and its absorbance was read at a wavelength of 412 nm. AChE % inhibition activity was calculated with the following equation.

% Inhibition = (Acontrol – Sample) / Acontrol x 100

Three parallel runs were performed for each sample.

1.5. BChE Activity Test

The method used in the AChE activity test was used. Differently, butyrylcholinesterase obtained from horse serum was used as enzyme and butyrylthiocholine iodide was used as substrate. BChE % inhibition activity was calculated with the following equation.

% Inhibition = (Acontrol – Sample) / Acontrol x 100

Three parallel runs were performed for each sample.

1.6. Antiurease Activity Test

In this test method, urease is used as an enzyme and urea is used as a substrate (Zahid et al. 2015). Firstly, 10 µL of boron compounds in 4000 ppm concentration solution prepared in ethanol and 25 µL of enzyme solution were added to the wells of the plate, then 50 µL of substrate (urea) was added. The first absorbance was read at 630 nm wavelength by placing the plate on the ELISA reader. This solution was incubated at 30ºC for 15 minutes. At the end of the period, 45 µL of phenol reagent and 70 µL of alkaline reagent were placed on this solution. After 20 minutes of incubation, a second reading was taken at 630 nm and absorbance was read. Tyrosinase % inhibition activity was calculated with the following equation. Thiourea was used as standard.

% Inhibition = (Acontrol – Sample) / Acontrol x 100

Three parallel runs were performed for each sample.

1.7. Antityrosinase Activity Test

In this test method, tyrosinase is used as the enzyme and L-DOPA is used as the substrate (Hearing and Jiménez 1987). 150 µL of phosphate buffer (pH = 6.8), 10 µL of 4000 ppm solutions of boron compounds prepared in ethanol and 20 µL of enzyme solution were added to the wells on the plate. The first absorbance was read at 475 nm wavelength by mixing the plate for 3 minutes in the ELISA reader. This solution was incubated at 37°C for 10 minutes. At the end of the period, 20 µL of substrate (L-DOPA) was added. The absorbance was read by making a second reading at 475 nm wavelength by incubating again at 37ºC for 10 minutes. Tyrosinase % inhibition activity was calculated with the following equation. Kojic acid was used as a standard.

% Inhibition= (Acontrol – Sample) / Acontrol x 100

1.8. Bacterial Strains and Culture Conditions

The bacterial strains used in the study was C. violaceum ATCC 12472 and C. violaceum O26. The wild-type strain C. violaceum ATCC 12472 (CV12472) was used as biosensor strain for QS. CV12472 and CVO26 was a kind gift from Prof. Dr Robert J.C. McLean (University of Texas, TX, USA). Before the experiments, the C. violaceum strain was cultured at the appropriate temperature of 30°C and inoculated on Luria Bertani medium for 16 hours in a shaking oven. E. coli ATCC 25922 and P. aeruginosa ATCC 27853 strains were acquired from Department of Biology/Biotechnology of Hacettepe Unıversity respectively. Cell culture maintenance of these strains was carried out in

BHI medium at 37 °C. Colonies were counted as needed by serial decimal dilution incubation in sterile distilled water and at optimum temperature suitable for biofilm and for 48 hours for this bacteria.

1.9. Assessment of Anti-Quorum Sensing (Anti-QS) Activity

Anti-quorum sensing (anti-QS) activities was assessed as described elsewhere (Erdönmez et al. 2018). Five mL of warm molten Soft Top Agar (1.3 g agar, 2.0 g Tryptone, 1.0 g sodium chloride and 200 ml deionized water) were seeded with 100 µL of overnight CV026 culture and 20 µL of 100 µg/ml C6HSL as a source of external AHL (acyl homoserine lactone) was added. This preparation was mixed and poured onto the surface of an immediately solidified Luria Bertani Agar (LBA) plate. After the poured mixture solidified, wells containing boron compounds with a diameter of 5 mm were opened on each plate. Each well was prepared with a sub-MIC concentration of 20 µL. A white or cream colored halo around an activated purple disc indicated QSI, despite the production of AHL-activated violasin pigment by CV026 bacteria (Zahin et al. 2010; Kalia et al. 2018). Each experiment was repeated 3 times. The assay plates were incubated at 30°C for 3 days and then the diameters of the quorum sensing inhibition zones were measured.

1.10. Violacein Pigment Inhibition

Boron compounds were subjected to qualitative analysis to find their QSI (Quorum-sensing inhibition) potential against C. violaceum ATCC 12472. 24-h fresh culture of C. violaceum (10 µL), (adjusted to an OD of 0.4 at 600 nm).) were incubated by adding 100 µL of LB medium and lower MIC concentrations of boron compounds. These plates were incubated at 30°C for 24 hours, after which a reduction in violacein pigment production was observed. First, 200 µL of treated and untreated cultures were placed in an Eppendorf tube and lysed by addition of 200 µL of 10% SDS, vortexed for 5 s and incubated at room temperature for 5 min. Sub¬sequently, 900 µL of water-saturated butanol (50 mL n-bu¬tanol mixed with 10 mL distilled water) were added to the cell lysate, followed by vortexing for 5 s and centrifugation at 13 000×g for 5 min. The upper (butanol) phase containing the violacein was collected and the absorbance was read at 585 nm in UV-Vis spectrophotometer (Packiavathy et al. 2012; Erdönmez et al. 2018). Each experiment was performed in triplicate and the percent inhibition of violacein was calculated by the formula:

Violacein inhibition (%) = OD 585 control−OD585 sample × 100

OD 585 control

1.11. Biofilm formation evaluation

The experiment was performed by crystal violet staining (CV) using 96-well cell culture plates. E. coli and P. aeruginosa were grown in LB broth to 0.5 McFarland turbidity (bacterial concentration 1,2 × 108 CFU/mL). Next, the concentration of bacterial suspension was adjusted to approximately 106 CFU/mL in liquid MH medium. 200 µL of the diluted bacterial solution was added to the 96-well plates. After the addition the boron compounds, 100 µL of the culture were pipetted into the wells of the microtiter plates and the plates were incubated for 48h at 37°C. When the appropriate incubation time was reached, the 96-well plates were removed from the incubator. Bacterial culture from the 96-well plate was discarded. 250 µL of sterile phosphate buffer (PBS) was gently added to the wells, rinsed repeatedly 3 times and discarded. The 96-well plates were dried and fixed at 65°C for 1 hour. Then, 200 µL of 0.1% (v/v) CV solution was added to each well and staining was performed for 20 minutes. Plates were rinsed 3 times with PBS and dried at room temperature. Finally, 95% (v/v) ethanol (200 µL) was added to the wells and left for 5 minutes. The OD value at 595 nm was measured with a microplate reader. Inhibitor-mediated reduction of biofilm formation was assessed by comparing it to the control without the boron compounds and the standart antibiotic, ampicillin (2µg/ml), was also used as a positive control (Wagh et al. 2013; Erdönmez et al. 2017). The percentage inhibition of biofilm was calculated as:

Biofilm inhibition (%) = (Control OD595nm-Test OD595 nm/Control OD595 nm)x100

1.12. Investigation of the Effects of Compound B1 on Cell Proliferation

The effects of one of the synthesized compounds, B2, on the proliferation of cancer cells (MCF-7 and HT-29) and healthy cell line (PDF) were performed using the MTT Cell Proliferation Kit (Sigma) and following the company's instructions for use(Mojarrab et al. 2013). Briefly, 1x104 cells were placed in a 96-well

plate and incubated for 24 hours in a humid environment at 37 ºC, 5% CO_2 for the cells to adhere to the plate. After 24 hours, cells were treated with samples at various concentrations for 48 hours. After 48 hours of treatment, cells were incubated with 10 µL of MTT solution for 4 hours. After incubation, a dark blue formazan dye was formed. After washing the cells with the washing solution that came with the kit and keeping them in the dark at room temperature for 2 hours, the absorbance of the formazan dye at 570 nm was measured with a plate reader (Thermo/MultiscanGo). The MTT assay was performed in triplicate for each concentration and each MTT assay was repeated 3 times.

1.13. Statistical Analysis

All the experiments were performed in triplicate and repeated at least twice. All values are expressed as the mean ± SD. The differences between the control and test samples were analysed using t-test and one-way ANOVA. Differences at $p<0.05$ were considered statis¬tically significant.

Results And Discussion

2.1. Structure Identification of B1 and B2 Compounds

In the ^{1}H NMR spectrum of B1, singlet peaks appearing at δ = 9.30, 10.74 and 12.47 ppm are peaks belonging to the -OH group. Multiple peaks in the range δ = 6.17-7.73 ppm are Ar-H peaks. In the ^{13}C NMR of B1, the peaks at 94, 97, 104 and 155 ppm have aromatic carbons in ring A. Peaks between 115-146 ppm Ar-C carbons in the C ring. The carbonyl peak was observed at 175 ppm. C-OH peaks in ring A and B were 161-164, 136 ppm, respectively. The exact mass was found as m/z 697 corresponds to the B1 structure with [M-H+] at negative scan by liquid chromatography mass spectrometry with ion trap time of flight (LC-MS-IT-TOF). In the FT-IR spectrum, 1355-1316 cm^{-1} B-O stretching, 1043 cm^{-1} and 1008 cm^{-1} B-C vibration, 879 cm-1 B-Ph vibration and Ar-O stretching vibration at 1160 cm^{-1} are observed. The vibration peaks seen in 1597 and 1649 cm^{-1} belong to the stretching of aromatic C = C and C = O, respectively. The free phenyl boronic acid peaks seen at 1089 cm^{-1} and 1071 cm^{-1} were slipped on the formed product and were seen at 1043 cm^{-1} and 1008 cm^{-1}. In the UV-Vis., the peaks observed at 256 and 302 nm belongs to the transitions of π →π* in the benzene ring. The peaks at 371 and 390 nm correspond to transitions in the n → π * free OH group.

In the ^{13}C NMR of B2, the peaks at 94, 99, 102 and 154 ppm have aromatic carbons in ring A. Peaks between 110-148 ppm Ar-C carbons in the C ring. To the carbon atom in the Ar-C-B structure at 122 ppm. The peak at 25 ppm belongs to the C atom in Ar-CH_3. The carbonyl peak was observed at 177 ppm. C-OH peaks in ring A and B were 160-163, 134 ppm, respectively. The exact mass was found as m/z 418 corresponds to the B2 structure with [M-H+] at negative scan by liquid chromatography mass spectrometry with ion trap time of flight (LC-MS-IT-TOF). In the FT-IR spectrum, 1316-1368 cm^{-1} B-O stretching, 1021 cm-1 B-C vibration, 811 cm-1 B-Ph vibration and Ar-O stretching vibration at 1162 cm^{-1} are observed. The vibration peaks seen in 1590 and 1639 cm^{-1} belong to the stretching of aromatic C = C

and C = O, respectively. The free phenyl boronic acid peaks seen at 1089 cm^{-1} and 1071 cm^{-1} were slipped on the formed product and was seen at 1021 cm^{-1}. In the UV-Vis., the peaks observed at 216-303 nm belongs to the transitions of π →π* in the benzene ring. The peaks at 368-387 nm correspond to transitions in the n → π * free OH group.

2.2. Antioxidant activity

ABTS cation radical scavenging activity; B1 compound was found to be more active at the studied concentrations (1-10 µg/mL) than α-TOC and BHT used as standard. In addition, B2 compound was determined to be more active at 5 and 10 µg/mL concentrations than the standards (Table 1.)

Table 1
ABTS cation radical scavenging activity

ABTS Radical Cation				
	Concentration (µg/mL)			
	1	2,5	5	10
B1	84,45±1,32	90,17±4,43	90,40±3,12	90,44±2,53
B2	28,06±0,93	78,06±2,58	87,31±0,58	88,19±0,20
α-TOC	76,47±0,35	77,42±0,61	78,84±1,01	79,32±0,89
BHT	82,59±0,86	83,81±0,96	84,58±1,03	86,13±0,97

DPPH in free radical scavenging activity; B1 compound was found to be more active at the studied concentrations (1-10 µg/mL) than α-TOC and BHT used as standard. However, B2 compound was determined to be active at concentrations of 1-5 µg/mL than the BHT standard (Table 2.)

Table 2
DPPH in free radical scavenging activity

DPPH Radical Free				
	Concentration (µg/mL)			
	1	2,5	5	10
B1	78,45±1,09	83,10±0,74	84,16±0,68	84,62±0,97
B2	21,75±0,3	29,27±0,6	51,41±0,2	-
α-TOC	32,36±0,25	40,26±1,32	57,85±2,38	65,41±0,98
BHT	18,85±0,26	24,01±0,14	36,82±0,65	63,58±0,61

In the CUPRAC method, it was determined that the B1 compound was more active than the standards at the concentrations studied. B2 compound was found to be less active than the standards (Table 3).

Table 3
CUPRAC method

CUPRAC				
	Concentration (µg/mL)			
	1	2,5	5	10
B1	0,431±0,005	0,678±0,006	0,965±0,028	1,570±0,038
B2	0,197±0,001	0,356±0,01	0,554±0,01	-
α-TOC	0,336±0,003	0,373±0,005	0,618±0,008	0,879±0,012
BHT	0,425±0,004	0,536±0,005	0,863±0,006	1,066±0,082

Table 5
Anticholinesterase and Butyrylcholinesterase Activity Results

Code	AChE (% inhibition)[a]	BChE (% inhibition)[a]
B1	79,14±2,25	114,57±6,76
B2	71,31±0,33	113,60±5,68
Galantamine	**61,03±1,46**	**59,51±1,16**
a: Values are given as the mean and standard deviation of 3 parallel measurements (100µg/mL).		
b: Standard item		

2.3. Anticholinesterase and Butyrylcholinesterase Activity Results

It was determined that the acetylcholinesterase and butyrylcholinesterase enzyme inhibition activities of B1 and B2 compounds were higher than galantamine used as a standard (Table 4.).

2.4. Antiurease and Antityrosinase Activity Results

It was determined that the urease enzyme inhibition of B1 of the compounds was close, although lower than the standard, but the tyrosinase enzyme inhibition was lower than the standard. It was determined that the urease and tyrosinase enzyme inhibitions of the B2 compound were lower than the standard (Table 6.)

Table 6
Antiurease and Antityrosinase Activity Results

Code	urease (% inhibition)[a]	Tyrosinase (% inhibition)[a]
B1	88,37±2,32	75,63±2,21
B2	28,81±0,40	29,05±0,36
Thiourea[b]	**97,46±2,01**	
Kojic Asid[c]		**75,79±0,96**

a: Values are given as the mean and standard deviation of 3 parallel measurements (100 µg/mL).

b: Standard substance (for urease)

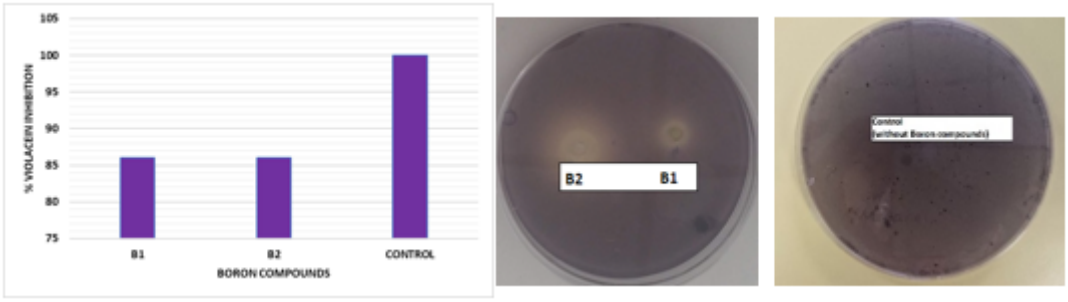
c: Standard substance (for Tyrosinase) A.D. = Inactive.

2.5. Antiquorum sensing activity

Both of the compounds, B1 and B2, exhibited 12.0±1.0 and 14.3±0.6 mm violacein inhibition zones, respectively. They also significantly ($p<0.05$) reduced the violacein pigment production (86.05±1.10% and 86.42±1.01%, respectively) and demonstrated powerful QS inhibition on CV12472 and cv026 biosensor system. B2 exhibited more powerful anti-QS effect than B1. The qualitative and quantitative results of the anti-quorum sensing potentials of the boron compounds, B1 and B2, exhibited in Table 7. as an inhibition zone (mm) and % inhibition amounts of violacein pigment production.

Table 7
Anti-quorum sensing potentials of the boron compounds

Boron Compounds (4mg/ml)	Inhibition Zone (mm)	Inhibition (%)
B1	**12.0±1.0**	**86.05±1.10***
B2	**14.3±0.6**	**86.42±1.01***



***:p <0.05 (statistically significant different from control)**

The effect of boron compounds on the biofilm formed by E. coli and P. aeruginosa was determined by CV staining. E. coli and P. aeruginosa were cultured in 96-well plates containing LB liquid medium for biofilm.

Figure .. First, OD595 and biofilm formation showed the same increasing trends and then decreased. The OD595 value reached its maximum at the end of 48 hours, and it was determined that this period was the time period when biofilm formation was strongest. After this period, the biofilm layer gradually decreased. Increasing the culture time may limit the nutrient content. The solution and bacteria begin to disperse at a later stage, so biofilm growth stops. biofilm begins to degenerate. In our study, the biofilm formation process of gram-negative bacteria progressed steadily from initial adhesion to maturity and then growth.

Boron derivatives can effectively inhibit the growth of planktonic bacteria E. coli and P. aeruginosa and even kill them. However, studies have shown that the dense and sheltered environment in which bacteria produce biofilms can protect them from the effects of antibacterial agents. Therefore, we further examined the effects of boron derivatives on E. coli and P. aeruginosa biofilms. All biofilms grown for 48 hours were stained with crystal violet. Biofilm layers formed spectrophotometrically were detected. Figure 3. revealed the results of the samples affecting the biofilms of E. coli and P. aeruginosa. Significantly, it was observed that the b1 boron compound was more effective on the biofilm formation of E. coli. It was determined that B2 boron compound was more effective on E.coli than the biofilm formed by P.aeruginosa.

2.6. Cytotoxicity results of compound B2

Toxic effects of compound B2 on healthy cell line (PDF), cytotoxic effects on cancerous MCF-7 (breast carcinoma) and HT-29 (colon carcinoma) were determined by MTT method. It has been determined that B2 does not have a toxic effect on living cells and cancer cells.

Conclusions

The high antioxidant activity of the new boronate ester compounds obtained from the study has revealed that such compounds can be used in medicine and cosmetics. The present study reveals that the boron compounds may become new AQS agents to control bacterial infection. However, more detailed studies need to be performed to show anti-QS activities of these boron compounds. In addition, antiquorum sensing and antibiofilm effects of boron derivatives may differ in gram negative pathogenic bacteria. The antibiofilm effect of B2 against E. coli was more pronounced than against P. aeruginosa. Based on the above results, boron derivatives can be considered as a potential new antibacterial agent in the treatment of biofilm-associated infections. The use of boron derivatives helps expand understanding of antibacterial agents in the food and pharmaceutical fields.

Declarations

Acknowledgments

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Tables

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Figures

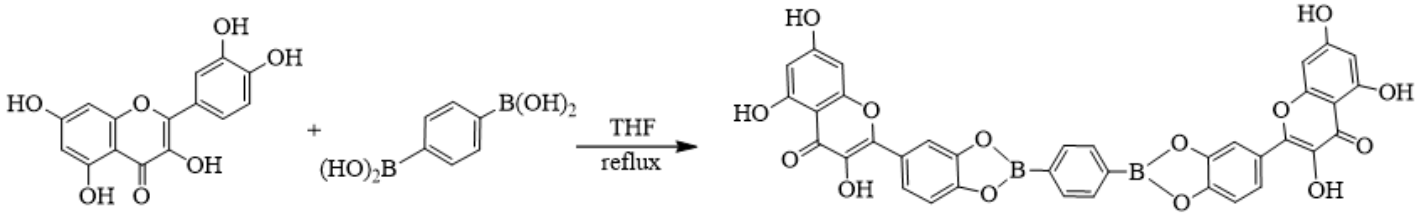

Figure 1

Synthesis of B1

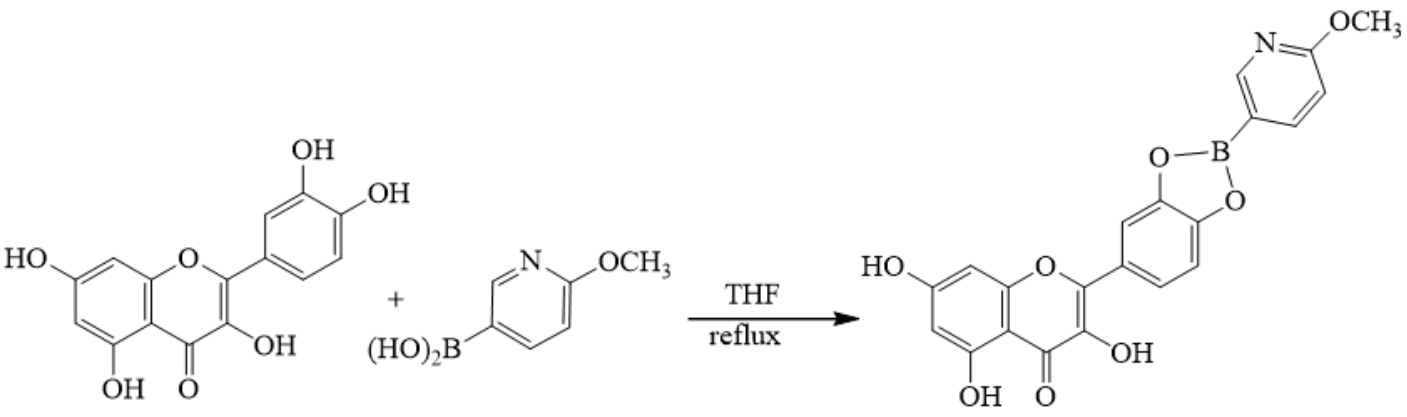

Figure 2

Synthesis of B2

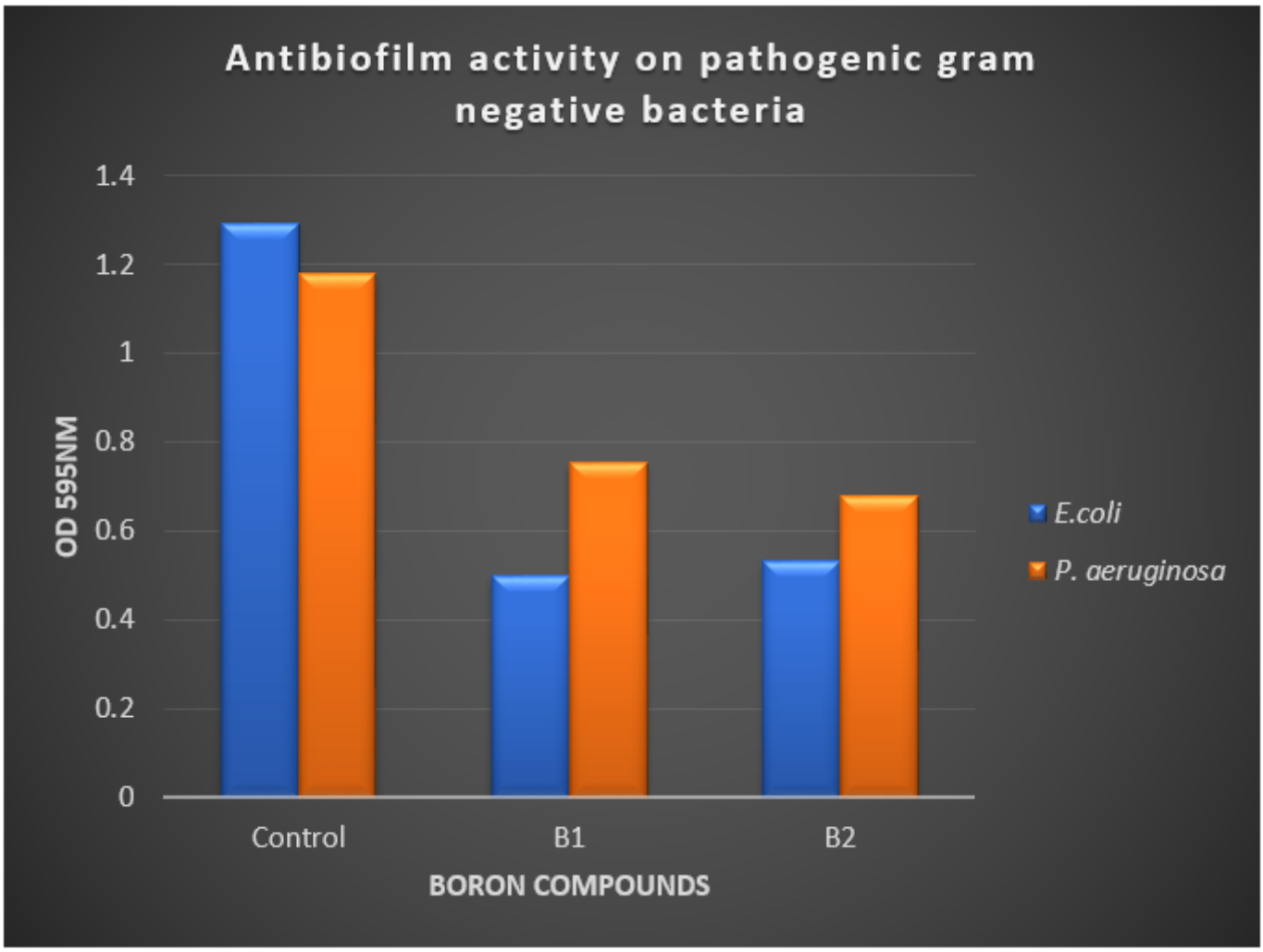

Figure 3

Antibiofilm activity on pathonergic gram negative bacteria

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