TENMAK BOREN BOR ARAŞTIRMA ENSTİTÜSÜ

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JOURNAL OF BORON
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Evaluation of 2-formylphenylboronic and 3-chlorophenylboronic acid derivatives for *in vitro* cytotoxicity and cell migration

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ARTICLE INFO

Article history:
Received May 31, 2024
Accepted November 13, 2024
Available online December 31, 2024

Research Article

DOI: 10.30728/boron.1493431

Keywords:
2-formylphenylboronic acid
3-chlorophenylboronic acid
In vitro cytotoxicity
Wound healing

ABSTRACT

Wound treatment and skin regeneration are complex processes, and non-healing wounds pose a major socioeconomic burden in terms of health. Effective and alternative approaches are needed for successful wound management. Although boronic acid derivatives have been reported to have positive and strong effects on wound healing, phenyl-substituted boronic acid derivatives can be used as more regenerative and effective compounds in healing. In this study, the *in vitro* cytotoxic effects of 2-formylphenylboronic acid and 3-chlorophenylboronic acid on L929 fibroblast cell lines were investigated using WST-8 analysis, and their wound healing effects were investigated by cell migration test. Our data reveal concentration-dependent effects of both boronic acid derivatives. For 2-formylphenylboronic acid, dosage applications between 3.90-31.25 µg/ml showed a viability of 84% and above, and at higher concentrations, the viability was found to be 5-10%. For 3-chlorophenylboronic acid, a viability rate of 64-109% is observed as a result of dosage applications between 3.90-250 µg/ml, while the percentage of viability decreases to 17% at a concentration of 500 µg/ml. Cell migration test data show that the effects of phenylboronic acid (PBA) derivatives in terms of cell migration increase as time increases, and the effect of 2-formylphenylboronic acid at the 24th hour is quite effective in terms of cell migration. Since the wound healing effect of PBA derivatives is concentration dependent, it should be taken into consideration that the use of high concentrations may be toxic.

1. Introduction

Wounds that disrupt the integrity of the skin and mucosa due to various factors can heal through a very complex process in the body. While factors such as age, nutrition, and smoking affect healing, infections caused by microorganisms in the wound area are also quite common. Nutrition of the scar tissue by ensuring perfusion and oxygen exchange in the wound area is a critical factor [1]. Therefore, wound management imposes a serious economic burden on healthcare systems and has dramatic effects on individuals with chronic, traumatic, or surgical wounds [2]. Various materials such as films, foams, hydrogels, and hydrocolloids are recommended for dressing purposes or as tissue adhesives in treatment, but problems such as poor biocompatibility or limited tissue adhesion have still not been fully overcome. New and effective alternatives that prevent wound healing are needed.

Boron is a trace element that is in group 3A of the periodic table and is considered a semi-metal and is found in nature as a compound with oxygen. While it is reported that boron is necessary in physiological events such as ensuring the rigidity of the cell wall in plants, pollination, growth, flowering, or seed formation, its roles in the human and animal bodies have not yet been fully clarified. Boron is found in a number of natural products isolated from bacteria and may show antibiotic activity. These natural products also support that boron can be tolerated in biological systems. Boron also attracts great attention in the field of health and has positive effects on cancer, bone health, wound healing, and the immune system. It is reported in the literature that boron plays a role in events such as ion transport, hormone production, calcium metabolism or bone development, and that it increases the healing rate in deep wounds and reduces the duration of stay in intensive care. It is also stated that boron derivatives increase keratinocyte migration and extracellular matrix turnover by increasing matrix metalloproteinase expression [3, 4]. Organic compounds containing boron moieties have a wide range of applications in synthetic organic chemistry. Boronic acid contains organic compounds containing a trivalent boron structure. One of them has an alkyl substitution and the other two have a hydroxyl group [5]. Boronic acids are not found in nature and are synthesized in the laboratory [6]. In addition to their high reactivity and stability, an important feature

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is that they have a very low toxicological profile [7]. They are also very popular because they are easy to synthesize and can be used in many chemical and biological reactions [8]. Boronic acids, with their 'saccharide binding' properties, attract great attention both in the research of biological systems and in the identification of metabolites in the pathology of diseases such as diabetes. Various studies state that they can bind to glycoproteins in the cell membrane, thus accelerating wound healing by proliferating cells such as lymphocytes. It is also known that boronic acid can bind to macromolecules containing cis-diol functional groups, thanks to its boronate ester rings. This situation causes boronic acid to covalently interact with components with cis-diol functionality, such as teichoic acid or lipopolysaccharide, found in the bacterial cell wall [9, 10]. Since phenyl-substituted boronic acid (PBA) derivatives exhibit interesting properties in terms of synthetic, application, and structural aspects, they are used in many areas such as sensors, receptors, polymers, drug active ingredients, cancer treatment, organic synthesis, and functionalized nanoparticles. There are many experimental and theoretical studies on the molecular structures and spectroscopic properties of PBA and its derivatives. Recent research reveals the important antibacterial and anticancer properties of PBA and its derivatives [5].

Formyl-substituted phenylboronic acids are noteworthy because they contain a reactive aldehyde group and can interact with the neighboring boronic unit in many ways thanks to the formyl group [11]. 2-formylphenylboronic acid shows many interesting properties in terms of synthetic, application, and structural aspects. Recent studies support the antimicrobial/antifungal activity of 2-formylphenylboronic acid and its anticancer or wound healing properties by adding it to the structure of various nanomaterials [12-14]. To our knowledge, there is no study yet on the biological effects of 3-chlorophenylboronic acid in the literature. In this study, the *in vitro* cytotoxicity of 2-formylphenylboronic acid and 3-chlorophenylboronic acid on L929 cell lines was evaluated and the effects of these components on cell migration were investigated in the light of the findings.

2. Materials and Methods

2.1. Cytotoxicity Test

The fibroblast L929 cell line of ATCC CCL-1 origin and passage number 16 was used in the study. Cells were grown in T25 flasks and EMEM medium containing 10% FBS (P30-1301, Pan Biotech, Germany) and checked twice a day. The cells were allowed to reach over 70 % confluency (Doubling time: 22-26 hours). After the samples were prepared at a concentration of 1 mg/mL, they were exposed to UV light for 30 minutes, dissolved in 100 µL DMSO and homogenized with the medium. Concentrations to be used later were diluted with complete medium. After adding 500 µL Trypsin-EDTA (15400054, Gibco, USA) to the cells that reached confluency, they were waited for 3-5 minutes at a 37°C and 5% CO_2 environment. The cells were examined under an inverted microscope (Zeiss Primovert, Germany), and when it was determined that they were dissociated, medium containing 10% FBS was added. It was centrifuged at 300xg for 5 minutes, the medium was discarded, and 1 mL EMEM medium was added onto the cells. Counting was done with trypan blue (1525061, Gibco, USA) on the Logos Luna II device (Logos Biosystems, South Korea), and 10% FBS medium was added to obtain 10^4 cells per well.

For the purpose of performing the cytotoxicity test, the medium in the culture plate was removed when 70% confluency was reached in each well. After the addition of fresh medium, samples were added to the cell dish. Three replicates were studied for each group. Eight different concentrations of both boronic acid derivatives were applied to the cells (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 µg/mL). Cells were kept in the incubator for 22 hours after exposure. Apart from the test samples, the so-called negative well contained only medium and cells (growth control). The positive well contained 2 µL H_2O_2 (a drug control with known toxicity) in addition to the medium and cells. Finally, blank wells were prepared by adding only the medium. At the end of 22 hours, 10% of the well volume (equal to 20 µL WST-8 in the experiment) of WST-8 solution was added. The cell culture container was wrapped with aluminum foil and kept in the incubator for another 2 hours, and at the end of 2 hours, spectrophotometric readings were taken at wavelengths of 450 nm and 630 nm. The results were formulated, and the % viability was determined (Equation 1):

$$\% \text{ Viability } = \frac{(\text{Sample Well} - \text{Blank})}{(\text{Negative Control} - \text{Blank})} \times 100 \quad (1)$$

2.2. In vitro Cell Migration Tests

In order to determine the cell migration of boronic acid cells and therefore the possibility of wounding, a wound healing model should be prepared by testing cell components, looking at L929, which is a fibroblast cell. The samples were added to 48-well tissue culture dishes in EMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 4 mM L-glutamine at a volume of $2x10^4$ cells/ml and kept until the grown monolayer was covered. Then, the surface was scratched from one end to the other in a single move with a sterile pipette tip (200 µl), and the *in vitro* wound model was recorded. To remove the shield protection during the scratching process, the upper medium was removed and washed with PBS. After the cytotoxicity experiment, effective substances containing boronic acid derivatives were applied at recorded doses and incubated for 24 hours at 37°C in a 5% CO_2 incubator. The negative control group was also included in the study, the created wound model was viewed on an inverted microscope (Zeiss Primovert, Germany) and

placed at hour 0 with at least two images from the well. Likewise, the samples were digitally photographed at the 12th and 24th hours to integrate the necessary programming. The captured images were analyzed using the Image J image analysis program. The entire area and wound areas of this intended program were calculated. The wound closure rate was calculated with the following formula (Equation 2):

$$\text{Wound closure rate} = [\frac{\text{Area}_{t0} - \text{Area}_{t24}}{\text{Area}_{t0}}] \times 100 \quad (2)$$

Here, Area_{t0} refers to the measured area of the photo taken at the beginning, and Area_{t24} refers to the area of the photo taken at the 12th-24th hour.

2.3. Statistical Analyses

All experiments were independently repeated three times, and the data were recorded as the mean value with standard deviation. In order to determine the significant differences among ANOVA, Tukey honest significance test (HSD) test, and single direction variance analysis. The wound closure rate between treatment groups and control groups was analyzed using the SPSS26 package (USA). All data analyses were assessed based on 0.05 and 0.01 significance.

3. Results

According to 24-hour WST-8 cytotoxicity analysis data, it was determined that both phenylboronic acid derivatives used in the study were not toxic to the cells. Table 1 and Figure 1 express the percentage viability of 2-formylphenylboronic acid on L929 cells. When the percentage viability values observed in the cells according to the concentrations studied were examined, the percentage viability rates were quite low at the concentrations of 2-formylphenylboronic acid applied between 500 and 62.5 µg/mL and were approximately 5-10%, but a significant increase in viability began to be observed at the concentration applied at 31.25 µg/mL ($p < 0.05$). This rate was found to be approximately 90.9%. In line with the data obtained at lower doses, the highest survival percentage was obtained at a concentration of 7.81 µg/mL, approximately 115%, and this concentration was chosen to be used in the wound healing study ($p < 0.05$).

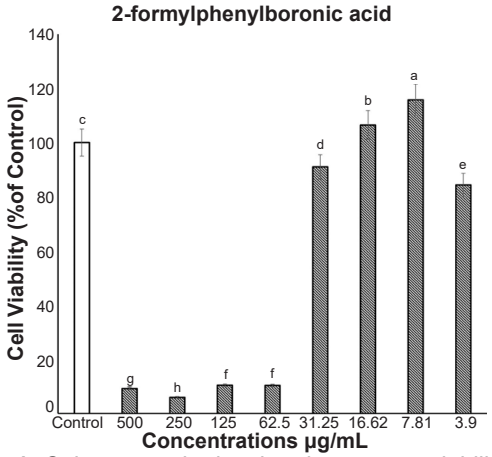

Figure 1. Column graph showing the percent viability rates as a result of applying 2-formylphenylboronic acid at concentration rates of 500-3.90 µg/mL on L929 fibroblast cells. Different lowercase letters indicate that the concentrations for each 2-formylphenylboronic acid were significantly different from each other according to Tukey's HSD test ($p < 0.05$)

Table 2 and Figure 2 express the percentage viability rates of 3-chlorophenylboronic acid on L929 cells. When the percentage viability values observed in the cells according to the concentrations studied

Table 1. Absorbance values (ABS), mean percent viability (MEAN), and percent standard deviation (STD) of viability values (24 hours) obtained as a result of applying 2-formylphenylboronic acid at concentration rates of 500-3.90 µg/mL on L929 fibroblast cells ($p < 0.05$)

Concentrations	ABS			MEAN	STD	Tukey's HSD test
500 µg/mL	0.372	0.394	0.405	9.219	1.482	g
250 µg/mL	0.360	0.366	0.354	5.942	0.529	h
125 µg/mL	0.384	0.420	0.400	10.407	1.591	f
62.5 µg/mL	0.390	0.404	0.408	10.335	0.834	f
31.25 µg/mL	1.232	1.110	1.100	90.997	6.483	d
15.62 µg/mL	1.311	1.321	1.240	106.482	3.895	b
7.81 µg/mL	1.438	1.401	1.292	115.808	6.695	a
3.90 µg/mL	1.107	1.059	1.092	84.372	2.166	e
POZ	0.531	0.507	0.528	23.443	1.153	
NEG	1.235	1.202	1.255	100	2.361	c
BLANK	0.309	0.294	0.312	0	0.851	

(*Different lowercase letters indicate that the concentrations for each 2-formylphenylboronic acid were significantly different from each other according to Tukey's HSD test.)

Table 2. Absorbance values (ABS), mean percent viability (MEAN), and percent standard deviation of viability values (24 hours) obtained as a result of applying 3-chlorophenylboronic acid at concentration rates of 500-3.90 µg/mL on L929 fibroblast cells ($p < 0.05$)

Concentrations	ABS			MEAN	STD	Tukey's HSD test
500 µg/mL	0.457	0.478	0.467	17.537	0.927	c
250 µg/mL	0.801	1.007	0.904	64.710	9.085	b, c
125 µg/mL	0.896	0.904	1.055	69.860	7.901	a, b, c
62.5 µg/mL	1.072	1.270	1.171	93.554	8.732	a, b, c
31.25 µg/mL	1.141	1.274	1.208	97.515	5.866	a, b
15.62 µg/mL	1.304	1.215	1.428	109.183	9.436	a
7.81 µg/mL	1.298	1.145	1.321	102.593	8.439	a
3.90 µg/mL	0.953	0.908	0.996	69.932	3.881	a, b, c
POZ	0.531	0.507	0.528	23.443	1.153	
NEG	1.235	1.202	1.255	100	2.361	a, b
BLANK	0.309	0.294	0.312	0	0.851	

(*Different lowercase letters indicate that the concentrations for each 2-formylphenylboronic acid were significantly different from each other according to Tukey's HSD test.)

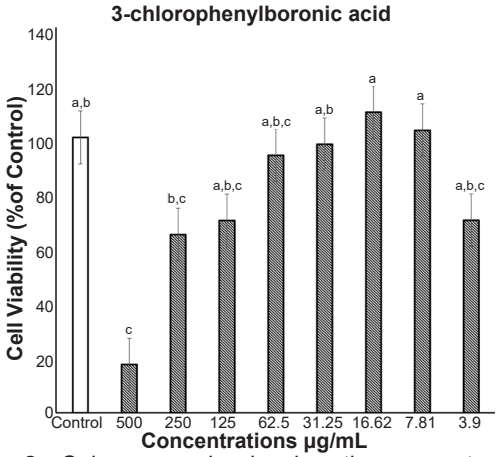

Figure 2. Column graph showing the percent viability rates as a result of applying 3-chlorophenylboronic acid at concentration rates of 500-3.90 µg/mL on L929 fibroblast cells. Different lowercase letters indicate that the concentrations for each 3-chlorophenylboronic acid were significantly different from each other according to Tukey's HSD test ($p < 0.05$)

were examined, when 3-chlorophenylboronic acid was applied at a concentration of 500 µg/mL, the percentage viability rate was quite low (17.5%), but a significant increase in viability was observed at the concentration applied at 250 µg/mL, and this rate was approximately 64% ($p < 0.05$). The concentration with a significant increase in viability was determined as 62.5 µg/mL (93%). The concentration that showed the highest cell viability at lower concentrations was 15.62 µg/mL (109%), and this concentration was chosen to be used in the wound healing study ($p < 0.05$).

In the cell stratch test, cell images obtained with an inverted microscope as a result of the 0th, 12th, and 24th hour applications of both boronic acid derivatives are presented in Figure 3, and the percent wound closure rates are presented in Figure 4. According to the data obtained, it was observed that there was a cell migration of 7.04 inches at the 0th hour, 6.81 inches at the 12th hour, and 6.55 inches at the 24th hour in the control

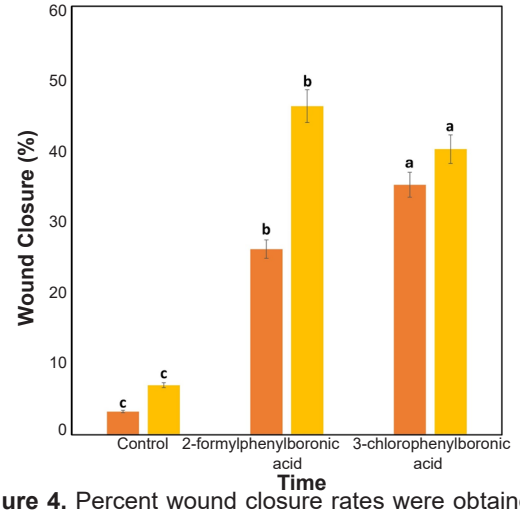

Figure 4. Percent wound closure rates were obtained as a result of 12, and 24 hour application of 2-formylphenylboronic acid (7.81 µg/mL) and 3-chlorophenylboronic acid (15.62 µg/mL) on L929 cell lines by cell stratch test. Different lowercase letters indicate that the concentrations for each 2-formylphenylboronic acid and 3-chlorophenylboronic acid were significantly different from each other according to Tukey's HSD test ($p < 0.05$)

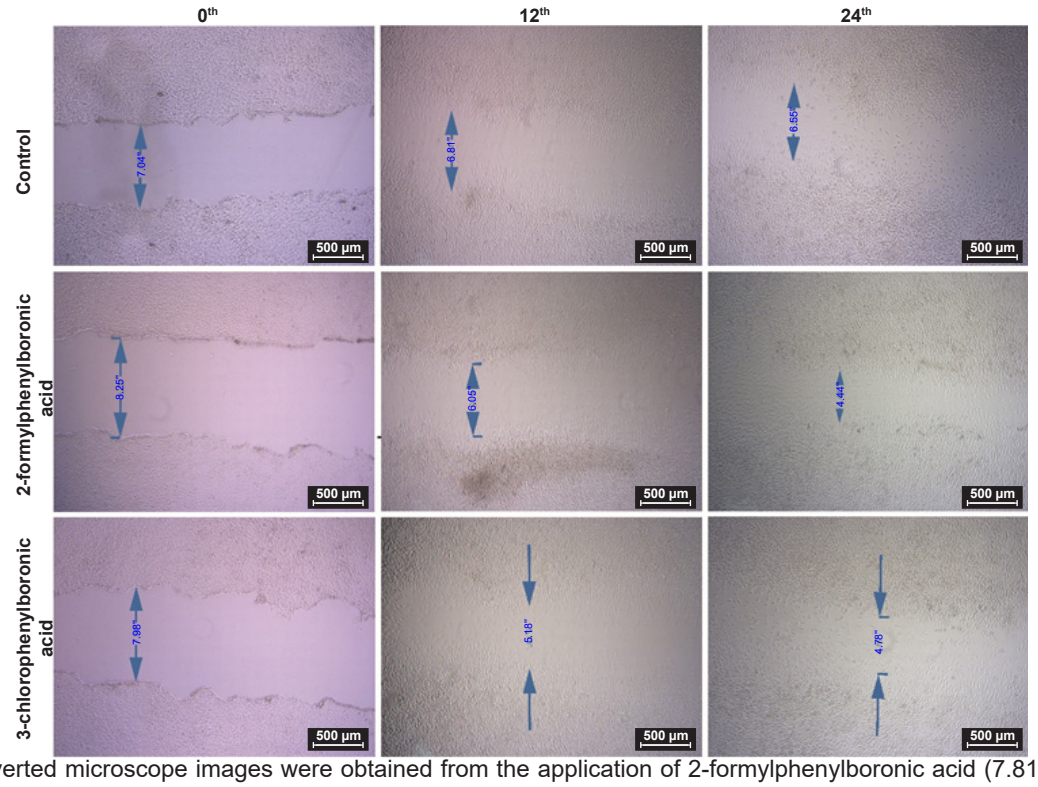

Figure 3. Inverted microscope images were obtained from the application of 2-formylphenylboronic acid (7.81 µg/mL) and 3-chlorophenylboronic acid (15.62 µg/mL) for 0, 12, and 24 hours using the cell stratch test on L929 fibroblast cell lines (Scale bar: 500 µm)

group. In the 2-formylphenylboronic acid-applied group, there was a cell migration of 8.25 inches at the 0th hour, 6.05 inches at the 12th hour, and 4.44 inches at the 24th hour, and this result was higher than the effect seen in the control. When evaluated statistically, there was a significant difference in wound healing between the control group and the group receiving 7.81 µg/mL 2-formylphenyl boronic acid in the 12 and 24 hour periods (P<0.01). In the 3-chlorophenylboronic acid-applied group, it was observed that there was a cell migration of 7.98 inches at the 0th hour, 5.18 inches at the 12th hour, and 4.76 inches at the 24th hour. This group also had a stronger effect compared to the control, but it was observed that there was a similar but slower effective cell migration to the 2-formylphenylboronic acid-applied group. When evaluated statistically, there was a significant difference in wound healing between the control group and the group receiving 15.62 µg/mL 3-chlorophenylboronic acid in the 12 and 24 hour periods (P<0.01).

In line with the data presented in Figure 4, a wound closure rate of 3.36% at the 12th hour and 6.96% at the 24th hour was achieved in the control group. In the 2-formylphenylboronic acid-applied group, there was a wound closure of 26% at the 12th hour and 46% at the 24th hour. As a result of the application of 3-chlorophenylboronic acid, a wound closure of 35% was detected at the 12th hour and 40% at the 24th hour. At the same time, with the multiple comparison test, there are significant differences between the groups, namely between the control and 2-formylphenylboronic acid, between 2-formylphenylboronic acid and 3-chlorophenylboronic acid, and between 3-chlorophenylboronic and the control group (P<0.01).

4. Discussion

Boron is absorbed into the human body in the form of boric acid and circulates. It has been reported in the literature that boric acid is protective against lung cancer, and some *in vitro* studies have reported that it reduces cell proliferation, migration, and invasion in tumors such as melanoma, prostate, breast cancer, colon cancer, and hepatocellular carcinoma [15-18]. In a previous study, the effects of boric acid on human non-small cell lung cancer cells (A549) were investigated through the TGF-β signaling pathway. The authors investigated cytotoxicity analysis with the MTT test, apoptosis test with Annexin V/PI and immunofluorescence analyses, and expression levels of TGF-β and SMAD2/3/4 genes with real-time polymerase chain reaction (RT-qPCR) and found that boric acid has both anti-proliferative and anti-proliferative properties. They also showed that it has anti-apoptotic activity [19].

Boronic acids, a group of boron compounds, are very popular with their properties, such as being stable, non-toxic, and easy to synthesize, and they can be used in many chemical and biological reactions [8]. They are of great importance due to their high reactivity and stability and are used in many areas such as sensors, receptors, polymers, drug active ingredients, cancer treatment, organic synthesis, and functionalized nanoparticles. Research on boronic acid today covers new compound classes and various application areas of these compounds. The tetrahedral boron atom geometry in boronic acid is very similar to the enzyme-catalyzed substrate tetrahedral transition state. This has enabled the biological activities of boron-containing compounds to be intensively examined [20]. Substituents in the phenylboronic acid structure greatly affect the molecular and chemical structure of the molecule, and therefore its properties [21]. It is reported in the literature that phenylboronic acid is much more potent than boric acid in targeting the metastatic and proliferative properties of cancer cells. Additionally, boronic acid and its esters are thought to have no intrinsic toxicity problems. However, it is still unknown how PBA inhibits cell growth *in vitro* and *in vivo* [22, 23]. Studies showing the potential effects of phenylboronic acid derivatives on wound healing are quite limited and detailed studies are needed. This study covers the evaluation of the *in vitro* cytotoxicity and effects of two different boronic acid derivatives (2-formylphenylboronic acid and 3-chlorophenylboronic acid) on cell migration.

Our cytotoxicity test data support that both phenylboronic acids used are not toxic to the L929 cell line, but high concentrations lead to a decrease in viability. In their study examining the cytotoxic activity of phenyl boroxine acid, Marasovic and colleagues used mouse mammary adenocarcinoma 4T1, mouse squamous cell carcinoma SCCVII, hamster lung fibroblast V79, and mouse dermal fibroblasts L929 cell lines. The cytotoxic effects on the tested tumor and non-tumor cell lines showed dose-dependent changes, among the concentrations studied (0.1, 1.0, and 10 mg/ml), a PBA dose of 10 mg/ml significantly reduced the survival of the cells compared to the control group [24]. In our study, much lower PBA concentrations were used (500-3.90 µg/ml), and the lowest viability observed concentration was determined as 500 µg/ml.

The majority of studies on the biological properties of phenylboronic acid in the literature include their antimicrobial and antifungal activities and their incorporation into various biopolymers, hydrogels or nanomaterial structures to reduce cytotoxicity [24]. PBA derivatives have the ability to transform from hydrophobic form to hydrophilic form by adjusting pH and diol concentration. This feature offers the ability to apply in many different areas, such as diabetes treatment [25]. Abid and colleagues investigated the antibacterial and wound healing effects of Quercetin-4-formyl phenylboronic acid (4FPBA−Q) against bacterial pathogens responsible for diabetic foot ulcers. 4FPBA−Q showed a significant effect on both gram-positive and gram-negative bacteria. In the experimental model, wound healing was observed to be increased after 10 days in diabetic rats [26]. Similar studies show the antimicrobial effects of

formyl phenylboronic acid and that it is beneficial for diabetic foot ulcers due to the presence of two hydroxyl groups [10]. In another study, a new boron-based compound was synthesized using PBA and quercetin, and its antioxidant, antibacterial, antiurease, anticholine esterase, antithyrosinase, and anticancer properties were investigated. This process was then tested dermatologically and biologically in an *in vivo* experiment to examine its effectiveness in cream formulation. The authors stated that the component synthesized using PBA-quercetin may have higher potential compared to the use of quercetin alone and suggested that the relevant component could be used in areas such as the food, pharmaceutical, or cosmetic industry [27].

Boron derivatives have important biological properties such as antimicrobial activity, keratinocyte migration, proliferation, vascularization, growth factor expression, and effectively accelerating the wound healing process [28]. It is also reported in the literature that boron derivatives improve extracellular matrix transformation, increase the release of proteoglycan, collagen, and proteins, which have important roles in the wound healing process, and also stimulate the synthesis of tumor necrosis factor (TNF-a) [29]. In a previous study, the effects of curcumin and PBA-linked hydrogel (GOHA-Cur) on diabetic wound healing were investigated, based on the dynamic interaction of hemoglobin and oxygen in red blood cells. *In vivo* data have shown that GOHA-Cur enhances wound healing by inhibiting inflammation [30]. Similarly, Demirci and colleagues developed a new antimicrobial carbopol-based hydrogel formulated with boron and pluronic block copolymers and examined the wound healing potential of this hydrogel on *in vitro* cell culture techniques and an experimental burn model. The authors reported that this formulation triggered wound healing through complex mechanisms by stimulating cell migration, growth factor expression, inflammatory response, and vascularization [16].

5. Conclusion

As a result, 2-formylphenylboronic acid and 3-chlorophenylboronic acid used in our study showed dose-dependent effects on L929 fibroblast cells and had a non-toxic effect unless very high concentrations were used. In fact, it has been observed that proliferation increases significantly at certain concentrations. In addition, according to the cell migration test results, which allow us to obtain rapid and preliminary information about the wound-closing feature, the effects of both PBA derivatives in terms of cell migration increased over time. In particular, the effect of 2-formylphenylboronic acid at the 24th hour was significantly increased compared to its effect at the 12th hour ($P<0.01$). The multiple comparison test showed that, there are significant differences between the groups, namely between the control and 2-formylphenylboronic acid, between 2-formylphenylboronic acid and 3-chlorophenylboronic acid, and between 3-chlorophenylboronic and the control group ($P<0.01$). The effect of 3-chlorophenylboronic acid also increased in a time-dependent manner ($P<0.01$), but the difference between the 12th and 24th hours was less than that observed in the 2-formylphenylboronic acid group. Our data support the concentration-dependent wound healing effects of both PBA derivatives. 2-formylphenylboronic acid exhibited more effective results in terms of cell migration, especially at the 24th hour, compared to 3-chlorophenylboronic acid. However, the concentration dose used here is extremely important, and if this dose is not taken into account, 2-formylphenylboronic acid may have a more toxic effect. Considering that boron is the most important strategic mineral of our country, the wound healing potential of boronic acid derivatives 2-formylphenylboronic acid and 3-chlorophenylboronic acid should be supported by detailed *in vitro* and *in vivo* studies. The data obtained may provide insight into the use of these components in the production of wound creams or dressings, but the possible toxic effects of high concentrations should also be carefully considered in the evaluation.

6. Acknowledgments

This work was studied at Eskisehir Osmangazi University Central Research Laboratory Application and Research Center (ARUM).

7. Authors' Contributions

Betül Yılmaz Öztürk: Conceptualization, data curation, formal analysis; methodology, writing-original draft, visualization, investigation, supervision, software, validation.

Bükay Yenice Gürsu: Writing-review and editing, supervision, project administration, formal analysis.

İlknur Dağ: Writing-original draft, conceptualization, formal analysis, methodology, supervision, writing-review and editing, resources.

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