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Evaluation of M86 and M87 compounds against lead, cadmium, and arsenic toxicity in 2D and 3D liver cell models

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ABSRACT

Increasing environmental pollution increases the risk of human exposure to toxic metals. Therefore, there is a need for substances to protect individuals against the harmful effects caused by toxic metals. This study investigates the 1:1 and 1:2 mole ratios of 3-methoxy catechol with 1,4-phenyl diboronic acid, resulting in the synthesis of 1,4-bis(4-methoxybenzo[d][1,3,2]dioxaborol-2-yl)phenyl)boronic acid (M86) and 1,4-bis(4-methoxybenzo[d][1,3,2]dioxaborol-2-yl)benzene (M87), and evaluates their protective effects against lead (Pb), cadmium (Cd), and arsenic (As) toxicity in the THLE-2 liver cell line. The structures of synthesized compounds M86 and M87 were characterized by 1 H, 13 C NMR, LC-MS-IT-TOF, UV-Vis., FTIR. The biological activities of these compounds were evaluated by DPPH, ABTS, CUPRAC, anticholinesterase, antiurease and antithyrosinase tests. 2D and 3D cell models were used in THLE-2 cell line. The protective effects of M86 and M87 against Pb, Cd and As toxicity were examined by XTT test and ATP colorimetric method and IC50 values were determined. In antioxidant tests, it was observed that M86 and M87 exhibited high activity in ABTS, DPPH and CUPRAC tests compared to standard antioxidants α-tocopherol (α-TOC) and butylated hydroxytoluene (BHT). Enzyme inhibition tests showed that M86 and M87 significantly suppressed acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme activities. These compounds were found to reverse the decrease in cell proliferation following Pb, Cd and As exposure. In conclusion, M86 and M87 have the potential to be versatile therapeutic agents that provide effective protection against metal toxicity. In the future, with the evaluation of the efficacy of these compounds in in vivo models and clinical studies, it is thought that their use against metal toxicity may be possible.

1. Introduction

With the increase in environmental pollution, pollutants have become a threat to human health. Human-induced activities, particularly industrialization, constitute one of the major causes of environmental pollution, leading to the release of heavy metals into environmental media such as air, water, and soil, thereby posing a significant threat to human health [1]. Heavy metals such as lead (Pb), cadmium (Cd), and arsenic (As) are among the most prominent environmental pollutants. The accumulation of these metals in ecosystems poses significant health risks to humans [2]. Pb exposure may lead to both acute and chronic toxic effects on the nervous, hepatic, hematologic, urinary, and cardiovascular systems. Clinically, Pb poisoning can result in various symptoms including mental dysfunction (e.g., memory loss), anemia, tubular atrophy, and even death in severe cases [3]. Cd exposure is also associated with a wide range of health effects, including osteoporosis, dental deterioration, and cancers such as lung and prostate cancer. Moreover, it may impair fetal development and reduce birth weight. Cd is classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC) due to its high toxicity [4–6]. As exposure is similarly dangerous, leading to conditions such as hyperpigmentation, keratosis, basal cell carcinoma, hepatomegaly, cirrhosis, cardiovascular complications, nephritis, urinary tract cancers, and a heightened risk of diabetes mellitus [7–15].

Chelation therapy is the standard treatment for heavy metal intoxication, aiming to promote the elimination of accumulated metals from the body. However, current chelating agents often show limited efficacy and can cause adverse effects. Agents like dithiocarbamates and

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monoisoamyl meso-2,3-dimercaptosuccinate (MiADMS) used in Cd poisoning may lead to nephrotoxicity and gastrointestinal disturbances [4,16]. Similarly, treatments for As poisoning such as meso-2, 3-dimercaptosuccinic acid, propane-1-sulfonate, and British Anti-Lewisite may induce hypertension, nausea, and elevated liver enzymes [17]. Ethylenediaminetetraacetic acid (EDTA) and dimercaptosuccinic acid (DMSA) chelating agents are widely used in the treatment of Pb poisoning [18–20]. EDTA can cause kidney toxicity and redistribution of Pb to the brain [18]. DMSA can cause side effects such as loss of appetite, nausea and diarrhea [21,22]. These limitations highlight the urgent need for safer and more effective alternatives.

Boronic acids, due to the vacant p-orbital of the boron atom, act as soft Lewis acids. This property makes them highly attractive as receptors, protecting groups, and biologically active compounds. Boronic acids have found broad applications in medicine, chemistry, biology, and materials science [23]. Catechol, an important precursor in pharmaceutical and food industries (e.g., in the synthesis of vanillin), has gained attention for the development of biologically active derivatives due to the physiological significance of its 3- and 4-substituted forms [24]. Recent studies have reported that boron-containing compounds exhibit antioxidant, antimicrobial, anticancer, and antiviral properties, contributing positively to human and animal health [25,26]. Despite growing interest, studies combining 3-methoxycatechol and boronic acid derivatives-especially in the context of antioxidant, anti-inflammatory, and metal toxicity-protective activities-remain limited and have primarily been carried out by our research group [27–29].

The selection of the boron-containing compounds M86 and M87 in this study was based on the promising biological potential of structurally related compounds reported in the literature. Boronic acids are known to exhibit significant pharmacological activities, including antioxidant, antimicrobial, anticancer, and enzyme-inhibitory effects. Recent studies have emphasized the growing importance of boron-containing heterocycles in medicinal chemistry due to their unique structural properties and favorable interactions with biological targets [30–32]. The specific molecular design of M86 and M87, which are derived from the reaction of 3-methoxycatechol and 1,4-phenyldiboronic acid, was aimed at enhancing antioxidant activity and enzyme inhibition potential. These activities are particularly relevant in the context of mitigating oxidative damage and enzymatic dysregulation caused by heavy metal toxicity.

Therefore, this study aims to explore the dual biological role of M86 and M87: (i) their antioxidant and enzyme-inhibitory activities, and (ii) their capacity to counteract lead (Pb), cadmium (Cd), and arsenic (As) toxicity in liver cells. The integration of both mechanistic pathways is expected to provide a more comprehensive evaluation of their hepatoprotective potential.

The two boron-containing compounds (M86 and M87) differ only in their stoichiometric synthesis: M86 was obtained with a 1:1 molar ratio of 3-methoxycatechol to 1,4-phenyldiboronic acid, whereas M87 was synthesized using a 1:2 ratio. This deliberate variation allows us to investigate how increasing the number of boronate moieties influences the biological activity. In particular, the study was designed to explore two complementary mechanisms: the antioxidant potential of the compounds in mitigating oxidative stress induced by heavy metals, and their enzyme-inhibitory effects, especially on AChE and BChE. These two pathways are closely interconnected in the context of metal-induced cellular damage, and their coupling may lead to synergistic hepatoprotective outcomes.

The liver, as a central organ in the body's detoxification processes, is particularly vulnerable to heavy metal toxicity. It plays a major role in biotransformation and elimination of toxic substances, making it a primary target for damage [33]. The hepatotoxic effects of metals such as Pb, Cd, and As are mainly driven by oxidative stress, which involves excessive generation of reactive oxygen species (ROS), lipid peroxidation, and damage to proteins and DNA damage [34]. In particular, metals such as Pb, Cd and As can cause severe hepatotoxic effects by inhibiting liver detoxification enzymes and impairing cellular functions [4,35]. The THLE-2 human liver cell line, which closely mimics human hepatocyte characteristics, serves as a reliable in vitro model for evaluating both the toxicity of metals and the protective efficacy of potential therapeutic compounds [3].

In this study, novel boron derivatives were synthesized by reacting 3-methoxycatechol, known for its strong antioxidant activity, with 1,4-phenyldiboronic acid in specific stoichiometric ratios. The resulting compounds 4-(4-methoxybenzo[*d*][1,3,2]dioxaborol-2-yl)phenyl) boronic acid (M86) and 1,4-bis(4-methoxybenzo[*d*][1,3,2]dioxaborol-2-yl)benzene (M87) were evaluated for their antioxidant capacity, enzyme inhibitory activity (anticholinesterase, antiurease, and antithyrosinase), and protective effects against Pb, Cd, and As toxicity.

2. Metarials and methods

2.1. Synthesis of M86 and M87

2.1.1. Synthesis of compound M86 (4-(4-(4-methoxybenzo[d][1,3,2] dioxaborole-2-yl)phenyl)boronic acid)

1 mmol of 3-methoxycatechol (0.140 g) was dissolved in 25 mL of THF and refluxed at 120 °C. After approximately 10 minutes, a solution of 1 mmol (0.165 g) of 1,4-phenylenediboronic acid in 15 mL of THF was added to the mixture. The reaction was refluxed for 24 hours. Afterward, the reaction mixture was evaporated to remove the solvent and water. The resulting precipitate was dissolved in ethanol and crystallized. The final product was collected by filtration and dried in an oven.

2.1.2. Synthesis of compound M87 (1,4-bis(4-methoxybenzo[e][1,3,2] dioxaborole-2-yl)benzene)

2 mmol 3-methoxy catechol (0.280 g) was dissolved in 25 mL THF and refluxed at 120 °C. After about 10 min, a solution of 1 mmol (0.165 g) 1,4-phenyl diboronic acid in 15 mL THF was added to this mixture. Reflux for 24 hours was continued. The reaction mixture was then evaporated to remove the solvent and water. The solid obtained was recrystallized by dissolving in ethanol and dried in an oven after filtration.

2.2. Determination of the biological activities of M86 and M87

2.2.1. DPPH free radical scavenging activity

The free radical scavenging activities of the synthesized boron-derived compounds were evaluated using DPPH by the method proposed by Blois (1958) and Boğa et al. (2016). Each sample was analyzed in triplicate. The standards used were α-Tocopherol (α-Toc) and BHT [36,37].

2.2.2. ABTS cation radical removal activity

The ABTS cation radical scavenging activity of 3-methoxy catecholated boronic acid derivatives was investigated using the method described by Re et al. (1999) [38]. All experiments were performed in triplicate. The standards used were α-Tocopherol (α-Toc) and BHT.

2.2.3. CUPRAC (Copper(II) Ion Reducing Antioxidant Capacity) test

According to the method of Apak et al. (2004), the absorbance at 450 nm of the Cu(I)-Nc complex, formed by reduction of the synthesized compounds with the copper(II)-neocuproine complex, was measured [39]. The results were evaluated in triplicate by comparison with the standards α-Tocopherol (α-Toc) and BHT.

2.2.4. Anticholinesterase activity

Colorimetric analysis of the product resulting from the breakdown of acetylcholine to thiocholine by the enzyme AChE was performed according to the method of Ellman et al. (1961) [40]. The assays were conducted using 96-well microplates.

2.2.5. Antiurease activity

The anti-urease activities of the compounds were determined using the urease enzyme and urea substrate, according to the method of Zahid et al. (2015) [41]. Each sample was tested in triplicate.

2.2.6. Antithyrosinase activity

The tyrosinase inhibitory activities of the compounds were evaluated spectrophotometrically using fungal tyrosinase, according to the method of Hearing and Jiménez (1987) [42]. Inhibitory kinetic constants were calculated using Lineweaver-Burk diagrams with various concentrations of compounds (5–50 μM). Absorbance measurements were recorded at 475 nm.

2.3. Determination of the efficacy of M86 and M87 on Pb, As and Cd exposure in THLE-2 cell line

2.3.1. Development of two-dimensional (2D) and three-dimensional (3D) cell lines

In this study, THLE-2 (Transformed Human Liver Epithelial-2) cells were used to investigate the potential toxicity of the compounds M86 and M87. THLE-2 cells were cultured in a two-dimensional (2D) format in 75 cm^2 flasks under specific conditions using Dulbecco's Modified Eagle Medium (DMEM, High Glucose) supplemented with 10 % fetal bovine serum (FBS), 1 % gentamicin, and 1 % penicillin, in an incubator with 5 % CO_2 at 37°C. When cells covered approximately 80 % of the flask surface, they were passaged using trypsin. Additionally, THLE-2 cells were cultured in a three-dimensional (3D) format as spheroids using the hanging drop method. This 3D culture technique was used to determine whether it elicited a different response to the administered compounds compared to 2D cultures, and to serve as an alternative approach to in vivo studies. The cells were maintained in an incubator with 5 % CO_2 at 37°C throughout the experiment.

2.3.2. Cytotoxicity assays

Pb, As, Cd, M86, and M87 were applied to all treatment groups for 24, 48 and 72 hours. The cytotoxic effects on the cells were evaluated using the XTT assay. Cells were seeded into 96-well plates at a density of 5000 cells per well. M86 and M87 were administered to the cells by adding serial dilutions of the prepared solutions (maximum dose: 1000 μM) to the wells. Subsequently, the XTT kit containing formazan dye was added to each well and allowed to incubate for 2–5 hours. Optical densities were then measured using a BIOTEK ELISA reader. Optical densities were measured using a BIOTEK ELISA reader, and the data were analyzed to determine the IC_{50} values for cells treated with M86 and M87. For 3D cell cultures, the Elabscience ATP Colorimetric Assay Kit (E-BC-K157-S) was used according to the manufacturer's instructions. Accordingly, the protective effects of M86 and M87 against Pb, Cd, and As toxicity were evaluated.

2.4. Statistical analysis

Statistical analysis of the data was performed using SPSS (Statistical Package for the Social Sciences) version 23.0. All data are presented as mean ± standard error (SE). The Kolmogorov-Smirnov test was used to assess the normality of the data distribution. For data that met the assumption of normality, one-way analysis of variance (ANOVA) was applied. In cases where significant differences were found between groups, Tukey's multiple comparison test was used for post hoc analysis. For data that did not meet the assumption of normality, the Kruskal-Wallis test was employed. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Synthesis of M86 and M87

Fig. 1 shows the synthesis schemes of compounds M86 (4-(4-methoxybenzo[*d*][1,3,2]dioxaaborol-2-yl)phenyl)boronic acid) and M87 (1,4-bis(4-methoxybenzo[*c*][1,3,2]dioxaaborol-2-yl)benzene) respectively.

3.2. Spectroscopic properties of compound M86

In the mass spectrum of compound M86, the molecular ion peak was observed at *m/z* 269 $[M86–H+]^-$ (MW: 270.09 g/mol), confirming the structural identity of the compound (Fig. 2a). The ^1H NMR spectrum showed aromatic proton (Ar–H) signals in the range of δ = 6.39–8.74 ppm, and a methoxy (–OCH_3) group signal at δ = 3.71 ppm (Fig. 2e). The $^{1\,3}C$ NMR spectrum revealed aromatic carbon (Ar–C) signals between δ = 103.78–148.80 ppm, a –OCH_3 group at δ = 55.98 ppm, and an Ar–C–B group at δ = 118.41 ppm (Fig. 2d). In the FT-IR spectrum, characteristic peaks were identified as follows: υ(B–O) at 1324–1365 cm^{-1} , υ(B–C) at 1013–1085 cm^{-1} , υ(B–Ph) at 769 cm^{-1} , υ(Ar–O) at 1172 cm^{-1} , and υ(C═C) at 1627 cm^{-1} (Fig. 2c). UV–Vis analysis showed absorbance peaks at 203 nm, 206 nm, 229 nm, and 273 nm (Fig. 2b).

3.3. Spectroscopic properties of compound M87

In the mass spectrum of compound M87, the molecular ion peak was observed at *m/z* 373 $[M87–H^+]^-$ (MW: 374.11 g/mol), confirming the structural identity of the compound (Fig. 3a). The ^1H NMR spectrum displayed aromatic proton (Ar–H) signals between δ = 6.38–7.72 ppm and methoxy (–OCH_3) group signals at δ = 3.71 and 3.78 ppm (Fig. 3d). The $^{1\,3}C$ NMR spectrum revealed aromatic carbon (Ar–C) signals at δ = 103.77–152.80 ppm, a –OCH_3 group signal at δ = 55.66 ppm, and an Ar–C–B group signal at δ = 118.80 ppm (Fig. 3e). In the FT-IR spectrum, characteristic bands were observed at υ(B–O) = 1322–1367 cm^{-1} , υ(B–C) = 1015–1070 cm^{-1} , υ(B–Ph) = 769 cm^{-1} , υ(Ar–O) = 1176 cm^{-1} , and υ(C═C) = 1628 cm^{-1} (Fig. 3c). UV–Vis analysis showed absorbance peaks at wavelengths of 203 nm, 209 nm, 228 nm, and 271 nm (Fig. 3b).

3.4. Biological properties of compounds M86 and M87

In the ABTS assay, both M86 and M87 showed high activity, especially at a concentration of 10 μg/mL, comparable to α-TOC and BHT. In the ABTS assay, M86 showed 89.04 ± 0.86 % and M87 showed 88.36 ± 0.19 % activity at 10 μg/mL, very close to the 86.13 ± 0.97 % activity of BHT. In the DPPH test, M86 and M87 showed 63.82 ± 2.64 % and 78.43 ± 0.59 % inhibition, respectively, while BHT showed 63.58 ± 0.61 % inhibition. M86 and M87 showed similar levels of activity with BHT in both tests ($p > 0.05$). In the CUPRAC test, M86 and M87 showed activity of 0.804 ± 0.02 and 1.671 ± 0.01 at 10 μg/mL, respectively. BHT had a value of 1.066 ± 0.082 and both compounds were observed to have high CUPRAC capacity. However, the CUPRAC value of M87 was found to be significantly higher than that of BHT ($p < 0.05$). IC_{50} and A0.5 values showed that the free radical scavenging capacity of M86 and M87 surpassed α-TOC and BHT. In the ABTS (IC_{50}) test, the values of 3.80 ± 0.08 μg/mL and 2.36 ± 0.06 μg/mL of M86 and M87, respectively, were much lower than the 62.17 ± 0.15 μg/mL of BHT ($p < 0.05$). In DPPH (IC_{50}) test, M86 and M87 showed IC50 values of 7.88 ± 0.08 μg/mL and 4.46 ± 0.13 μg/mL, respectively, indicating that they were more effective than BHT's 12.68 ± 0.17 μg/mL ($p < 0.05$). In enzyme inhibition tests, M86 showed 68.51 ± 1.12 % and 71.03 ± 1.63 % inhibition of AChE and BChE, respectively, while M87 showed 85.18 ± 3.64 % and 60.37 ± 0.76 % inhibition, respectively. These results revealed that M86 and M87 exhibited higher

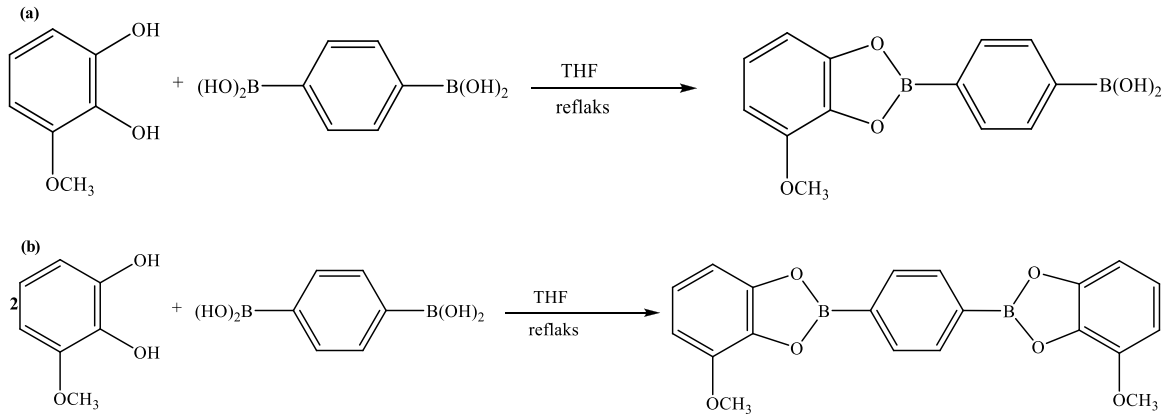

Fig. 1. Synthesis scheme of M86 and M87 (a) M86, (b) M87.

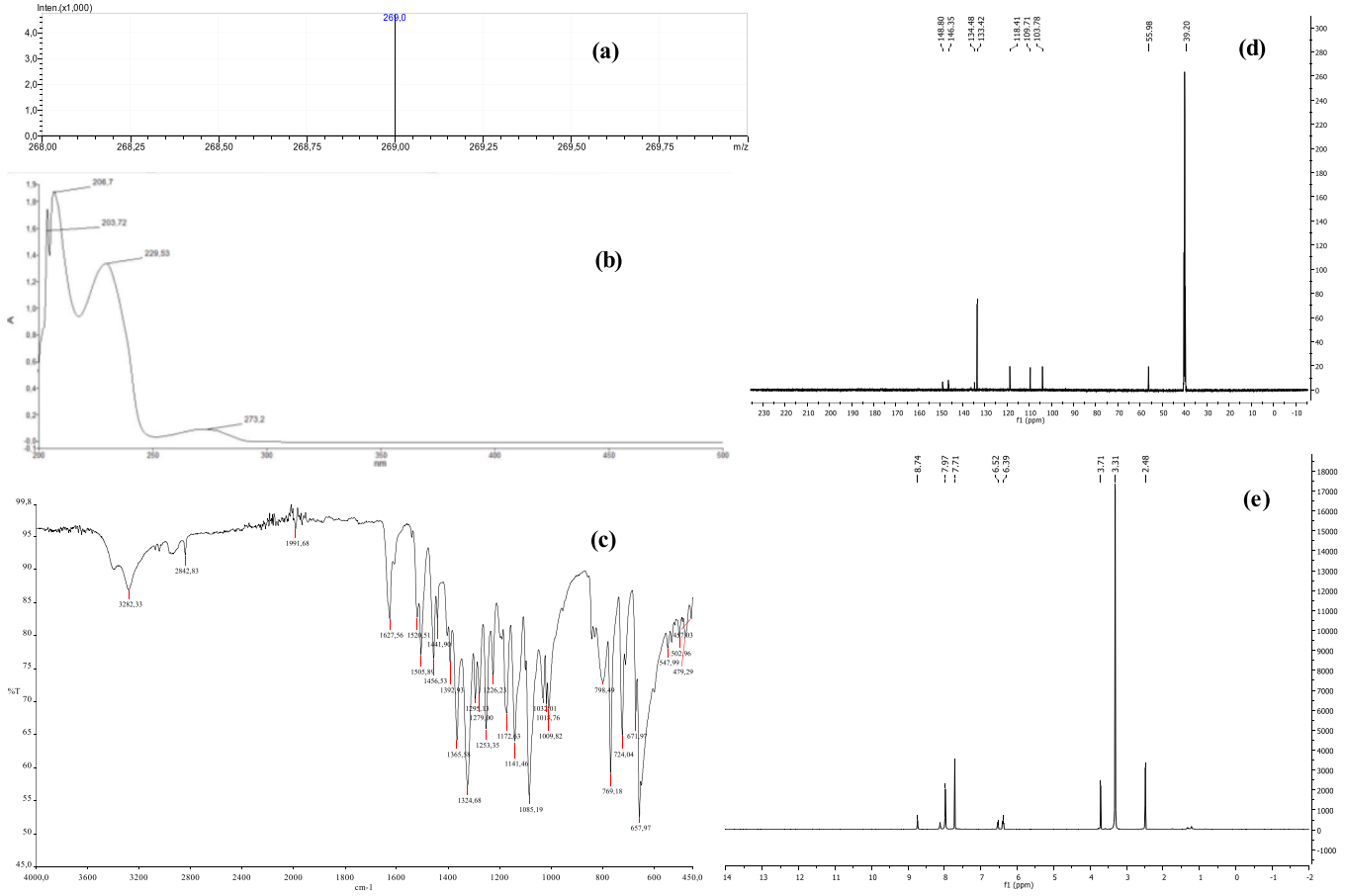

Fig. 2. Spectra of M86 (a) Mass spectrum, (b) UV-Vis spectrum, (c) IR spectrum, (d) ^{13}C NMR spectrum, (e) ^{1}H NMR spectrum.

activity ($p < 0.05$) in AChE and BChE inhibition than galantamine. In the urease inhibition test, M86 and M87 showed 20.26 ± 0.02 % and 34.20 ± 0.06 % inhibition, respectively. Although the inhibition levels of M86 and M87 were lower than that of thiourea, both compounds still exhibited potential inhibitory effects ($p < 0.05$). Table 1 shows the biological activity values of compounds M86 and M87.

3.5. *Efficacy of M86 and M87 on Pb, As and Cd exposure in THLE-2 cell line*

In order to determine the protective activity of M86 and M87, these compounds were co-administered with Pb, As, and Cd, and their effects on cell viability were evaluated. For this purpose, M86 and M87 were applied to the cells together with the IC50 concentrations of Pb, Cd, and As determined from the cytotoxicity analysis (Figs. 4–5).

The results of the XTT analysis following M86 and M87 treatment in 2D cells, and the ATP assay following treatment in 3D cells, are presented in Fig. 5. No toxic effects on the cells were observed.

In the initial phase of the study, the toxicity of Pb, Cd, and As was evaluated using the XTT assay in 2D THLE-2 cells and the ATP assay in 3D THLE-2 cells. According to the results, the IC_{50} value of Pb was 10 μM, As was 8 μM, and Cd was 5 μM in 2D cell lines. In 3D cell lines, the IC_{50} value of Pb was 25 μM, As was 15 μM, and Cd was 10 μM (Fig. 6).

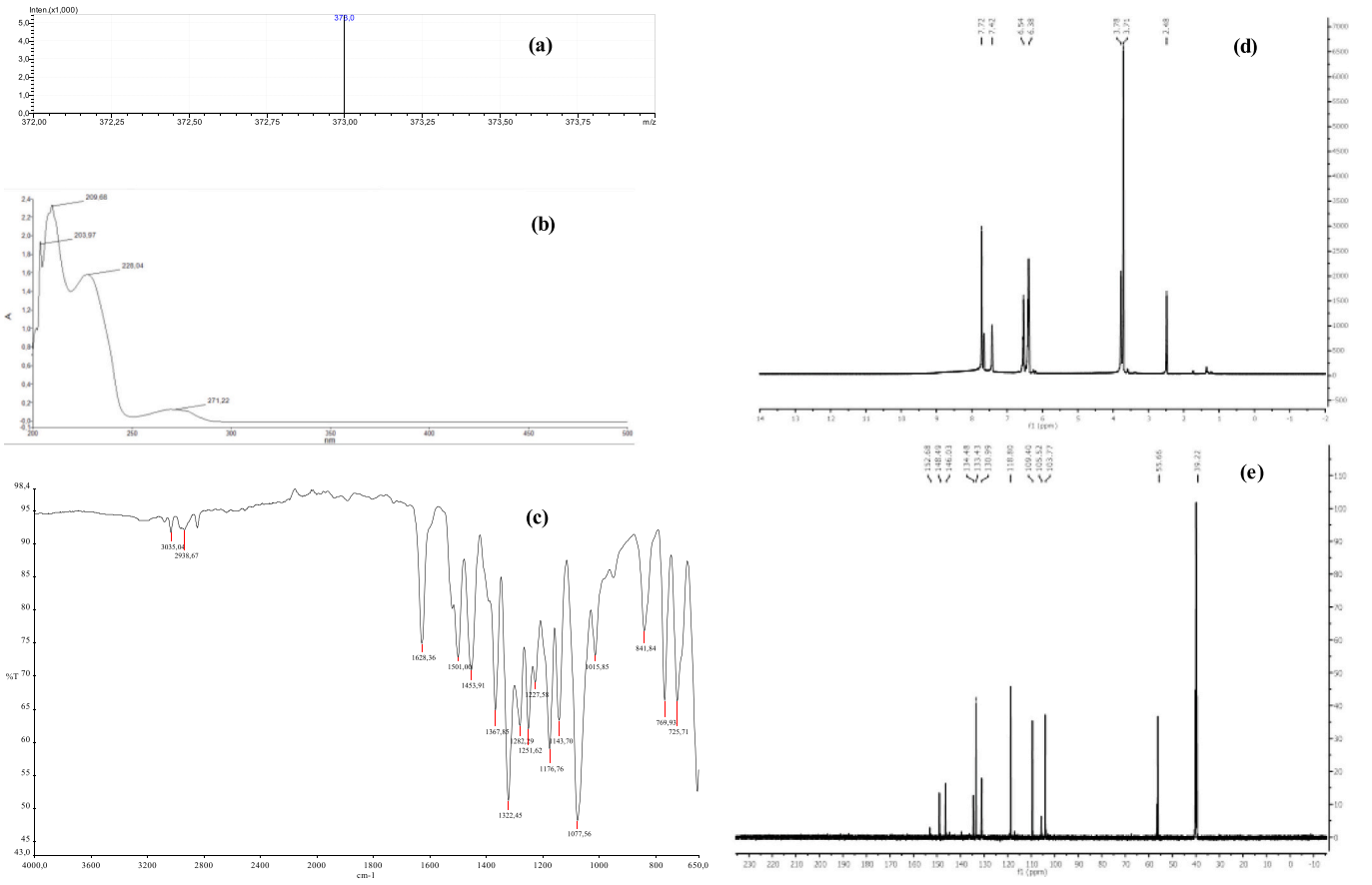

Fig. 3. Spectra of M87 (a) Mass spectrum, (b) UV-Vis spectrum, (c) IR spectrum, (d) ^{1}H NMR spectrum, (e) ^{13}C NMR spectrum.

Table 1
Biological activity values of M86 and M87.

Activity		M86	M87	α-TOC	BHT	Galantamine	Thiourea	Kojic Acid
ABTS Cation Radical	1 μg/mL	22,78 ± 1,14[a,b]	16,48 ± 0,78[a,b]	76,47 ± 0,35	82,59 ± 0,86	-	-	-
	2,5 μg/mL	44,38 ± 1,14[a,b]	62,23 ± 2,45[a,b]	77,42 ± 0,61	83,81 ± 0,96	-	-	-
	5 μg/mL	80,53 ± 0,93	87,26 ± 2,78	78,84 ± 1,01	84,58 ± 1,03	-	-	-
	10 μg/mL	89,04 ± 0,86	88,36 ± 0,19	79,32 ± 0,89	86,13 ± 0,97	-	-	-
DPPH Free Radical	1 μg/mL	9,28 ± 0,10[a,b]	22,55 ± 1,90[a]	32,36 ± 0,25	18,85 ± 0,26	-	-	-
	2,5 μg/mL	12,00 ± 0,81[a,b]	35,37 ± 0,27[b]	40,26 ± 1,32	24,01 ± 0,14	-	-	-
	5 μg/mL	32,38 ± 0,41[a]	67,80 ± 0,90[a,b]	57,85 ± 2,38	36,82 ± 0,65	-	-	-
	10 μg/mL	63,82 ± 2,64	78,43 ± 0,59[a,b]	65,41 ± 0,98	63,58 ± 0,61	-	-	-
CUPRAC	1 μg/mL	0255 ± 0,02[a,b]	0218 ± 0,02[a,b]	0336 ± 0003	0425 ± 0004	-	-	-
	2,5 μg/mL	0341 ± 0,04[b]	0418 ± 0,01[a,b]	0373 ± 0005	0536 ± 0005	-	-	-
	5 μg/mL	0452 ± 0,01[a,b]	0739 ± 0,01[a,b]	0618 ± 0008	0863 ± 0006	-	-	-
	10 μg/mL	0804 ± 0,02[a,b]	1671 ± 0,01[a,b]	0879 ± 0012	1066 ± 0082	-	-	-
ABTS (IC_{50}) (μg/mL)		3,80 ± 0,08[a,b]	2,36 ± 0,06[a,b]	13,20 ± 0,05	62,17 ± 0,15	-	-	-
DPPH (IC_{50}) (μg/mL)		7,88 ± 0,08	4,46 ± 0,13	12,49 ± 0,06	12,68 ± 0,17	-	-	-
CUPRAC ($A_{0.5}$) (μg/mL)		5,24 ± 0,11[a]	3,02 ± 0,05[a,b]	9,38 ± 0,05	4,90 ± 0,12	-	-	-
AChE (% Inhibition)		68,51 ± 1,12[c]	85,18 ± 3,64[c]	-	-	61,03 ± 1,46	-	-
BChE (% Inhibition)		71,03 ± 1,63[c]	60,37 ± 0,76	-	-	59,51 ± 1,16	-	-
Urease (% Inhibition)		20,26 ± 0,02[d]	34,20 ± 0,06[d]	-	-		97,46 ± 2,01	-
Tyrosinase (% Inhibition)		7,06 ± 0,12[e]	A.D	-	-		-	75,79 ± 0,96

Abbreviations: M86; 4-(4-methoxybenzo[*d*][1,3,2]dioxaborol-2-yl)phenyl)boronic acid, M87; 1,4-bis(4-methoxybenzo[*d*][1,3,2]dioxaborol-2-yl)benzene, ABTS; 2,2′-azino-bis(3-ethylbenzothiazoline-6- sulfonic acid), DPPH; 2,2-diphenyl-1-picrylhydrazyl, α-TOC; α-Tocopherol, BHT; Butylated Hydroxy Toluene, CUPRAC; Cupric Ion Reducing Antioxidant Capacity, BChE; Butyrylcholinesterase.

[a] indicates the group different from α-TOC in each row ($p < 0.05$).
[b] indicates the group different from BHT in each row ($p < 0.05$),
[c] indicates the group different from galantamine in each row ($p < 0.05$),
[d] indicates the group different from thiourea in each row ($p < 0.05$),
[e] indicates the group different from colic acid in each row ($p < 0.05$).

In the second phase of the study, compounds M86 and M87 were applied to THLE-2 cell lines together with the heavy metals Pb, As, and Cd. The IC50 doses of these heavy metals, determined in the first phase for both 2D and 3D cell lines, were used in the treatments. Fig. 7 shows images of the untreated 2D and 3D cell models.

In the THLE-2 cell line 2D model, co-treatment of Pb, As and Cd with

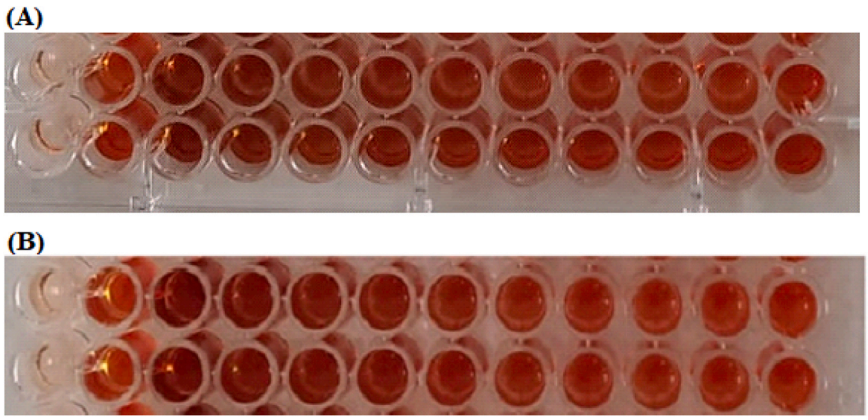

Fig. 4. Cytotoxic assays of compounds M86 (A) and M87 (B) in THLE-2 cell line.

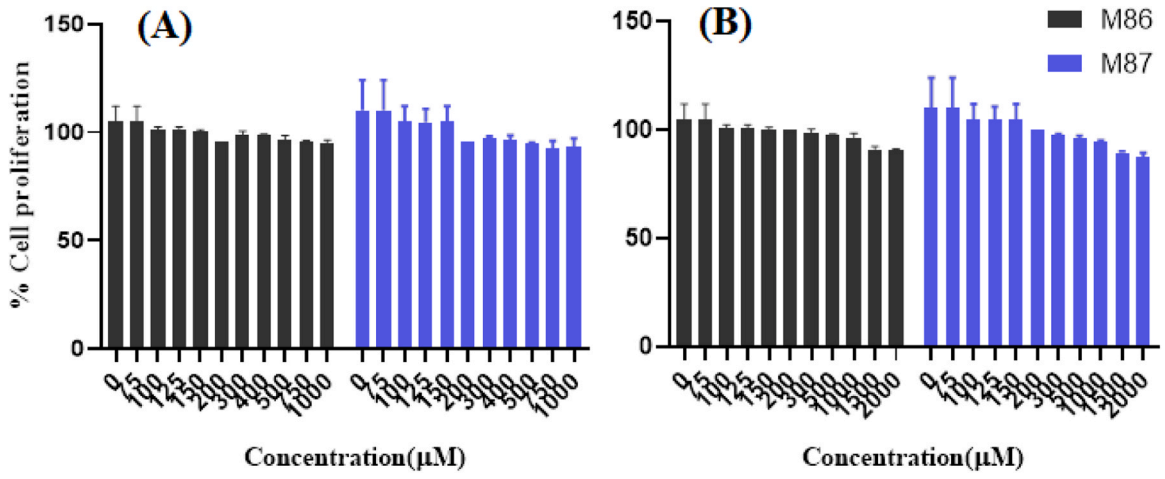

Fig. 5. Cytotoxic value of synthesized compounds M86 and M87 in (A) 2D and (B) 3D THLE-2 cell line.

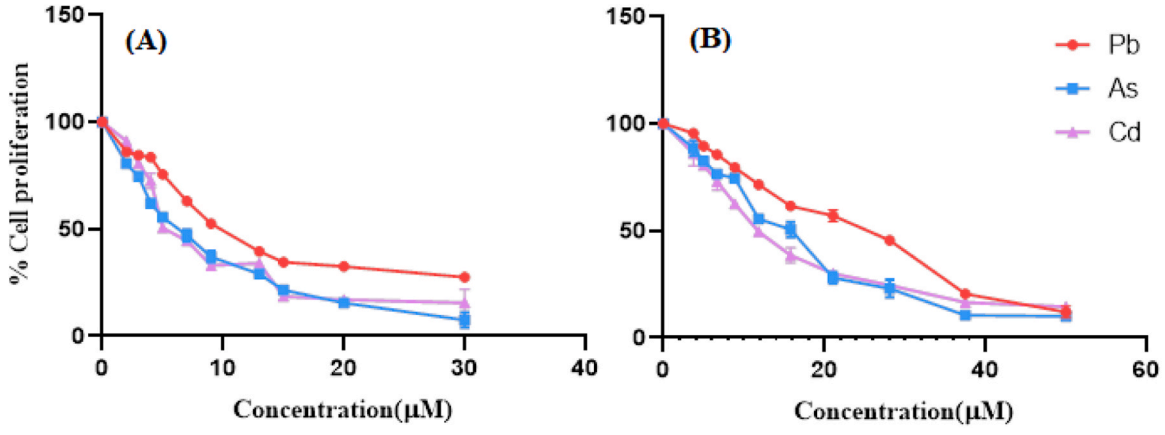

Fig. 6. Graph of cytotoxicity analysis of Pb, As and Cd (A) in 2D Thle-2 cell lines (Pb IC_{50}: 10 μM, As IC_{50}: 8 μM, Cd IC_{50}: 5 μM), (B) Graph of cytotoxicity analysis in 3D Thle-2 cell lines (Pb IC_{50}: 25 μM, As IC_{50}: 15 μM, Cd IC_{50}: 10 μM) ($p < 0.05$).

M86 and M87 prevented the damage caused by these metals (Fig. 8).

In the THLE-2 3D cell model, when Pb, As and Cd were applied both alone and in combination with M86 compound, it was found that the cell density in the group treated with Pb, As and Cd alone was higher than the group treated with M86 (Fig. 9).

When THLE-2 cell line groups in which Pb was applied alone and M86 and M87 were applied together at doses of 25 μM/ 50 μM were compared in terms of cell proliferation, it was found that M86 and M87 significantly increased cell proliferation in a dose-dependent manner ($p < 0.05$) (Fig. 9 (A)). When As was applied alone and in combination with M86 and M87 at doses of 25 μM/ 50 μM, it was found that M86 and M87 dose-dependently reversed the negative effect of As on cell proliferation in THLE-2 cell line ($p < 0.05$) (Fig. 9 (B)). When Cd was applied alone and in combination with M86 and M87 at doses of 25 μM / 50 μM, it was found that M86 and M87 dose-dependently reversed the decrease in cell proliferation caused by Cd in the THLE-2 cell line ($p < 0.05$)

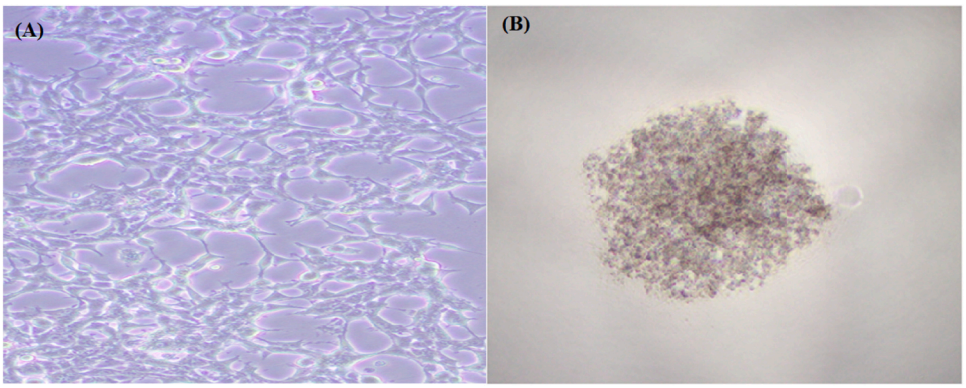

Fig. 7. THLE-2 control cells (A) 2D, (B) 3D model.

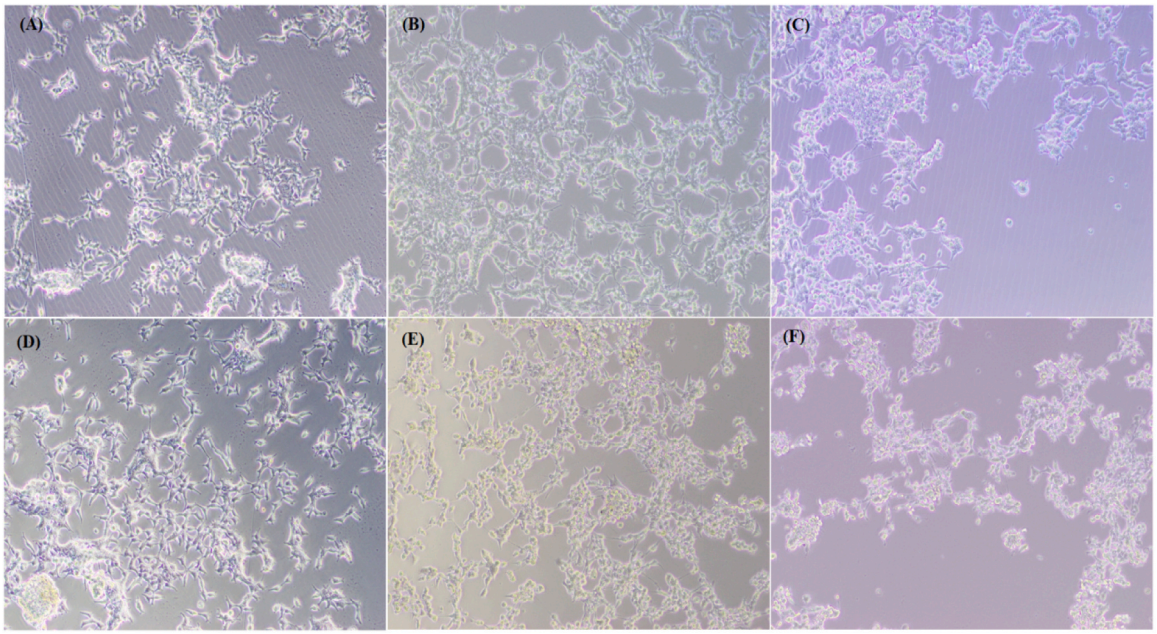

Fig. 8. 48 h image of THLE-2 2D cell lines treated with (A) Pb +M86 (25 μM), (B) As +MK86 (10 μM), (C) Cd +MK86 (25 μM), (D) Pb +M87 (25 μM), (E) As +M87 (10 μM), (F) Cd +M87 (25 μM).

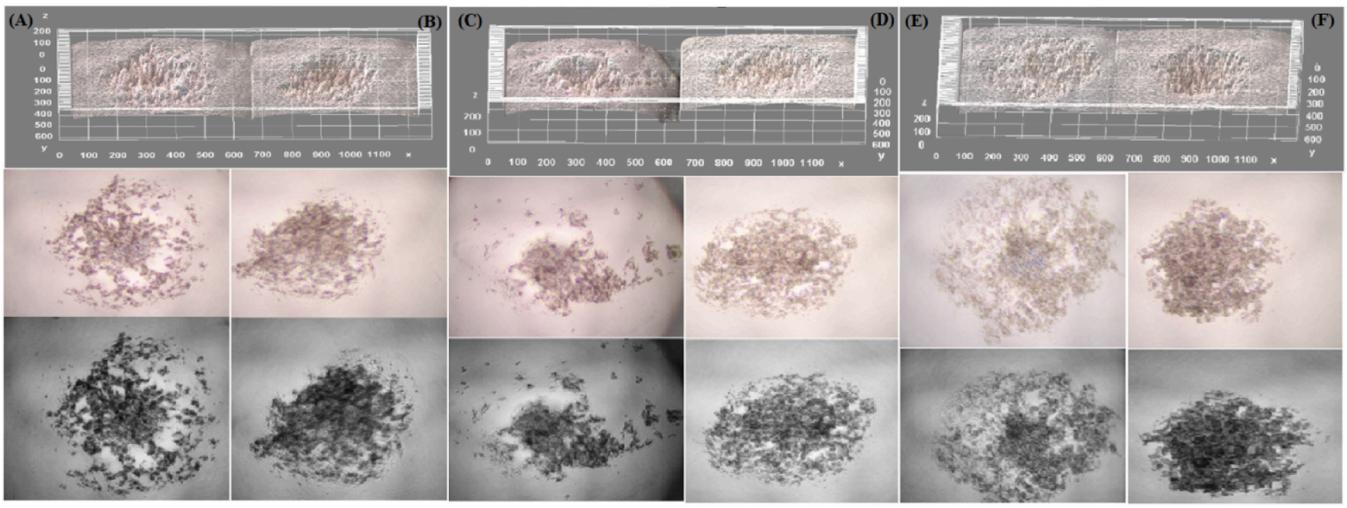

Fig. 9. 48 h image of THLE-2 3D cell lines treated with (A) Pb, (B) Pb +MK86 (25 μM), (C) As, (D) As +MK86 (10 μM), (E) Cd, (F) Cd +MK86 (25 μM).

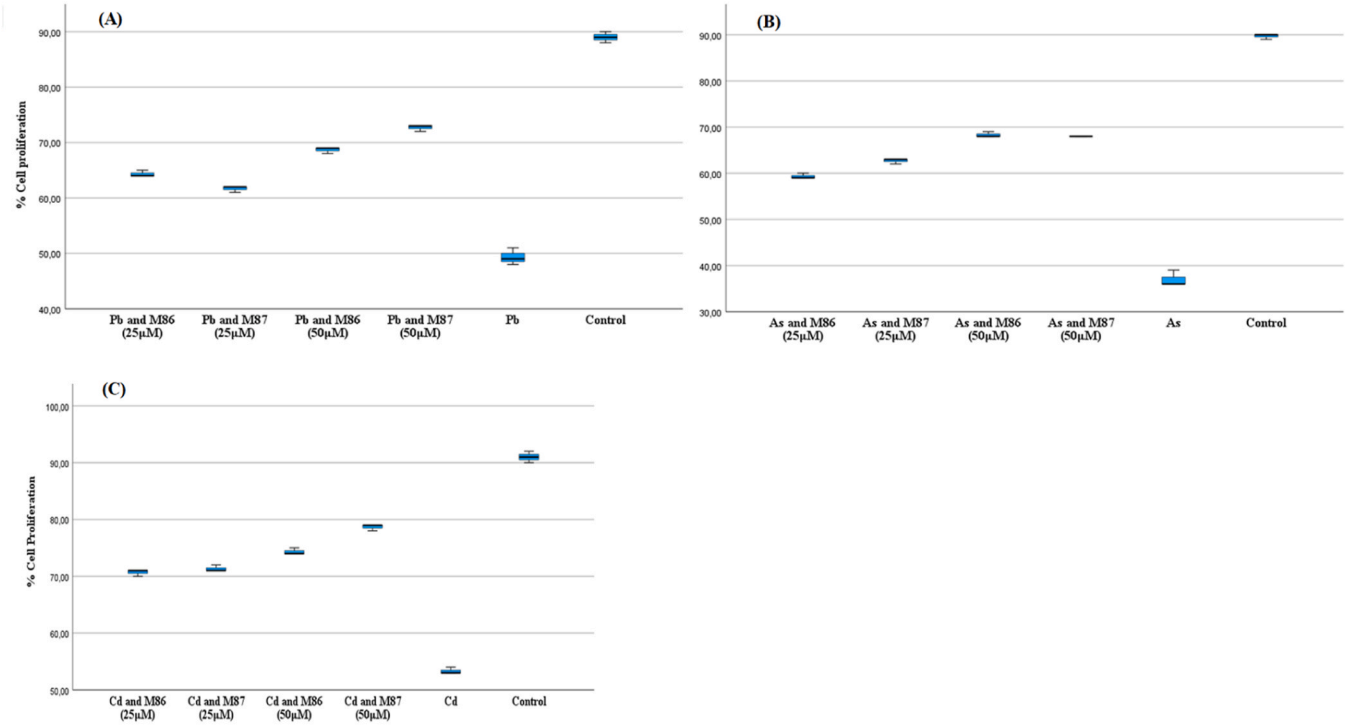

Fig. 10. Cell proliferation graph in THLE-2 3D cell models after (A) Pb, M86 and M87, (B) As, M86 and M87, (C) Cd, M86 and M87 combinational treatment.

(Fig. 10 (C)).

4. Discussion

This study was carried out to investigate the protective effects of newly synthesized boronic acid derivatives M86 and M87 against Pb, Cd and As toxicity in the THLE-2 liver cell line and to reveal the therapeutic potential of these compounds. The findings revealed that these compounds are particularly effective against oxidative stress and enzyme inhibition and have potential therapeutic properties in Pb, Cd and As toxicity.

In ABTS, DPPH and CUPRAC tests, the antioxidant activities of M86 and M87 were found to be at high levels compared to standards such as BHT and α-TOC. In particular, in the ABTS test, the activity values of M86 and M87 at 10 µg/mL concentration were 89.04 ± 0.86 % and 88.36 ± 0.19 %, respectively, close to the 86.13 ± 0.97 % value of BHT. These results indicate that the free radical scavenging capacity of these compounds is similar to that of traditional antioxidants such as BHT. In the DPPH test, M86 exhibited a higher antioxidant activity with an inhibition value of 78.43 ± 0.59 %, surpassing the 63.58 ± 0.61 % value of BHT. In the CUPRAC test, the value of 1.671 ± 0.01 of M87 at 10 µg/mL concentration was significantly higher than the value of 1.066 ± 0.082 of BHT. Considering these test results, it can be said that M86 and M87 are compounds with high antioxidant capacity.

The structural features of M86 and M87, including the presence of two boronate ester moieties and a methoxy group on the aromatic rings, may underlie their strong biological activity. These features are known to enhance the lipophilicity of the molecules, promoting cellular uptake and bioavailability. Additionally, the electron-donating methoxy substituents may stabilize the boron centers and enhance free radical scavenging activity. The conjugated system between aromatic rings and boronate groups may facilitate electron delocalization, further contributing to antioxidant properties. Thus, the observed biological activity, particularly in antioxidant and enzyme inhibition assays, can be closely associated with these chemical modifications [30,43].

Anticholinesterase activity tests showed that M86 and M87 have the potential to inhibit AChE and BChE enzymes. M86 was found to be more effective than galantamine with 68.51 ± 1.12 % in AChE inhibition and 71.03 ± 1.63 % in BChE inhibition. Similarly, M87 also showed a high activity in AChE inhibition with 85.18 ± 3.64 %. When these findings are evaluated, it can be stated that both compounds have the potential to produce neuroprotective effects through anticholinesterase activity. In the anti-urease activity test, M87 exhibited higher activity compared to M86 (20.26 ± 0.02 %) with 34.20 ± 0.06 % inhibition. However, these values were lower compared to the 97.46 ± 2.01 % inhibition value of thiourea. In tyrosinase inhibition, M86 showed a limited effect with a value of 7.06 ± 0.12 %, while M87 was not active in this assay. These results indicate that M86 and M87 show limited activity in the areas of urease and tyrosinase enzyme inhibition, but require further optimization. Considering these tests and the literature, it can be said that the biological activity of the synthesized compounds M86 and M87 is due to the high biological activity of boronic acid and catechols [26,28,29,44–46].

Boron atoms in M86 and M87 are central to their pharmacological effects. Due to their Lewis acid nature and vacant p-orbital, boron atoms form reversible covalent bonds with electron-rich biological targets such as diols in enzymes or reactive oxygen species. This enables boron-containing compounds to modulate enzyme activities, as observed in the inhibition of AChE and BChE in this study.

Additionally, the presence of boron enhances the ability of the compounds to interact with cellular thiol groups, which may contribute to their protective effects against metal-induced oxidative damage. The role of boron in biological systems is becoming increasingly recognized, and M86 and M87 represent novel scaffolds to exploit these interactions [31,32,44].

Studies in the literature have shown that boronic acid derivatives possess antioxidant, anticancer, antibacterial, and antiviral properties [26,43]. Additionally, one study reported that a boronic acid derivative exerted a protective effect against lipopolysaccharide (LPS)-induced liver damage [47]. However, to date, no study has evaluated the efficacy of boronic acid derivatives with different therapeutic potentials against the toxicity of heavy metals such as Pb, Cd, and As. In this context, the present study, conducted in the THLE-2 cell line, demonstrated that the newly synthesized compounds M86 and M87 exhibited significant

protective activity against Pb, Cd, and As-induced toxicity.

Pb induces oxidative stress in biological systems, leading to cellular dysfunction. Its toxicity is primarily mediated by the overproduction of reactive oxygen species (ROS), resulting in lipid peroxidation, impairment of enzymatic activity, protein oxidation, DNA damage, and reduced antioxidant defense mechanisms [3,48]. In our study, Pb exposure alone significantly reduced cell proliferation in both 2D and 3D THLE-2 liver cell models. However, co-treatment with the M86 and M87 compounds effectively prevented this Pb-induced decline in cell proliferation. Previous studies have indicated that boric acid can reduce oxidative stress and induce apoptosis, thereby exhibiting anticancer properties [49]. Given that boric acid derivatives are known to support cellular regeneration by modulating apoptotic pathways, it is plausible that M86 and M87 contribute to cellular protection and detoxification through similar mechanisms. Considering the high antioxidant capacities of M86 and M87 observed in DPPH, ABTS, and CUPRAC assays, it can be inferred that their protective effects against Pb toxicity are at least partly attributable to their antioxidant properties.

Cd, one of the most toxic heavy metals, causes severe oxidative stress and cellular damage in the liver and other organs [33]. At the cellular level, Cd induces oxidative stress by increasing the production of reactive oxygen species (ROS), leading to DNA damage, protein oxidation, and lipid peroxidation. This oxidative stress impairs the antioxidant defense systems of cells, activates apoptotic pathways, inhibits DNA repair mechanisms, and may result in genetic instability and carcinogenic effects [50]. Wang et al. evaluated the protective role of boron against trichloroacetic acid-induced hepatotoxicity and oxidative stress, demonstrating that boron inhibits cell apoptosis by reducing oxidative damage and exerts its protective effect via the p38 MAPK pathway [51]. Similarly, boric acid has been shown to mitigate alcohol-induced oxidative stress and apoptosis [52]. In human blood cells, boric acid significantly reduced the cytotoxicity, oxidative stress, and genotoxicity induced by 3-chloro-1,2-propanediol (3-MCPD) [53]. Moreover, boric acid provided effective protection against DNA strand breaks and micronucleus formation induced by cadmium chloride ($CdCl_2$) in V79 cells [54]. In our study, the reduction in cell proliferation caused by Cd exposure in the THLE-2 cell line was reversed by the boronic acid derivatives M86 and M87. These protective effects may be attributed to mechanisms similar to those previously reported for boron and boric acid. However, further comprehensive studies are needed to clarify whether M86 and M87 mitigate Cd-induced toxicity in THLE-2 cells solely through antioxidant mechanisms or via additional pathways.

As is a metalloid element with significant toxic and carcinogenic properties. It is commonly found in nature in the form of oxides, sulfides, or salts formed with elements such as iron, sodium, calcium, and copper [33]. Inorganic arsenite [As(III)], with the general formula R-As^{2+}, and organic arsenic compounds tend to bind with high affinity to adjacent dithiol groups. This binding creates highly sensitive targets for the toxic effects of As on biomolecules, including certain selenoenzymes [55]. By interacting with proteins, As^{3+} alters their structure and function, thereby disrupting various cellular processes such as epigenetic modifications of DNA and histones, transcriptional regulation of mRNA, pre-mRNA splicing, DNA damage repair, oxidative stress response, ribosome-associated protein quality control, glycolysis, and microtubule dynamics. Studies on arsenic–protein interactions suggest that such disruptions may underlie the pathogenesis of diseases associated with chronic arsenic exposure [56]. Moreover, arsenic increases the generation of reactive oxygen species (ROS), leading to lipid peroxidation, protein oxidation, and DNA damage, while also impairing cellular detoxification mechanisms [55].

As evidenced by the literature, arsenic can induce cellular toxicity through multiple mechanisms. For instance, Manna et al. investigated the protective effect of arjunolic acid against As-induced cardiac oxidative damage and found that it protected cardiac tissue by reducing oxidative stress and hyperlipidemia due to its antioxidant properties [57]. Similarly, Ijaz et al. evaluated the efficacy of nobiletin against As-induced liver damage in rats, demonstrating that it prevented the As-induced decrease in antioxidant enzyme activities, reduction in oxidative stress markers, and the onset of inflammation, apoptosis, and histopathological damage in liver tissue [58]. In our study, the inhibition of arsenic-induced toxicity in the THLE-2 cell line by the boronic acid derivatives M86 and M87 may be attributed to their strong antioxidant properties. However, further comprehensive studies are required to elucidate whether the protective effects of M86 and M87 are mediated solely through antioxidant mechanisms or involve additional molecular pathways.

One of the noteworthy aspects of this study is the demonstration that M86 and M87 serve dual purposes: as enzyme inhibitors and hepatoprotective agents. Their ability to inhibit key enzymes such as AChE and BChE suggests potential applications in neuroprotection and metabolic regulation. Simultaneously, their strong antioxidant effects and capacity to reverse heavy metal-induced cytotoxicity in 2D and 3D THLE-2 liver models support their role as liver-protective agents. This dual functionality may render them attractive candidates for therapeutic development, particularly in complex pathologies where oxidative stress and enzymatic dysregulation coexist, such as metal-induced hepatotoxicity or neurodegenerative diseases [26,46,51].

5. Conclusion

This study demonstrated that the newly synthesized boronic acid derivatives, M86 and M87, exhibit strong antioxidant and enzyme-inhibitory activities, along with significant protective effects against Pb, Cd and As toxicity in 2D and 3D THLE-2 liver cell models. Both compounds were effective in restoring cell viability and counteracting metal-induced cytotoxicity, likely through their free radical scavenging abilities and interactions with key enzymes. The dual role of M86 and M87 as both enzyme inhibitors and hepatoprotective agents highlights their potential as promising therapeutic candidates for the treatment of heavy metal toxicity. Future studies, including in vivo models, are warranted to further elucidate their mechanisms of action and therapeutic relevance.

Author statement

All authors have made substantial contributions to the conception, design, execution, and interpretation of this study.

- **Hamdi Temel** contributed to the synthesis, characterization, biological activity assessment, interpretation, and writing of the new ligands.
- **Serap Yalçın Azarkan** contributed to the evaluation of the protective activity of the compounds against Lead (Pb), Cadmium (Cd), and Arsenic (As) toxicity in 2D and 3D model THLE-2 liver cell lines.
- **Serkan Şahin** contributed to manuscript writing, literature review, and toxicity studies.

All authors have reviewed and approved the final version of the manuscript and agree to be accountable for all aspects of the work. The authors declare that there are no conflicts of interest related to this study.

CRediT authorship contribution statement

Temel Hamdi: Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **Azarkan Serap Yalçın:** Investigation, Formal analysis, Data curation, Conceptualization. **Şahin Serkan:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis.

Ethical approval

Not applicable.

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Declaration of Competing Interest

The authors declare that there are no financial, personal, or professional conflicts of interest that could have influenced the research presented in this manuscript. The study was conducted independently, and no external funding, employment, consultancy, stock ownership, honoraria, patents, or other relationships could lead to a conflict of interest.

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Data Availability

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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