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# EFFECT OF HALOGENATED BOROXINE ON HEMATOLOGICAL PARAMETERS IN RATS AND ON CREATINE KINASE ACTIVITY *IN VITRO* RETROSPECTIVE REAL-WORLD ANALYSIS

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Halogenated boroxine is an important enzyme inhibitor with potential applications in cancer therapy. This study aimed to evaluate its effects on hematological parameters in rats *in vivo* and creatine kinase activity *in vitro*. Halogenated boroxine, administered either intraperitoneally (IP) or orally, induced route-dependent changes in hematological parameters compared with controls. IP administration significantly increased neutrophils, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), while decreasing hematocrit (HCT). In contrast, oral administration significantly decreased lymphocytes and increased basophils, while other hematological parameters remained unchanged. These effects were more pronounced following IP administration. *In vitro*, halogenated boroxine exhibited competitive inhibition of creatine kinase without pre-incubation, whereas after a 30-minute pre-incubation, the inhibition pattern indicated a non-competitive mechanism. Further analysis showed a time-dependent shift in creatine kinase inhibition mechanism following pre-incubation, characterized by a decrease in maximum reaction velocity ($V_{max}$), while the Michaelis constant ($K_m$) remained unaltered. These findings show that halogenated boroxine modulates specific hematological parameters and inhibits creatine kinase activity, with effects depending on the route of administration and experimental conditions.

Keywords: halogenated boroxine, hematological parameters, spectrophotometry, creatine kinase, inhibition

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## INTRODUCTION

Boroxins, which are cyclic oxides of boron, represent a class of compounds characterized by a stable core structure and the ability to form various coordination complexes. Their chemical modification, particularly through halogenation, changes their electronic and steric properties, often resulting in new biological activities. Consequently, boron-containing compounds have attracted considerable attention in biomedical research because of their antimicrobial, enzyme-inhibitory, and antitumor properties (1,2). Halogenated boroxine $K_2[B_3O_3F_4OH]$ is a specific boron-containing compound with pronounced antitumor potential that has been patented as a therapeutic agent for benign and malignant skin lesions (Figure 1). Previous studies have demonstrated significant antiproliferative effects of halogenated boroxine (HB) across several tumor cell lines, as well as distinct antitumor activity in experimental animal models. Notably, these effects are more pronounced in tumor cells than in normal cells. Recent investigations have shown that halogenated boroxine induces deregulation of genes involved in apoptosis and autophagy, thereby contributing to its selective antitumor activity. The core structure of boroxine, consisting of three boron atoms interconnected through oxygen bridges with fluorine substitutions on the ring, provides both the structural stability and chemical and biological reactivity necessary to interact in different cellular systems (3,4).

Evaluation of hematological parameters in experimental animals is a standard approach in toxicological and pharmacological research. Hematological indices, including erythrocyte, leukocyte, and platelet counts, together with hemoglobin concentration and hematocrit values, are sensitive indicators of systemic toxicity, inflammatory processes, immune responses, and bone marrow function. Previous studies have demonstrated that halogenated boroxine exhibits selective genotoxic and cytotoxic effects in malignant hematopoietic and tumor cells, whereas normal peripheral blood cells show substantially lower sensitivity. These findings suggest the potential utility of halogenated boroxine in treating hematological malignancies, while emphasizing the necessity of further *in vivo* safety evaluations. Although numerous studies have investigated the antitumor activity and toxicity profile of halogenated boroxine, data concerning its influence on hematological parameters *in vivo* remain scarce (3,5-7).

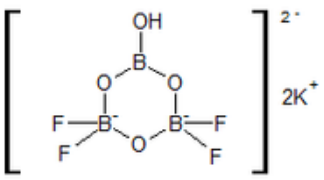



**Figure 1.** *Structure of dipotassium trioxohydroxytetrafluorotriborate, $K_2[B_3O_3F_4OH]$*

Since *in vitro* studies cannot fully reflect the complex interactions occurring within a living organism, an *in vivo* assessment of hematological parameters was included to evaluate the systemic biological effects of halogenated boroxine (HB). The selected parameters included white blood cell count (WBC), lymphocyte count (LYM) and granulocyte count (GRAN) as indicators of immune status and inflammatory response; platelet count (PLT) as a marker of coagulation and bone marrow activity; red blood cell count (RBC), hemoglobin concentration (Hb), and hematocrit value (HCT) as indicators of oxygen transport capacity; and mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) as indices of erythrocyte morphology and quality. These parameters provide a broader and more clinically relevant assessment of biological activity than enzyme testing alone (6).

In addition to hematological evaluation, investigation of the effects of halogenated boroxine on creatine kinase (CK) activity provides insight into its interaction with enzyme systems. Boron-containing compounds are known to act as enzyme inhibitors due to their ability to interact with nucleophilic groups and metal-containing active sites. Notably, $K_2[B_3O_3F_4OH]$ has previously been identified as a potent inhibitor of several human carbonic anhydrase isoenzymes, suggesting that its biological effects may partly arise from enzyme modulation. Therefore, it is reasonable to hypothesize that halogenated boroxine may also interact with other enzymes, including creatine kinase (2). Creatine kinase is a key enzyme in cellular energy metabolism responsible for maintaining adenosine triphosphate (ATP) homeostasis in tissues with high energy demand, particularly skeletal muscle. Elevated CK activity in serum is widely recognized as a biomarker of muscle injury, cellular damage, and drug-induced toxicity. Changes in CK activity may therefore indicate direct interactions between halogenated boroxine and cellular enzyme systems or reveal potential cytotoxic effects. Through spectrophotometric analysis, this study seeks to clarify the possible inhibitory action of halogenated boroxine on creatine kinase activity and to characterize the type of enzyme inhibition involved (8).

The study aimed to investigate the effects of dipotassium trioxohydroxytetrafluorotriborate, $K_2[B_3O_3F_4OH]$, on hematological parameters following intraperitoneal and oral administration, as well as its effect on creatine kinase activity *in vitro*. The obtained results may contribute to a better understanding of the biological relevance, safety profile, and potential therapeutic applications of this boron-containing compound in medicine and pharmacy.

## METHODS

Our *in vivo* studies were done on a total of 24 Wistar rats bred in the vivarium of the Department of Biology, Faculty of Science, University of Sarajevo. Housing conditions met standard regulatory criteria for experimental animals: temperatures of 20–24 °C, a standard light/dark cycle, and isolation from excessive noise. The animals were housed in stainless steel cages featuring solid, transparent, hard-plastic floors covered with suitable wood litter and equipped with a feeder and a waterer providing food and water ad libitum. Following acclimatization, 90-day-old rats weighing 180 ±12.31 g were randomly assigned to three groups: a control group (n = 6) and two experimental treatment groups (n = 9 each). Halogenated boroxine was dissolved in redistilled water and administered at a dose of 10 mg/kg of body weight. The first experimental group received halogenated boroxine intraperitoneally for 5 days at the same time, while the second experimental group received it orally (*per os*) for five days at the same time. Intraperitoneal application of the appropriate dose of halogenated boroxine was performed once daily for 5 days at a consistent time using a syringe and needle. The second experimental group received the same dose orally (*per os*) once daily for 5 days using a metal gavage probe with a rounded tip to minimize the risk of esophageal injury. Control animals were maintained under the same housing and dietary conditions as the experimental groups and received only an equivalent amount of redistilled water for 5 days without halogenated boroxine.

Animals in the control and experimental groups were of the same age. The control group provided baseline values for the examined hematological parameters, which were compared against the values from both experimental groups. Dosing criteria and exposure times were determined based on previous research by Haverić *et al*. (9). All animal care procedures adhered to the Principles of Laboratory Animal Care, established by the Universal Declaration of Animal Welfare, the UNESCO Declaration on the Rights of Animals, and the European Parliament's Directive 2010/63/EU on the protection of animals used for scientific purposes. This *in vivo* research was governed by the Law on the Protection and Welfare of Animals (Official Gazette of BiH, No. 25/2009 and 9/2018) and approved by the Ethics Committee of the Faculty of Science, University of Sarajevo (Approval No. 01/01-1945/2-2025, issued on October 23, 2025).

Halogenated boroxine, $K_2[B_3O_3F_4OH]$, is a water-soluble, white, inorganic boron powder (7.1% solubility at 20 °C) with a purity of 99.99%. It was synthesized by reacting potassium hydrofluoride ($KHF_2$) with boric acid at a 2:3 molar ratio at room temperature, as reported in the literature (10).

Before a cardiac puncture, an intraperitoneal dosage of xylazine (20 mg/kg) and ketamine (100 mg/kg) was administered, and a total of 4 mL of blood was taken 24 hours after the treatment. Blood sampling was performed as a terminal procedure. The collected blood was immediately mixed with EDTA for hematological analysis. The examined hematological parameters included WBC, LYM, GRAN, PLT, RBC, Hb, HCT, MCV, MCH, and MCHC. These parameters were determined using a Mythic 5VET PRO hematology analyzer, which utilizes multi-dimensional laser light cell classification for WBC differentials, impedance for WBC, RBC, and PLT counts, and spectrophotometry for Hb determination.

For *in vitro* studies, a spectrophotometric method was used with measurements made at 340 nm and a temperature of 37 °C, using a commercial assay kit (Creatine Kinase Activity Assay Kit, MAK116; Sigma-Aldrich, USA) according to the manufacturer's instructions. The concentration of halogenated boroxine used was 10 mg/mL (11).

### Statistical methods

Data were analyzed using descriptive statistics and presented as mean ± standard deviation ($\bar{X}$ ± SD). Differences between groups were analyzed using Student's t-test for independent samples and one-way ANOVA. Comparisons were made between the control group and each experimental group, as well as between the two experimental groups. Differences were considered statistically significant at $p < 0.05$. Statistical analysis was performed using SPSS software.

## RESULTS

*In vivo*

The control group consisted of 6 rats, and their basic hematological parameters and differential blood count were analyzed. Individual values, calculated mean values ($\bar{X}$), and standard deviations (SD) for the analyzed parameters are shown in Table 1. The parameters evaluated included total leukocyte count (WBC), differential leukocyte formulas (LYM%, MON%, NEU%, EOS%, BASO%), erythrocyte indicators (RBC, Hb, HCT, MCV, MCH, MCHC), and platelet count (PLT). The average value of leukocyte count (WBC) in the control group was 6.95 × $10^9$/L, with a standard deviation of 1.66 × $10^9$/L. Within the differential leukocyte formula, the average values were: lymphocytes 58.68%, monocytes 14.49%, neutrophils 22.69%, eosinophils 4.08%, and basophils 0.05%. Erythrocyte parameters of the control group showed an average erythrocyte count (RBC) of 8.30 ×$10^{12}$/L. The average hemoglobin concentration (Hb) was 147.90 g/L, while the hematocrit (HCT) value was 0.43 L/L. The erythrocyte indices values were: MCV 51.85 fL, MCH 17.91 pg, and MCHC 347.07 g/L. The average platelet count (PLT) in the control group was 413.00 ×$10^9$/L. All obtained control values were consistent with established baseline reference intervals for Wistar rats shown in Table 2 (12,13). The greatest variability between individual animals was recorded in platelet values (SD = 100.88 ×$10^9$/L), MCV (SD = 5.06 fL), total leukocyte count (SD = 1.66 ×$10^9$/L), and erythrocyte count (SD = 1.01 ×$10^{12}$/L). In contrast, smaller variations were recorded in the differential leukocyte formula parameters, especially basophil values. Overall, the control group established a baseline range of individual values, mean values , and standard deviations for all analyzed hematological indicators.

The first experimental group consisted of 9 Wistar rats that were administered halogenated boroxine intraperitoneally at a dosage of 10 mg/kg of body weight for five consecutive days. The results of hematological analyses are shown in Table 3. Analysis of the hematological parameters indicated that certain values deviated from the control group. The average WBC value in the IP group was lower than that of the control group, but this difference was not statistically significant ($p > 0.05$). Analysis of the differential leukocyte formula showed a statistically significant increase in the proportion of neutrophils in the IP group compared to the control (28.62 ± 2.33% vs. 22.69 ± 4.87%, $p < 0.05$). The values of lymphocytes, monocytes, eosinophils, and basophils did not exhibit statistically significant differences compared to the control group. Regarding erythrocyte parameters, both the erythrocyte count and hemoglobin concentration were slightly lower in the IP group, but these differences were not statistically significant ($p > 0.05$). However, the hematocrit value was significantly lower compared to the control group (0.36 ± 0.07 vs. 0.43 ± 0.03 L/L, $p < 0.05$). The value of MCV showed no significant changes, while the values of MCH and MCHC were significantly increased in the IP group ($p < 0.05$). The platelet count was slightly higher in the IP group compared to the control, but this difference was not statistically significant ($p > 0.05$).

**Table 1.** *Values of hematological parameters of the control group without halogenated boroxine*

| | WBC $10^9$/L | LYM% | MON% | NEU% | EOS% | BASO% | RBC$10^{12}$/L | Hb g/L | HCT L/L | MCV fL | MCH pg | MCHC g/L | PLT$10^9$/L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Countrol group | 3.94 | 60.09 | 17.37 | 13.16 | 9.38 | 0 | 8.25 | 152 | 0.457 | 55.5 | 18.4 | 332 | 236 |
| | 8.8 | 53.53 | 17.05 | 26.89 | 2.39 | 0.14 | 10.28 | 157 | 0.437 | 42.6 | 15.2 | 359 | 494 |
| | 7.13 | 60.12 | 13.46 | 23.23 | 3.03 | 0.16 | 7.75 | 141 | 0.395 | 51 | 18.1 | 356 | 524 |
| | 6.56 | 58.4 | 13.9 | 23.34 | 436 | 0 | 7.98 | 146 | 0.41 | 51.4 | 1,829 | 356 | 423 |
| | 7.38 | 58.75 | 12.74 | 25.38 | 3.11 | 0 | 7.43 | 143 | 0.4 | 53.83 | 19.24 | 357.5 | 389 |
| | 7.88 | 61.24 | 12.41 | 24.13 | 2.22 | 0 | 8.13 | 148.4 | 0.461 | 56.76 | 18.25 | 321.9 | 412 |
| X̄ | 6.95 | 58.68 | 14.49 | 22.69 | 4.08 | 0.05 | 8.3 | 147.9 | 0.43 | 51.85 | 17.91 | 347.07 | 413 |
| Sd | 1.66 | 2.73 | 2.17 | 4.87 | 2.7 | 0.08 | 1.01 | 5.92 | 0.03 | 5.06 | 1.39 | 15.95 | 100.88 |

**Table 2.** *Reference values for hematological parameters of Wistar rats (12,13)*

| WBC $10^9$/L | LYM % | MON % | NEU % | EOS % | BASO % | RBC $10^{12}$/L | Hb g/L | HCT % | MCV fL | MCH pg | MCHC g/L | PLT $10^9$/L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3.7-5.8 | 54.9-65.3 | 7.0-8.3 | 23.4-40.5 | 0.3-3.4 | 0–0.8 | 6.1–8.5 | 118-162 | 32.6-46 | 51-62 | 17.7-20.0 | 327-362 | 315-512 |

**Table 3.** *Hematological parameters of experimental group 1 which received halogenated boroxine intraperitoneally (IP) 10 mg/kg*

| | WBC $10^9$/L | LYM% | MON% | NEU% | EOS% | BASO% | RBC $10^{12}$/L | Hb g/L | HCT L/L | MCV fL | MCH pg | MCHC g/L | PLT $10^9$/L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Experimental group 1 (IP) | 2.79 | 55.71 | 8.13 | 28.69 | 7.47 | 0 | 3.65 | 75 | 0.186 | 51.2 | 20.5 | 403 | 459 |
| | 5.95 | 66.59 | 0.67 | 27.48 | 5.26 | 0 | 6.95 | 135 | 0.367 | 52.9 | 19.4 | 367 | 467 |
| | 4.76 | 41.82 | 22.6 | 28.75 | 6.51 | 0.32 | 7.82 | 147 | 0.423 | 54.1 | 18.7 | 347 | 469 |
| | 7.82 | 45.51 | 20.9 | 25.94 | 7.61 | 0.04 | 7.27 | 134 | 0.368 | 50.7 | 18.4 | 364 | 487 |
| | 5.21 | 49.91 | 13.49 | 33.1 | 3.09 | 0.41 | 7.36 | 137 | 0.385 | 52.4 | 18.6 | 355 | 461 |
| | 6.11 | 56.34 | 14.61 | 24.59 | 4.46 | 0 | 6.56 | 134 | 0.37 | 52.9 | 19.4 | 367 | 455 |
| | 5.84 | 48.78 | 15.98 | 30.48 | 4.45 | 0.31 | 6.24 | 131 | 0.31 | 49.6 | 20.99 | 422.5 | 463 |
| | 4.87 | 55.17 | 10.18 | 29.87 | 4.54 | 0.24 | 7.23 | 140 | 0.35 | 49.79 | 19.36 | 400 | 424 |
| | 5.12 | 56.22 | 10.88 | 26.31 | 6.45 | 0.14 | 7.78 | 142 | 0.38 | 48.88 | 18.25 | 373.68 | 476 |
| $\bar{X}$ | 5.66 | 52.78 | 11.94 | 28.62 | 5.6 | 0.16 | 7.03 | 134.3 | 0.36 | 51.9 | 19.6 | 378.9 | 465.4 |
| Sd | 1.47 | 7.46 | 6.93 | 2.33 | 1.47 | 0.14 | 1.42 | 22.1 | 0.07 | 1.7 | 0.8 | 19.7 | 19.6 |

The second experimental group consisted of 9 male Wistar rats to which halogenated boroxine was administered orally (*per os*) at a dose of 10 mg/kg of body weight for 5 consecutive days. The obtained hematological parameters are presented in Table 4.

The average value of WBC in the OG group was lower compared to the control group, but the difference was not statistically significant ($p > 0.05$). Analysis of the differential leukocyte formula showed a statistically significant decrease in the percentage of lymphocytes compared to the control group (53.58 ± 4.63% vs. 58.68 ± 2.73%, $p < 0.05$). The proportion of monocytes and neutrophils did not show statistically significant differences compared to the control group ($p > 0.05$). A statistically significant increase in basophils was recorded in the OG group compared to the control group (0.50 ± 0.25% vs. 0.05 ± 0.08%, $p < 0.05$) and compared to the IP group ($p < 0.05$). Eosinophil values showed individual variations, but without statistically significant differences ($p > 0.05$). Erythrocyte parameters did not show statistically significant differences compared to the control group. The RBC count, Hb concentration, and HCT values remained relatively preserved. Erythrocyte indices (MCV, MCH and MCHC) did not show significant changes. The platelet count showed an increase in mean value in the OG group compared to the control, but the difference was not statistically significant ($p > 0.05$).

Comparative statistical analysis given in Table 5 revealed that halogenated boroxine administration induced selective changes in certain hematological parameters, highly dependent upon the route of administration.

The total leukocyte count (WBC) remained statistically unaltered among the control, intraperitoneal (IP), and oral (OG) groups ($p > 0.05$). However, differences were observed in the differential leukocyte formula. The IP group showed a significant elevation in neutrophil percentage relative to the control group (28.62 ± 2.33% vs. 22.69 ± 4.87%, $p < 0.05$). Conversely, the OG group showed a significant decline in lymphocyte percentage compared to controls (53.58 ± 4.63% vs. 58.68 ± 2.73%, $p < 0.05$), alongside a significant increase in basophil proportions compared to both the control and IP cohorts ($p < 0.05$). IP administration significantly reduced hematocrit (HCT) levels compared to the control group (0.36 ± 0.07 vs. 0.43 ± 0.03 L/L, $p < 0.05$) while significantly elevating MCH and MCHC values ($p< 0.05$). No statistically significant differences were found in the number of erythrocytes, hemoglobin concentration, and MCV values among the groups. Platelet values showed an increasing trend in both treated groups compared to the control, but these differences were not statistically significant ($p > 0.05$).

*In vitro*

Halogenated boroxine was evaluated at a concentration of 10 mg/mL, both with and without a 30-minute pre-incubation step. Enzyme activity was assessed before and after incubation to determine whether the incubation period modulated changes in enzymatic activity. The Lineweaver–Burk plot obtained without pre-incubation demonstrates that halogenated boroxine competitively binds to the enzyme, as shown in Figure 2. The obtained kinetic values of maximum velocity ($V_{max}$) remained constant regardless of the halogenated boroxine addition at 0.0003 mM $s^{-1}$. Conversely, the Michaelis constant ($K_m$) shifted significantly, increasing from 0.0004 mM in the absence of the inhibitor to 0.007 mM upon the addition of halogenated boroxine. Measurements of creatine kinase activity prior to incubation confirmed that halogenated boroxine induces significant enzyme inhibition at a concentration of 10 mg/mL, yielding an $IC_{50}$ value of 42%.

**Table 4.** *Values of hematological parameters of experimental group 2, halogenated boroxine administered orally (OG)*

| | WBC $10^9$/L | LYM% | MON% | NEU% | EOS% | BASO% | RBC $10^{12}$/L | Hb g/L | HCT L/L | MCV fL | MCH pg | MCHC g/L | PLT$10^9$/L |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Experimental group 2 (OG) | 5.57 | 58.47 | 15.87 | 22.35 | 3.14 | 0.17 | 8.36 | 154 | 0.436 | 52.2 | 18.4 | 353 | 680 |
| | 7.93 | 60.53 | 15.19 | 21.58 | 2.06 | 0.64 | 7.88 | 139 | 0.401 | 50.9 | 17.6 | 346 | 445 |
| | 3.81 | 50.37 | 12.24 | 26, 79 | 10.14 | 0.46 | 5.48 | 105 | 0.274 | 50 | 19.1 | 383 | 359 |
| | 3.76 | 46.32 | 20.92 | 30.1 | 2.08 | 0.58 | 6.3 | 119 | 0.318 | 50.6 | 18.8 | 374 | 710 |
| | 5.08 | 52.42 | 14.26 | 29.5 | 3.41 | 0.41 | 10.77 | 186 | 0.557 | 51.8 | 17.2 | 333 | 445 |
| | 4.88 | 48.23 | 19.25 | 29.66 | 2.45 | 0.41 | 4.98 | 119 | 0.25 | 50.2 | 23.89 | 476 | 550 |
| | 4.56 | 55.12 | 14.32 | 26.34 | 3.12 | 0.96 | 5.9 | 128 | 0.29 | 49.15 | 21.69 | 441 | 414 |
| | 6.78 | 54.34 | 15.11 | 27.22 | 2.98 | 0.44 | 6.68 | 146 | 0.31 | 46.4 | 21.85 | 470 | 489 |
| | 6.23 | 52.45 | 13.89 | 24.65 | 3.56 | 0.38 | 7.76 | 147 | 0.4 | 51.54 | 18.94 | 367 | 507 |
| $\bar{X}$ | 5.62 | 53.58 | 15.45 | 26.44 | 3.67 | 0.5 | 7.13 | 138.1 | 0.37 | 50.54 | 19.59 | 385.4 | 511 |
| Sd | 1.42 | 4.63 | 2.87 | 3.01 | 2.49 | 0.25 | 1.77 | 24.1 | 0.09 | 1.74 | 2.12 | 47.8 | 118 |

**Table 5.** *Comparative presentation of statistical analysis of the examined parameters*

| Parametar | Control $\bar{X} \pm Sd$ | IP $\bar{X} \pm Sd$ | OG $\bar{X} \pm Sd$ | T test IP vs Control | T test OG vs Control | T test IP vs OG |
|---|---|---|---|---|---|---|
| WBC ($10^9$/L) | 6.95 ± 1.66 | 5.66 ± 1.47 | 5.62 ± 1.42 | 0.154 | 0.14 | 0.954 |
| LYM (%) | 58.68 ± 2.73 | 52.78 ± 7.46 | 53.58 ± 4.63 | 0.054 | 0.019* | 0.789 |
| MON (%) | 14.49 ± 2.17 | 11.94 ± 6.93 | 15.45 ± 2.87 | 0.327 | 0.475 | 0.189 |
| NEU (%) | 22.69 ± 4.87 | 28.62 ± 2.33 | 26.44 ± 3.01 | 0.029* | 0.133 | 0.106 |
| EOS (%) | 4.08 ± 2.70 | 5.60 ± 1.47 | 3.67 ± 2.49 | 0.248 | 0.772 | 0.067 |
| BASO (%) | 0.05 ± 0.08 | 0.16 ± 0.14 | 0.50 ± 0.25 | 0.076 | <0.001* | 0.004 * |
| RBC ($10^{12}$/L) | 8.30 ± 1.01 | 7.03 ± 1.42 | 7.13 ± 1.77 | 0.064 | 0.128 | 0.897 |
| Hb (g/L) | 147.90 ± 5.92 | 134.30 ± 22.10 | 138.10 ± 24.10 | 0.111 | 0.272 | 0.732 |
| HCT (L/L) | 0.43 ± 0.03 | 0.36 ± 0.07 | 0.37 ± 0.09 | 0.021* | 0.093 | 0.796 |
| MCV (fL) | 51.85 ± 5.06 | 51.90 ± 1.70 | 50.54 ± 1.74 | 0.982 | 0.565 | 0.113 |
| MCH (pg) | 17.91 ± 1.39 | 19.60 ± 0.80 | 19.59 ± 2.12 | 0.030* | 0.087 | 0.99 |
| MCHC (g/L) | 347.07 ± 15.95 | 378.90 ± 19.70 | 385.40 ± 47.80 | 0.005 * | 0.049* | 0.713 |
| PLT ($10^9$/L) | 413.00 ± 100.88 | 465.40 ± 19.60 | 511.00 ± 118.00 | 0.262 | 0.111 | 0.284 |

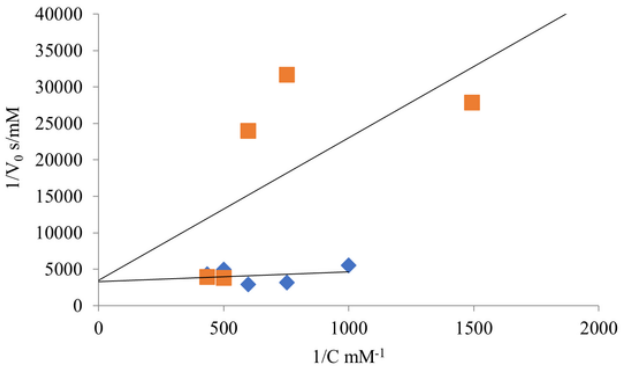



**Figure 2.** *Lineweaver-Burk plot for determining $V_{max}$ and $K_m$ in the absence or presence of various concentrations of halogenated boroxine; ◆ 0 mg/mL; ◆ 10 mg/mL (without incubation)*

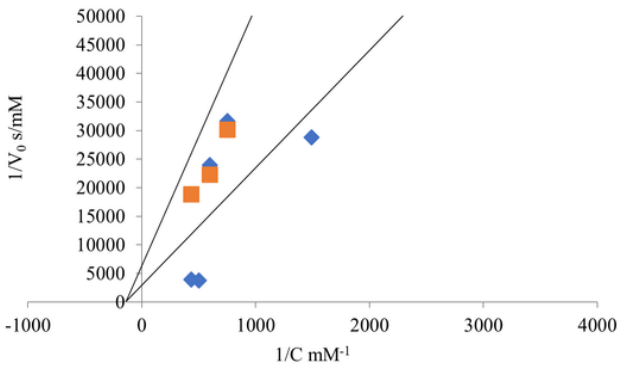



**Figure 3.** *Lineweaver-Burk plot for determining $V_{max}$ and $K_m$ in the absence or presence of different concentrations of halogenated boroxine; ◆ 0 mg/mL; ◆ 10 mg/mL (with incubation)*

Analysis of the Lineweaver–Burk plot following a 30-minute pre-incubation with halogenated boroxine revealed a non-competitive mechanism of inhibition, as shown in Figure 3. In the presence of halogenated boroxine, the maximum reaction velocity ($V_{max}$) decreased to 0.0001 mM $s^{-1}$, whereas the Michaelis constant ($K_m$) remained unaltered at 0.007 mM. In comparison, control measurements in the absence of halogenated boroxine yielded a $V_{max}$of 0.0003 mM $s^{-1}$, while the $K_m$ value remained the same, amounting to 0.007 mM.

## DISCUSSION

*In vivo*

The hematological profile of the control group fell within established reference limits for Wistar rats, confirming the validity of the experimental model. Erythrocyte parameters, including RBC, hemoglobin, hematocrit (HCT) values, and erythrocyte indices, as well as platelet (PLT) counts, were entirely consistent with physiological baseline values reported for laboratory rats. Minor variations observed within the differential leukocyte formula reflect expected biological variability. These variations align with the previous literature demonstrating that hematological parameters in laboratory animals are dynamically influenced by genetic strain, housing environments, and experimental conditions (14–16).

Following intraperitoneal (IP) administration of potassium hydrofluoroboroxine ($K_2[B_3O_3F_4OH]$) at a dose of 10 mg/kg of body weight, the majority of hematological parameters remained unaltered relative to the control group. The absence of significant shifts in overall leukocyte (WBC), erythrocyte (RBC), hemoglobin (Hb), and platelet (PLT) counts indicates that the 5-day administration of the tested compound neither caused general hematological suppression nor disruption of basic hematopoietic functions.

The most significant change in the IP group involved an elevated neutrophil percentage with a simultaneous decrease in hematocrit. An increase in neutrophils, occurring independently of any increase in the total number of leukocytes, represents a shift in the relative proportions of leukocyte subsets and potentially signifies a mild activation of the innate immune response. Similar shifts in the differential leukocyte formula have been described in laboratory animals after exposure to various chemical substances and most often represent a transient adaptive response, especially when they are not accompanied by other indicators of inflammation (15,17). Furthermore, because the HCT reduction in the IP group was not accompanied by declines in either RBC counts or Hb concentrations, nor by shifts in MCV, it cannot be linked to erythroid pathologies or the development of anemia. This pattern indicates an isolated change in the HCT value without any evidence of a disorder of erythropoiesis. Similarly, the isolated elevation in MCH, occurring while all other erythrocytic markers remained preserved, may indicate a localized shift in hemoglobin content per cell, though it lacks apparent pathological relevance. Upon oral administration of $K_2[B_3O_3F_4OH]$, most hematological parameters also did not show significant changes compared to the control group. The stability of erythrocyte parameters and platelets indicated the preserved function of the erythrocyte and platelet lineages after systemic absorption of the compound. However, the OG group was uniquely characterized by a statistically significant contraction in lymphocyte percentages alongside an increase in the percentage of circulating basophils.

A decrease in the relative share of lymphocytes, without a change in the total number of leukocytes and without pronounced neutrophilia, does not indicate a systemic stress response, but rather a change in the ratio of different leukocyte forms. Similarly, the observed basophilia must be interpreted with caution given the naturally low abundance of basophils in the peripheral blood of healthy rats. In the absence of other hematological deviations, these changes may indicate mild immunomodulatory activity, but not the development of a pathological immune process.

A comparison of the two routes of administration shows that the hematological response depended on the route of administration. Intraperitoneal administration was associated with a more pronounced change in the neutrophil population, while oral administration led to changes in the lymphocytic and basophilic components of the differential leukocyte formula. These differences may be due to different routes of absorption, distribution, and interaction of the compounds with the immune system. It is known that the route of administration significantly affects the intensity of the biological response after exposure to chemical substances in experimental animals (18).

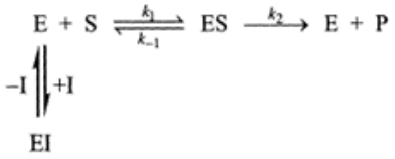
The obtained results should be viewed in the context of biological significance, and not just the statistical significance of individual changes. Although certain parameters showed statistically significant differences between the groups, their values mostly remained within the reference physiological limits, without simultaneous changes in other hematological indicators that would indicate toxic effects. Boron compounds represent a group

of substances whose biological effects depend on their chemical structure, dose, and time of exposure. Previous research indicates that boron compounds can participate in the regulation of various cellular processes and exhibit biological activity without the necessary development of toxic effects at moderate doses (19-21).

Accordingly, the changes in this study, limited primarily to the differential leukocyte formula, can be interpreted as a mild adaptive immune response after administration of $K_2[B_3O_3F_4OH]$. Overall, administration of dipotassium trioxohydroxytetrafluorotriborate ($K_2[B_3O_3F_4OH]$) at a dose of 10 mg/kg body weight for 5 consecutive days in rats did not cause marked hematological disorders 24 hours after the last application. The observed changes were selective, dependent on the method of administration, and mostly limited to individual components of the differential leukocyte formula and erythrocyte indices. Preservation of basic hematological parameters indicates the absence of significant hematological toxicity at the examined dose and period of exposure.

*In vitro*

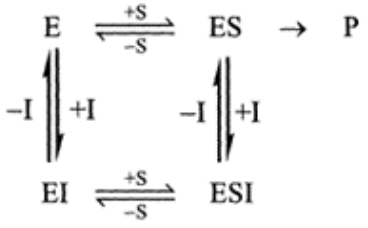
Halogenated boroxine showed a dual mechanism of creatine kinase inhibition, depending on the time of contact with the enzyme. Without prior incubation, Lineweaver–Burk analysis indicated competitive inhibition, which was confirmed by an unchanged $V_{max}$ value (0.0003 mM $s^{-1}$) and a significant increase in $K_m$ from 0.0004 to 0.007 mM. An increase in $K_m$ with a constant $V_{max}$ is a characteristic indicator of competitive inhibition, in which the inhibitor and substrate compete for the same or overlapping binding site on the enzyme, according to the following mechanism:




This pattern indicates that halogenated boroxine reduces the affinity of the enzyme toward the substrate, but does not affect the maximum catalytic capacity of the enzyme when the substrate concentration is high enough. This finding is in accordance with the known properties of boronic compounds, which, due to the electron-deficient boron atom, can achieve specific interactions with the catalytic centers of enzymes and act as reversible competitive inhibitors (22,23). Additionally, the measured inhibition of creatine kinase activity at 42% at a halogenated boroxine concentration of 10 mg/mL confirms that this compound possesses a significant ability to modulate enzyme activity. Although $IC_{50}$ was not fully reached at the tested concentration, the degree of recorded inhibition indicates a pronounced interaction between the inhibitor and the enzyme.

Similar inhibitory effects of halogenated boroxine were previously suggested for other enzymes, including human catalase and carbonic anhydrase, where molecular docking experiments showed binding of the compound within the active sites of the enzymes and potential inhibition of their activity (22).

Notably, after a 30-minute pre-incubation, the change of type of inhibition from competitive to non-competitive occurred. In this case, $K_m$ value remained practically unchanged, while $V_{max}$ decreased from 0.0003 to 0.0001 mM $s^{-1}$. Such a kinetic pattern indicates that the inhibitor no longer acts primarily on substrate binding, but instead affects the catalytic function of the enzyme by forming a complex with the enzyme or an enzyme–substrate complex at an allosteric site, according to the following mechanism:




An unchanged $K_m$ suggests that the affinity of the enzyme for the substrate remains the same, while a decrease in $V_{max}$ reflects a decrease in the number of functionally active enzyme molecules. This result indicates that a certain time of interaction between halogenated boroxine and the enzyme is required to achieve a full inhibitory effect (24). The change in the mechanism of inhibition after incubation can be explained by the gradual formation of a more stable complex between the inhibitor and the enzyme. In the case of numerous boron derivatives, time-dependent inhibition of enzymes caused by the formation of reversible covalent or very strong non-covalent interactions has been described. Such inhibitors often show initial competitive binding, which over time develops into a complex of higher stability, which results in a drop in $V_{max}$ and the appearance of kinetic characteristics of non-competitive or mixed inhibition (24,25).

The obtained results indicate that the mechanism of action of halogenated boroxine is complex and probably involves multiple sites of interaction with creatine kinase. Such behavior is not unusual for boron-containing compounds, as they can act as analogs of the transition state of the reaction and form stable complexes with amino acid residues inside

or outside the active site of the enzyme. It is known that boron compounds have the ability to reversibly bind to enzymes and can significantly change their kinetic parameters (2,4,24). Overall, the results of this research show that halogenated boroxine at a concentration of 10 mg/mL acts as an efficient creatine kinase inhibitor. Without prior incubation, the inhibitor behaves as a competitive inhibitor, while after 30 minutes of incubation, it shows the properties of non-competitive inhibition. This time-dependent change in the mechanism of inhibition suggests the gradual establishment of more stable interactions between halogenated boroxine and the enzyme, which may be of importance for understanding its biological action and potential pharmacological application.

The findings of this study demonstrate that halogenated boroxine modulates specific hematological profiles, depending on the method of application and experimental conditions. Under *in vitro* conditions, halogenated boroxine inhibited creatine kinase activity. Furthermore, enzyme kinetic analysis revealed a distinct, time-dependent shift in the mechanism of inhibition following a 30-minute pre-incubation period, characterized by a significant decrease in the maximum reaction velocity, $V_{max,}$, while the Michaelis constant, $K_m$, remains unaltered.

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## Author Contributions

Conceptualization, S.H., E.H. (First author), and E.H. (Second author); Formal analysis, E.H. (First author), M.F., and A.B.; Investigation, E.H. (First author), M.F., and A.B.; Methodology, S.H., E.H. (First author), and E.H. (Second author); Supervision, S.P., and M.H.; Writing – original draft, S.H., E.H. (First author), M.F., A.B., S.P., M.H., and A.D.Z.; Writing – review & editing, S.H., N.V., and A.D.Z. All authors have read and approved the published version of the manuscript.

## Statement of Ethics

This study protocol was reviewed and approved by the Ethics Committee, University of Sarajevo Faculty of Science, approval number 01/01-1945/2-2025, issued on 23.10.2025.

## Statement of Competing Interest

The authors declare no relevant conflicts of interest.

## Statement of Data Availability

Not applicable.

## Statement of Generative AI Use

No generative AI was used.

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