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Effects of a Novel Benzenesulfonamide 4-(3-(4-Bromophenyl)-5-(2,4-dimethoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl) on Antioxidant Enzymes and Hematological Parameters of Rainbow Trout (*Oncorhynchus mykiss*)

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Authors' Contribution
DOO, SB, HIG and CY performed synthesis of the B4 compound. GA, AU, MK, VP, MA and TY tested B4 compound. AM and TY evaluated the data and wrote the manuscript.

Key words
CAT, SOD, GPx, rainbow trout, pyrazoline, B4

ABSTRACT

In this study, a mixture of a suitable chalcone and *p*-hydrazinobenzenesulfonamide hydrochloride in 1:1.1 mol ratio in ethanol (25 ml) in the presence of glacial acetic acid (0.05 ml) was refluxed for synthesis of 4-(3-(4-Bromophenyl)-5-(2,4-dimethoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl) benzenesulfonamide, B4, for 7h at first experiment. Effects of B4 on antioxidant enzymes of gill and liver, and changes in hematological parameters were determined in rainbow trout. For this purpose, rainbow trout alevins were exposed to 0, 0.25, 0.5, 1 and 2.5 mg/L of B4 and 1 ml of DMSO for 96 hours. Blood samples were collected for hematological analyzes and mortalities were recorded daily. Exposure to B4 caused significant changes in antioxidant enzymes of gill and liver of alevins ($p<0,05$). Catalase, superoxide dismutase (SOD) and glutathione peroxidase activities were decreased, while MDA values increased significantly ($p<0.05$) in gill and liver of fish compared to control group. SOD activity was time dependent; it decreased initially, but then take increased. WBC, MCV, MCH and MCHC levels increased, while RBC Hb, Htc and PLT levels decreased with increasing dose from 0.25 to 2.5 mg/L of B4 ($p<0.05$) compared to control. There were no differences between control and DMSO group ($p>0.05$) with respect to all tested properties. It is suggested that B4 should be investigated further for possible usage in aquaculture industry to produce healthy fish.

INTRODUCTION

There are many derivatives of pyrazoline synthesized from intermediate chalcone (Khedkar *et al*., 2018). The activity of pyrazolines was reported as an antioxidant (Silva *et al*., 2015), antibacterial (Ozbey *et al*., 2005; Akyıldız *et al*., 2018), anti-inflammatory (Cristina *et al*., 2018; Ugvu *et al*., 2018), cytotoxic, anticancer (Gul *et al*., 2016, 2017a Kucukoglu *et al*., 2016; Custodio *et al*., 2019; Ozgun *et al*., 2019) analgesic, anticonvulsant (Mishra *et al*., 2018), anxiolytic and anthelmintic (Debnath and Devanna, 2013), antimalarial Silveira *et al*., 2018), herbicidal (Xie *et al*., 2018), antifungal (Al-Fahemi *et al*., 2018, enzyme inhibitors such as carbonic anhydrase (Gul *et al*., 2016, 2017a, b; Kucukoglu *et al*., 2016; Mete *et al*., 2016; Yamali *et al*., 2018; Ozgun *et al*., 2019) and acetyl choline esterase (Yamali *et al*., 2018; Ozgun *et al*., 2019) fluorescent pH sensor (Bozkurt *et al*., 2018a), fluorometric "turn-off" sensing for Hg2+ (Bozkurt *et al*., 2018b) and antimicrobial activity (Alyar *et al*., 2018). Effects of pyrazoline derivatives on glaucoma (Abdel-Aziz *et al*., 2017) and anticancer activity (Ghorab *et al*., 2017) were investigated.

Antioxidant effects of pyrazoline on catalase (CAT),

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peroxidase (POD), glutathione reductase (GSSG-Rx) and superoxide dismutase (SOD) enzymes have been reported in living organisms (Nimse and Pal, 2015). After normal cellular metabolism, reactive oxygen species (ROS) are produced in living organisms. Enzymatic antioxidants, CAT, glutathione peroxidase (GSH-Px) and SOD is used for the protection of cells from the destructive effects of ROS in the body. Nonenzymatic antioxidants, glutathione, ascorbic acid, α-tocopherol, carotenoids, and phenolic compounds are used also for this purpose (Koruk *et al.*, 2004). Antioxidant defense systems control the negative effects of free radicals in a cell (Gülçin, 2002). Cellular defense enzymes SOD, CAT, and GSH-Px prevent the reduction of oxygen metabolism products (Rahimi *et al.*, 2005). Toxic substances have some adverse effects on the hematology of fishes (Atamanalp and Yanik, 2003).

There is, however, very little information about the toxicity of novel pyrazoline derivatives in fish. Therefore, the present study was conducted to test the toxicity of newly synthesized novel pyrazoline derivative, B4, on hematological parameters and enzymatic antioxidants namely SOD, CAT and glutathione peroxidase (GPx) and lipid peroxidation malondialdehyde (MDA) in gill and liver in rainbow trout.

MATERIALS AND METHODS

Synthesis of the A4 compound

4-bromoasetophenone and 2,4 dimethoxyasetaldehyde 1:1 mol ratio were dissolved in ethyl alcohol (10 ml), a 20 ml solution of cold NaOH (10%) was added dropwise onto the mixture. The mixture was stirred at room temperature. Progress of the reaction. followed by T.L.C. At the end of the reaction, the balloon contents were poured into ice water and neutralized with enough HCl solution (10%). The purity of the obtained solid was determined by T.L.C. After the second stage of B4 synthesis was used without further purification. The method of synthesis of chalcone is presented in Figure 1.

Synthesis of the B4 compound

B4 were synthesized according to works of (Bozkurt *et al.*, 2018; Gul *et al.*, 2018; Yamali *et al.*, 2018; Ozgun *et al.*, 2019). In brief, a mixture of 3-(2.4-dimethoxyphenyl)-1-(4-bromophenyl)-2-propen-1-on and p-hydrazinobenzenesulfonamide hydrochloride in 1:1.1 mol ratio in ethanol (25 ml) in the presence of glacial acetic acid (0.05 ml) was refluxed for 4-(3-(4-bromophenyl)-5-(2.4-dimethoxyphenyl)-4.5-dihydro-1H-pyrazol-1-yl) benzenesulfonamide namely B4 for 7h ((Fig. 2). Reactions were monitored by TLC using chloroform: methanol (4.8:0.2) as a solvent system. When the reactions were stopped, the precipitate obtained was filtered, dried and recrystallized from ethanol.

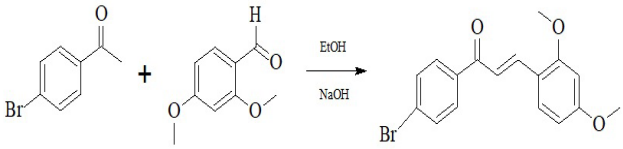

Fig. 1. 3-(2.4-dimethoxyphenyl)-1-(4-bromophenyl)-2-propen-1-on and *p*-hydrazinobenzenesulfonamide hydrochloride (A4).

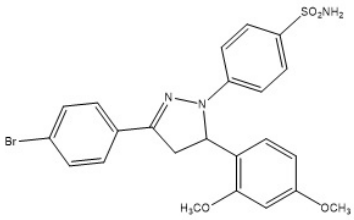

Fig. 2. 4-(3-(4-bromophenyl)- 5- (2.4- dimethoxyphenyl)-4.5- dihydro- 1*H*-pyrazol-1-yl) benzenesulfonamide (B4).

The chemical structure of the compound was determined by ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectroscopies using a Varian Mercury Plus spectrometer (Varian Inc., Palo Alto, California, U.S.). Chemical shifts (δ) are reported in ppm and coupling constants (*J*) are expressed in hertz (Hz). Mass spectra of the compounds were undertaken on a HPLC-TOF Waters Micromass LCT Premier XE (Milford, MA, USA) mass spectrometer using an electrospray ion source (ESI). Melting points were determined using an Electrothermal 9100/IA9100 instrument (Bibby Scientific Limited, Staffordshire, UK) and are uncorrected. The reactions were monitored using silica gel HF254-366 Thin Layer Chromatography (TLC) plates (E. Merck, Germany).

Cream color solid, mp 225 °C, yield 55 %. ^{1}H NMR (DMSO-d_6, ppm, 400 MHz) δ 7.69 (d, J = 8.4 Hz, 2H, Ar-H), 7.61 (d, J = 8.4 Hz, 2H, Ar-H), 7.57 (d, J =8.8 Hz, 2H, Ar-H), 7.01 (d, J = 6.2 Hz, 2H, Ar-H), 6.98 (s, 2H, -SO_2NH_2), 6.75 (d, J = 8.4 Hz, 1H, Ar-H), 6.62 (d, J = 2.3 Hz, 1H, Ar-H), 6.39 (dd, J = 8.3, 2.3 Hz, 1H, Ar-H), 5.64 (dd, J = 12.1, 5.0 Hz, 1H, H-5 (pyrazoline)), 3.85 (s, 3H, -OCH_3), 3.69 (s, 3H, -OCH_3), 3.04 (dd, J = 17.6, 5.0 Hz, 1H, H-4 (pyrazoline)). ^{13}C NMR (DMSO-d_6, ppm, 100 MHz) δ 160.8, 157.8, 149.8, 146.3, 133.7, 132.3, 131.9, 128.6, 127.9, 127.4, 122.9, 120.9, 112.5, 105.7, 99.7, 57.9 (C-5 pyrazoline), 56.5 (-OCH_3), 55.9 (-OCH_3), 42.3 (C-4 pyrazoline). HRMS (ESI-MS) $C_{23}H_{23}N_3O_4SBr$, Calculated $[M+H]^+$: 516.0593; Found $[M+H]^+$: 516.0605.

Experimental design

A set of 6 aquaria with 48 fish (25 ± 2 g) were used for testing the B4 compound at 11 ± 1.5 °C during a 96 h study in the Aquarium Fish Production Unit of Ataturk University. Fish were acclimated for 14 days before the testing experiments. Studied doses were chosen based on the study done by Khedkar *et al*. (2018). B4 compound was dissolved in dimethyl sulfoxide (DMSO). Exposure doses were 0.00 (control), 1 ml DMSO, 0.25, 0.5, 1 and 2.5 mg / L.

Mortalities were recorded daily. Fish were offered a commercial feed (Sibal INC.) (Bricknell *et al*., 1999).

At the end of the experimental period, blood samples were collected and subjected to hematological analyzes by using Prokan PE 6800 blood analyzer device. Liver and gill tissue samples, kept at -86 ºC, were used for determination of antioxidant enzyme activities -SOD (Sun *et al*., 1988), CAT (Aebi, 1984), GPx (Beutler, 1975), total protein (Bradford, 1976) and lipid peroxidation (MDA) (Luo *et al*., 2006).

The determined data was analyzed statistically by SPSS software. Duncan test was used for the determination of differences between groups at alpha 0.05 level.

RESULTS

Different doses of pyrazoline compound B4; 4-(3-(4-bromophenyl)-5-(2,4-dimethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl) benzenesulfonamide; caused significant effects on antioxidant enzyme activity in gill (Table I), liver (Table II) and hematology of fish (Table III).

Table I shows decreases of CAT, GPx and SOD levels in gill with increasing concentration of B4 up to 1 mg/L compared to control. MDA levels increased significantly ($p<0.05$) at 0.5 and 1 mg/L then drop to insignificant level ($p>0.05$) compared to control. There were no differences between the control and DMSO group ($p>0.05$) for all tested properties.

Table II shows significant reductions in CAT, GPx and SOD values in the liver significantly at 0.5 mg / L of B4 ($p<0.05$) compared to control. MDA levels did not differ ($p>0.05$) compared to control except for the dose of 1 mg / L. There were no differences between control and DMSO group ($p>0.05$) for all tested properties. After exposition to B4 substance, fish mortalities varied 0% in control and DMSO group, 25.5% in 0.25 mg / L group and 25% in 0.5, 1 and 2.5 mg / L group.

Table III shows increases in WBC, MCV, MCH and MCHC levels and decreases in RBC Hb, Htc and PLT levels in blood with the increasing concentration of B4 ($p<0.05$) compared to control. There were no differences between the control and DMSO group ($p>0.05$) for all tested properties.

Table I. Effects of different doses of pyrazoline derivative, B4, on specific antioxidant enzyme activities (CAT, GPx and SOD) and lipid peroxidation (MDA) levels in gills of rainbow trout after 96 h exposure.

B4 (mg/L)	CAT (EU/ mgprot.)	GPx (EU/ mgprot.)	SOD (EU/ mg prot.)	MDA (nmol/ml)
0	0.82±0.22^{a}	0.54±0.23^{a}	1.13±0,14^{a}	0.17±0.32^{b}
0.25	0.41±0.19^{b}	0.19±0.20^{bc}	0.65±0.14^{bc}	0.26±0.32^{ab}
0.5	0.13 ±0.13^{c}	0.02 ±0.01^{c}	0.39±0,14^{c}	0.32±0.32^{a}
1	0.24 ±0.16^{bc}	0.01 ±0.04^{c}	0.47±0,14^{c}	0.31±0.32^{a}
2.5	0.68 ±0.28^{a}	0.24±0.26^{bc}	0.73±0,14^{abc}	0.20±0.32^{b}
DMSO	0.71±0.13^{a}	0.41±0.21^{ab}	1.04±0,14^{ab}	0.20±0.32^{b}

Table II. Effects of different doses of pyrazoline derivative B4 on specific antioxidant enzyme activities (CAT, GPx and SOD) and lipid peroxidation (MDA) levels in liver of rainbow trout after 96 h exposure.

B4 (mg/L)	CAT (EU/mg)	GPx (EU/mg)	SOD (EU/mg)	MDA (nmol/ml)
0	0.86±0.09^{a}	0.64±0.036^{a}	1.63±0.29^{a}	0.19±0.05^{b}
0.25	0.56±0.09^{ab}	0.34±0.036^{b}	0.74±0.29^{ab}	0.26±0.05^{b}
0.5	0.43 ±0.09^{b}	0.37 ±0.036^{b}	0.65±0.29^{b}	0.37±0.05^{b}
1	0.65 ±0.09^{ab}	0.43 ±0.036^{ab}	0.83±0.29^{ab}	0.64±0.05^{a}
2.5	0.68 ±0.09^{ab}	0.47 ±0.036^{ab}	0.92±0.29^{ab}	0.22±0.05^{b}
DMSO	0.83±0.09^{a}	0.59±0.036^{ab}	1.02±0.29^{ab}	0.21±0.05^{b}

DISCUSSION

Considering all enzymes, CAT is the one, which has the highest turnover numbers. It was reported that millions of hydrogen peroxide molecules can be converted into water and oxygen in a second by one-catalase molecule (Koruk *et al*., 2004; Nimse and Pal, 2015). Mao *et al*. (1993) hypothesized that the amount of H_2O_2 should be reduced by CAT and the toxicity of SOD is reduced by catalase at high concentrations. CAT protects cells from oxidative damage by reactive oxygen species (ROS) (Koruk *et al*., 2004). Winston *et al*. (2001) reported that SOD, CAT, and glutathione peroxidase (GPx) enzymes can conjugate with antibodies. Therefore, it is thought that having low, SOD and GPx values both in the gill and liver of fish might be due to the negative effects of B4 chemical caused oxidative stress. In the present study, low SOD values may be due to the consumption of the enzyme in converting oxygen to

Table III. Effects of different doses of pyrazoline derivative, B4, on haematology of rainbow trout after 96 h exposure.

Dose (mg/L)	WBC (10^4/ mm^3)	RBC (10^4/ mm^3)	Hb (g/dl)	Htc(%)	PLT (10^4/ mm^3)	MCV (µm3)	MCH (pg)	MCHC (g/100ml)
0	124.50±3.34^{c}	1.68±0.09^{b}	12.17±0.336^{c}	27.85±1.45^{b}	13.00±0.87^{a}	168.20±9.82^{a}	79.50±5.71^{a}	46.80±3.71^{a}
0.25	137.83±3.34^{a}	1.14±0.09^{a}	9.77±0.336^{bc}	20.42±1.45^{a}	9.88±0.87^{b}	179.86±9.82^{a}	86.20±5.71^{a}	47.96±3.71^{a}
0.5	142.50±3.34^{a}	1.09 ±0.09^{a}	9.00±0.336^{c}	18.97±1.45^{a}	8.17±0.87^{b}	174.53±9.82^{a}	82.38±5.71^{a}	49.49±3.71^{a}
1	142.98±3.34^{a}	1.13 ±0.09^{a}	9.55±0.336^{bc}	18.77 ±1.45^{a}	8.83±0.87^{b}	167.92±9.82^{a}	85.19±5.71^{a}	50.96±3.71^{a}
2.5	136.12±3.34^{ab}	1.13 ±0.09^{a}	10.08±0.336^{b}	21.78±1.455^{a}	9.50±0.87^{b}	185.99±9.82^{a}	85.96±5.71^{a}	46.38±3.71^{a}
DMSO	127.40±3.34^{bc}	1.22±0.09^{a}	11.30±0.336^{c}	20.42±1.45^{a}	8.17±0.87^{a}	168.77±9.82^{a}	92.82±5.71^{a}	55.78±3.71^{a}

hydrogen peroxide. As the reduction of oxygen caused by the B4 substance might result in the formation of many reactive oxygen species (ROS) including superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion and nitric oxide (Hancock *et al*., 2001; Forman and Torres, 2002).

An increase in the formation of MDA levels shows that there was overwhelming stress in which free radicals suppressed the antioxidant system and caused low antioxidant levels in gill and livers. From the present study, it can be seen that the SOD activity is time-dependent as it was reduced first and then increased again.

Cellular damage in lipid, DNA, RNA, and proteins is caused by ROS. ROS are produced as a normal product of cellular metabolism. It is mentioned that plasma MDA level was increased by oxidative damage in DNA (8-oxodG, 1, N6-εdA, 1,N2-εdG) (Malavolta and Mocchegiani, 2016). Measurements of higher values in WBC, MCV, MCH, and MCHC, and low values of RBC Hb, Htc and PLT levels compared to the control group shows that fish had great stress after exposition to B4. There are many controversial results in fish hematology after expositions to chemical substances (Atamanalp and Yanik, 2003). Hamed and El-Sayed (2019) reported that exposure to pendimethalin (PM) caused decreases in SOD, CAT, total antioxidant capacity (TAC) and glutathione peroxidase (GSH-Px) levels, and increases in MDA as well as DNA fragmentation in the liver in Nile tilapia, *Oreochromis niloticus* (L.). Singh *et al*. (2019) reported that exposure to iron resulted in the accumulation of iron in fish tissues and caused significant fluctuations in erythrocyte and leukocyte counts, hemoglobin, lipid peroxidation, antioxidant enzyme activity (SOD and CAT). It was found that while lipid peroxidation was increasing SOD and CAT was decreasing double in the exposure of excess iron. There were a time and dose-dependent tissue injuries in gills and liver in freshwater fish *Labeo rohita* (Singh *et al*., 2019).

CONCLUSION

SOD, CAT, and GPX are the major antioxidant enzymes that protect cells against the harmful effects of ROS to maintain the health of organisms. Activators and inhibitors of these enzymes, therefore, are really of vital importance. The first line of the antioxidant defense system is comprised of the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Ighodaro and Akinloye, 2018). Low values of these enzymes show that B4 has negative effects on the total defense system of fish and, therefore, it might be useful testing its microbial properties in further studies before using it as the active substance of medicines.

Statement of conflict of interest

The authors have declared no conflict of interest.

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