

Novel Carbonic Anhydrase Activators in the Rats Exposed to H₂O₂

H₂O₂ Uygulanan Sıçanlarda Yeni Karbonik Anhidraz Aktivatörleri

Research Article

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ABSTRACT

Carbonic anhydrase has an important role in attentional gating of memory storage, signal process and long-term synaptic transformation beside its pH regulation, HCO₃⁻ reabsorption and CO₂ expiration tasks. Within the study, in vivo effects of (Benzofurane-2-yl)(3-phenyl-3-methylcyclobutyl)ketoxime (A), (Benzofurane-2-yl)(3-methyl-3-mesitylcyclobutyl)ketone thiosemicarbazone (T), 1,3-bis(2-chlorbenzoyl)imidazoline-2-thione (B) which are synthetic products and H₂O₂ (H) on the carbonic anhydrase activity in the liver, erythrocyte, heart and kidney tissues of male *Wistar* rat were analyzed. For this purpose, control group, separate groups for each of the synthetic products, H₂O₂ group and H₂O₂ combination group with synthetic products were created. As a result of the obtained evidence, a decrease in carbonic anhydrase activity was observed in examined tissues exposed to H₂O₂, but A compound has activation effect on enzyme activity in liver, T compound has it in erythrocyte and heart tissues, B compound has it in liver and erythrocytes.

Key Words

Activation, Carbonic Anhydrase, H₂O₂, In vivo, thiosemicarbazone.

ÖZET

Karbonik anhidrazın pH düzenlenmesi, HCO₃⁻ geri emilimi, CO₂'in solunumla atılması gibi görevlerinin yanısıra, hafıza, sinyal prosesleri, uzun dönem sinaptik transformasyon olaylarında önemli rolleri vardır. Yapılan çalışmada, sentetik ürünler olan (Benzofuran-2-il)(3-fenil-3-metilsiklobutil) ketoksım (A), (Benzofuran-2-il)(3-metil-3-mezitilsiklobutil)keton tiyosemikarbazon (T), 1,3-bis(2-klorobenzoil)imidazolin-2-tiyon (B) ve H₂O₂'in karbonik anhidraz aktivitesi üzerine erkek *Wistar* karaciğer, eritrosit, kalp ve böbrek dokularında etkileri incelendi. Bu amaçla, kontrol grubu, her bir sentetik ürün için bir grup, H₂O₂ grubu ve sentetik ürünler ile H₂O₂ kombinasyon grupları oluşturuldu. Elde edilen verilerden H₂O₂ uygulan grupta bütün dokularda karbonik anhidraz aktivitesinde azalma olduğu görüldü. Bununla birlikte A maddesi karaciğerde aktivasyona neden olurken T maddesi eritrosit ve kalp dokularında, B maddesi ise karaciğer ve eritrositlerde aynı etkiyi gösterdi.

Anahtar Kelimeler

Aktivasyon, Karbonik Anhidraz, H₂O₂, In vivo, tiyosemikarbazon.

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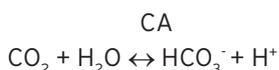
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INTRODUCTION

Carbonic anhydrase (carbonate hydrolyase E.C.4.2.1.1) is a metalloenzyme which is present commonly in every organisms and consists Zn²⁺ ion in its active region. Carbonic anhydrase (CA) explored in cattle's erythrocytes for the first time is an important enzyme that catalyzes reversibly the reactions of hydration of CO₂ and dehydration of HCO₃⁻:



Carbonic anhydrase also catalyzes cyanate's carbamic acid or urea's cyanamide, aldehyde's geminal diol hydration reactions besides hydration reaction of CO₂. This enzyme also catalyzes carboxylic, sulfonic, and phosphoric acid ester hydrolysis [1].

CA enzyme is characterized as an enzyme that regulates pH in various tissues along with erythrocytes. It has a role in various metabolic issue notably acid-base balance. It takes charge in the critic physiological process related to CO₂/bicarbonate respiration and transport between tissues/organs and lung, in pH and CO₂ hemostasis, electrolyte secretion, biosynthetic reactions (gluconeogenesis, lipogenesis and urea synthesis), ostosis, calcification, tumorigenesis and in many other physiological and pathological process [2,3].

Benzofuranes which are present in many natural products attract the attention of chemistry because of their biological activities. It is known that there are many natural products consisting of benzofurane rings. These are particularly the products isolated from the species like *Machilus glaucescens*, *Ophryosporus charua*, *Ophryosporus lorentzii*, *Krameria ramosissima* and *Zanthoxylum ailanthoidol* [4].

Benzofurane-derived products are important molecules in terms of pharmaceuticals. It is known that benzofurane-derivatives constituting a considerable part of heterocyclic compound have important biological features. Many studies have still be conducted to produce more active structures of these molecules by diversifying

them in medical word [5,6]. Benzofurane-derived products are stated to be important biologically and their features like antimicrobial agent, enzyme inhibitor and activator, agonist and antagonist receptor, anti-inflammatory agent, anticancer, antiviral, antitubercular, antioxidant, diagnostic imaging in Alzheimer's disease, complement system inhibitor, anti-ulcerogenic, ischemic cell death inhibitor and dopamine uptake inhibitor have been expressed [7].

Imidazole ring is present in many compounds in the nature and in the structure of various drugs. Imidazole is present in histidine -an amino acid, adenine and guanine bases and uric acid. This compound can be seen in the structure of many enzymes and have a role in the active center of these enzymes [8]. Imidazole-derived products have been reported to have antioxidant activities besides their anti-parasite, antiviral, antibacterial, antihypertensive, analgesic effects [9].

Thiosemicarbazones present their biological activities by inhibiting ribonucleotide reductase enzyme which is necessary for synthesizing DNA precursors. The inhibition is achieved by that the non-heme subunit of the enzyme is inhibited/inactivated by thiosemicarbazones. Because of these features, it is reported that thiosemicarbazones show antitumor activity and also antifungal features [10]. In another study, it is determined that thiosemicarbazone-derivatives have metal chelation features and antimicrobial effect [11].

Hydrogen peroxide (H₂O₂) is among the potential reactive oxygen species sources. Reactive oxygen species damage enzymes, cell membrane, DNA, proteins and lipids from the macro molecules. H₂O₂ causes oxidative stress in metabolism by transforming into hydroxyl radical (.OH). As a result of oxidative stress, many diseases such as cardiovascular, diabetes and cancer occur [12,13].

Within this study, CA activity in the erythrocyte, liver, heart and kidney tissues of the rat groups that were treated with H₂O₂, synthetic products {(Benzofurane-2-yl)(3-phenyl-3-methylcyclobutyl) ketoxime, (Benzofurane-2-yl)(3-methyl-3-

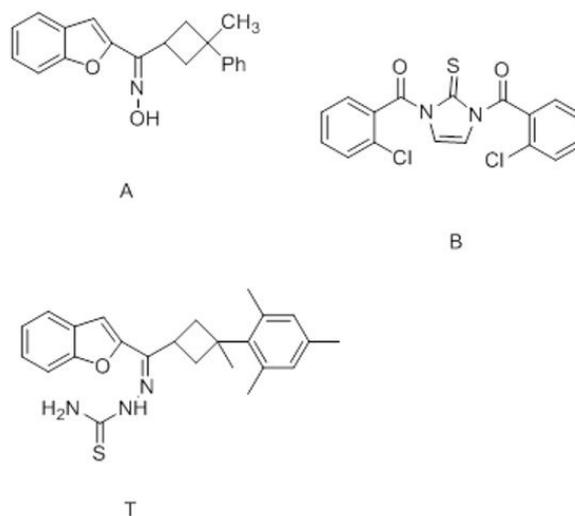


Figure 1. Structure of organic synthesis matters.

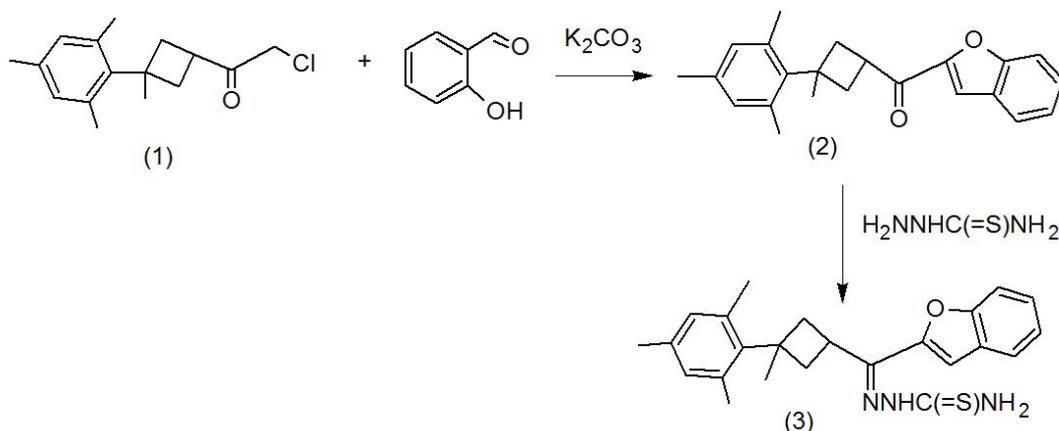


Figure 2. Synthesis of (Benzofuran-2-yl)(3-mesityl-3-methylcyclobutyl)ketone thiosemicarbazone.

mesitylcyclobutyl) ketone thiosemicarbazone and 1,3-bis(2-chlorobenzoyl)imidazole-2-thione (Figure 1) and the combination of these compounds.

MATERIALS AND METHODS

Chemical

Protein assay reagents and 4-nitrophenylacetate were obtained from Sigma-Aldrich Co. The other chemicals were of analytical grade and obtained from Merck.

The Synthesis of the Compounds

The synthetic products used in this study were synthesized before with 1,3-bis(2-chlorobenzoyl)imidazole-2-thione [14] and (Benzofuran-2-yl)(3-phenyl-3-mesitylcyclobutyl)ketoxime [4].

(Benzofuran-2-yl)(3-mesityl-3-methylcyclobutyl) ketone thiosemicarbazone which is another compound used in the study was synthesized with the method summarised in Figure 2.

All the chemicals were reagent grade as received from commercial sources (Sigma-Aldrich and Fluka) unless otherwise stated. 1-mesityl-1-methyl-3-(2-chloro-1-oxoethyl)cyclobutane 1 and (benzofuran-2-yl)(3-mesityl-3-methylcyclobutyl)methanone 2 were prepared according to the literature [4,15,16].

Melting points (uncorrected) were determined with a Gallenkamp apparatus. The IR spectra were measured with Mattson 1000 FT-IR spectrophotometer (potassium bromide disks).

The $^1\text{H-NMR}$ spectra were recorded on a Varian-Gemini 200 MHz spectrometer and were reported in ppm (d) relative to tetramethylsilane (TMS) as the internal standard and $^{13}\text{C NMR}$ (50.34 MHz) were referenced to deuteriochloroform (CDCl_3).

Synthesis of (Benzofuran-2-yl)(3-mesityl-3-methylcyclobutyl) Ketone Thiosemicarbazone

A mixture of (2) (0.3324 g, 1.0 mmol), thiosemicarbazide (0.091 g, 1.0 mmol) and p-toluene sulphonic acid (0.010 g, as catalyst) in absolute ethanol (150 mL) was refluxed. The progress of reaction was monitored by FT-IR by following the disappearance of 1687 cm^{-1} ketone (carbonyl) peak. After the solvent was removed under reduced pressure, the residue was treated with water and the final crude product (3) thus obtained by recrystallizing from ethanol [4,15,16]. Spectral data: (benzofuran-2-yl)(3-mesityl-3-phenylcyclobutyl) ketone thiosemicarbazone (3). Yield: 0.39 g (97%); m.p.: $256\text{-}258^\circ\text{C}$; IR (KBr cm^{-1}): $3432\text{-}3155$ (four sharp peaks, thiosemicarbazide), 1660 (C=N), 1251 (C-O, on furan ring), 1048 (C=S). $^1\text{H-NMR}$ (200 MHz; CDCl_3): 1.63 (s, 3H, CH_3), 2.24 (s, 9H, CH_3), 2.59-2.80 (m, 4H, $-\text{CH}_2-$), 3.52-3.75 (p, 1H, $>\text{CH}-$), 6.29-6.32 (broad s, 2H, $-\text{NH}_2$), 6.78 (s, 2H, mesityl), 7.11-7.70 (m, 5H, benzofurane), 10.93 (s, 1H, $-\text{NH}-$); $^{13}\text{C-NMR}$ (50.34 MHz; CDCl_3): 181.45 (C=S), 156.63, 150.83, 151.87, 145.66 (2C), 141.77 (2C), 137.03, 136.99, 132.97, 129.20, 128.37, 126.24, 124.14, 114.07, 112.75, 44.00, 42.68 (3C), 35.72, 26.90, 23.48, 22.38.

Preparation of the Hemolysate and Lysate

Fresh rat blood samples were collected in tubes containing EDTA, then centrifuged (15 min, $2.500\times g$) and plasma and buffy coat (leucocytes) were removed. The packed red cells were washed three times with physiological serum, homolyzed with 5 volume of ice-cold water and then centrifuged ($10.000\times g$, for 30 min) to remove the ghosts and intact cells.

Tissue samples were lysed with liquid nitrogen, then taken into 2-3 ml per gram tamp (50 mM Tris-SO_4 pH: 7.4). Then, they were centrifuged (30 min, $10.000\times g$), collected for supernatant experiments, and the remaining cell trashes were removed.

Laboratory Animals

In the study, male *Wistar* albino rats were used. Laboratory animals were obtained from Firat University Experimental Research Center (FUERC) and within this unit the experimental applications were done. The rats were fed in cages which were peculiarly designed and their daily cleanings were done regularly in a special environment with ventilation system. The rats were given tap water with nursing bottles with stainless steel balls. They were fed with special steel dishes by giving pellet feed.

Before starting experimental studies, preparatory study was done. The environment in which the animals were kept stabilized to $22\text{-}25^\circ\text{C}$. The rats were observed for 12 hours under the light and for 12 hours in the dark. A total of 56 *Wistar* albino male rats whose average weight is between 200-250 g were used in the study. The rats were divided into 8 groups. These groups and the matter concentrations given to these groups are indicated below:

1. Control group (K)
2. H_2O_2 group (H)
3. Benzofurane-2-il)(3-phenyl-3-methylcyclobutyl) ketoxime (A)
4. (Benzofurane-2-il)(3-methyl-3-methylcyclobutyl) ketone thiosemicarbazone (T)
5. 1,3-bis(2-clorbenzoyl)imidazoline-2-tion (B)
6. A + H group
7. T + H group
8. B + H group

The synthetic products were dissolved in DMSO. Control group was given DMSO. H_2O_2 in 12 mg/kg distillate waters, A in 12 mg/kg, B in 12 mg/kg and C in 12 mg/kg DMSO were dispersed and were administrated to the rats via intraperitoneally. After the 30-day period, the rats were decapitated and the organs (heart, liver, etc. and tissues and plasma) were kept in -50°C after being removed surgically until the analysis.

Protein Determination

Quantitative protein determination was spectrophotometrically measured at 595 nm

according to Bradford's method [17], with bovine serum albumin being used as a standard.

Esterase Activity Assay

The esterase activity was assayed by following the change in absorbance of 4-nitrophenylacetate to 4-nitrophenylate ion at 348 nm over a period of 3 min at 25°C using a spectrophotometer (Shimadzu UV-VIS Spectrophotometer, UV-1800) according to the method described by Verpoorte et al. [18]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL 0.05 M Tris-SO₄ buffer (pH 7.4), 1.0 mL 3 mM 4-nitrophenylacetate, 0.5 mL H₂O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution.

RESULTS AND DISCUSSION

CA activity of H group was decreased in all tissues compared to C group ($p < 0.05$, $p < 0.01$, $p < 0.001$). The enzyme activity of A and B groups was increased in liver tissue compared to C group ($p < 0.01$, $p < 0.001$). With the addition of A and B compounds, enzyme activities of HA and HB groups were increased compared to H group ($p < 0.05$, $p < 0.001$). In erythrocytes, enzyme activities of B and T groups were increased compared to C group ($p < 0.001$). In erythrocytes, enzyme activities of all combination groups were observed to increase compared to H group ($p < 0.001$). It was observed that the enzyme activity of T group was increased in heart tissue compared to C group ($p < 0.05$). It was determined that the enzyme activities of the heart tissue combination groups were increased compared to H group ($p < 0.05$). It was determined that there is no statistically difference between the CA activity of C group and A, B and T groups ($p > 0.05$). It was determined that the CA activity of kidney tissue HB group was increased compared to H group ($p < 0.001$), however it was relatively decreased compared to HA and HT groups ($p > 0.05$, Table 1).

Discussion

The synthetic products used in this study are thiosemicarbazone, benzofurane and imidazole-derived compounds and these structures have oxygen, sulfur and nitrogen within themselves. In a study conducted with thiosemicarbazone

oximes, it was found that such organic matters have a higher interaction capacity with Fe²⁺ in liver, kidney and brain homogenates of rats. It was explained that the reason of this is the reactions and bonding of such compounds with the oxygen, sulfur and nitrogen atoms in the electron-donor center of such compounds [19]. Besides, it was reported that benzofurane-derived compounds have anticancer, antimicrobial [20], antitumor [21,22], anti-HIV [23] and antioxidant [24] activities. Also, it was determined that imidazole-derived compounds have antihypertensive, antiviral, antibacterial and antioxidant activities [9]. Thiosemicarbazone-derived products were reported to have antiviral, anticancer, antitumor, antibacterial, anti-inflammatory and anti-amibic effects [25].

Memory acquisition and strengthening recollection are an important pharmacological objective in the treatment of cognitive diseases. CA has an important role in attentional gating of memory storage, signal process and long-term synaptic transformation beside its pH regulation, HCO₃⁻ reabsorption and CO₂ expiration tasks. CA function disorders lead to cognitive disorders and this is related to Alzheimer, mental retardation and aging [26].

CA inhibitors were analyzed in detail in many studies and it was determined that these have been used to prevent some diseases and clinical treatments. Yet, the studies on CA activators are quiet limited in the literature [27]. However, it was reported that the three series of the derivatives obtained through the reaction of amino-5(2-aminoethyl)- and 2-amino-5(3-aminopropyl)-1,3,4-thiadiazolün 2,4,6-trisubstituted pyrylium salts activates CA II enzyme [28]. It is also known that many amine and amino acids such as noradrenaline, adrenaline, histamine, hystidine, imidazoles, phenylalanine and 5-Ht are CA activators. It was also reported that CA activators may have an important use in the treatment of genetic CA deficiency and mental disorders [26]. The central administration of MCD (mast-cell-degranulating peptide) which is a neurotoxin from bee venom that releases histamine was reported to induce a quasi-permanent hippocampal waves and generate arousal at low concentrations. A

Table 1. CA enzyme activities in tissues.

Groups	Liver	Erythrocyte	Heart	Kidney
C	0.649035±0.03	0.119346±0.006	0.039445±0.002	0.654126±0.02
A	0.866513±0.02 ^{bt}	0.128330±0.005 ^t	0.035886±0.001	0.678422±0.02 ^t
B	1.421233±0.04 ^{ct}	0.150378±0.008 ^{ct}	0.037168±0.004	0.706442±0.03 ^t
T	0.616211±0.05	0.153321±0.005 ^{ct}	0.045016±0.002 ^{at}	0.737921±0.04 ^t
H	0.504052±0.01 ^a	0.099572±0.003 ^a	0.033351±0.001 ^b	0.374625±0.07 ^c
HA	0.669332±0.03 ^y	0.145819±0.003 ^{bt}	0.040145±0.001 ^y	0.312100±0.01 ^c
HB	1.214423±0.02 ^{ct}	0.130479±0.004 ^t	0.047586±0.006 ^{ct}	0.695647±0.03 ^t
HT	0.566532±0.03	0.130109±0.006 ^t	0.038508±0.001 ^y	0.248609±0.03 ^c

Statistical significance compared to the C group: a:P<0.05, b:P<0.01, c:P<0.001

Statistical significance compared to the H group: y:P<0.05, z:P<0.01, t:P<0.001

MCD-like endogenous was determined in extract of rat brain [29]. It was determined that the concentration of nerve endings in hippocampus containing histamine was decreased in the brain tissues of Alzheimer patients [30]. The importance of GABAergic synaptic transformation in the control of signal process in hippocampal network was determined with conducted studies showing that strengthening of these transformations can lead to enhance memory and learning [31]. Besides, in the study conducted by Sun and Alkon, it was reported that the strengthening of these transformation was done for the first time by using CA activators [26].

The critic role of CA activation which is the observed effect of CA activators was determined directly with the effect of acetazolamide -a CA inhibitor- in stopping the synaptic transformation. It was shown that HCO₃⁻ flow can be eliminated in hippocampal pyramidal neurons [32]. In CA₁ pyramidal cells, CA activation was reported to be essential in intracellular applications of benzolamide due to blocking GABAergic synaptic transformation [33].

The evidence as a result of the conducted studies supports that A matter statistically activates carbonic anhydrase enzyme in liver in a considerable extent (p<0.05), B matter substantially activates it in liver and erythrocytes (p<0.001), and T matter show quiet more activation effect in erythrocytes (P<0.001) compared to heart tissue (P<0.05) (Figure 3). Moreover, it was

determined that H₂O₂ statistically inhibits CA enzyme in liver and erythrocytes in a considerable extent compared to control group (P<0.05), and this inhibition effect was observed more in kidney and heart tissues (P<0.01, P<0.001, respectively). As a result of the evidence obtained through the study, it is thought that the synthetic products used in the study can be used as CA activators and found a base for the further therapeutic-intended CA activator synthesis studies.

Reactive oxygen species like H₂O₂ are produced in normal cell metabolism process and they get important roles in signal paths. However, H₂O₂ shows toxicological effects and induces the damage on the cell components as it causes new radicals to occur. Beyond this, it was determined that exogen H₂O₂ disrupt the balance between formation of reactive oxygen species and transformation to the pro-oxidative state [34]. Hydrogen peroxide forms hydroxide radical by reacting with metal ions like ferrous, copper as an oxidizing species. When H₂O₂ reacts with ferro (Fe²⁺) which is present in the heme group of proteins radical reactions start. H₂O₂ forms the most harmful radical, hydroxyl radical, by reacting with superoxide radical. This reaction is called as Haber-Weiss reaction. If this reaction is with catalyzer, the reaction is faster. Ferri (Fe³⁺) is degraded to ferro (Fe²⁺) by superoxide. With the present ferro (Fe²⁺), hydroxyl radical is produced from H₂O₂ by Fenton reaction [35].

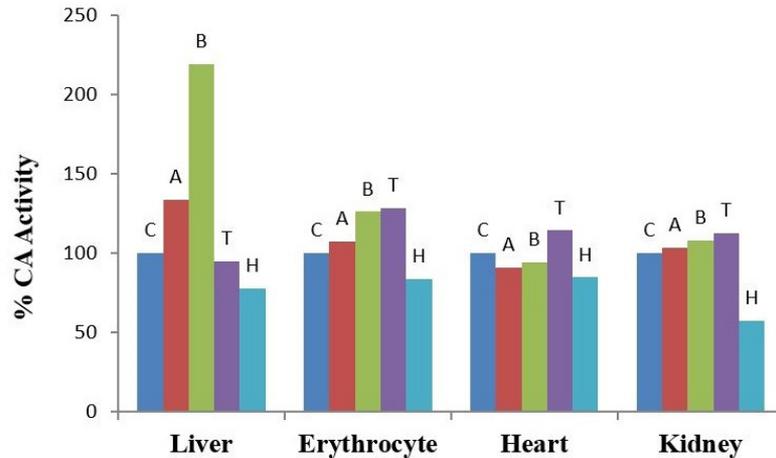


Figure 3. % Activity in different tissues of the group compared to the control group (The control group activity was taken as 100%).

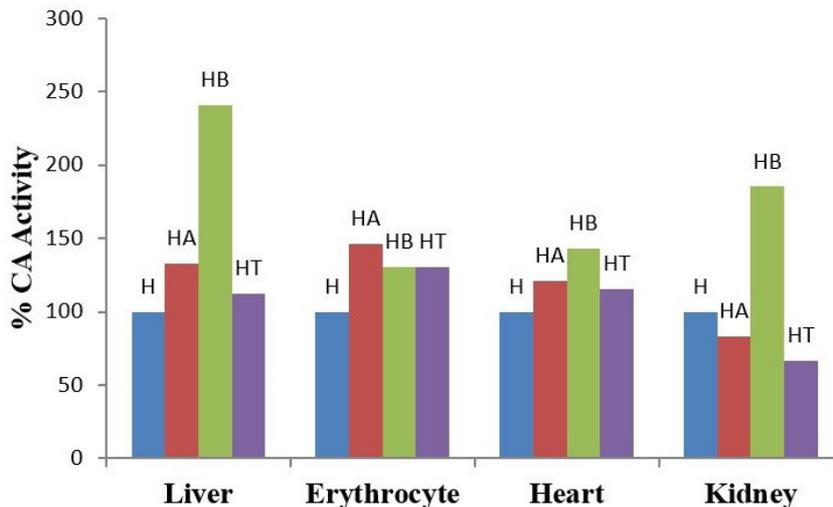


Figure 4. % Activity in different tissues of the group compared to the H group (The H group activity was taken as 100%).

Within the study, in vivo effect of H_2O_2 on CA was analyzed and for this purpose, H_2O_2 was administrated to healthy adult male *Wistar* albino rats. It was determined that administration of H_2O_2 inhibits CA in all tissues. H_2O_2 statistically inhibits CA enzyme in liver and erythrocytes in a considerable extent compared to control group ($P < 0.05$), and this inhibition effect was observed more in kidney and heart tissues ($P < 0.01$, $P < 0.001$, respectively). However, it was observed that when the rats on which H_2O_2 administration was done were administered with A matter, CA activity was increased in a considerable extent in liver ($P < 0.05$), erythrocyte ($P < 0.001$) and heart

($P < 0.05$) tissues compared to H group. It was determined that B matter increased CA activity ($P < 0.001$) in all tissues compared to H group while T matter increased the activity only in erythrocyte ($P < 0.001$) and heart ($P < 0.05$) tissues (Figure 4).

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