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PURIFICATION, CHARACTERIZATION OF GLUTATHIONE S-TRANSFERASE FROM THE LIVER TISSUE OF GOOSE (*Anser anser Domesticus*) AND INHIBITION EFFECTS OF SOME METAL IONS AND CHEMICALS

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ABSTRACT

The GST enzyme is an antioxidant that protects organs against toxic substances. It reduces reactive oxygen species to less reactive metabolites. The GST enzyme has been studied in many living creatures such as sheep, birds, fish, bacteria, cattle, plants and humans. However, no studies on the Goose (*Anser anser Domesticus*) liver GST enzyme (E.C. 2.5.1.18) have been found in the literature. This study demonstrates the purification and characterization of the GST enzyme from Goose (*Anser anser Domesticus*) liver tissue by affinity chromatography. Besides, inhibitory effects of Hg^{2+}, Ag^{2+} and Fe^{2+} cations and Oxytetracycline HCl, Tylosin Tartrate, Enrofloxacin and Doxycycline Hyclate chemicals on GST enzyme activity were investigated. The research was conducted under *in vitro* conditions using 1-Chloro-2,4-dinitrobenzene method. The degree of purity of the enzyme solution was verified by SDS-PAGE. With the characterization of the enzyme, the optimal pH, optimal ionic strength and optimal temperature values were found to be 7.0, 100 mM and 40 °C, respectively. Separate inhibition graphs of Hg^{2+}, Ag^{2+}, Fe^{2+}, Oxytetracycline HCl, Tylosin Tartrate, Enrofloxacin and Doxycycline Hyclate inhibitors were drawn. IC_{50} levels of inhibitors were found from the Activity% - [Inhibitor] graphs. As a result, the inhibition order of the inhibitors was found to be $Hg^{2+} > Ag^{2+} > Fe^{2+}$ for cations, and Enrofloxacin < Tylosin Tartrate < Doxycycline Hyclate < Oxytetracycline HCl for chemicals.

Keywords: Affinity, Antioxidant, Goose, Nutrient, GST.

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INTRODUCTION

Glutathione-S-Transferases (GSTs) protect cells from the toxic effects of drugs and environmental electrophiles. They are xenobiotic enzymes. Additionally, these enzymes affect oxidative stress and toxicity regulators (Awasthi *et al.,* 2017). Due to technological reasons; air, water, soil and other natural resources can be polluted with different chemicals. At the top of these chemicals; heavy metals and pesticides are causing pollution and contamination. These chemicals entering the metabolism by respiration, water and foods lead to toxic effects in the body (Ceyhun *et al.,* 2011). Most living creatures have preventive mechanisms such as detoxification and antioxidant systems. This system involves GSSG-Rx, GSH-Px and GSH-Tr versus xenobiotics and oxidative stress (Burdon, 1999). GSTs have an important role in the detoxification of electrophilic xenobiotics like pesticides, anti-cancer drugs, herbicides, environmental pollutants and carcinogens (Masella *et al.,* 2005). It is anticipated that these enzymes protect the cells against pesticides, drugs and foreign substances such as carcinogenic (Huston *et al.,*1976). The GSTs enzymes catalyze the reactions between lipophilic compounds and glutathione. In this way, they ensure the neutralization of toxic compounds, xenobiotics and oxidative stress products (Economopoulos, 2010). Tissues with a high concentration of GSTs enzymes were liver, large intestine, kidney, small intestine, placenta, spleen, erythrocyte, testis, gill, lung and breast. In these organs, the GSTs enzymes constitute 5% of the protein of the cytosol of the organs. GSTs, which are also considered as one of the natural protective systems, are responsible for excretion of substances such as drugs (lactones, alkyls, arylhalides, quinones, epoxides, herbicide, peptide, esters etc.), anticancer drugs (cisplatin, fosfomycin, chlorambucil, melphalan, cyclophosphamide and mitoxantrene etc.), chemical carcinogens (adeninepropenal, dopominocrene, aminochrome, hydrocarbons, ethacrynic acid etc.) and environmental pollutants (butadiene, acrolein, ethylene oxide etc.)

(Gyamfi *et al.,* 2004). To protect duck spermatozoa from the effect of reactive oxygen species, antioxidants must be added along with semen extenders.Because antioxidants protect spermatozoa from such effects both *in vivo* and *in vitro* (Taskın *et al.,* 2023).

The inhibitory effects of drugs, herbicides, metals, anions-cations and pesticides frequently used in treatment have been demonstrated in many studies. The required enzyme activities may be negatively affected by the chemicals encountered. Chemicals seriously affect metabolism by reducing the activities of critical enzymes. These effects can be very distressing (Demirdag *et al.,* 2012; Raspanti *et al.,* 2009). The detoxification mechanism of aquatic creatures is polluted with pesticides, metals, anion-cation and herbicides (Kirici *et al.,* 2016). Poultry has a very important place in production of animal origin. Considering the rearing and consumption of poultry, the priority is chicken, turkey, goose and duck, respectively. Because of the cheap production and low cost of meat obtained from poultry, they have priority in the consumption of animal proteins as a protein source (Aksoy and Arikan, 1995). Goose meat is considered as a healthy meat due to its high nutritional value and low fat and cholesterol levels (Diker and Deniz, 2017). Goose meat is also rich in minerals such as calcium, phosphorus, potassium, selenium, zinc and iron. One hundred grams of skinless goose meat can meet approximately 14% of the daily iron requirement and 31% of phosphorus in humans (Vatansever *et al.*, 2020).

The aim of this research is to purify the GST enzyme from the liver tissue of Goose (*Anser anser Domesticus*), characterize it and examine the inhibitory effects of Hg^{2+}, Ag^{2+} and Fe^{2+} cations and Tylosin Tartrate, Oxytetracycline HCl, Enrofloxacin and Doxycycline Hyclate chemicals.

MATERIALS AND METHODS

Chemicals: N,N,N',N'-Tetramethyl ethylenediamine, Glutathione−Agarose, CDNB (1-Chloro-2,4-dinitrobenzene), L-Glutathione reduced (GSH), Coommassie Brilliant Blue R-250, Bromine Thymol Blue, protein experiment reagents were purchased from Sigma–Aldrich (Steinheim, Germany). Other chemicals were purchased from Merck (Darmstadt, Germany).

Preparation of the purification column: One gram of lyophilized glutathione-agarose was taken and washed several times with 200 mL of distilled water. After the impurities were removed, it was loaded into the column with a bed volume of 10 ml. The air of the gel was removed using a water trompe. The gel was cleaned with washing buffer (10 mM KH_2PO_4/0.1 M KCl, (pH = 8.0)). The gel was made ready for use with equilibration buffer (10 mM KH_2PO_4/150 mM NaCl, (pH = 7.4)). Then, the gel was packed into a cooled column with a 1x10 cm closed system. Equilibration buffer was passed through the column using a peristaltic pump. For the purification process, the absorbance, pH of the eluate and the equilibration buffer should be the same. All of the purification steps were performed at 4°C.

Purification of the enzyme: The experimental part of the research was carried out at Agrı Ibrahim Cecen University, Central Research and Application Laboratory. The liver tissues of goose were brought to our research laboratory according to the cold chain rules. The liver was separated into small pieces. The liver cell membrane was lysed by the freeze-thaw method. Cell contents were diluted using 2 times buffer solution. The cell contents were centrifuged at 12500 rpm for 60 min. The plasma and precipitate were separated from each other. Plasma was run through the previously equilibrated affinity column. Wash buffer was then run through the column.The Goose liver tissue GST enzyme was eluted with 50 mM KH_2PO_4/2.5–10 mM GSH (pH = 7.5). A cold room was used while the procedures were carried out.

Measuring enzyme activity: Enzyme activity was performed by CDNB methode by using a spectrophotometer (Shimadzu, UVmini-1240 UV-VIS spectrophotometer, Kyoto, Japan). The aromatic electrophile CDNB substrate is one of the most frequently used substrates for the determination of GST enzyme activity. It is based on the fact that the dinitrobenzene S-glutathione showing max absorbance at a wavelength of 340 nm. Thus, activity measurements were performed at this wavelength (Habig *et al.,* 1974). For the aim of finding K_M and V_{max} for GSH and CDNB substrates of GST enzyme purified from the liver tissue of Goose (*Anser anser Domesticus*), activity measurement was performed with five different CDNB concentration at the stable GSH concentrations. 1/[CDNB]-1/V Lineweaver - Burk graphs were prepared using the findings (Lineweaver and Burk, 1934). Similarly by performing the activity measurements with GSH's five different concentrations at the stable CDNB concentration 1/[GSH]-1/V Lineweaver - Burk graphs were drawn.

Protein detection: Bovine albumin was used as the standard in the purification stages. Protein detection was carried out at 595 nm wavelength using Bradford reagent (Bradford, 1976).

SDS-PAGE: Following the purification steps, the degree of purity of the enzyme solution was verified by SDS-PAGE. It was prepared in 10% and 3% acrylamide (acrylamide/bisacrylamide 29:1 (w/w)) for the stacking and separating gel, with 0.1 percent SDS. 25 µg enzyme solution was loaded into the electrophoresis well. Gel was painted for 120 min in 0.1% Coommassie Brilliant

Blue R-250 in 50% methanol and 10% acetic acid (Laemmli, 1970).

Optimal pH, optimal temperature and optimal ionic strength detection: To detection the optimal pH, potassium-phosphate buffers were preferred in the pH range of 5.0 to 7.5. For detection of the optimal temperature, GST activity was tested at various temperatures in the range from 5 ºC to 50 ºC. The target temperatures were reached with the help of a water bath. For the detection of optimal ionic strength, activity was detected using various concentrations of potassium-phosphate buffer, pH: 7.3, in the range from 1 mM to 200 mM.

Effects of inhibitors: *In vitro* effects of the Hg^{2+}, Ag^{2+} and Fe^{2+} cations and Oxytetracycline HCl, Tylosin Tartrate, Enrofloxacin and Doxycycline Hyclate chemicals were examined at different concentrations. Inhibitors were tested in triplicate at each concentration usage. GST activity was analyzed in the presence of inhibitor. Without inhibitor, the control activity was considered as 100%. [Inhibitor] - Activity% graphs were prepared for each inhibitors. The IC_{50} levels were determined in this way.

RESULTS AND DISCUSSION

In this experimental study, Goose (*Anser anser Domesticus*) liver tissue GST enzyme was characterized for first time. It was achieved at ~48.4-fold with a yield of 70.51 percent, and a specific activity of 0.121 EU/mg proteins. (Table 1)

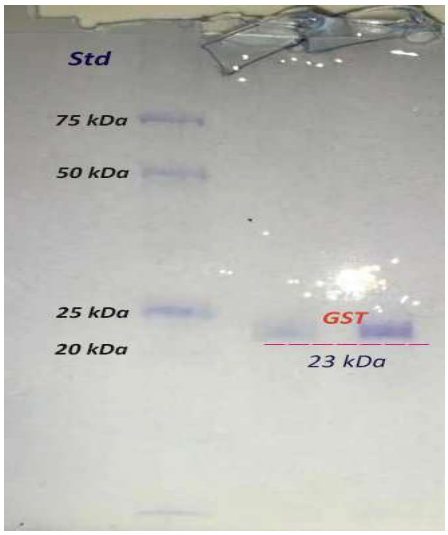
Table 1. Summary of purification procedure for Goose (*Anser anser Domesticus*) liver tissue GST enzyme.

Purification Steps	Activity (EU/mL)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity	Specific activity (EU/mg protein)	Yield %	Purification fold
Homogenate	1.65	18	64.2	1155.6	29.7	0.00257	100.0	1.0
Glutathione-Agarose affinity chromatography	3.49	6	28.84	173.04	20.94	0.121	70.51	48.4

Similar results were found for the enzyme in other researched creatures. For instance, rainbow trout erythrocytes GST was purified 11026-fold with a yield of 59%, and a specific activity of 16.54 EU/mg proteins (Comakli *et al*., 2011) (*monopterus albus*) fishy gill GST was achieved 300-fold with a yield of 14%, and a specific activity of 13.07 EU/mg proteins (Huang *et al*., 2008), human erythrocytes GST was purified 1143-fold with a yield of 80%, and a specific activity of 16.00 EU/mg proteins (Erat and Sakiroglu, 2013). In another study, the same enzyme was achieved 6800 times from bovine erythrocytes with a yield of 97% (Guvercin *et al*., 2008). In addition, the same enzyme was purified 2300 times from *E. coli* bacteria with a yield of 7.5% (Iızuka *et al*., 1989). When other studies in the literature are analyzed, it can be seen that the enzyme has been purified at low yields in 2-3 steps. However, we purified the enzyme with higher efficiency in a single step.

With the aid of 1/[CDNB]-1/V Lineweaver - Burk graphs, K_M and V_{max} of GST enzyme were found to be 0.68 mM and 0.5 EU/mL, for CDNB. With the aid of 1/[GSH]-1/V Lineweaver - Burk graphs, K_M and V_{max} of GST enzyme were found to be 1.34 mM and 1.14 EU/mL, for GSH. In the study of Guvercin *et al.* in 2008 for GST purified from bovine erythrocytes, the K_M for CDNB and GSH substrates are 0.186 mM and 0.423 mM, respectively. V_{max} is 116 EU/mL and 192 EU/mL, respectively (Guvercin *et al*., 2008).

The degree of purity of the enzyme solution was verified by SDS-PAGE. The MW was determined approximately to be 23 kDa (Fig. 1).



Lane (1) Lane (2, 3)

Figure 1: SDS-PAGE analysis of purified Goose (*Anser anser Domesticus*) liver tissue GST enzyme. Lane (1) standard proteins. Lane (2,3) GST enzyme purified from the liver tissue of Goose (*Anser anser Domesticus*).

Almost the same results were found for the GST enzyme in other researched creatures. For instance, rainbow trout liver GST is 23 kDa (homodimer), Atlantic salmon GST and brown trout GST are approximately 23.7 kDa (Novoa Valinas *et al.,* 2002), Lettuce (*Lactuca sativa*) GST is 23 kDa (Hee-Joong *et al.,* 2005), Tilapia (*Oreochromis niloticus*) fishy gill GST is 25.46 kDa and (*monopterus albus*) fishy gill GST is 26 kDa (Huang *et al*., 2008). In the study of Akkemik *et al.* in 2012, the MW of the enzyme achieved from turkey liver was found to be 26 kDa (Akkemik *et al.,* 2012). In the study of Guvercin *et al.* in 2008, the MW of the enzyme purified from bovine erythrocytes was found to be 27 kDa (Guvercin *et al*., 2008). In a study on *E. coli*, it was found that the MW of the GST enzyme is 24 kDa (Arca *et al.,*1990).

In the study conducted at temperatures between 5 ºC-50 ºC, it was seen that the GST showed the highest activity at 40 ºC. Huang *et al.* in 2008 reported that the optimal temperature of (*monopterus albus*) fishy gill GST enzyme as 45 ºC (Huang *et al*., 2008). Comakli *et al.* reported that the optimal temperature of rainbow trout erythrocytes GST enzyme as 30 ºC (Comakli *et al*., 2011). Taysı reported the optimal temperature of 55 ºC for the GST enzyme achieved from quail liver in his master's thesis in 2018 (Taysı, 2018). In the study of Akkemik *et al.* in 2012, the optimal temperature of the enzyme achieved from turkey liver was reported to be 50 ºC (Akkemik *et al.,* 2012).

As a result of the characterization of the Goose (*Anser anser Domesticus*) liver tissue GST enzyme, optimal pH, optimal ionic strength and optimal temperature were found as 7.0, 100 mM and 40 °C, in order.

Unfortunately, with the uncontrolled development of industry and technology, all living things are negatively affected by various chemicals. Chemical pollution is seen as a result of people's using chemicals unconsciously. People and other organisms are affected not only by the chemicals used but also by the waste created by humans. Particularly, in many countries, waste materials from factories are buried in the ground or released into the atmosphere. Accordingly, many toxic substances such as heavy metals and harmful chemicals negatively affect soil and water. These harmful compounds enter the food chain and accumulate in plants, animals and humans. For this reason, enzyme activity studies using these substances are very popular today (Ekinci *et al.,* 2007). Especially, researchers have reported that metal ions, which play an important role in carrying out metabolic activities in living things, cause toxic effects such as inhibition effects on different enzymes in the protein structure (Comakli *et al.,* 2015).

Metals are present in the aquatic environment in amounts that do not harm living creatures. However, with the developments in areas such as industry, agriculture and mining, metals are becoming capable of harming living things (Kalay and Canlı, 2000). This situation takes the first place on the agenda of environmental toxicology. It poses a serious threat to living organisms in aquatic environments. Enzymes catalyze every chemical reaction in living organisms. Chemicals such as metals, etc., affect enzyme activities positively or negatively even in very low amounts (Ekinci *et al.,* 2007). Metals that aquatic creatures are exposed to accumulate in their tissues. It is possible that other creatures in the food chain may experience health problems as a result of consuming these creatures (Kaya *et al.,* 2015). In a study by Ozaslan and colleagues (Ozaslan *et al.,* 2017), it was reported that Cd^{2+}, Ag^{+}, Zn^{2+} and Cu^{2+} metals inhibited Lake Van Fish Gill Tissue GST enzyme. In the study of Akkemik *et al.*, the effects of Cu^{2+}, Hg^{2+}, Fe^{2+}, Zn^{2+}, Ag^{2+}, Mg^{2+}, Ni^{2+} and Mn^{2+} metals on GST enzyme achieved from turkey liver were investigated (Akkemik *et al.,* 2012). In the study of Comakli *et al.* (2011) were investigated the effects of some metal ions on trout (rainbow) erythrocytes (Comakli *et al.,* 2011). When the IC_{50} values of the ions in question are listed, they appear as $Ag^{2+}<Cd^{2+}<Cr^{2+}<Mg^{2+}$. Hg^{2+}, Cu^{2+}, Se^{4+}, and Al^{3+} metals inhibited the GST enzyme achieved from the liver tissue of trout (Comakli *et al.,* 2015).

GSTs have an important role in the metabolism of a wide group of electrophilic substrates, including antibiotics, analgesics and anticancer drugs (Hodgson and Levi, 1994). They are a multifunctional group of enzymes involved in both endogenous and exogenous conjugation (Celik *et al.,* 2003). The properties of GSTs which have been purified from mammals such as human, mouse, bovine and rabbit have been characterized in a detailed way. But, the GST enzyme in Goose is not as well-characterized as the GST enzymes in living organisms. Besides, the purification of different kinds of enzymes, the determination of structural and kinetic features are going on today. Studies on drug design and elucidation of enzyme mechanisms have attracted much attention in recent years. Therefore, this research, which will contribute to the literature, is very important.

Consequently, we purified Goose (*Anser anser Domesticus*) liver tissue GST enzyme in a single step and analyzed characteristic features. IC_{50} values of Hg^{2+}, Ag^{2+} and Fe^{2+} metals were calculated as 0.015, 0.2, and 0.288 mM, respectively. IC_{50} values of Oxytetracycline HCl, Tylosin Tartrate, Enrofloxacin and Doxycycline Hyclate chemicals were calculated as 0.046, 3.5, 4.3, and 0.46 mM, respectively. (Figure 2a, 2b, 2c, 2d) As a result, *in vitro* inhibition rank order was determined as $Hg^{2+} > Ag^{2+} > Fe^{2+}$ for metal ions, Enrofloxacin < Tylosin Tartrate < Doxycycline Hyclate < Oxytetracycline HCl for chemicals.

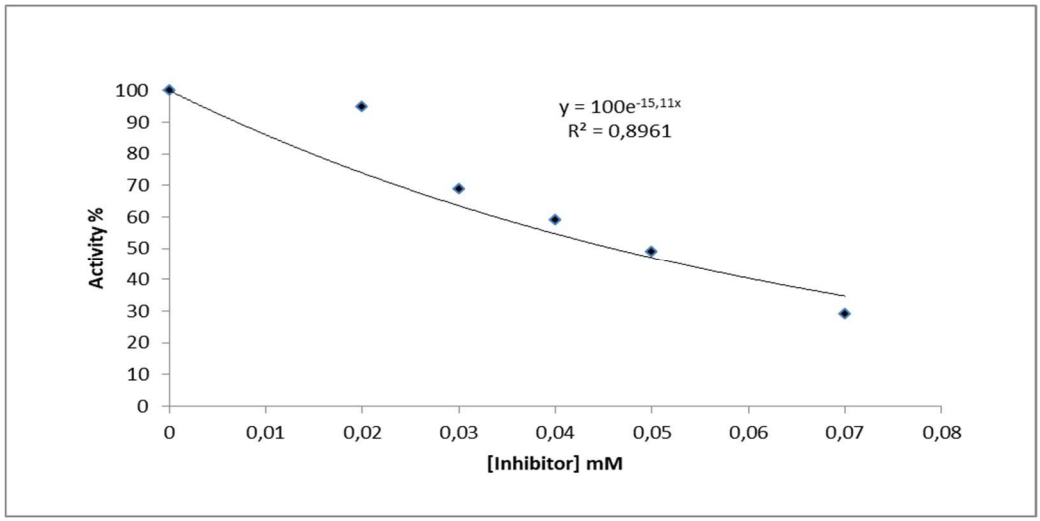


Figure 2a: Activity% - [Inhibitor] graphs to determine the IC_{50} value of Oxytetracycline HCl.

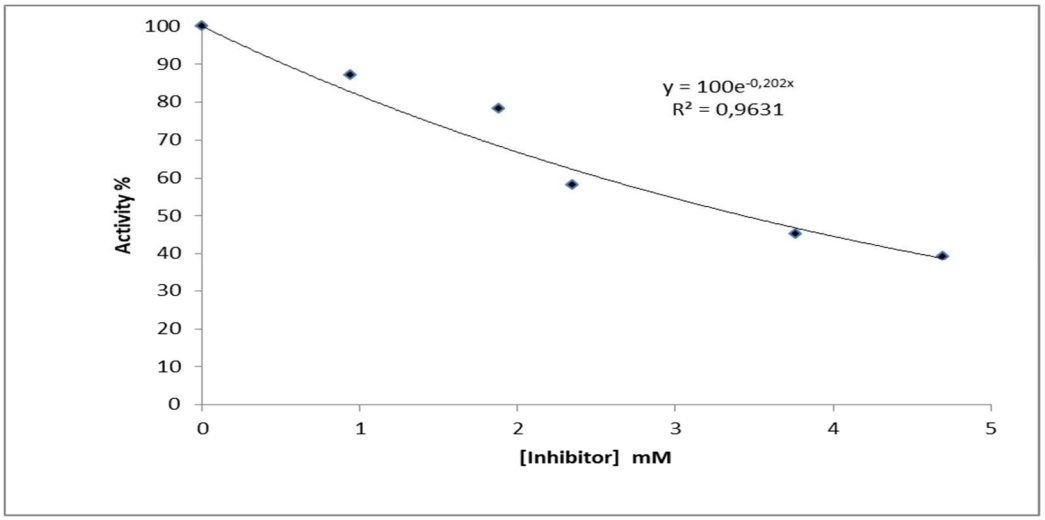


Figure 2b: Activity% - [Inhibitor] graphs to determine the IC_{50} value of Tylosin Tartrate.

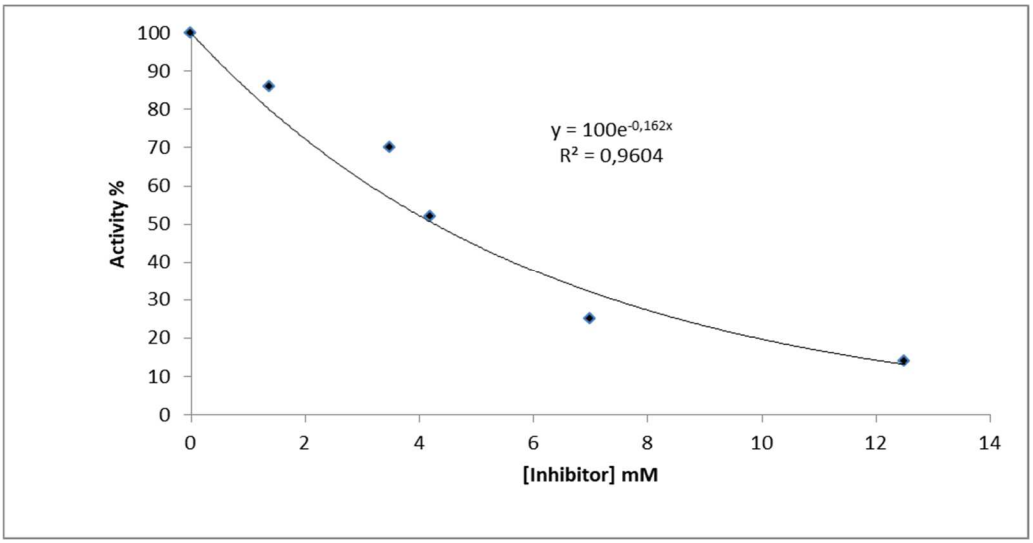


Figure 2c: Activity% - [Inhibitor] graphs to determine the IC_{50} value of Enrofloxacin.

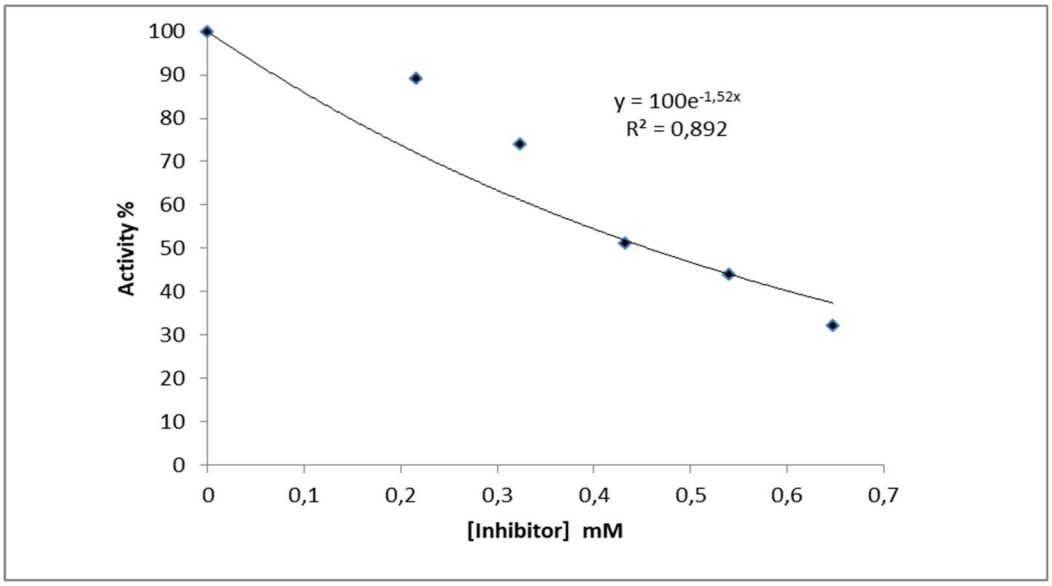


Figure 2d: Activity% - [Inhibitor] graphs to determine the IC_{50} value of Doxycycline Hyclate.

According to this study Hg^{2+}, Ag^{2+} and Fe^{2+} metals and Oxytetracycline HCl, Tylosin Tartrate, Enrofloxacin and Doxycycline Hyclate chemicals were found to inhibit the enzyme at much lower concentrations. (Table 2)

Table 2. IC_{50} values for some metal ions and chemicals of Goose (*Anser anser Domesticus*) liver tissue GST enzyme.

Type of chemicals	*Average values of IC_{50} (mM)*	*Type of metals*	*Average values of IC_{50} (mM)*
Oxytetracycline HCl	0.046	Hg^{2+}	0.015
Tylosin Tartrate	3.5	Ag^{2+}	0.200
Enrofloxacin	4.3	Fe^{2+}	0.288
Doxycycline Hyclate	0.46		

It has also been reported in the literature that metals have adverse effects on metabolism and embryonic development and have genotoxic effects (Teta *et al.,* 2017). Today, it has been reported that industrial development, rapid population growth, agricultural activities and mining have a negative impact on the water environment (Kaya *et al.,* 2015). In geese that are at food chain, chemicals such as heavy metals accumulate in body tissues and show toxic effects (Sorsa *et al.,* 2016). Considering its nutritional properties, goose meat has been shown to be an important alternative animal protein source in terms of dry matter, protein and mineral substance levels (Sahin *et al.,* 2022). There are important studies in the literature on the preservation of the generations of geese whose meat and liver are consumed as food. In the study conducted in 2022, it was examined how goose semen was affected by toxic chemicals. It has been suggested to freeze and preserve goose semen in order to improve the reproductive characteristics of geese and preserve the high genetic potential of the goose (Taskın *et al.,* 2022). Another study conducted on webbed-footed ducks, which play an important role among poultry animals that constitute a significant part of the world's biodiversity, emphasized the need for antioxidant systems to protect spermatozoa (Taskın *et al.,* 2023).

Researchers need to find ways to eliminate problems in protecting the health of geese in the food chain. We can conclude from this study that metals and chemicals have negative effects on health. Because of this,more attention should be paid to industrial and metal residues.

Conflict of Interest: None of the authors have any potential conflict of interest to declare.

Authors' contribution: Concept: RD, ; Literature Search: YS, EY, RD, ; Data Collection and Processing: YS, EY, RD,
Analysis or Interpretation: YS, EY, RD,; Written: EY.

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