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Purification and characterization of glutathione S-transferase from blueberry fruits (*Vaccinium arctostaphylos* L.) and investigated of some pesticide inhibition effects on enzyme activity

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Abstract

Pesticides cause pollution by remaining in water, soil, fruits and vegetables for a long time and also reach human through the food chain. It was thought that some pesticides used in agriculture could adversely affect the antioxidant enzyme system and the minimum inhibition values were studied. glutathione s-transferase (GST), an important antioxidant enzyme, catalyzes the conjugation of glutathione with toxic metabolites. It was purified from the blueberry fruits. The purification of the enzyme was performed separately by affinity and gel filtration

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chromatography. The purity of the enzyme was determined by SDS-PAGE electrophoresis. Characterization studies were done for the enzyme. For this purpose, optimal pH, temperature, K_m and V_{max} values for GSH and CDNB were also determined for the enzyme as 7.2 in K-phosphate buffer, 50 °C, 1.0 M, 7.0 in K-phosphate buffer, 1.57 mM; 0.17 mM and 0.048 EU/mL, 0.0159 EU/mL, respectively. Additionally, inhibitory effects of some pesticides; dichlorvos, acetamiprid, cyhalothrin, haloxyfop-p-Methyl, 2,4 dichlorophenoxy acetic acid, cypermethrin, imidacloprid, fenoxaprop-p-ethyl, glyphosate isopropylamine salt were examined the enzyme activity *in vitro* by performing Lineweaver−Burk graphs and plotting activity % IC_{50} and K_i values were calculated for each of pesticides. All of the pesticides inhibited the GST enzyme at millimolar level. Pesticide showing the best inhibitory effect was found as dichlorvos. The Ki value which is the inhibition constant of this pesticide was 0.0175 ± 0.005.

Keywords: Natural product chemistry, Food science

1. Introduction

Blueberries are rich from anthocyanins and tannins. Anthocyanins [1] are powerful antioxidants containing ions required to remove radicals. The flavonoids in plants form insects attracting colors and anti-microbial compounds [2, 3, 4]. Tannins prevent the feeding of animals and insects from the plant and protect the plant against fungal and bacterial attacks [5]. These phenolic compounds found in the relevant plants have an effect on the antioxidant properties as well as the blue/black color of the plant. Due to these properties, it is interpreted as a bioactive key in health [6, 7].

Glutathione S-transferase (GST; EC 2.5.1.18) is a broad and comprehensive enzyme that removes a large number of electrophilic xenobiotics from the biological systems by binding to tripeptide glutathione (GSH; g-glu-cys-gly) and participates in the detoxification system by various mechanisms [8]. The GST enzyme removes some toxic substances in the circulation by covalent or non-covalent bonding, as well as incorporation into phase II reactions in the detoxification of xenobiotics and produces antioxidant activity against the stress caused by organic hydroperoxides with peroxidase activity [9, 10]. The isoenzymes of the GST are found in all organisms as diverse as microbes, insects, fungi, fish, birds, mammals and plants [11, 12]. The most common tissues of the GST are the liver, especially the organs such as kidney, small intestine, lung and breast [13, 14]. The GST enzyme constitutes 5% of the total protein content of the cytosols of these organs.

As a result of the high presence of GST in these organs, it is directly in contact with xenobiotics taken from the outside [15, 16]. The living things try to minimize the

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negative effects of the internal and external environment with their antioxidant enzymes and non-enzymatic molecules [17]. One of the important enzymes that increase the importance of biotransformation in pesticide toxicology is the GST. Therefore, the study of oxidative stress enzymes is very important in terms of ecological, toxicological and evalutional aspects.

Pesticides are chemicals used in agriculture to prevent or control the negative effects of harmful organisms. These substances increase the production and cause contamination in the products and environment which are used as food raw materials at the same time. These contaminants are reduced to a certain extent in technological processes and remain in the foodstuffs produced. This situation severely affects human health and the environment [18]. Many pesticides are used for agricultural products. Some of them are dichlorvos, acetamiprid, imidacloprid, cypermethrin, lambda-cyhalothrin, fenoxaprop-p-ethyl, haloxyfop methyl ester, 2,4 dichlorophenoxy acetic acid (2,4 D) and glyphosate.

Dichlorvos, the chemical name 2,2-dichlorovinyl dimethyl phosphate is a broad-spectrum organic phosphorus compound that is used as an antihelmintic and insecticide in both agricultural and veterinary medicine. It is a colorless amber liquid with an aromatic smell. Organophosphorus (OP) compounds are a widely used group of pesticides for controlling agricultural pests and disease-causing vectors. It is also an acetylcholine esterase (AChE) enzyme inhibitor [19].

Acetamiprid is a group of neonicotinoid pesticides. The chemical name is N - [(6-chloro-3-pyridyl) methyl] -N′-cyano-N-methyl-acetamidine. It is in the form of colorless crystals and is an acetylcholine receptor. Aphids on many cultivated plants are effective on absorbent insects such as whiteflies [20].

Imidacloprid, chemical name N- {1 - [(6-Chloro-3-pyridyl) methyl] -4,5-dihydroimidazol-2-yl} nitramide, is a systemic insecticide that acts as a neurotoxin [21]. It is an acetylcholine receptor, an insecticide with a wide range of users worldwide.

Cypermethrin, chemical formula is [Cyano- (3-phenoxyphenyl) methyl] 3- (2,2-dichloroethenyl) -2,2-dimethylcyclopropane-1-carboxylate, is the pyrethroids of the synthetic organophosphorus group. It is light-resistant and rapidly disintegrated by soil bacteria and also has a nourishing ability. It is an effective insecticide on the nervous system of insects [22].

Cyhalothrin, Lambda-cyhalothrin, etc., chemical formula of 3- (2-chloro-3,3,3-trifluoro-1-propenyl) -2,2-dimethyl-cyano (3-phenoxyphenyl) methyl cyclopropane-carboxylate is an insecticide. That are preferred insecticides because of their long-term effects. It is a colorless liquid, and its solubility in water is low. Lambda-cyhalothrin causes organisms to die by destroying the nervous system [23]. It is indicated on many insects in rural agriculture.

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Fenoxaprop-p-ethyl, Its chemical formula is (R) -2- [4- (6-chloro-1,3-benzoxazol-2-yloxy) phenoxy] propionic acid is a herbicide of the aryloxyphenoxypyrrolidone class. It inhibits acetyl CoA carboxylase and prevents the synthesis of fatty acids [24].

Haloxyfop methyl ester, chemical formula is methyl (R) -2- [4- (3-chloro-5-trifluoromethyl-2-pyridyloxy) phenoxy] propionate, is a herbicide including to the class of arylphenoxypyropionic acid. That is a clear white liquid with very low volatility and readily soluble in water. In biochemistry, it stops the synthesis of fatty acids by inhibiting acetyl CoA carboxylase [25].

2,4 Dichlorophenoxy acetic acid (2,4 D) is a herbicide that causes uncontrolled cell division of plants in vascular tissues. It is a synthetic auxin derivative [26]. 2.4 D causes uncontrolled cell division in vascular tissues of plants [27].

Glyphosphate is a natural amino acid glycine analogue and is an aminophosphonic material. Glyphosate blocks the synthesis of aromatic amino acids of tyrosine, tryptophan, and phenylalanine, causing the death of many plants [28].

The purpose of this study to investigate the inhibitory effects of some pesticides (Fig. 1) on the activity of GST enzyme isolated from blueberry fruits (*Vaccinium arctostaphylos* L.*)*, which has a considerably healthier aspect.

2. Experimental

2.1. Chemicals

All chemical were bought from Fluka (Munich, Germany) or Sigma Chem. Company (St. Louis, MO), E. Merck AG and Pharmacia.

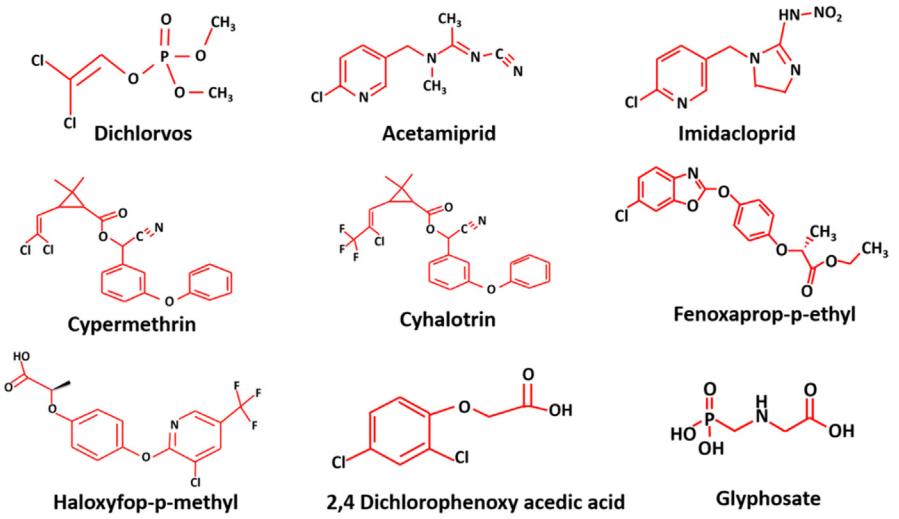

Fig. 1. Structure of tested some pesticides.

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2.2. Plant material

Vaccinium arctostaphylos L. berries were collected from Erbaa, Tokat, Turkey, after full ripening in September 2013. They were kept in cool bags for transport to the laboratory. The fruits were stored as packed in freezer bags at -20 °C until tested [29].

2.3. Preparation of the homogenates

First, *V. arctostaphylos* L. berries (20 g) powdered using liquid nitrogen were suspended in 0.2 M Tris-HCl buffer (pH 7.8 containing 5 mM PMSF, 1mM DTT, 1 mM EDTA) [29]. The mixture was filtered with double layer filter paper. The filtrate was centrifuged at 20.000 g for 30 min at + 4 °C. Subsequently; the mixture was filtered again with a thin band filter paper, thereby removing solid particles from the protein solution and the supernatant was collected for experiments [30].

2.4. Sephadex G-100 gel filtration chromatography

The concentrated culture filtrate was passed through a Sephadex G-100 column. Fractions (2 mL each) were collected by eluting with the buffer, and then the absorbance values were measured at 280 nm for determination of enzyme activity values [30].

2.5. Glutathione-agarose affinity chromatography

The most commonly used method in enzyme purification is the affinity chromatography method. The affinity column was prepared and then, high activity tubes that were combined at the end of gel filtration chromatography were applied to glutathione-agarose affinity column. Subsequently, the column was washed with 20 mM Na_2HPO_4, pH 7.2 This was followed by the spectrophotometer and the absorbance values were equal to the curvature. After the column was equilibrated, the gradient elution was carried out and the enzyme was purified. The elution solution was formed from the gradient of the solution containing 50 mM Tris-HCl and 5mM GSH (1.25−10 mM GSH, pH 9.5). With the aid of fraction collectors, eluates were taken into 1.5 mL tubes and their absorbance at 340 nm was examined. All of the procedures were performed at 4 °C [13].

2.6. Protein determination

The samples were taken from homogenate and affinity chromatography. Later, they were put into 0.1 mL tubes and added 5 mL of Coomassie brilliant blue G-250 each of tubes. After mixed with vortex and incubated for 10 minutes. Then, the absorbance values were measured at 595 nm wavelength at 25 °C. Three trials were made from each sample, and the true value was determined from the arithmetic mean of these three values. Protein quantities were determined using this standard value [31].

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2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE)

After the enzyme was purified, the purity of the enzyme was checked by 3−8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method (Fig. 2) [32].

2.8. Characterization studies

2.8.1. Study of optimum pH

To determine the optimum pH of GST enzyme, 0.1 M phosphate buffers with pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and pH 7.0, 7.5, 8.0, 0.1 M Tris-HCl buffer were prepared. Enzyme activity measurements were performed with each buffer (Fig. 3).

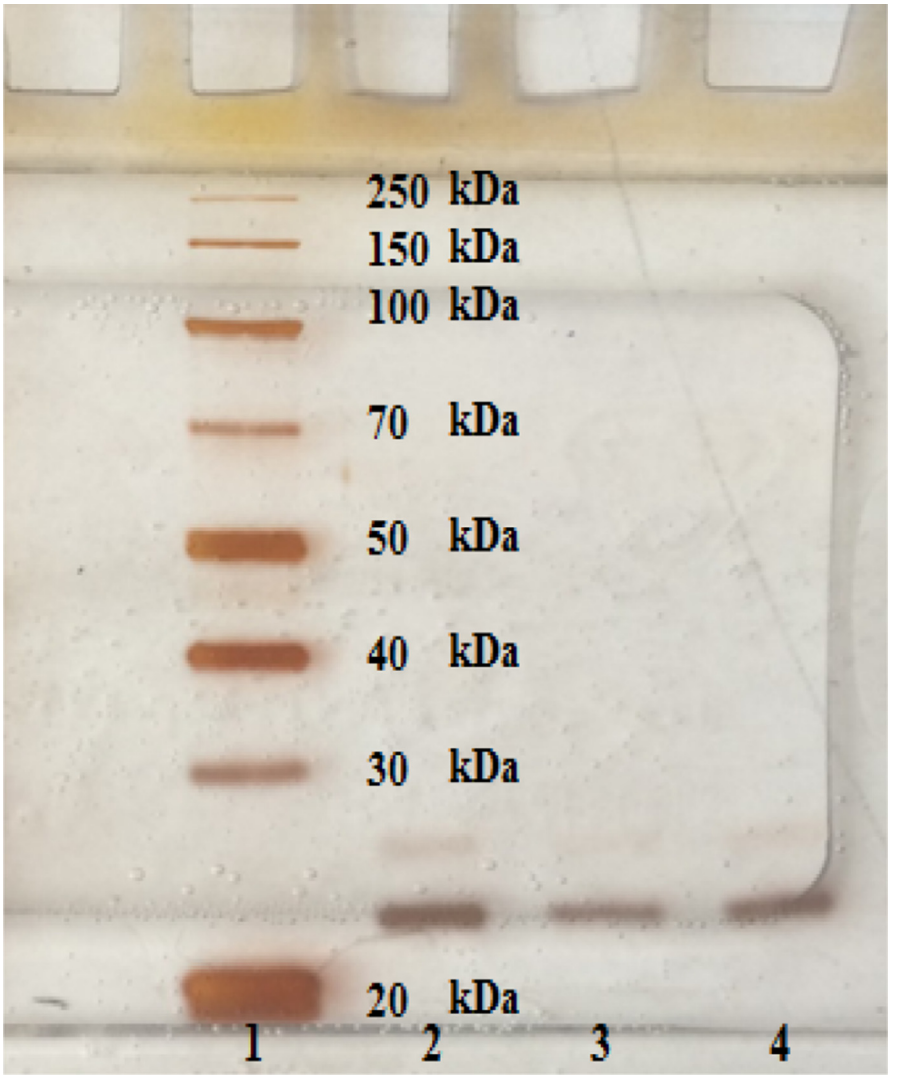

Fig. 2. SDS- PAGE photograph: line 1, standart proteins (MA: 20, 30, 40, 50, 70, 100, 150, 250); line 2, 3, 4 purified enzyme from glutathione-agarose matrix.

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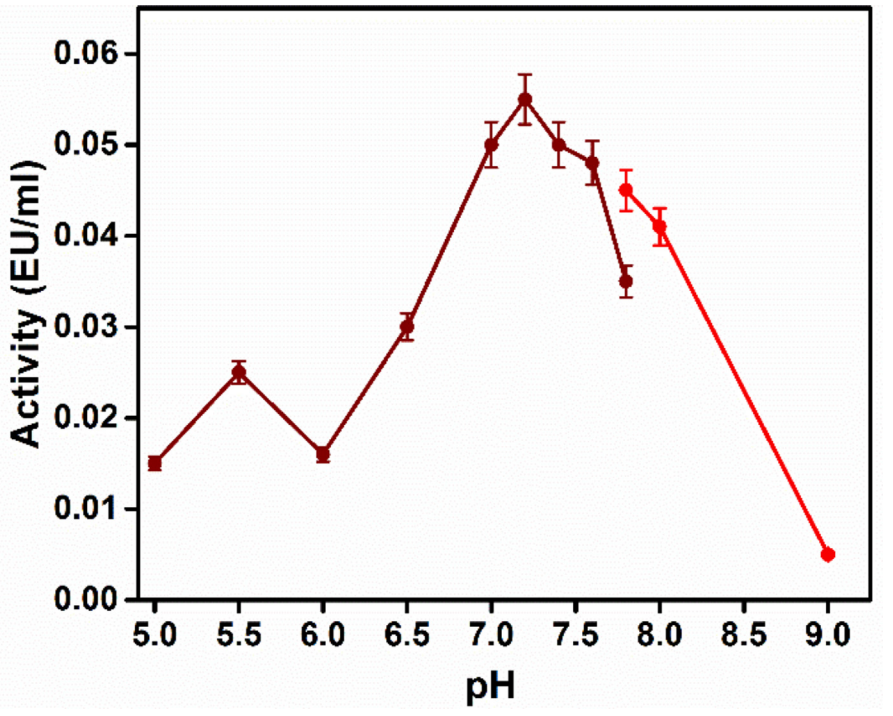

Fig. 3. Effect of optimum pH on the activity of blueberry fruit GST.

2.8.2. Study of optimum ionic strength

The optimum pH of the enzyme was determined, at different pH levels (5.0−8.0) with 0.1M phosphate and pH (7.0−8.0) 0.1 M Tris-HCl buffers (Fig. 4). Enzyme activity measurements were made with each buffer.

2.8.3. Study of optimum temperature

To determine the optimum temperature, enzyme activity was assayed in 0.1 M K-phosphate buffer (pH 7.0) at different temperatures in a range from 0 °C to 70 °C (Fig. 5). The desired temperature was provided by using a water bath (Nuve; model BM-302).

2.8.4. Kinetic studies

The kinetic parameters, V_{max} and K_M for CDNB were determined using a CDNB range from 0.05 mM to 1.6 mM and a fixed GSH concentration of 20 mM. The apparent V_{max} and K_m values for GSH were also calculated by using a GSH range from 0.05 mM to 1.6 mM and a fixed CDNB concentration of 25 mM.

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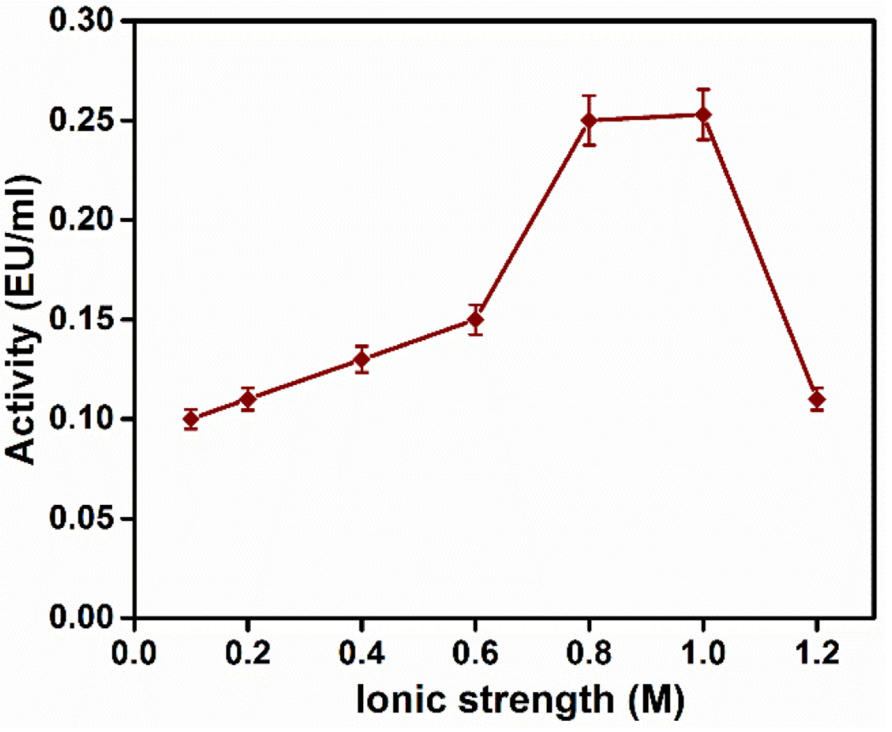

Fig. 4. Effect of ionic strength on the activity of blueberry fruit GST.

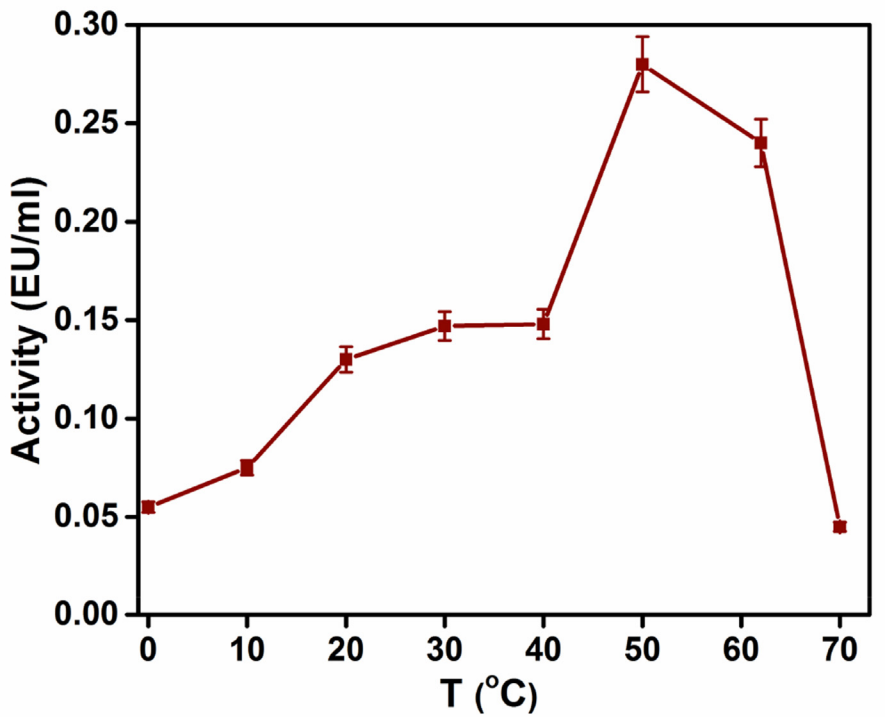

Fig. 5. Effect of optimum temperature on the activity of blueberry fruit GST.

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2.9. Biological activity

2.9.1. Purification of GST

Firstly, homogenate (5 mL) was applied gel filtration column, then high activity tubes combined and were applied to GSH-agarose affinity column equilibrated with 10 mM K-phosphate buffer (pH 7.4) including 150 mM NaCl. The column was washed with equilibrium buffer and then enzyme was eluted with gradient of 5−10 mM GSH in 50 mM Tris/HCl (pH 9.0), 1.5 mL fractions were collected [33, 34].

2.9.2. Glutathione S-transferase activity

The glutathione S-transferase (GST) enzyme catalyzes the conjugation of an aromatic electrophile with tripeptide glutathione molecule. The most widely used aromatic electrophile is 1-chloro-2,4-dinitrobenzene. The dinitrobenzene S-glutathione (DNB-SG) product formed by using this substrate shows a maximum absorbance at 340 nm. So, this activity can be measured by utilizing the increase in absorbance in the wavelength. It is also measured enzyme activity according to this method by us. In the absence of GSH, CDNB rapidly inactivates glutathione S-transferase [35]. Due to this reason, it is not necessary to initiate the reaction with CDNB. The mixture was prepared as a total volume of 1 mL for activity measurement. For this, 25 mM 20 μl of 95% CDNB (in ethanol), 20 mM 50 μl GSH and 730 μl of purified water were added to the control 0.1 μl 200 μl of phosphate buffer (K_2HPO_4/KH_2PO_4 pH: 7.0). The baths in which the activity measurement was performed were thoroughly mixed by adding 200 μl of 0.1 M (K_2HPO_4/KH_2PO_4 pH: 7.0) 25 mM 20 μl of 95% CDNB (in ethanol) and 20 mM 50 μl GSH. After 20 μl enzyme sample was added rapidly, the sample was placed into the spectrophotometer with a cuvette and the absorbance values were measured for 3 minutes [36].

2.9.3. In vitro inhibitors effects

The inhibition measurements of some pesticides were used at a minimum five different concentrations on GST enzyme. Without pesticides were defined as control (100% activity). IC_{50} values were calculated from activity (%) - pesticides concentration graphs. GSH was used as a substrate. Lineweaver - Burk graphs were drawn using 1/V versus 1/[GSH] values, K_i graphs and K_i constants and inhibition types were determined. The best inhibitory showing of pesticides is dichlorvos (Fig. 6) [37].

3. Results and discussion

The world population is growing rapidly and uncontrollably. For this reason, the need for food is increasing but sufficient amount of product can not be provided.

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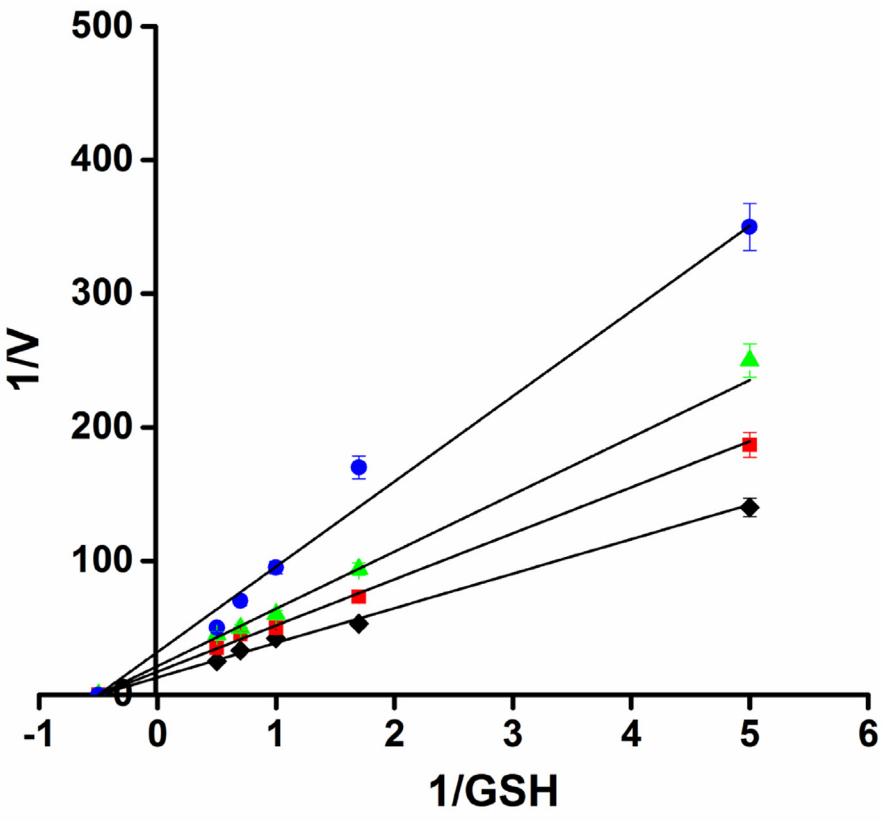

Fig. 6. Dichlorvos Ki values.

It is possible to eliminate this problem by increasing efficiency and quality in unit area, reducing cost and most importantly taking measures to minimize the environmental pollution. "Pesticides" are used in the fight against various pests to increase the production in our country and in the world. Thanks to these substances a remarkable increase in production is observed. However, these pesticides cause pollution by remaning in water, soil, fruits and vegetables for a long time and also reach human through the food chain [38].

The isoenzymes of the glutathione S-transferase are antioxidant enzymes, are found in all organisms as diverse as microbes, insects, fungi, fish, birds, mammals and plants. Plants also contain various GSH-dependent detoxfying enzymes, most specially GST [39]. GST, known as phase II detoxification enzymes, function in biotransformation in plants by catalyzing nucleophilic conjugation of the reduced tripeptide glutathione with electrophilic, hydrophobic and cytotoxic substrates as well as growth and development [40]. Although, GST are antioxidant enzymes toxic substances may affect the amount of glutathione in the cells and may reduce the antioxidant capacity [41]. Because of this, enzyme inhibition studies are important for toxicology studies.

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The GST enzyme was first purified, from *V. arctostaphylos* L. berries. Purification was carried out prepearing homogenate, sephadex G-100 gel filtration and glutathione-agarose affinity chromatography. At the end of these steps the enzyme was obtained with a yield of 23.33%, approximately 189.19-fold, and had a specific activity of 28.00 EU/mg (Table 1). GST enzyme has been isolated from diverse other sources, including fish, plants, erythrocytes etc. using different methods.

In previous studies, GST was isolated from human erythrocytes, bovine erythrocytes, corn and Laurel fruit (*Laurocerasus officinalis Roem.*) with spesific activities of 301.5, 164.31, 66.00, 0.176 EU mg^{-1} protein and purification factors were determined 1143, 252.7, 6800, 60, 30.34- fold, respectively [13, 29, 34, 42]. The purity of the enzyme was determined by the SDS-PAGE method and showed (Fig. 2). The enzyme optimum pH was obtained at approximately pH: 7.2 in 0.1 M K_2HPO_4 buffer (Fig. 3). In previous studies, the optimum pH of the GST enzyme obtained from the Velvet Leaf (*velvetleaf)*, maize plants and *Laurocerasus officinalis Roem.* in the pH range of 6.6−6.8 and 8.0−8.5, 7.0 respectively [29, 42, 43].

Enzyme activities were measured between 0.1 and 1.2 M K_2HPO_4 concentrations in order to determine effects of ionic strength on the enzyme activity. Maximum enzyme activity was obtained in 1 M K-phosphate buffer (pH 7.2) that is the optimum ionic strength of the enzyme activity (Fig. 4).

In this study, enzyme activity was measured spectrophotometrically at 0 °C−70 °C every 10 °C. The optimum temperature of GST enzyme from blueberry fruits was determined as 50 °C (Fig. 5). In previous studies, the optimum temperature of GST enzyme was obtained from (*Oryza sativa)* rice at 55 °C, human blood serum at 65 °C and *Laurocerasus officinalis Roem.* at 30 °C [29, 44, 45]. The fact that the enzyme does not lose activity at high temperature indicates that the enzyme is stable to temperature in some living tissues. These results support this situation.

The kinetic parameters of K_m and V_{max} were determined using CDNB and GSH as co-substrates. K_m values were measured as 5.681 and 0.635 mM, and also V_{max} values were determined as 0.159 and 0.048 EU/ml for CDNB and GSH, respectively. Similarly, in the GST enzyme kinetic studies purified from bovine

Table 1. Purification of GST from blueberry fruit.

Purification step	Total volume (mL)	Activity (U/mL)	Total activity (U/mg)	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Homogenate	30	0.120	3.600	0.806	24.180	0.148	100	1.00
Sephadex G-100 gel filtration chromatography	15	0.153	2.295	0.408	6.120	0.375	63.75	2.53
GSH agarose affinity chromatography	5	0.168	0.006	0.006	0.030	28.00	23.33	189.19

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erythrocytes, the K_M values for GSH and CDNB substrates obtained for the black-grass, rainbow trout erythrocytes, turkey liver and Van Lake fish as 0.3325 mM and 0.744 mM, 1.07 and 1.56, 0.0395 mM, 0.2590 mM, 0.380 and 0.154 and 1.59 and 0.53, respectively [34, 46]. In addition to the above studies, GST enzyme has been reported to be more interested in the GSH substrate [37, 47, 48, 49, 50] and these situations support our work.

K_{cat} values were also determined as 50,210 min^{-1} for CDNB and 15,410 min^{-1} for GSH. Because of K_{cat} and K_{cat}/K_m values of GSH are higher than CDNB, it may be said that GSH is a better substrate for the enzyme. The K_{cat} and K_m values obtained in this study were similar to that studied in GST from human erythrocytes [51].

The purity of the enzyme was determined by SDS-PAGE and showed one band on the gel (Fig. 2). The result indicates that the purification process is sufficient, can be used for further studies and not time-consuming.

K_i and IC_{50} values were shown in Table 2, that are the most suitable parameters for seeing inhibitory effects. Here, *in vitro* studies showed that the enzyme activity was inhibited by dichlorvos, glyphosate-isopropylamine salt, fenoxaprop-p-ethyl, cypermethrin 2,4 dichlorophenoxy acetic acid, haloxyfop-p-methyl, imidacloprid, lambda-cyhalothrin and acetamiprid.

K_i values of the pesticides are ordered that the dichlorvos $<$ fenoxaprop-p-ethyl$<$ 2,4- dichlorophenoxy acetic acid $<$ glyphosate-isopropylamine salt $<$ cypermethrin haloxyfop-p-methyl $<$ imidacloprid $<$ lambda-cyhalothrin $<$ acetamiprid. The results show that the best inhibitor of bluberry GST enzyme is dichlorvos (K_i: 0.0175 $\pm$ 0.005mM) because of its the smallest K_i value.

The results of this study, it is clear that the GST enzyme, which plays a vital role in the detoxification system, causes considerable damage to its activity as a result of the

Table 2. The IC_{50} and K_i values as well as inhibition types on GST enzyme activity for some pesticide compounds.

Inhibitor	K_i mM	IC_{50}(mM)	Inhibition type
Glifosat- izopropilamine salt	0.266 $\pm$ 0.100	0.16	competitive
Fenoxaprop-p-ethyl	0.133 $\pm$ 0.4	0.23	competitive
Cypermethrin	0.310 $\pm$ 0.088	0.32	uncompetitive
Dichlorvos	0.0175 $\pm$ 0.005	0.024	uncompetitive
Imidacloprid	0.630 $\pm$ 0.283	0.49	uncompetitive
Acetamiprid	1.56 $\pm$ 0.733	4.10	competitive
Lambda-Cyhalothrin	0.633 $\pm$ 0.115	0.53	uncompetitive
Haloxyfop-p-Methyl	0.583 $\pm$ 0.087	0.47	uncompetitive
2,4 Dichlorophenoxy acetic acid	0.188 $\pm$ 0.085	0.46	competitive

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use of pesticides, metal ions and organic compounds in very small quantities, resulting in significant damage to the live defense system. Therefore, paying attention to the findings obtained in kinetic studies will be very important for the defense systems in the metabolism.

4. Conclusions

When we look at the study results, it is clear that the GST enzyme, which plays a vital role in the detoxification system, has significantly lost its activity as a result of the use of very small amounts of pesticides, resulting in significant damage to the live defense system. Therefore, paying attention to the findings obtained in kinetic studies will be very important for the defense systems in the metabolism. We observe that pesticides exhibit both competitive and non-competitive inhibition effect with the substrate of the enzyme. In addition, we can say that pesticides show these effects by binding to the active center of the enzyme. It is necessary to prevent the enzymes which are used in food industry to inhibit enzymes. We suggest that the amount of pesticide used should be kept to a minimum.

Declarations

Author contribution statement

Fatih Sen, Fikret Türkan, Halis Şakiroğlu: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ayşenur Aygün, Neslihan Balcı: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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